Role of thyroid hormones in the neuroendocrine control of seasonal reproduction in red deer hinds

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A series of eight experiments was conducted to investigate the requirement for thyroid hormones in neuroendocrine processes which lead to the seasonally anoestrous state in red deer hinds.

The first two experiments used thyroidectomized, ovariectomized, oestradiol-treated hinds which received various thyroid hormone replacement treatments ($n = 5$ per group) to investigate the timing and dose-responsiveness of thyroid hormones in bringing about seasonal oestradiol-induced suppression of plasma LH concentration. A significant seasonal decline in mean plasma LH concentration during September (coinciding with the onset of anoestrus in entire cycling hinds in New Zealand) was observed in all thyroidectomized hinds in both experiments regardless of T_4 or T_3 treatment. When oestradiol implants were removed in November or December, mean plasma LH concentrations increased significantly in all but one of hinds in which T_4 had been administered at very low doses by subcutaneous implants, and mean plasma LH concentrations and LH pulse amplitude increased in approximately half of hinds administered T_3 at varying doses by subcutaneous injections over a one-week period in October. These results suggested that thyroid hormones are not required for steroid-dependent reproductive suppression, but could possibly play a role in steroid-independent suppression of LH secretion. Because problems were encountered in delivering appropriate doses of thyroid hormones in both experiments, further confirmation of these findings was required. Therefore in the next experiment the role of thyroid gland secretions was examined in euthyroid ($n = 5$) and thyroidectomized ($n = 4$) ovariectomized hinds treated with oestradiol implants. These implants were removed for about one month on three occasions to examine the effect of thyroidectomy on steroid-independent control of seasonal LH secretion. During the non-breeding season basal and GnRH-induced plasma LH concentrations declined in

all hinds in the presence of oestradiol, but returned to breeding season levels when oestradiol was withdrawn in November. In a concurrent experiment, thyroidectomy of ovary-entire hinds (n = 7) during the breeding season prevented the cessation of oestrous cyclicity in spring; this was in contrast to oestrous cyclicity in T_4 replaced (n = 4) or euthyroid control (n = 5) hinds which ceased to occur in early September. Collectively, these results indicate that thyroid hormones are required for the termination of the breeding season in cycling red deer hinds and that this action occurs via steroid-independent neuroendocrine pathways.

Two experiments were conducted using neurotransmitter receptor agonists and antagonists to identify neural pathways in the brain which mediate LH suppression by oestradiol and by steroid-independent mechanisms, and to test if the thyroid gland is required for activation of these pathways during the non-breeding season. It was concluded from the lack of plasma LH responses to dopaminergic and opioidergic agonists and antagonists in ovariectomized and ovariectomized, thyroidectomized hinds (n = 5) that neural pathways involving dopamine-D_2 receptors do not mediate oestradiol-induced seasonal suppression of plasma LH concentrations, and neither dopaminergic or opioid neural pathways mediate non-steroidal suppression of plasma LH concentrations. However preliminary evidence was obtained for a stimulatory role of serotonergic neural pathways in controlling LH secretion.

Another experiment was conducted to identify when the steroid-independent mechanisms which suppress LH concentrations during the non-breeding season are responsive to thyroid hormones. T_4 treatment at the beginning of or during the non-breeding season was effective in bringing about suppression of plasma LH concentration in thyroidectomized, ovariectomized hinds (n = 5 per group), but this action of thyroid hormones did not occur during the breeding season. These results show that the steroid-independent mechanisms which contribute to seasonal suppression of plasma gonadotrophin concentrations require thyroid hormones to be present only from around the time of the end of the breeding season for their normal expression, and they remain responsive to thyroid hormones after this period.

Lastly, the feasibility of achieving out-of-season breeding using thyroidectomized hinds (n = 9) was evaluated by comparing oestrous behaviour, ovulation and

pregnancy rates to those of euthyroid control hinds ($n = 7$) following synchronization of oestrous cycles. There was a non-significant trend for a greater occurrence of oestrous behaviour and ovulation in thyroidectomized hinds compared with euthyroid controls during the non-breeding season, but the pregnancy rate following out-of-season mating with a thyroidectomized stag was low, suggesting that a side effect of thyroidectomy may be impaired fertility. Six out-of-season pregnancies were obtained from eight matings, however because three of these pregnancies occurred in euthyroid control hinds no improvement in out-of-season reproductive performance could be attributed to thyroidectomy. It is likely that if the actions of the thyroid glands are to be exploited as a tool for achieving out-of-season breeding in this species, techniques will have to be developed for specifically blocking or overcoming the effects of thyroid hormones on the reproductive neuroendocrine centres without causing general hypothyroidism and its associated side-effects.

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Chapter 1

Introduction

Seasonal breeding is crucial to the survival and reproductive success of the vast majority of vertebrate species inhabiting temperate regions. Seasonal changes in environmental factors such as photoperiod interact with an underlying endogenous rhythm in many long-lived species to bring about alternate periods of reproductive activity and quiescence, the timing of which ensures that the birth of young occurs at a season when environmental conditions most favour neonatal survival (Farner, 1985; Gwinner, 1986; Nicholls *et al.*, 1988a, Lincoln, 1992). In farm animals, our understanding of the neuroendocrine mechanisms that control reproductive seasonality comes predominantly from studies in the ewe. A current hypothesis for control of reproductive seasonality arising from such studies is that the level of responsiveness of the hypothalamus to the negative feedback of oestradiol is the main factor determining the frequency of gonadotrophin secretion and hence the ability to ovulate (e.g. Karsch *et al.*, 1984). However these mechanisms may differ in other species such as the red deer (*Cervus elaphus*), which has not been subjected to genetic modification by centuries of selective breeding (Fisher and Bryant, 1993). It is therefore important that our understanding of reproductive function is not confined to a single species.

Red deer exhibit a rigidly programmed seasonal physiology and a relatively short mating season, timed to synchronise the production of offspring with the season of food abundance in the wild (Lincoln and Short, 1980; Lincoln, 1992). They comprise the prevalent deer species in New Zealand, and along with fallow deer (*Dama dama*), form the basis of a rapidly growing deer farming industry within the world's temperate regions (Asher *et al.*, 1991). Calving of deer in New Zealand occurs between the months of November and January following mating in April and May. In agricultural systems however, maximal grass production rates occur several weeks before the peak in food requirement of the lactating hind. Pasture production in most parts of New Zealand peaks in late October to early December (Lynch, 1949; Korte *et al.*, 1987), after which time a decline in both quality and growth rate occurs principally due to

low rainfall in mid summer. The mismatch of plant production and animal feed requirements has profound effects on the economics of farmed red deer, such as the need for supplementary feeding and early weaning. Considerable research effort has therefore been devoted to studying the physiological mechanisms underlying the timing of the breeding season in domesticated deer species.

Seasonal changes in reproductive state have been shown within the last two decades to be dependent on the presence of thyroid hormones in a variety of species including certain birds, sheep and red deer (Karsch *et al.*, 1995). In the ewe, the profound suppression of the release of gonadotrophin-releasing hormone (GnRH) and luteinizing hormone (LH) by oestradiol does not occur in the absence of thyroid hormones (Webster *et al.*, 1991b). In the red deer hind, suppression of LH by mechanisms not involving oestradiol appear to contribute to the non-breeding state to a much greater degree than is the case for the ewe (Meikle and Fisher, 1996), and thus the hind provides a unique model for studying seasonal reproductive transitions and the role of the thyroid glands in mediating these processes. In addition, a better understanding of this role could lead to the development of techniques which exploit the actions of thyroid hormones to enable manipulation of the timing of mating on commercial deer farms, thus allowing calving to occur at a more favourable time of the year for lactation and rearing.

This thesis includes a review of the physiological basis for seasonality and the oestrous cycle of red deer hinds and the present state of advanced breeding technology, as well as detailing a series of experiments which investigate the role of thyroid hormones and various neuronal pathways in bringing about the non-breeding state. Chapter 4 briefly describes two experiments which investigated the timing and dose-responsiveness of thyroid hormones in suppressing neuroendocrine reproduction in ovariectomized oestradiol-treated hinds. Because these experiments were impaired by problems encountered in delivering appropriate doses of thyroid hormones and by use of an inappropriate model animal (as shown by subsequent experiments), they are presented in summary form only. Chapters 5 - 7 describe experiments which examined the neuromodulation and role of the thyroid glands in steroid-dependent and steroid-independent control of LH secretion, the time of year when the steroid-independent gonadotrophin suppression mechanisms are responsive to thyroid

hormones, and the role of thyroid hormones in seasonal patterns of oestrous cyclicity. These three chapters are largely based on papers which have been submitted for publication in international journals. Chapter 8 describes an attempt at out-of-season breeding in thyroidectomized red deer hinds.

Chapter 2

Literature Review

2.1 Neuroendocrine control of seasonal breeding in temperate animals

It has long been recognised that many seasonally breeding species exhibit an annual cycle of reproduction in is regulated principally by environmental photoperiod (Yeates, 1949; Hafez, 1952; Turek and Campbell, 1979). The link between photoperiodic signals and reproductive responses involves the pineal gland and its secretory product, melatonin, which in turn modifies the pulsatile secretion of the pituitary gonadotrophins via their hypothalamic releasing factor, gonadotrophin releasing hormone (Karsch *et al.*, 1984).

Under natural environments, the annual cycle of reproductive activity in sheep, deer and other seasonally breeding animals operates in close synchrony with annual fluctuations in daily photoperiod (Robinson *et al.*, 1985; Robinson and Karsch, 1988; Loudon and Brinklow, 1992). The effect of photoperiod is to entrain a built in or endogenous rhythm, which occurs in many species even under constant daylengths (Farner, 1985; Gwinner, 1986; Karsch *et al.*, 1989b). The mechanisms by which a change in photoperiod is transformed into an alteration in gonadotrophin secretion can be divided into three major steps. First, the length of the day is perceived and an appropriate neural signal is transmitted to the pineal gland. The second step is transduction of this neural information into an endocrine signal by the pineal gland. Finally, the endocrine signal from the pineal is translated into a change in gonadotrophin secretion by the hypothalamo-hypophyseal axis (Goodman, 1988).

2.1.1 Hormonal control of seasonality in sheep

Role of melatonin

Light and dark photoperiodic signals are transmitted from the eyes first to the suprachiasmatic nuclei and then to the paraventricular nuclei of the hypothalamus. Sympathetic innervation then transmits the signal to the pineal gland, which

synthesises and secretes melatonin during darkness (for reviews see Karsch *et al.*, 1984; Arendt, 1986, Malpaux *et al.*, 1996). The duration of secretion of melatonin therefore indicates the length of each night and is thus a measure of daily photoperiod. The nature of the interaction between melatonin and the hypothalamus, pituitary and gonads however, is not yet well understood. While it is thought that endogenous melatonin operates centrally in the mediobasal hypothalamus to alter pulsatile secretion of gonadotrophin releasing hormone (GnRH) (Malpaux *et al.*, 1996), it has recently been shown that melatonin can act at the level of the anterior pituitary gland to alter the secretion of prolactin in hypothalamo-pituitary disconnected rams (Lincoln and Clarke, 1994; 1995). It is still unclear whether melatonin has other sites of action in ruminant animals at the level of the gonads to either activate or inhibit their function (Kennaway and Rowe, 1995).

Role of photoperiod and the endogenous rhythm

One hypothesis which has been suggested for species with a mating season in the autumn (such as sheep, goats and deer) is that the change from long to short periods of daylight following the summer solstice leads to an increase in the pulsatile secretion of GnRH, which in turn activates the reproductive axis (Lincoln and Short, 1980; Lincoln, 1992). However Malpaux *et al.* (1989) showed that the time of onset of the breeding season in the Suffolk ewe did not depend on decreasing daily photoperiod after the summer solstice or on the cessation of increasing photoperiod as this solstice approaches, but rather was initiated several months before the breeding season by lengthening daily photoperiods during late winter and early summer. It has recently been demonstrated in pinealectomized ewes that delivery of melatonin signals which mimic those normally secreted by the pineal gland during spring and summer is sufficient to synchronise the endogenous reproductive rhythm in the absence of any other photic cues (Woodfill *et al.*, 1994). It appears that although shortening daylengths prior to the transition into the breeding season do not time the onset of reproductive activity in the ewe, they do contribute to maintaining its full duration (Wayne *et al.*, 1990; Malpaux and Karsch, 1990; O'Callaghan *et al.*, 1991).

Although the transition to anoestrus can be driven by exposure to artificially elevated photoperiods (Dahl *et al.*, 1994), studies have shown that increasing daylengths are not required for this reproductive transition to occur at the normal time (e.g. Robinson

and Karsch, 1984; Malpaux *et al.*, 1988). Rather, it appears as if the end of the breeding season is associated with an inability to respond to the stimulatory effects of short days. This loss of reproductive response to inductive photoperiod (termed 'photorefractoriness') is thought to play a critical role in the natural transition to seasonal anoestrus in certain breeds of sheep. Reproductive refractoriness can be defined as the physiological state which develops following prolonged exposure to a fixed photoperiod, and in most species it manifests itself as a spontaneous reversal in the prevailing neuroendocrine state (Nicholls *et al.*, 1988a). While the lack of decrease in day length around and after the winter solstice may play some role in timing the end of the breeding season, the primary reason for the transition into anoestrus has been shown to be an obligatory turn-off, i.e. the expression of the underlying endogenous rhythm (Malpaux *et al.*, 1988). Refractoriness in the Suffolk ewe to short (inductive) photoperiod is not due to an inappropriate secretory pattern of melatonin (Malpaux *et al.*, 1987); however relatively little is known about the specific neuroendocrine mechanisms involved in developing and expressing photorefractoriness. Responsiveness to short or long photoperiods can be restored in ewes that have become photorefractory to either of these regimes by exposure to a brief period (30 days) of long or short photoperiods, respectively (Jackson *et al.*, 1988).

These conclusions support an hypothesis of an endogenous rhythm of reproduction in which the increasing photoperiods of late winter and early summer synchronise the process that leads to reproductive onset and the long days around the summer solstice are necessary to delay the breeding season until early autumn.

Role of non-photoperiodic factors

Nutrition, temperature and rainfall are believed to cause some entrainment of the endogenous rhythm, however because the annual change in photoperiod is so regular between years it is undoubtedly the predominant factor for temperate species (Lincoln and Short, 1980). Variations in the expression of reproductive responses to nutritional alterations among sexes, breeds and species probably reflects variations in the role of this environmental factor as a modulator of reproductive function (for review see Martin and Walkden-Brown, 1995).

Reproduction is also influenced by social factors. For example, oestrus and ovulation can be induced in anovulatory females by exposure to ewes induced to oestrus (Zarco *et al.*, 1995). Introduction of rams is also able to increase LH pulse frequency and induce ovulation in ewes during the non-breeding season, a phenomenon known as the 'ram effect' (Martin *et al.*, 1986).

Role of pituitary gonadotropins and ovarian steroids

Seasonal changes in the secretion of the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland influence the timing of the onset and termination of the breeding season. Both hormones are known to be regulated by the hypothalamic decapeptide, GnRH (Martin, 1984). These hormones are secreted in discrete pulses, each LH episode being temporally related to (and presumably the direct consequence of) an episode of GnRH (Levine *et al.*, 1982; Clarke and Cummins, 1982, Karsch *et al.*, 1987; Barrell *et al.*, 1992; Karsch *et al.*, 1993). Feedback effects of ovarian steroids (predominantly negative) control the pulsatile secretion of GnRH from the hypothalamus and LH from the anterior pituitary gland (Hauger *et al.*, 1977, Karsch *et al.*, 1977; Goodman and Karsch, 1980; Goodman and Karsch, 1981).

The key observation that laid the foundation for much of the recent progress in elucidating the physiological mechanisms by which changes in day length alter ovarian function was the demonstration of dramatic seasonal variations in the negative feedback actions of oestradiol on tonic LH secretion (Legan *et al.*, 1977; Karsch *et al.*, 1980). Compelling evidence was obtained from a study in which ovariectomized ewes were treated with oestradiol-containing implants which maintained stable physiological oestradiol levels (Legan *et al.*, 1977). Under this treatment, there was a striking seasonal change in circulating LH which was uniformly undetectable in anoestrus and greatly elevated during the breeding season. In contrast, there was no major variation in serum LH concentrations in a group of ovariectomized ewes not treated with oestradiol. The seasonal shifts in LH coincided with transitions between breeding and anoestrus in intact ewes. Subsequent findings indicated that this seasonal change also occurs in the intact ewe (Karsch *et al.*, 1979) and that it is controlled by environmental photoperiod (Legan and Karsch, 1979, 1980).

Although the original work by Legan, Karsch and coworkers indicated that photoperiod exerts effects only on oestradiol negative feedback, more detailed analysis revealed seasonal alterations in LH secretion in ovariectomized ewes not treated with oestradiol (Goodman *et al.*, 1982; Robinson *et al.*, 1985). A small but significant decrease in LH pulse frequency and an increase in pulse amplitude occurs during the non-breeding season in the absence of oestradiol (Goodman *et al.*, 1982), so that mean LH concentrations are not lower at this time compared to the breeding season (Legan *et al.*, 1977) and may even be higher (Pau and Jackson, 1985). These seasonal changes in LH pulse frequency in ovariectomized animals, which are directly proportional to the length of the prevailing photoperiod (Robinson *et al.*, 1985), are often referred to as the 'steroid-independent' actions of photoperiod.

Within the last decade, the thyroid gland has been increasingly implicated in the control of seasonal transitions in mammals. The role of thyroid hormones in seasonal reproduction is reviewed in detail in Section 2.2.

2.1.2 Hormonal control of seasonality in red deer

As is the case with the majority of species that have evolved in temperate and cold climates, red deer rely principally on fluctuations in environmental photoperiod to entrain their annual reproductive cycles. This was first noted as a rapid adaptation of seasonal reproductive cycles to changes in ambient photoperiod when deer were transported between the northern and southern hemispheres (Marshall, 1937; Otway, 1985). The importance of photoperiod has since been demonstrated in several studies in red deer stags by altering the timing of the antler cycle in response to artificially manipulated photoperiods (e.g. Jaczewski, 1954; Suttie *et al.*, 1989). In pubertal red deer hinds subjected to artificially shortened photoperiods, the onset of the breeding season was significantly advanced (Webster and Barrell, 1985). As in sheep, the effects of photoperiod are mediated by the pattern of melatonin secretion from the pineal gland, and treatment of anoestrous hinds with exogenous melatonin by daily injection, orally or by subcutaneous implants can advance reproductive activity (Webster and Barrell, 1985; Adam *et al.*, 1986; Wilson, 1992).

Episodic secretion of LH in entire red deer hinds is greater in early winter (1-2 pulses/4 h) than in the non-breeding season (<1 pulse/4 h), probably due to a seasonal

increase in episodic GnRH secretion (Duckworth, 1992; Limsirichaikul, 1992). LH pulse frequency has been measured in more detail in Père David's deer hinds. McLeod *et al.* (1992) reported a varying pulse frequency throughout the non-breeding season of Père David's hinds. Mean LH pulse frequency in early anoestrus (2.3 pulses/12 h) was significantly lower than in mid and late anoestrus (3.7 and 4.4 pulses/12h) and during the oestrous cycle (follicular phase: 7.1 pulses/12 h; luteal phase: 2.9 pulses/12 h). These authors suggested that there is a period of deeper anoestrus early in the non-breeding season in Père David's deer than there is after the middle of the non-breeding season. The ability of Père David's hinds to ovulate following pulsed or continuous administration of GnRH is also reduced during early anoestrus (McLeod *et al.*, 1991; Brinklow *et al.*, 1992).

While administration of exogenous gonadotrophins such as pregnant mare serum gonadotrophin (PMSG) or of GnRH is sufficient to overcome the seasonal inhibition of ovulation and oestrus in anoestrous sheep (Smith *et al.*, 1988), seasonally anoestrous deer hinds do not always ovulate upon similar treatment (Fisher *et al.*, 1986, 1989; McLeod *et al.*, 1991; Duckworth and Barrell, 1988; 1991). This suggests that insufficient GnRH release may not be the only factor limiting ovulatory activity in anoestrous deer hinds (Duckworth, 1992). In addition to this, it has been shown that Père David's deer hinds which do ovulate return to anoestrus immediately after the withdrawal of exogenous GnRH treatment (McLeod *et al.*, 1991; Brinklow *et al.*, 1992).

Photoperiodic entrainment of the endogenous reproductive rhythm may occur differently for red deer than for sheep (see Section 2.1.1), since Duckworth (1992) showed that the onset of seasonal breeding activity in mature red deer hinds was not affected by the lengthening daily photoperiods in spring (which are important for entraiment of the endogenous rhythm in ewes) but was delayed if the autumnal decrease in photoperiod is delayed. Deer also appear to differ from sheep with regard to the relative importance of seasonal changes in pituitary responsiveness to GnRH and steroid-independent mechanisms governing LH secretion; both of which have recently been shown to decline dramatically around the time of the summer solstice in red deer hinds (Meikle and Fisher, 1996).

2.1.3 Neuronal control of seasonal LH and prolactin secretion

As outlined in the preceeding sections, changes in the length of daily photoperiod apparently alter the sensitivity of the hypothalamo-hypophyseal system to the negative effects of gonadal steroids, but the neural pathways by which photoperiod exerts these effects are still poorly understood despite the increasing research into this area over the last decade.

Since most GnRH neurons do not themselves possess oestradiol receptors in the rat (Shivers *et al.*, 1983), ewe (Karsch and Lehman, 1988; Lehman and Karsch, 1993), guinea-pig (Watson *et al.*, 1992) or rhesus monkey (Sullivan *et al.*, 1990) it is generally accepted that the increasing negative effects of oestradiol at the termination of the breeding season must be conveyed to the GnRH secretory neurons via other neurons which are afferent to them. Several potential neurotransmitters have been implicated in mediating this effect of photoperiod, based mostly on studies involving either lesions to specific areas of the brain, pharmacological administration of neurotransmitters or their antagonists, or immunocytochemical investigations of the distribution of neurons and oestrogen receptors in brain regions. There are a lot of apparent discrepancies in the results of these studies; these may be accounted for by differential stimulation or inhibition of a particular pathway or pathways depending on the specificity and potency of the pharmacological agents used and their ability to cross the blood-brain barrier, dose, route of administration and physiological status of the animal. Some drugs, for example, have agonistic effects at low concentrations but antagonistic effects at high concentrations. It should also be remembered that pharmacological experiments in whole animals may assess the summation of drug actions at multiple sites, so that the observed response probably selects for the dominant pathway (Weiner *et al.*, 1993). In this regard, it is not known whether or to what extent the various types of neurons which have been shown to be involved in seasonal reproductive changes are connected in parallel or in series. If the former is the case, blocking or activating only one neuronal pathway may have a relatively small effect on LH secretion.

Endogenous opioid peptides

Most, but not all, of the experiments using opioid agonists or antagonists have concluded that endogenous opioid peptides are relatively unimportant in suppression of the GnRH pulse generator during the non-breeding season by oestradiol (e.g. Meyer and Goodman, 1985; Brooks *et al.*, 1986b; Yang *et al.*, 1988; Currie *et al.*, 1991) or testosterone (Lincoln *et al.*, 1987). The regional distribution and concentrations of ß-endorphin, one of the opioid peptides, did not vary in the hypothalami of sexually active and inactive rams (Ebling *et al.*, 1987). However Shen *et al.* (1993) demonstrated that steroid hormones modulate opioid receptors in ovariectomized oestradiol-implanted ewes during the non-breeding season in a receptor subtype- and region-dependent manner, and suggested that these steroid-induced changes in receptor characteristics could be at least partly involved in the negative feedback regulation of LH by ovarian steroids. Moreover, Currie *et al.* (1993) have recently shown that the opioid antagonist naloxone is able to overcome suppression of LH in entire anoestrous ewes when perfused into the median eminence, but not when administered intravenously.

Opioid peptides have been shown to be important modulators of progesterone suppression in ewes during either season (Brooks *et al.*, 1986b; Yang *et al.*, 1988; Whisnant and Goodman, 1988; Currie *et al.*, 1991) and in luteal phase cows (Stumpf *et al.*, 1993). The opioid neurons concerned probably exist primarily in the medial basal hypothalamus (Whisnant and Goodman, 1994). Limited data suggests that opioid peptides mediate oestradiol effects in the breeding season during the luteal, and possibly the follicular phases of the oestrous cycle (Brooks *et al.*, 1986a; Whisnant and Goodman, 1988; Currie and Rawlings, 1989; Cosgrove *et al.*, 1993), possibly by inhibiting GnRH pulse size (Goodman *et al.*, 1995). Such inhibitory actions of opioid peptides during the breeding season may be inhibited by dopamine during the non-breeding season (Tortonese and Lincoln, 1995). Opioid peptides have been unambiguously shown to suppress LH in prepubertal ovariectomized oestradiol-implanted ewe lambs (Schall *et al.*, 1991) and heifers (Wolfe *et al.*, 1991) and prepubertal human females (Genazzani *et al.*, 1993). Contacts between GnRH neurons and nearby opioid neurons have been observed in juvenile female rhesus monkeys (Thind and Goldsmith, 1988). This suppression of LH by opioids declines as animals mature following puberty (Brooks *et al.*, 1986a; Wolfe *et al.*, 1991), and

other neuronal pathways such as catecholaminergic pathways appear to become more important in subsequent anoestrous seasons (Schall *et al.*, 1991). However some results indicate that opioid pathways still modulate the steroid-independent effects of photoperiod in mature animals (Shillo *et al.*, 1985; Yang *et al.*, 1988; Schall *et al.*, 1991; Evans *et al.*, 1994) while others disagree with this finding (Brooks *et al.*, 1986b; Meyer and Goodman, 1986; Currie *et al.*, 1991).

The opiate drug morphine increases prolactin concentrations in sheep (Schillo *et al.*, 1985, Parrott and Goode, 1992). In the rat, opioids are thought to stimulate prolactin secretion by reducing dopamine release into the hypothalamo-pituitary portal circulation (Gudalsky and Porter, 1979; Van Loon *et al.*, 1980a; 1980b; Wilkes and Yen, 1980). The influence of opioid peptides on prolactin secretion may vary with season, since in Holstein calves naloxone suppressed prolactin concentrations during spring, but increased prolactin concentrations during early winter (Johnson *et al.*, 1990). Naloxone was shown to act centrally in this experiment, since another antagonist which does not readily cross the blood-brain barrier, methyl levallorphan mesilate, was without effect on prolactin concentrations.

Serotonin

Until recently, the relatively few studies which had investigated serotonergic pathways in seasonal reproduction were in general agreement. Using the $5HT_2$ receptor antagonist cyproheptadine, it has been demonstrated that serotonin is not involved in suppression of LH in intact anoestrous ewes (Meyer and Goodman, 1985). This finding was later verified and extended to show that, out of the 7 different neurotransmitter antagonists tested, only cyproheptadine was able to reverse the steroid-independent actions of photoperiod in ovariectomized ewes (Meyer and Goodman, 1986). Because cyproheptadine is not very specific, binding to histamine H_1 receptors with a higher affinity than to $5HT_2$ receptors, the same research group then repeated these results in ovariectomized ewes using methysergide (which is a more specific antagonist than cyproheptadine, but may also be a dopamine agonist) and parachlorophenylalanine (which blocks serotonin synthesis) (Whisnant and Goodman, 1990). Using the latter drug, it was also demonstrated that the steroid-dependent actions of photoperiod could operate independently of the steroid independent actions. These results are in general agreement with those of Riggs and

Malven (1974), who showed that intracerebroventricular infusion of serotonin depressed LH release in castrate male sheep.

Research from three other groups has shown contradictory findings. Deaver and Dailey (1982) showed that intravenous infusion of serotonin stimulates LH secretion in a dose-dependent manner. Kao *et al.* (1992), reported little effect of cyproheptadine at all under any photoperiod in ovariectomized ewes, and some suppressive effects of this drug in ovariectomized oestradiol-implanted ewes. It was concluded that serotonin had little importance in control of seasonality. In contrast, Le Corre and Chemineau (1993a) reported an increase in LH in ovariectomized oestradiol-implanted ewes under short day refractoriness conditions in response to cyproheptadine. These results were verified using three different antagonists with different specificities for 5HT (and other) receptors; the general conclusion being that serotonin plays a major inhibitory role in control of LH secretion during short day refractoriness in ovariectomized oestradiol-implanted ewes, and that $5HT_2$ receptors are probably involved.

Serotonin is also involved in regulation of prolactin secretion. Using hypothalamo-pituitary disconnected ewes which were administered intravenously with serotonin, it was shown that serotonin itself is not a direct prolactin-releasing factor in sheep, but exerts an effect via the hypothalamus or neural lobe (Thomas *et al.*, 1988). Studies in rats also suggest against a direct effect of serotonin on pituitary prolactin secretion (López *et al.*, 1987). Serotonin is thought to stimulate prolactin secretion by inhibiting hypothalamic dopamine release (Pilotte and Porter, 1981) as well as by stimulating the release of prolactin-releasing factors into hypophyseal portal blood (Kaji *et al.*, 1986).

Dopamine

Most studies of dopaminergic involvement in control of GnRH secretion suggest that such neurons mediate gonadal steroid suppression of LH during anoestrus. Dopamine infusion decreased mean LH in ovariectomized anoestrus ewes (Deaver and Dailey, 1982). Tortonese and Lincoln (1994a) showed in a simple experiment using entire rams that bromocriptine (agonist) could suppress LH on short days but had no effect during long days, while sulpiride (antagonist) increased LH on long, but not short

days. These results suggest an inhibitory dopaminergic system participates in GnRH suppression during anoestrus. Pimozide, another antagonist, increased LH in entire or ovariectomized oestradiol-implanted, but not ovariectomized non-implanted ewes during anoestrus or in luteal phase ewes (Meyer and Goodman, 1985; 1986). Dopaminergic suppression of pulsatile LH secretion appears to be mediated via the D_2 rather than the D_1 receptor (Curlewis *et al.*, 1991).

Local injection of pimozide (Halvern *et al.*, 1991) or the neurotoxin 6-hydroxydopamine (which specifically destroys catecholaminergic cells) (Thiéry *et al.*, 1989) and radiofrequency lesions (Halvern *et al.*, 1994) in specific hypothalamic sites suggest the inhibitory dopaminergic system acts in the medial basal hypothalamus (retrochiasmatic area or median eminence) during anoestrus. The inhibition of LH secretion by oestradiol under long days is accompanied by an increase in the rate-limiting dopamine biosynthesis enzyme, tyrosine hydroxylase, in the neurons of the retrochiasmatic area (A15 nucleus) (Gayrard *et al.*, 1994; 1995), and transfer to short days or treatment with exogenous melatonin is associated with a dramatic decrease in dopamine content and tyrosine hydroxylase activity in the median eminence (Viguié *et al.*, 1996; 1997). Blockade of tyrosine hydroxylase activity stimulates pulsatile LH secretion during long days (Viguié *et al.*, 1995). Dopamine probably acts at the level of the GnRH terminals in the median eminence rather than on the cell bodies in the preoptic area (Kuljis and Advis, 1989; Clarke and Scott, 1993). Melatonin implants in the medial basal hypothalamus appear to counteract the suppressive effects of bromocriptine on FSH secretion in rams (Tortonese and Lincoln, 1994b). Recent experiments involving administration of pimozide to ovariectomized oestradiol-treated ewes with anterior hypothalamic knife cuts suggests oestradiol exerts its effects via dopaminergic input from the rostral hypothalamus (Whisnant and Goodman, 1994).

It is possible that noradrenergic neurons operating in the preoptic area (Halvern *et al.*, 1991) may stimulate the inhibitory effects of dopaminergic structures which are intermediate to the noradrenergic and GnRH secretory neurons (Goodman, 1989). These findings have been reinforced by recent results showing that the inhibitory action of noradrenaline implants placed in the preoptic area on LH pulse frequency

was blocked by pimozide implants in the medial basal hypothalamus (Goodman *et al.*, 1994).

In contrast, some experiments in ovariectomized oestradiol-implanted ewes have demonstrated a role for dopamine under conditions of short day photorefractoriness, but not long day photosuppression (Kao *et al.*, 1992; Le Corre and Chemineau, 1993b). Such findings could be explained by the relatively low doses of pimozide used in these experiments compared to many of the previously mentioned studies. Riggs and Malven (1974) were not able to consistently alter mean plasma LH concentrations following intracerebroventricular administration of dopamine at five different dosages to castrate male sheep. Tilbrook and Clarke (1992) found no response to pimozide in entire, castrate or castrate testosterone injected rams, but a significant increase in LH pulse frequency in entire ewes following pimozide, during the non-breeding season. These results are supported by the findings of Lubbers and Jackson (1993), which suggest a profound sexual differentiation of the neuroendocrine mechanisms controlling seasonal breeding in sheep.

Immunocytochemical investigations have revealed synaptic contacts between dopaminergic (tyrosine hydroxylase positive) and GnRH containing neurons at electron microscopic level in mid-luteal phase ewes (Kuljis and Advis, 1989). Others have failed to verify this finding (Karsch *et al.*, 1989). Tyrosine hydroxylase positive neurons in the arcuate nucleus and periventricular anterior hypothalamus have, however, been shown to possess oestrogen receptors in anoestrus ovariectomized oestradiol implanted and non-implanted ewes (Karsch and Lehman, 1988; Lehman *et al.*, 1993).

In vitro, dopamine has been shown to *increase* GnRH release via ß-adrenergic receptors on GnRH cells cultured from a transgenic mouse tumour (Weiner and Martinez de la Escalera, 1993).

In sheep and red deer under both long and short days, dopamine or its agonists suppress prolactin secretion while antagonists increase it (Deaver and Dailey, 1982; Deaver *et al.*, 1987; Milne *et al.*, 1990; Regisford and Katz, 1993), and endogenous opioids appear to augment dopaminergic inhibition under short days (Ssewannyana

and Lincoln, 1990). In red deer hinds, the termination of the breeding season has been delayed by administering a long-acting form of bromocriptine to suppress circulating prolactin (Curlewis *et al.*, 1988). However the decrease in dopaminergic activity in the median eminence following transfer of ewes to short days appears to be unrelated to prolactin inhibition (Viguié *et al.*, 1997). Activation of central dopamine D_1 and D_5 receptors has been shown to *stimulate* prolactin release (Porter *et al.*, 1994; Curlewis *et al.*, 1995).

Noradrenaline

Noradrenaline is able to cause the release of LH in oestrogen suppressed ewes in the non-breeding season, but not in the breeding season (Scott *et al.*, 1992; Clarke and Scott, 1993). One explanation of these results is that the negative feedback effect of oestradiol in the non-breeding season involves the withdrawal of the noradrenaline drive to GnRH neurons, so that replacement with exogenous hormone would overcome the negative influence. The α-adrenergic antagonists phenoxybenzamine and phentolamine reduced mean LH levels in ovariectomized ewes, but did not consistently block oestradiol-induced LH release in these animals (Jackson, 1977). Season was not mentioned in this study. However other results strongly suggest that noradrenergic pathways are inhibitory, since injection of the α-adrenergic antagonist phenoxybenzamine increased LH pulse frequency during anoestrus in intact, but not ovariectomized, ovariectomized oestradiol-implanted or luteal phase ewes (Meyer and Goodman, 1985; 1986). Intravenous noradrenaline infusion decreased mean LH concentration in ovariectomized anoestrous ewes (Deaver and Dailey, 1982), and intracerebroventricular infusion of noradrenaline also lowered mean LH concentration in castrate male sheep (Riggs and Malven, 1974). Halvern *et al.* (1991) suggested an inhibitory noradrenergic neural system operates in the preoptic area in intact anoestrus ewes, based on local injection of phenoxybenzamine into specific hypothalamic sites. LH pulses were also elevated in ovariectomized oestradiol-treated compared with untreated ewes after injection of the catecholaminergic neurotoxin 6-hydroxydopamine into the retrochiasmatic area during anoestrus, although this probably reflects destruction of dopaminergic pathways as well (Thiéry *et al.*, 1989). As mentioned previously, there is evidence that noradrenergic neurons may stimulate the inhibitory effects of dopaminergic cells which in turn innervate the GnRH secretory neurons (Goodman, 1989; Goodman *et al.*, 1994).

In vitro studies using pituitary tissue from castrated ram lambs have suggested that adrenaline, acting via a β_2-adrenergic receptor, may modulate the pituitary gonadotrope's response to GnRH (Swartz and Moberg, 1986).

Gamma-aminobutyric acid (GABA)

Preoptic GABAergic cells in sheep are oestrogen-sensitive, and may be part of an interconnected network of preoptic and hypothalamic neurons which mediate the influence of oestradiol upon GnRH secretory activity and reproductive behaviour (Herbison *et al.*, 1993). Suppression of pulsatile LH secretion in ovariectomized ewes with oestradiol is associated with an increase in GABA concentrations in the retrochiasmatic area and mediobasal hypothalamus (Gallegossanchez *et al.*, 1996). GABA is known to inhibit LH release via hypothalamic action in the rat (Jarry *et al.*, 1993), and the activity of GABAergic neurons terminating in the rostral hypothalamus and median eminence is positively regulated by testosterone in male orchidectomized rats (Grattan and Selmanoff, 1994). GABAergic neurons in the preoptic area of the rat have been shown to synapse directly on GnRH neurons (Leranth *et al.*, 1985). Mixed findings have been obtained from GABA agonist and antagonist studies in sheep (Meyer and Goodman, 1985; 1986; Scott and Clarke, 1993; Clarke and Scott, 1993). One possible interpretation of the data is that the seasonal 'switch' involves the induction of $GABA_B$ receptors during anoestrus, and that effects mediated via this subtype are the reverse of those obtained through $GABA_A$ receptors at this time (Scott and Clarke, 1993; Clarke and Scott, 1993).

Neuropeptide Y

Neuropeptide Y (NPY) is probably involved in positively mediating the LH surge at both GnRH secretory and pituitary levels (Freeman, 1993; Bauer-Danton *et al.*, 1993; Barker-Gibb and Clarke, 1994; Besecke and Levine, 1994). NPY positive cells are closely apposed to GnRH somas and dendrites (Karsch *et al.*, 1989). During anoestrus, however, oestradiol does not influence expression or release of NPY from terminals within the preoptic area or organum vasculosum (regions of GnRH cell bodies) or median eminence (region of GnRH cell terminals (Barker-Gibb and Clarke, 1993). Intracerebral infusion of NPY has been reported to transiently *suppress* episodic LH secretion in ovariectomized ewes (Malven *et al.*, 1992).

Other Neurotransmitters

Pharmacological evidence suggests that excitatory amino acids such as glutamate, aspartate, and *N*-methyl-D-aspartate (NMDA, a synthetic analogue of aspartate) stimulate GnRH and LH release in rats (Farah *et al.*, 1991; Mahachoklertwattana *et al.*, 1994; Ping *et al.*, 1994) and male calves (Shahab *et al.*, 1994). Abundant glutamate receptors are present throughout the rat hypothalamus and preoptic area, albeit in densities considerably lower than those in other brain regions (Meeker *et al.*, 1994). In ovariectomized ewes, administration of NMDA at three different doses did not alter LH secretion; however all doses were able to overcome suppression of LH release by exogenous oestradiol implants (Estienne *et al.*, 1990). Chronic stimulation of a glutaminergic pathway with NMDA can overcome the inhibitory effect of short day exposure on LH secretion and testis size in the male Siberian hamster (Ebling *et al.*, 1994).

In the last few years it has become increasingly apparent that the short-lived gas nitric oxide acts as a major endocrine regulator. Nitric oxide may act as a neurotransmitter which is involved in initiating the GnRH surge in rats, possibly by stimulating excitatory amino acid activation of GnRH release (Bonavera *et al.*, 1993). Recent *in vitro* studies have suggested both stimulatory (Moretto *et al.*, 1993) and inhibitory (Sortino *et al.*, 1994) roles for nitric oxide in the control of GnRH secretion.

Studies using an H_1-receptor antagonist have shown that histamine also affects LH during anoestrus and during the oestradiol-induced surge in ovariectomized ewes (Van Kirk *et al.*, 1989). However Alexander *et al.* (1994) observed inconsistent effects of the same antagonist during anoestrus, and concluded that histamine was probably not a direct modulator of GnRH release.

2.2 Role of the thyroid glands in seasonality

There is increasing evidence that the thyroid hormones are also components of the system which regulates the seasonal patterns of reproductive activity in mammals and birds. This phenomenon was first discovered 50 years ago in birds by Woitkewitsch (1940), who reported that thyroidectomized European starlings remained in the breeding season indefinitely. 30 years later, Wieselthier and Van Tienhoven showed that thyroidectomy of starlings prevented the development of anoestrus. Most reports concerning the role of thyroid hormones in seasonal reproduction of mammals have used the thyroidectomized ewe as a model (e.g. Nicholls *et al.*, 1988b; Webster *et al.*, 1991a; 1991b), but other species are now being investigated and a considerable body of evidence has accumulated to document that the thyroid glands are required for the seasonal decline in reproductive function at the termination of the breeding season.

The role of the thyroid glands in seasonal reproduction in ewes was first reported by Nicholls *et al.* (1988b), who showed that thyroidectomy late in the non-breeding season did not alter the transition into the breeding season but prevented the subsequent termination of reproductive activity, so that regular oestrous cycles were exhibited for more than one year.

Different mammalian species studied

A role for the thyroid glands in seasonal reproduction has been extended from birds over the last decade to include golden hamsters (Vriend, 1985), mink (Jacquet *et al.*, 1986), sheep (Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Parkinson and Follett, 1994) and red deer (Shi and Barrell, 1992; 1993). Some species and gender differences regarding the role of thyroid hormones are apparent in the results of these studies. For example, while removal of the thyroid glands caused ewes to remain in the reproductive state indefinitely (Nicholls *et al.*, 1988b), in the mink thyroidectomy merely delayed the onset of sexual quiescence (Jacquet *et al.*, 1986). Data from two studies in golden hamsters (which exhibit reproductive activity under long photoperiods) are conflicting. While Vriend (1985) reported that melatonin-induced gonadal regression in long day photoinduced hamsters could be prevented by treatment with a goitrogenic compund, Champney (1988) was unable to prevent or

attenuate the occurrence of gonadal regression induced by short photoperiods by thyroidectomizing hamsters.

The effects of thyroidectomy on seasonal reproduction in ewes appear to be confined to the transition to anoestrus in late winter; the timing of the onset of the breeding season in autumn is not affected regardless of the time of thyroidectomy relative to the breeding season (Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Thrun *et al.*, 1997). In contrast, thyroidectomy of rams at the start of the non-breeding season caused premature entry into the next breeding season (Parkinson and Follett, 1994).

Effects of thyroidectomy on photoperiodically mediated seasonal processes

Thyroidectomy has been shown not to disrupt sensitivity to photoperiodic stimuli in several species, since appropriate prolactin and melatonin responses to fluctuating photoperiods are preserved following thyroidectomy (Jacquet *et al.*, 1986; Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Shi and Barrell, 1992; Dahl *et al.*, 1994a). However, thyroidectomized ewes failed to exhibit a reproductive neuroendocrine response to an abrupt switch from short to long photoperiods (Dahl *et al.*, 1994a). These findings indicate that the influence of thyroidectomy on seasonal reproduction is not due to a general disturbance of seasonal phenomena or photoperiodic perception, but rather is caused by a disruption of the normal response of the hypothalamo-hypophysial axis to photoperiodic stimuli.

Role of thyroid hormones in steroid-dependent seasonal suppression of reproduction

In ovariectomized ewes treated with constant-release oestradiol implants, thyroidectomy late in the non-breeding season did not alter the pronounced increase in serum LH concentration at the onset of the breeding season but completely obliterated the subsequent fall in both mean serum LH concentration and pulsatile LH secretion at the end of the breeding season (Moenter *et al.*, 1991; Webster *et al.*, 1991a). This effect was prevented by replacement of thyroxine (T_4) to physiological serum concentrations (Webster *et al.*, 1991a). Results of another study by the same workers showed that thyroidectomy was also able to prevent the seasonal decline in pulsatile GnRH secretion in ovariectomized oestradiol-implanted ewes (Webster *et al.*, 1991b).

The role of thyroid hormones in steroid-independent seasonal LH secretion in the ewe has received no direct attention; however the results of Moenter *et al.* (1991) suggest that LH pulse frequency and amplitude remain at breeding season values in thyroidectomized, ovariectomized ewes during the non-breeding season.

Permissive mode of action of thyroid hormones

A seasonal cycle of circulating thyroid hormone concentrations exists for many species (Karsch *et al.*, 1995). There is some suggestive evidence that the seasonal rise in thyroid hormone concentration around the time of the end of the breeding season could actively drive the transition to anoestrus. In ewes made hypothyroid with a goitrogenic substance, the breeding season was extended by one or two oestrous cycles (Follett and Potts, 1990). In another experiment, ewes made hyperthyroid by injection of large doses of T_4 during the breeding season terminated seasonal reproductive activity prematurely (O'Callaghan *et al.*, 1993). However more recent experiments in red deer stags and ewes have shown that prematurely increasing circulating thyroid hormone concentrations during the breeding season to values similar to seasonal peak concentrations, by exogenous T_4 treatment, does not result in an early transition to the non-breeding season (Shi and Barrell, 1994; Dahl *et al.*, 1995). Furthermore, in thyroidectomized ewes treated with low doses of T_4 (which produced circulating thyroid hormone concentrations equivalent to the seasonal nadir) the seasonal decline in serum LH concentration was not delayed relative to euthyroid control ewes (Dahl *et al.*, 1995). These findings strongly suggest that, within the normal physiological ranges, thyroid hormones act permissively to bring about the non-breeding season rather than in a dose-dependent manner.

Timing of thyroid hormone action

The observation that thyroidectomy in the middle of the breeding season, after the time when the photoperiodic cues for synchronization of the endogenous rhythm are perceived (Woodfill *et al.*, 1994), is effective in preventing the onset of anoestrus (Webster *et al.*, 1991) led to the hypothesis that thyroid hormones may act only during a short 'window' of time to bring about the seasonal decline in reproductive function (Thrun *et al.*, 1993; Karsch *et al.*, 1995). Support for this hypothesis comes from the recent finding that thyroid hormones need only be present for a brief period of time near the end of the breeding season for the neuroendocrine changes that lead to

anoestrus (Thrun *et al.*, 1996). Subsequent experiments showed that the minimum effective period of exposure to thyroid hormones for the transition to anoestrus was 60-90 days and that exposure to T_4 in autumn, just prior to the start of the normal breeding season, failed to provoke development of neuroendocrine anoestrus in thyroidectomized ewes (Thrun *et al.*, 1997). However it remains to be tested whether thyroidectomized animals remain responsive to thyroid hormones in early summer, when the prevailing photoperiods are inhibitory to reproduction.

2.3 Neuroendocrine control of the oestrous cycle

For a portion of each year seasonally breeding, spontaneously ovulating females exhibit periodic cyclicity of two temporally related events, oestrus and ovulation. These cycles are referred to as 'oestrous cycles' and are maintained only in the presence of stimulatory environmental cues (e.g. photoperiod) and in the absence of pregnancy. The term oestrus describes the period of receptivity of the female towards the sexual advances of the male, whereas ovulation refers to follicular maturation, rupture of the follicle wall and exodus of oocytes from the ovary. These two phenomena are controlled by interrelated hormonal events. For successful fertilisation and conception, oestrus and ovulation must occur within 2-3 days of each other (Asher, 1986).

2.3.1 Hormonal control of oestrus and ovulation in the ewe

The functioning of the ovary is dependent on secretion of gonadotrophins from the anterior pituitary gland. The secretion of these gonadotrophins is in turn regulated by feedback effects (both positive and negative) at the hypothalamic and pituitary levels of steroids from the ovary. This feedback loop is often referred to as the hypothalamo-hypophyseal-ovarian axis. FSH and LH are secreted by the anterior pituitary gland in response to stimulation by hypothalamic GnRH, although inhibin also plays an important role in control of FSH secretion (Clarke *et al.*, 1986; Tortonese and Gomez-Brunet, 1996). The secretion of GnRH by the hypothalamic neurons is intermittent and hence gonadotrophin release is pulsatile in nature. The ovary is therefore exposed to a fluctuating rather than constant concentrations of LH and FSH (Baird, 1984).

LH and FSH bind to receptors on theca cells and granulosa cells respectively of developing preantral follicles (Richards *et al.*, 1976). The binding of LH and FSH initiates the production of androgens and oestradiol respectively, with the thecal androgens being aromatised to oestradiol (Ruckebusch *et al.*, 1991). Antral follicles also have granulosa cell LH receptors (Richards *et al.*, 1976) and produce inhibin (Findlay *et al.*, 1986). FSH stimulates growth and development of the preovulatory

follicles and plays a key role in determining how many follicles are rescued from atresia and are allowed to ovulate (Baird *et al.*, 1991). FSH is greatly influenced by the negative feedback effects of oestradiol and inhibin (Martin *et al.*, 1988). As preantral follicles develop their steroidogenic capacity increases, thus increasing the level of negative feedback to the hypothalamus. However, in the absence of progesterone, oestradiol feedback changes from being inhibitory to being stimulatory on GnRH and LH secretion (Evans *et al.*, 1994). This change from inhibition to stimulation occurs approximately 2-3 days after the decline in circulating progesterone concentration from the previous luteal phase, and results in a sustained increase in oestradiol, secreted from the developing follicles in response to increased LH secretion. This positive feedback ultimately culminates in the GnRH 'surge' and the subsequent LH and FSH surges (Baird and McNeilly, 1981; Evans *et al.*, 1994), which in turn induces ovulation (Martin, 1984). Oestrous behaviour is also triggered by the increase in oestradiol concentration (Karsch *et al.*, 1980).

Caraty *et al.* (1995) have reviewed the pattern of GnRH and LH secretion in the ewe. The initial increment in GnRH secretion precedes or coincides with the onset of the LH surge. The GnRH surge is non-pulsatile in nature and of extended duration, lasting far longer than the preovulatory LH surge. The resulting LH surge is characterised by changes in LH concentration which may be up to one hundred-fold higher than basal levels. This surge system is separate from the 'tonic' background secretion of LH which occurs throughout the oestrous cycle and is important for steroid synthesis and follicular development in the ovary (Baird *et al.*, 1976; Baird and McNeilly, 1981). The peak of the surge occurs about 4-8 hours after the onset, while the decline back to basal levels occurs over the next 10 hours. For the ovine follicle to rupture, blood concentrations of LH need to increase approximately 40-fold for at least 4 hours (McNatty, 1983). Following ovulation, the GnRH pulse generator is 'clamped' by exposure to *negative* feedback effects of ovarian steroids (principally progesterone, although oestradiol also plays a role) and episodic secretion of LH is reduced (Karsch *et al.*, 1977; Hauger *et al.*, 1977; Goodman and Karsch, 1980). Thus oestradiol acts on two functionally independent feedback systems; a negative feedback system controlling tonic LH secretion and a positive feedback system governing the preovulatory LH surge. Oestradiol has also been shown to act directly at the pituitary level, by either *decreasing* pituitary responsiveness to GnRH over a short term

following a bolus injection of oestradiol (Clarke and Cummins, 1984) or by *increasing* pituitary responsiveness to GnRH during the preovulatory surge (Reeves *et al.*, 1971; Kaynard *et al.*, 1988; Clarke; 1995).

Ovulation occurs approximately 24 hours after the onset of the surge. Oestrus, on the other hand, usually begins around the onset of the surge and may last for well over a day, although this varies with breed of sheep. At ovulation, the mature follicle releases its oocyte from the ovary and the theca and granulosa cells proliferate within the ovary, become invaded with blood vessels and undergo structural and biochemical changes. This mass of modified follicular cells is the corpus luteum, which is the major source of progesterone during the oestrous cycle. During the next phase of the oestrous cycle (the luteal phase), the hormonal environment is dominated by increasing concentrations of progesterone secreted from the developing corpus luteum, which suppresses hypothalamic (and therefore pituitary) function via negative feedback (Goodman, 1988).

Under most natural or farmed situations, mating occurs at the first oestrus. In the absence of pregnancy, the corpus luteum is terminated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which is secreted by the uterus (Goding, 1974; Scaramuzzi and Baird, 1976). Destruction of the corpus luteum, or luteolysis, is a key event for control of ovulation and provides the basis for artificial synchronisation techniques. After luteolysis, the negative influence of progesterone is removed, GnRH, LH and FSH are again secreted in response to oestradiol, and the follicular phase begins again to be followed by another ovulation.

2.3.2 Hormonal control of oestrus in the red deer hind

The oestrous cycles of red deer hinds usually first occur around late March each year in New Zealand (Asher *et al.*, 1991), and have a duration of approximately 18 days each (Guinness *et al.*, 1971; Kelly *et al.*, 1985). It has been observed that in several species of deer the first oestrus of the breeding season may be preceeded by one or more 'silent' ovulations (during which overt oestrus is not expressed) associated with the formation of short-lived corpora lutea (fallow: Asher, 1985; white-tailed: Harder and Moorhead, 1980; black-tailed: Thomas and Cowan, 1975), which may serve to synchronise the first overt oestrus within a herd. Silent ovulations may also occur in

red deer (Webster and Barrell, 1985; Asher *et al.*, 1991), although Jopson *et al.* (1990) suggested that the first 6-11 day increase in plasma progesterone observed at the beginning of the breeding season originated from the adrenal gland rather than a short-lived corpus luteum. The first oestrus and mating immediately followed the decline of these plasma progesterone concentrations. Guinness *et al.* (1971) and Adam *et al.* (1985) also found no evidence for silent ovulations in red deer hinds.

These studies have also shown that most red deer hinds conceive at the first ovulation of the breeding season. Hinds which are not mated may continue to cycle for up to 150 days (6-8 oestrous cycles) (Kelly *et al.*, 1985; Guinness *et al.*, 1971; Duckworth, 1992).

Data on episodic secretion of gonadotrophins during the oestrous cycle are few for red deer. The mean amplitude of endogenous LH episodes measured by McLeod *et al.* (1992) in entire female Père David's deer did not differ significantly between stages of oestrous cycle, in contrast to the ewe in which there is a dramatic increase in LH episode amplitude during the luteal phase compared with the follicular phase (Goodman and Karsch, 1980). However in red and fallow deer an increase in pulse frequency and amplitude of LH follows the decline in circulating progesterone concentration caused by luteolysis (Asher and Fisher, 1991). This is associated with increasing blood concentrations of oestradiol and androstenedione (Asher *et al.*, 1986), and eventually culminates in the preovulatory LH surge (Asher *et al.*, 1986; Asher and Fisher, 1991; Asher *et al.*, 1992). In red deer hinds, the onset of oestrus occurred within 8 hours either side of the LH peak, with the mean interval from oestrus onset to ovulation being 28 hours in hinds synchronised by withdrawal of exogenous progesterone treatment (Asher *et al.*, 1992a). In fallow deer, ovulation occurs approximately 24 hours after the onset of oestrus and 16-20 hours after the peak of the preovulatory LH surge (Asher *et al.*, 1992b).

Injections of exogenous GnRH stimulate release of LH (Kelly *et al.*, 1982; Manly *et al.*, 1989), an LH surge and ovulation in red deer hinds (Fisher *et al.*, 1986, 1989; Duckworth and Barrell, 1991), apparently in a manner dependent on the dose of GnRH and the responsiveness of the pituitary gland (Limsirichaikul, 1992). These findings indicate that LH secretion is controlled in a similar manner as in sheep.

Limsirichaikul (1992) demonstrated that exogenous oestradiol will elicit a biphasic response of LH secretion in ovariectomised red deer hinds, as occurs in sheep. Initial suppression (negative feedback) is followed by an increase in LH pulse frequency (positive feedback) culminating in a preovulatory surge-like secretion pattern 17 hours after oestradiol injection. Progesterone had an inhibitory effect on LH secretion, but a 'priming' effect on subsequent LH secretion in response to the stimulatory effect of oestradiol.

At the onset of the breeding season, plasma progesterone concentrations rise from <3 $nmol.l^{-1}$ to peak levels of around 14 $nmol.l^{-1}$ (Adam *et al.*, 1985) during the luteal phase of the oestrous cycle. Progesterone comes mainly from the corpus luteum; however the adrenal gland may also be a significant source (Meikle, 1988; Jopson *et al.*, 1990). Progesterone profiles are similar to those of the ewe, being low at the time of oestrus and ovulation and gradually rising thereafter until luteolysis, when concentrations of progesterone rapidly decline (Adam *et al.*, 1985; Jopson *et al.*, 1990; Duckworth, 1992). However Kelly *et al.* (1985) observed extremely varied progesterone patterns and an absence of sustained low levels about oestrus. It was suggested that this may have been due to the presence of an accessory corpus luteum which had persisted from the previous cycle.

Little information is available on patterns of FSH, oestradiol and prostaglandin secretion in red deer hinds. Cervine FSH cannot be assayed currently, and the methodology for measurement of plasma oestradiol in deer is not well established. However, Kelly *et al.*, (1985) reported varied and comparatively high (up to 148 $pg.ml^{-1}$) concentrations of circulating oestradiol during the oestrous cycle of red deer hinds. In fallow deer, oestradiol may gradually increase up to 25 $pg.ml^{-1}$ at the onset of oestrus (Asher *et al.*, 1986). Elevated concentrations of oestradiol prior to ovulation appear to be necessary for the expression of oestrous behaviour in red deer hinds (Meikle and Fisher, 1990; Duckworth and Barrell, 1991).

Administration of prostaglandin to cycling red deer hinds is known to bring about luteolysis (Fennessy *et al.*, 1986; 1994), associated with a rapid decline in plasma progesterone concentrations followed by oestrous behaviour 2-3 days later (Haigh *et al.*, 1988; Asher *et al.*, 1991). Spontaneous luteolysis in the red deer hind is preceded

by an increase in endometrial oxytocin sensitivity; however in pregnant hinds luteolysis may be averted by secretion of an interferon by the conceptus (Bainbridge *et al.*, 1996).

2.3.3 Detection of oestrus and mating in red deer

Detection of spontaneous oestrus in farmed deer has generally proven difficult to achieve because of the limited ability to inspect closely females within a pastoral environment. Direct observation of oestrous behaviour can be unreliable because overt oestrus in female deer tends to be rather passive compared with other livestock and is often terminated at copulation within minutes of its onset (Asher, 1986, Asher *et al.*, 1991). Veltman (1985) identified four stereotypical phases of the courtship sequence and copulation in red deer: (a) an olfactory inspection phase by the stag, which was often followed by flehmen if the hind urinated in response; (b) repeated bouts of chasing, with the stag running behind with his head low and tongue extended, often punctuating these bouts with roaring; (c) 'low mounting' by the stag (non-ejaculatory posture), often interspersed with mounting and riding of the stag by the hind; and (d) copulation, with the hind adopting a hunched posture and the hind feet of the stag momentarily leaving the ground. Immediately afterwards, the hind usually squatted and urinated. Oestrus can extend for up to 24 hours (Guiness *et al.*, 1971) and hinds may mate more than once with the same or different males (Loudon and Brinklow, 1992).

Vasectomized stags have been used to enable courtship and mating behaviour to be observed without insemination taking place (Duckworth and Barrell, 1991). The use of a ram mating harness fitted to the stag so as to avoid the need for intensive observation has proved very effective for fallow deer (Asher, 1985, 1986; Asher *et al.*, 1986), but its effectiveness for oestrous detection in red deer is variable. While Guinness *et al.* (1971) and Haigh *et al.* (1988) achieved successful marking of red deer hinds with the device, Duckworth and Barrell (1988) observed no relationship between marking of hinds and the estimated date of conception. This may be due to the fact that red deer stags appear to have a lower mount-to-serve ratio than fallow deer (Veltman, 1985; Asher, 1986), affording little opportunity for marking, and

because red deer stags frequently wallow in mud, which renders the crayon ineffective (Asher *et al.*, 1991).

2.3.4 Synchronisation of oestrus in red deer

Artificial synchronisation of oestrus in farmed deer is useful for artificial insemination, embryo transfer (Fennessy *et al.*, 1989b) and experimental studies of their reproductive physiology. As with other domestic ruminants, synchronisation can be achieved either by stimulating the activity of the corpus luteum through the administration and withdrawal of progestagens, or by administering a luteolysin to shorten the luteal phase of the cycle (Asher *et al.*, 1991).

CIDR devices and progestagen sponges

The oestrous cycle can be artificially synchronised in both red deer and wapiti females using a 12-15 day progesterone treatment delivered by silastic rubber Controlled Internal Drug Releasing devices (CIDRs), which are inserted into the vagina of the hind (Fisher and Fennessy, 1985). Peripheral plasma progesterone profiles of red deer receiving single CIDR devices are comparable to those observed during the oestrous cycle for the first six days of insertion (6-10 $nmol.l^{-1}$), but thereafter levels may decline to less than 3 $nmol.l^{-1}$ by day 14 (Jopson *et al.*, 1990). To overcome this decline in progesterone concentrations, two CIDRs have been used together (Duckworth and Barrell, 1991) or in some cases the CIDR has been replaced on day 9 (Fennessy *et al.*, 1990), however these techniques do not appear to improve the success rates of artificial insemination programmes (Bowen, 1989; Fennessy *et al.*, 1990). Upon withdrawal of the CIDR, plasma progesterone concentrations return to basal levels within about a day (Jopson *et al.*, 1990; Duckworth and Barrell, 1991). Oestrus occurs at about 48-72 hours after CIDR withdrawal, with the LH peak occurring at about 48 hours (Fennessy *et al.*, 1989a).

Progestagen impregnated polyurethane sponges have also been used to synchronise oestrus in red deer (Kelly *et al.*, 1982; Adam *et al.*, 1985; Haigh *et al.*, 1988), though they have largely been superseded by the more effective CIDRs as excessive sponge loss rates have been observed in some cases (Haigh *et al.*, 1988). In both sheep and deer, administration of an oestrogen, LH, FSH, GnRH, or of any compounds which

behave like these hormones (such as pregnant mare serum gonadotrophin, PMSG, which has FSH-like properties) at about the time of progestagen withdrawal will enhance the occurrence and synchrony of oestrus in response to CIDR or progestagen sponge treatment (Barrell, 1985).

Prostaglandins

The ability of prostaglandin administration to synchronise oestrus is dependent on the presence of an active corpus luteum at the time of treatment, which means that hinds must be injected during the luteal phase of the oestrous cycle. This has been achieved in wapiti cows by administering $PGF_{2\alpha}$ at 13-day intervals (Haigh *et al.*, 1984), after which they were successfully inseminated. In a similar experiment involving red deer hinds, the pregnancy success rate to natural mating was only 7.7 % compared with 90.9 % of hinds synchronised with intravaginal sponges (Haigh *et al.*, 1988). However it is likely that this latter experiment was conducted too early in the breeding season for prostaglandins to be effective for synchronising oestrus in non-progesterone-primed hinds. Better results have been reported from matings following double prostaglandin injections later in the breeding season (see Barrell, 1985). Incidence of oestrus in fallow deer hinds was higher following treatment with CIDR devices than with injection of a prostaglandin analogue (96.7 % compared with 41.4 %) (Jabbour *et al.*, 1991). A high dose (750 μg per hind) of the prostaglandin analogue cloprostenol has been shown to be more efficacious in inducing luteolysis than lower doses (100-500 μg per hind) (Fisher *et al.*, 1994).

2.4 Manipulating the breeding season of red deer.

2.4.1 Manipulating photoperiod

Since photoperiod ultimately governs the time of the breeding season by entraining the endogenous rhythm (see Section 2.1.1), housing deer indoors under artificial lighting regimes can be used to manipulate the onset of the breeding season. Although this is not a feasible option for commercially farmed deer in New Zealand, the technique has been used successfully to produce early-born calves under experimental conditions. Webster and Barrell (1985) subjected prepubertal red deer hinds to shortened daily photoperiod (8 hours light per day) for 83 days following the summer solstice, causing calving to be advanced by one month. Red deer stags, when kept on artificial photoperiod such that two full cycles of day length occurred during one calender year, showed two cycles of gonadal activity, antler growth and intake (Simpson *et al.*, 1983), though there was a lag of 3-4 months between the occurrence of these seasonal events and the time they would have been expected to occur relative to the artificial day length cycle.

2.4.2 Effects of lactation and nutrition

It is generally accepted that lactation and undernutrition of hinds prior to and during the rut delays the onset of oestrus, though little work has been done to investigate the effects of these factors in New Zealand (Wilson, 1989). While the plane of nutrition (and hence hind body condition) at the time of mating can be manipulated by good management, any treatment which is to advance the breeding season by more than a month or two must usually overcome the effects of lactational as well as seasonal anoestrus, unless calves from the previous season are early-weaned.

Nutrition

Few studies have investigated the importance of nutrition on reproductive performance of deer (Heydon *et al.*, 1992). A low plane of nutrition can reduce the percentage of adult females which show oestrus (Ortavant *et al.*, 1985). In ewe lambs, plasma FSH concentrations and LH pulse frequency were reduced by restricted feeding (Foster *et al.*, 1989).

Condition score at calving appears to have a large influence on the time until the onset of cycling in dairy cows (Jolly *et al.*, 1995). However Heydon *et al.* (1992) found no affect of level of food intake on the time of onset of oestrous cycles in red deer hinds.

Lactation

Guinness *et al.* (1971) studied the duration of lactational anoestrus (the interval between calving and subsequent oestrus) by spacing out matings so that calving occurred within the following mating season. The shortest interval between calving and oestrus in a lactating hind was 10 weeks, but if the calf was lost at birth so that the hind stopped lactating, this interval was as short as 20 days. The difference may have been a more general effect of body condition however, rather than lactation itself, as most lactating hinds were in relatively poor condition and supplementary feeding appeared to advance the onset of oestrus. Adam *et al.* (1985) reported a delayed onset of ovulation and a still further delayed date of conception in hinds that were not weaned prior to mating, compared with hinds weaned for 5 weeks. Hinds from both groups were in good condition and showed insignificant weight loss during the trial.

Nowak *et al.* (1985) induced early ovarian activity in non-lactating, but not in lactating, red deer hinds by feeding melatonin. Melatonin treatment depressed plasma prolactin levels similarly for both groups compared with lactating and non-lactating controls, indicating that lactational infertility was not primarily due to elevated prolactin levels. In contrast, Adam *et al.* (1986) showed that the breeding season of lactating and non-lactating hinds could be advanced equally (by 5 weeks) with melatonin. Further, there was no significant difference between the mean dates of first ovulation and conception in untreated lactating and non-lactating controls. Heydon *et al.* (1992) noted that lactation only affected the time of onset of oestrus on a restricting low-sward height pasture; under this situation some lactating hinds failed to cycle at all (probably due to low body condition). Differences in fertility of lactating, non-lactating and previously barren hinds in an earlier study could all be explained by live weight at the time of the rut (Hamilton and Blaxter, 1980). Loudon *et al.* (1983) carried out experiments from which they hypothesised that the major determinant of reproductive failure or of the length of lactational infertility under low

planes of nutrition was the increase in suckling frequency of the calf in response to decreased milk yield, and perhaps also the associated increase in prolactin levels.

2.4.3 Hormonal treatments

Considerable research effort in the United Kingdom and New Zealand has been devoted to establishing practical and reliable techniques for advancing calving in farmed deer. There are several potential techniques being evaluated which involve hormonal treatment of hinds and/or stags; the general approach being to utilise techniques based on those already employed successfully for other species of livestock such as sheep and cattle (Barrell, 1985; Wilson, 1989).

Progesterone/PMSG

The use of intravaginal progesterone treatment (usually by CIDRs), followed by parenteral administration of PMSG has proven to be a reasonably reliable technique for advancing ovulation in red deer hinds (Adam *et al.*, 1985; Fisher *et al.*, 1986; Moore and Cowie, 1986; Bringans and Lawrence, 1988), though fertility to induced ovulations has been poor in many cases (Fisher *et al.*, 1986; Moore and Cowie, 1986), possibly due in part to the use of sub-fertile stags prior to the rut. Hinds which do not conceive to the induced oestrus often conceive to the subsequent natural oestrus (Wilson, 1989).

A slightly increased incidence of multiple ovulations has been observed following PMSG administration to red deer hinds, occasionally resulting in conception and birth of twins to artificial insemination (Asher, 1991) or natural mating (Moore and Cowie, 1986; Bringans and Lawrence, 1988).

Progesterone/GnRH

A similar form of treatment to that described above utilises a period of gonadotrophin stimulation with GnRH followed by progesterone withdrawal. This technique has been used to induce ovulation in both lactating and non-lactating hinds prior to the normal breeding season in several experiments (Fisher and Fennessy, 1985; Fisher *et al.*, 1986; Moore and Cowie, 1986; Duckworth and Barrell, 1988, 1991), although the fertility to the induced ovulations has been very low. The requirement for continuous

infusion of GnRH, which precludes this method for on-farm use, has been overcome by using a recently developed GnRH analogue which is more potent and longer lasting than synthetic GnRH and can be administered by a series of five intramuscular injections (Duckworth and Barrell, 1988, 1991).

It is not known why GnRH-induced ovulations should have such low fertility, though one possibility is that the induced ovulations were silent and therefore not accompanied by overt oestrus (Duckworth and Barrell, 1988). However Duckworth and Barrell (1991) showed that treatment with oestradiol in conjunction with GnRH to induce oestrous behaviour still did not overcome the state of infertility imposed by seasonal anoestrus, although the number of matings following this treatment was improved.

In the Père David's hind, more animals ovulate in response to exogenous GnRH in late anoestrus rather than in early anoestrus (McLeod *et al.*, 1991, Brinklow *et al.*, 1992), suggesting that in this species at least GnRH treatment is more efficacious at this time.

Melatonin

Administration of melatonin (either orally, subcutaneously or intramuscularly) in a manner designed to mimic photoperiodic changes during the breeding season has been used experimentally to modify the timing of the onset of the breeding season in red deer hinds (Webster and Barrell, 1985; Adam *et al.*, 1986; Adam, 1992; Wilson, 1992). The degree of advancement achieved by these workers ranged from about 12 days (using melatonin implants) to 5 weeks (using melatonin given daily with feed for 3 months). Fennessy and Fisher (1988) observed that for every 5 days earlier the melatonin treatment of the hinds started, onset of calving was advanced by about 1 day. Although it is technically feasible to manipulate breeding using melatonin so that red deer calve at a time of year suitable to management conditions, critical economic evaluations will be required before the true commercial value of this type of treatment can be assessed (Adam, 1992).

Currently the most practical and cost-effective method for administration of melatonin on commercial deer farms is by subcutaneous implants (Wilson, 1989). Implants are available commercially as 'Regulin' implants, and the effectiveness of these has been

intensively investigated during recent years (Fennessy *et al.*, 1986; Fennessy *et al.*, 1986; Wilson, 1992).

Regulin implants are also used to advance the rut in stags (Fennessy and Fisher, 1988; Wilson, 1992). Several studies have shown that exposure of hinds to stags with advanced rutting behaviour following this treatment is enough to advance the onset of oestrus by 8-10 days or more (Moore and Cowie, 1986; Wilson, 1992).

2.5 Summary

Red deer exhibit a rigidly programmed seasonal breeding cycle that is entrained by photoperiodic cues which are relayed to the hypothalamic GnRH neurosecretory system via the pineal hormone melatonin. Suppression of plasma LH concentration during the non-breeding season involves inhibitory feedback by oestradiol, as well as steroid-independent pathways. These inhibitory pathways presumably involve intermediary neuronal systems as in sheep, but the identity of such neurons has not been described for deer. In the absence of pregnancy, hinds may have up to 8 oestrous cycles during the breeding season, starting in late March under New Zealand conditions. These cycles are normally characterised by low plasma progesterone concentrations and an LH surge during the follicular phase, and increasing plasma progesterone concentrations during the luteal phase followed by a return to basal levels by the next oestrus and ovulation. Oestrus can be synchronised in deer by treatment and withdrawal of progestagens, or by administration of a luteolysin. Hormonal treatments to advance the breeding season include progesterone/PMSG, progesterone/GnRH and melatonin treatment. While all of these techniques will induce ovulation prior to the normal breeding season, only the latter has proved successful in advancing the date of calving with any degree of reliability.

Recent research on birds and mammals has shown that thyroid hormones are necessary for seasonal reproductive transitions, particularly the transition from the breeding to the non-breeding state. This switch will not occur if thyroid hormones are absent around the time of the end of the breeding season. Thyroid hormones are necessary for seasonal decline in gonadal function and antler casting in red deer stags, but the role of thyroid hormones has not yet been investigated in the seasonal breeding cycles of red deer hinds.

This thesis describes a series of experiments designed to elucidate the role of thyroid hormones and neuronal pathways in bringing about the non-breeding state in red deer hinds.

Chapter 3

General materials and methods

3.1 Animals and management

Location of field trials

All the field work for the experiments described in this thesis was conducted at the Deer Unit of the Lincoln University Research Farm (latitude 43° 39'S, longitude 172° 28'E, altitude 10 m above sea level). The annual photoperiodic cycle for this locality ranges from 10 hours of light at the winter solstice to 16.5 hours of light at the summer solstice. Red deer (*Cervus elaphus*) hinds were maintained outdoors on pasture consisting mainly of a ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) sward in 0.5 - 2.2 ha paddocks. Ryegrass silage supplementation normally provided 80-90% of the total energy intake during winter. Any hinds exhibiting excessive live weight loss or general debilitation during winter were preferentially grazed on lush pasture. Unlimited access to water was provided at all times.

All hinds in the experiments described in Chapter 4 and approximately 20% of hinds in later experiments were sourced from stock maintained at the Lincoln University Deer Unit, which were derived from feral red deer hinds captured in the Rakaia River area during 1979 and 1980. Other hinds were purchased from outside the Deer Unit for the purposes of the experiments.

All experimental procedures were carried out in enclosed pens within a deer shed located on the Deer Unit. Animals were mustered usually by 2 people on foot and occasionally using a motorcycle.

Animal models

Ovariectomized red deer hinds bearing slow-release subcutaneous oestradiol 17β-impregnated implants were used to study the role of the thyroid glands in oestradiol-induced (steroid-dependent) seasonal suppression of LH secretion. To study the role

of the thyroid glands in steroid-independent seasonal suppression of LH secretion, ovariectomized hinds without oestradiol replacement were used. Ovary-entire, non-pregnant (except where pregnancy occurred as result of the experiment) hinds were used to study the role of the thyroid glands in the seasonal cessation of oestrous cyclicity.

In all experiments, blockade of thyroid function was achieved by thyroidectomy, with euthyroid hinds or thyroidectomized hinds with exogenous thyroid hormone replacement usually serving as positive controls for seasonal reproductive suppression.

Animal welfare

All procedures used in these studies were approved by the Ethics of Experimentation on Animals Committee of Lincoln University.

3.2 Field data collection

Live weight measurements

Deer were weighed to the nearest 0.5 kg without fasting while restrained in a manual side loading deer crush (M. Keans, Rangiora, NZ) mounted on electronic scales (Tru-Test Model 700, Tru-Test, Auckland, NZ). Accuracy of the scales was checked regularly with a standard weight.

Blood sampling

Infrequent blood sampling was by jugular venepuncture. Conscious deer were manually restrained by one person holding the head of the animal while another collected blood (5-10 ml) via a 20 G hypodermic needle into an evacuated glass tube containing 110 I.U. sodium heparin (Heparin (Mucous) Injection BP, Leo Pharmaceutical Products, Ballerup, Denmark). Blood samples were centrifuged (1500 x *g* at 4°C for 20 minutes) and the plasma decanted into 10 x 75 mm polystyrene tubes (Galanti Group Ltd, Auckland, NZ) which were stored at -20°C within 10 hours after sampling until assayed.

For measurement of pulsatile LH secretion patterns, blood samples were collected via indwelling jugular cannulae. One or 2 days prior to intensive sampling blood sampling, animals were lightly sedated using 0.3-0.5 ml i.v. of 5 % w/v xylazine hydrochloride (Thiazine 50, RWR Veterinary Products Pty Ltd, NSW, Australia). A 14 G x 5 cm i.v. catheter (Surflo i.v. Catheter, Terumo, Japan) was inserted into an external jugular vein. A solution containing 150 I.U. sodium heparin (Heparin (Mucous) Injection BP, Leo Pharmaceutical Products, Ballerup, Denmark) per ml of 0.9 % w/v sterile saline solution was flushed through the catheter to prevent blood clotting. A plastic stopper was fitted to the external end of the catheter, which was removed for collecting blood. Flexible fabric tape, wound around the external end of the catheter, was used to enable it to be sutured to the skin. All hinds were injected s.c. with 500,000 I.U. procaine penicillin, 500,000 I.U. benzathine penicillin and 1250 mg dihydrostreptomycin base (5 ml Penstrep LA, A/S Rosco, Denmark) immediately after cannulation.

During intensive sampling periods, hinds were divided into groups of 6-9 animals and penned in 2 m x 3 m handling areas. Blood samples were collected into 10 ml syringes while hinds were manually restrained, and transferred into heparinized glass tubes which were then treated as described above. Catheters were not flushed with sodium heparin solution between samples.

Pituitary responsiveness to GnRH

Changes in plasma LH concentration following injections of GnRH were used as an index of the responsiveness of the anterior pituitary gland to GnRH. GnRH was dissolved in sterile 0.9% saline solution and stored at -20°C until required. Initially (Chapter 4), hinds received 10 μg GnRH (LH-RH/FSH-RH amide form, NIAMDD, Bethesda, Maryland, USA) intravenously; this was modified in following experiments to 5 μg GnRH (LH-RH acetate salt, Sigma Chemical Co., St Louis, MO, USA) intravenously. The response was calculated as the plasma LH concentration measured at 10 minutes after injection of GnRH minus the plasma LH concentration immediately prior to injection (Suttie *et al.*, 1989; Meikle and Fisher, 1996). In Chapter 7, the plasma LH response was measured at 13 minutes after injection for practical reasons relating to the number of hinds in the experiment to be blood sampled.

Pelage scoring

The proportion of summer coat:winter coat was visually scored on a scale of 1-5 by a single person for all experiments. Pelage scoring was as follows: 1, complete winter coat; 2, predominantly winter coat with some summer fibres showing; 3, approximately half winter and half summer coats; 4, predominantly summer coat with some winter fibres showing; 5, complete summer coat (Curlewis *et al.*, 1988).

Assessment of ovarian status by laparoscopy

Approximately 24 h prior to laparoscopy, hinds were removed from pasture and kept in yards without access to food or water. Laparoscopy was performed under sedation induced with 0.8-1.0 ml i.v. of 5 % w/v xylazine hydrochloride (Thiazine 50, RWR Veterinary Products Pty Ltd, NSW, Australia). Sedated hinds were blindfolded and restrained in a dorsally recumbent position on deer laparoscopy cradles, raised 40° to the horizontal with the head lowered and limbs secured by straps. A 25 x 25 cm area immediately anterior to the udder was shorn and the skin swabbed with 70% ethanol. Local anaesthesia was achieved by a s.c. injection of 1 ml 2% lignocaine hydrochloride (Local, Techvet Laboratories Ltd., Auckland, New Zealand) at each incision site 10 cm on either side of the mid-ventral line, 12 cm anterior to the udder. General anaesthesia was induced with 4 ml i.v. of 5% sodium thiopentone (Pentothal, Techvet Laboratories Ltd, Auckland) if required (approximately 10% of all cases). A small (5 mm) scalpel incision was made through the skin, subcutaneous tissue and peritoneum on the right side, and these layers were punctured with a 6 mm diameter trocar and on the left side. The trocar was removed and the abdomen inflated with food grade carbon dioxide gas through the cannula. The 5 mm diameter laparoscope (Karl Stolz GmbH & Co., Tuttlingen, Germany) was introduced through the cannula and a manipulating probe was inserted through the right side scalpel incision. The ovaries were located, inverted to enable viewing of all surfaces and the presence of corpora lutea recorded. After removal of instruments, incisions were dusted with oxytetracycline (Terramycin Powder, Pfizer Laboratories, Auckland, NZ), and each hind received a s.c. injection of 800,000 I.U. procaine penicillin, 800,000 I.U. benzathine penicillin and 2000 mg dihydrostreptomycin base (8 ml Penstrep LA, A/S Rosco, Denmark). Normally animals were allowed to recover without reversal of sedation before making their way back to pasture, but if a hind was slow in recovering

2 ml i.v. of 10% yohimbine hydrochloride (Reversal, Phoenix Pharmaceutical Distributors Ltd, Auckland, NZ) was administered intravenously to speed recovery.

Pregnancy diagnosis

Hinds were pregnancy diagnosed between 44 days and 6 months after mating while restrained in the deer crush using a trans-rectal real-time ultrasonagraphic transducer (Aloka Echo Camera, model SSD-210DXII, Medtel Telectronics LTD, Auckland, NZ) fitted with an 15 mm diameter probe (model USY-658-5) with an operating frequency of 5 MHz. The presence of placentomes or a foetus was taken to be indicative of pregnancy.

Heart rate

Heart rate was measured by auscultation using a stethoscope (Phoenix, Kobayashi Shoji, Tokyo, Japan) while deer stood quietly under manual restrained by one person holding the head of the animal. Data from any hinds which became obviously agitated during this process were discarded.

Oestrus and mating behaviour observation

For observation of mating-related behaviour, a number was painted on both flanks of each hind with white spray paint to enable easy identification. All hinds and the stag were observed in a 0.5 ha paddock from a shed in an adjacent paddock using binoculars, from 15-100 hours following progesterone withdrawal, or from 10-80 hours following progesterone withdrawal if PMSG was used in conjunction with progesterone treatment. A night, temporary electric fencing was used to restrict animals to half of the paddock, which was illuminated with 2 x 500 W floodlights mounted on poles 4 m above ground level. This lighting had no obvious effects on behaviour of the animals. Stags and hinds were observed for typical oestrus and mating behaviour described by Veltman (1985) (see section 2.3.x).

Hinds were deemed to have exhibited overt oestrus if mounted by the stag. Where this occurred, the onset and offset of oestrus for a hind was taken to be the first and last mounting interaction respectively for that hind (whether by mounting other hinds or the stag or by standing to be mounted by other hinds or the stag). The hind was

considered to be mated if the stag thrust forward with feet off the ground and nose pointed upward while mounting the hind.

3.3 Surgery

Approximately 24 h prior to surgery, hinds were removed from pasture and kept in yards without access to food or water. Sedation and recumbency were induced 30 minutes prior to surgery by 2 ml i.m. of 5% xylazine hydrochloride (Thiazine 50, RWR Veterinary Products Ltd, NSW, Australia). Ovariectomy and thyroidectomy were performed aseptically under general anaesthesia induced 10 minutes prior to surgery by 5 ml i.v. of 5% sodium thiopentone (Pentothal, Techvet Laboratories Ltd, Auckland, NZ). If required, a further 2-5 ml of 5% sodium thiopentone was administered i.v. during surgery. Wherever possible, ovariectomy and thyroidectomy were performed on a hind on a single occasion to minimize use of anaesthetics. Hinds received a s.c. injection of 1000,000 I.U. procaine penicillin, 1000,000 I.U. benzathine penicillin and 2500 mg dihydrostreptomycin base (10 ml Penstrep LA, A/S Rosco, Denmark) at the time of surgery. Normally animals were allowed to recover without reversal of anaesthesia and sedation before making their way back to pasture, but in very cold weather or if a hind was slow in recovering, 2 ml i.v. of 10% yohimbine hydrochloride (Reversal, Phoenix Pharmaceutical Distributors Ltd, Auckland, NZ) was administered to speed recovery.

Ovariectomy

Hinds were blindfolded following sedation and induction of general anaesthesia and transferred in a dorsally recumbent position to deer laparoscopy cradles, which were raised 40° to the horizontal with the head lowered and the limbs secured by straps. A 25 x 25 cm patch immediately anterior to the udder was shorn, the skin was swabbed with 30% chlorohexidine digluconate solution (Savlon, ICI New Zealand Ltd., Wellington, NZ) and iodine was applied to the site of incision. A 6 cm midline incision was made through the skin, subcutaneous tissue and peritoneum, extending cranially 7 cm from the udder. The uterus and ovaries were located and exteriorised, all blood vessels supplying or draining the ovary ligated and the ovary removed. Peritoneal muscle layers and skin were sutured with 3 interruptions using synthetic

absorbable suture (Coated Vicryl, Ethicon Ltd., Edinburgh, Scotland), and the incision site dusted with oxytetracycline (Terramycin Powder, Pfizer Laboratories, Auckland, New Zealand).

Thyroidectomy

Hinds were blindfolded following sedation and transferred to operating cradles in a dorsally recumbent position with limbs secured by straps following induction of general anaesthesia. The hair over the larynx and trachea was shorn and the skin swabbed with 30% chlorohexidine digluconate solution (Savlon, ICI New Zealand Ltd., Wellington, New Zealand), and iodine tincture was applied to the site of incision. A 7 cm midline incision was made on the ventral surface of the neck, extending caudally from the larynx. The trachea was exposed by dissection of the subcutaneous tissue. The thyroid glands were located on either side of the trachea, and blunt dissected from the surrounding tissue. If the isthmus was identifiable, this was also removed. All blood vessels supplying or draining the thyroid glands were ligated. The skin (and muscle tissue if required) was then continuously sutured with synthetic absorbable suture (Coated Vicryl, Ethicon Ltd., Edinburgh, Scotland), and the incision site dusted with oxytetracycline (Terramycin Powder, Pfizer Laboratories, Auckland, New Zealand).

Because thyroidectomy inhibited hair growth, a few long-term (> 2 years) thyroidectomized hinds developed very sparse pelage cover which was often associated with excessive live weight loss. During winter, these hinds were preferentially fed on lush pasture and wore woollen covers, tied beneath the abdomen and with holes for the front legs (Plate 3.1).

Placement and removal of subcutaneous implants

For placement of subcutaneous implants, hinds were lightly sedated using 0.3-0.5 ml i.v. of 5 % w/v xylazine hydrochloride (Thiazine 50, RWR Veterinary Products Pty Ltd, NSW, Australia). The site of implantation (25 x 25 mm) was plucked free of hair and the skin swabbed with 30% chlorohexidine digluconate solution (Savlon, ICI New Zealand Ltd., Wellington, New Zealand).

Plate 3.1 Thyroidectomized hind fitted with a woollen cover.

Plate 3.2 Subcutaneous implantation with T_4 tablets in the base of the ear.

Slow release (200 days) silicone rubber oestradiol-impregnated implants (Compudose 200, Elanco Animal Health, Auckland, New Zealand), cut transversely so that each hind received one third of an implant containing 8 mg oestradiol 17β, were implanted s.c. in the ear using a 6 mm diameter trocar (Compudose implanter, Elanco Animal Health, Auckland, New Zealand), and renewed within 150 days. Following induction of local anaesthesia at the incision site by a s.c. injection of 1 ml 2% lignocaine hydrochloride (Local, Techvet Laboratories Ltd., Auckland, New Zealand), implants were removed while hinds were restrained in the deer crush by making a small scalpel incision (5 mm) in the skin at one end of the implant and applying pressure to the other end to force the implant out.

Sodium L-thyroxine (T_4) tablets (Glaxo Laboratories Ltd, Middlesex, England) were implanted subcutaneously using a 4 mm diameter trocar and renewed within 100 days. Initially (Chapter 4) each hind received 2 x 20 mg tablets at the base of the ear (Plate 3.2); this was modified in later experiments to 4 x 25 mg tablets in the side of the neck, 10 cm lateral and 5 cm cranial to the larynx. For T_4 tablet removal, a 10-25 mm scalpel incision was made alongside the implants following sedation and site preparation as for implant placement. Fibrous tissue encapsulating the tablets was dissected free from the surrounding tissue and removed as one piece containing the intact tablets. Where it was not possible to remove all tablets cleanly, care was taken to remove all residual tablet material and the wound was flushed with sterile 0.9% saline. The incision site was closed with 1 or 2 Michelle clips (15 mm, Aesculap, Germany) and dusted with oxytetracycline (Terramycin Powder, Pfizer Laboratories, Auckland, New Zealand).

3.4 Hormone assays

3.4.1 Luteinizing hormone (LH) RIA

Plasma LH concentrations were measured in duplicate 100 µl aliquots by heterologous double antibody radioimmunoassay, similar to that described previously (Scaramuzzi *et al.*, 1970) and validated for cervine LH (Kelly *et al.*, 1982).

Antibodies and antigens

Highly purified antigen for iodination and primary antiserum were gifted by NIDDK. Values are expressed in terms the ovine standard, NIAMDD-LH-S20 (biological activity 1.8-2.8 times that of NIH-LH-S1). Iodinated ovine LH (NIDDK-oLH-I-2, AFP-7071B) was used as tracer, and primary antiserum was NIDDK-anti-oLH-1 (AFP-192279). Goat anti-rabbit gamma globulin (Calbiochem, La Jolla, CA, USA) was used as the secondary antibody to precipitate the anti-ovine LH antigen complex. Standards, which ranged in concentration from 0.25-32 $ng.ml^{-1}$, were prepared in plasma collected from a hind which had been treated two weeks previously with 150 mg i.m. medroxyprogesterone acetate (Promone E, Upjohn Inter-American Corporation, Auckland, NZ) to suppress circulating LH concentrations to undetectable levels (as measured in another laboratory).

Buffer solutions

0.5 M phosphate buffer solution (PB) was prepared by combining 0.5 M $NaH_2PO_4.H_2O$ and 0.5 M Na_2HPO_4 at a ratio of approximately 1:7 v/v, to achieve a pH of 7.4. This was diluted to 0.1 M or 0.05 M as required for the iodination. Iodinated oLH was eluted with 0.05 M PB containing 1.5 % bovine serum albumin (BSA, Fraction V, powder, Sigma Chemical Co, St. Louis, MO, USA) and 0.02% NaN_3 (transfer buffer). 0.01 M phosphate buffered saline solution (PBS, assay buffer) consisted of 0.01 M Na_2HPO_4, and 0.001 M NaH_2PO_4, 0.15 M NaCl, 0.01 % thiomersal. The pH was adjusted to 7.4. Spinning down buffer (pH 7.4) consisted of 0.01 M PB , 0.1 % NaEDTA, 0.3 % flake egg albumin (BDH Chemicals Ltd, Poole, England) and 0.1 % NaN_3.

Radioiodination

Iodination of ovine LH was conducted using a modification of the chloramine T method of Greenwood *et al.* (1963). In a 1.5 ml polypropylene microcentrifuge vial (LabServe Products Ltd, Auckland, NZ) 6 µl ^{125}I (1 mCi in 0.1 M PB, pH 7.4, Amersham International, UK) was added to approximately 5 µg oLH freshly dissolved in 25 µl of 0.1 M PB. Ten µl of chloramine T (BDH Chemicals Ltd, Poole, UK) solution containing chloramine T at a ratio of 1:5 oLH and dissolved approximately 30 seconds previously in 0.1 M PB, was added and mixed gently to oxidize the reaction. After 35 seconds the reaction was stopped by the addition of 50 µl of a 2.4 mg/ml solution of sodium metabisulphite (BDH Chemicals Ltd, Poole, UK), dissolved in 0.1 M PB approximately 30 seconds previously. Immediately after gentle mixing, 100 µl of transfer buffer was added and the mixture pippetted onto a 1 x 25 cm G-75

Sephadex (Sigma Chemical Co, St Louis, MO, USA) gel filtration column (prepared in water, and prewashed with transfer buffer).

Labelled oLH and free iodine were eluted with transfer solution. Fractions (0.5 ml) were collected into 10 x 75 mm polystyrene tubes (Galanti Group Ltd, Auckland, NZ) and a 10 µl aliquot from each fraction was monitored for radioactivity. Peak radioactivity corresponding to iodinated LH usually eluted in fractions 8-13, and these fractions were stored at -20°C for use in the assay within 3 weeks of iodination.

Radioimmunoassay

Each assay included a set of 4 tubes from which the primary antibody was omitted, to enable correction for non-antibody-bound counts in the final precipitate, and 4 tubes which contained only 100 µl of tracer to measure total radioactivity added to each tube. Aliquots of standard, cervine control or sample (100 µl) were added 10 x 75 mm polystyrene tubes followed by 100 µl of assay buffer, 100 µl of primary antibody (diluted 1:350 000 with assay buffer) and 100 µl of tracer (approximately 10 000 c.p.m.) in assay buffer containing 0.275 % normal rabbit serum (Rapid Rabbits, Leeston, NZ)). After vortex mixing, tubes were incubated at room temperature for at least 20 hours before addition of 100 µl of secondary antibody diluted to 1.25% in assay buffer.

Tubes were again vortex mixed and incubated for at least 6 h at room temperature before addition of 1.5 ml spinning down buffer. After centrifuging at 1800 x *g* for 20 minutes at 4°C, the supernatant was decanted and the tubes inverted on absorbent paper to allow the remaining supernatant to drain from the pellet. Radioactivity (c.p.m.) in the precipitate was counted in a gamma counter (CliniGamma LKB Wallac, Turku, Finland). An attached computer was used to calculate unknowns from the standard curve using spline curve fitting method described by Rawlins and Yrjönen (1978).

Validation of the assay

Specificity of the primary antiserum, expressed in terms of cross-reactivity with pituitary hormones other than LH, has been determined by NIDDK to be 5.4 % for ovine FSH, 0.6 % for ovine growth hormone, 0.1 % for bovine thyroid stimulating hormone, and < 0.01 % for ovine prolactin, arginine vasopressin and adrenocorticotrophic hormone.

The sensitivity (95% confidence limit at 0 $ng.ml^{-1}$) averaged 0.4 $ng.ml^{-1}$ over 61 assays. Intra-assay CV averaged 16.1%, 12.3% and 7.1% respectively for plasma pools displacing radiolabelled oLH to approximately 82%, 65% and 38% of the total bound, and inter-assay CV were 23%, 16.7% and 12.5% for the same plasma pools. Serially diluted deer plasma produced a binding curve which was parallel to that of the ovine standard (Figure 3.1).

3.4.2 Thyroid stimulating hormone (TSH) RIA

Plasma TSH concentrations were measured in duplicate 200 µl aliquots by heterologous double antibody radioimmunoassay, similar to that described previously (Fraser *et al.*, 1985).

Antibodies and antigens

Highly purified antigen for iodination and primary antiserum were provided by NIDDK. Values are expressed in terms the bovine standard, bTSH-I-2 (AFP-9074C; biopotency 31 I.U. mg^{-1}). Iodinated bTSH-I-2 was also used as tracer, and primary antiserum was NIDDK-anti-oTSH-I1 (AFP-C33815). Goat anti-rabbit gamma globulin (Calbiochem, La Jolla, CA, USA) was used as the secondary antibody to precipitate the anti-ovine TSH-antigen complex. Standards, which ranged in concentration from 38-10,000 $pg.ml^{-1}$, were prepared in assay buffer. Horse plasma (200 µl) was added to all standard tubes to correct for the effects of cervine sample or control plasma.

Buffer solutions

All buffer solutions used in this assay were identical to those used in the LH RIA, except that 5% polyethylene glycol 6000 in 0.1 M PBS was used to aid precipitation instead of spinning down buffer.

Radioiodination

Iodination of bovine TSH was by the lactoperoxidase method of Thorell and Johansson (1971). In a 1.5 ml polypropylene microcentrifuge vial (LabServe Products Ltd, Auckland, NZ) 6 µl ^{125}I (1 mCi in 0.1 M PB, pH 7.4, Amersham International, UK) was added to approximately 6 µg bTSH freshly dissolved in 40 µl of 0.1 M PB. Twenty µl of lactoperoxidase (Sigma Chemical Co., St Louis, MO, USA) solution containing lactoperoxidase at a ratio of 1:5 bTSH in 0.1 M PB was added. Twenty µl H_2O_2 was added immediately after lactoperoxidase and again after 5 minutes of

gentle mixing. The reaction was stopped by the addition of 20 µl of 0.0001% thiomersal (BDH Chemicals Ltd, Poole, UK) in 0.1 M PB. Immediately after gentle mixing, 100 µl of transfer buffer was added and the mixture was pippetted onto a 1.5 x 8 cm G-25 Sephadex gel filtration column for elution with transfer buffer (PD-10 Column, Pharmacia Biotech AB, Uppsala, Sweden).

Labelled bTSH and free iodine were collected in 0.5 ml fractions into 5 ml polystyrene 10x75 mm tubes (Galanti Group Ltd, Auckland, NZ) and a 10 µl aliquot from each fraction was monitored for radioactivity. Peak radioactivity corresponding to iodinated TSH was eluted in fractions 8-10, and these fractions were then pooled and further purified on a 1 x 25 cm G-75 Sephadex (Sigma Chemical Co, St Louis, MO, USA) gel filtration column (prepared in water, and prewashed with transfer buffer). Eluted fractions (0.5 ml) were collected into polystyrene tubes (as above) and a 10 µl aliquot from each fraction was monitored for radioactivity. Peak TSH elution was in fractions 15-19, and these fractions were stored at -20°C for use in the assay within 3 weeks of iodination.

Radioimmunoassay

Each assay included a set of 4 tubes from which the primary antibody was omitted, to enable correction for non-antibody-bound counts in the final precipitate, and 4 tubes which contained only 100 µl of tracer to measure total radioactivity added to each tube. Aliquots of standard (100 µl), cervine control or sample (200 µl) were added to 10 x 75 mm polystyrene tubes followed by 100 µl of assay buffer (or 200 µl of horse plasma for standard tubes), 200 µl of primary antibody (diluted 1:200 000 with assay buffer) and 100 µl of tracer (approximately 10 000 c.p.m., in assay buffer). After vortex mixing, tubes were incubated at room temperature for at least 24 hours before addition of 200 µl of secondary antibody diluted to 1.25% in assay buffer containing 0.275 % normal rabbit serum (Rapid Rabbits, Leeston, NZ).

Tubes were again vortex mixed and incubated for 1 h at room temperature before addition of 1.0 ml polyethylene glycol and incubation at 4°C for 30 minutes. After centrifuging at 1800 x *g* for 25 minutes at 4°C, the supernatant was decanted and the tubes inverted on absorbent paper to allow the remaining supernatant to drain from the pellet. Radioactivity (c.p.m.) in the precipitate was counted in a gamma counter (CliniGamma LKB Wallac, Turku, Finland). An attached computer was used to calculate unknowns from the standard curve using spline curve fitting method described by Rawlins and Yrjönen (1978).

Validation of the assay

Specificity of the primary antiserum, expressed in terms of cross-reactivity with pituitary hormones other than TSH, has been determined by NIDDK to be 0.1 % for ovine luteinizing hormone and ovine growth hormone, and < 0.01 % for ovine FSH, ovine prolactin, arginine vasopressin and adrenocorticotrophic hormone.

The sensitivity (95% confidence limit at 0 $ng.ml^{-1}$) was 45 $pg.ml^{-1}$. Intra-assay CV was 16.8% and 4.0% respectively for plasma pools displacing radiolabelled bTSH to 92% and 11% of the total bound. All samples were analysed in a single assay. Serially diluted deer plasma produced a binding curve which was parallel to that of the bovine standard (Figure 3.1).

3.4.3 Thyroxine (T_4) and Triiodothyronine (T_3) RIAs

Plasma total T_4 and total T_3 concentrations were measured in duplicate 25 and 100 µl aliquots respectively using commercial solid-phase radioimmunoassay kits (Coat-A-Count Total T4 and Total T3, Diagnostic Products Corporation, Los Angeles, CA, USA). Standards, which were prepared in human serum and ranged in concentration from 12.9-309.0 $nmol.l^{-1}$ and 0.3-9.2 $nmol.l^{-1}$ for the total T_4 and total T_3 assays respectively, were supplied with the assay kits as were all other reagents.

Radioimmunoassay

Each assay included a set of two 10 x 75 mm polystyrene tubes (Galanti Group Ltd, Auckland, NZ) tubes which contained no antibody, to enable correction for non-antibody-bound counts in the final precipitate, and 2 tubes which contained only 100 µl of tracer to measure total radioactivity added to each tube. Aliquots of standard, cervine control or sample were added to 12 x 75 mm antibody-coated polypropylene tubes followed by 1 ml of ^{125}I-T_4 (approximately 46,000 c.p.m.) or ^{125}I-T_3 tracer (approximately 30,000 c.p.m.) containing blocking agents to circulating thyroid hormone-binding proteins. After vortex mixing, tubes were incubated at 37°C in a water bath for 1 hour (T_4) or 2 hours (T_3), before the supernatant was decanted and the tubes inverted on absorbent paper to allow the remaining supernatant to drain from sides of the tubes. Radioactivity (c.p.m.) in the tubes was counted in a gamma counter (CliniGamma LKB Wallac, Turku, Finland). An attached computer was used to calculate unknowns from the standard curve using spline curve fitting method described by Rawlins and Yrjönen (1978).

Validation of the assay

Specificity of the T_4 primary antiserum, expressed in terms of cross-reactivity with hormones other than L-T_4, was reported by the manufacturers to be 64% for D-T_4, 2% for L-T_3 and undetectable for D-T_3, diiodo L-tyrosine and monoiodotyrosine. Specificity of the T_3 primary antiserum, expressed in terms of cross-reactivity with hormones other than L-T_3, was reported by the manufacturers to be 100% for D-T_3, 1.1% for D-T_4, 0.5% for L-T_4, 0.01% for reverse T_3 and undetectable for diiodo L-tyrosine and monoiodotyrosine.

The sensitivity (95% confidence limit at 0 nmol.l^{-1}) was 3.0 nmol.l^{-1} for total T_4 in 1 assay, and 0.04 nmol.l^{-1} for total T_3 in 5 assays. Intra-assay CV was 7.9% for a plasma pool displacing radiolabelled T_4 to 46% of the total bound. Average intra- and inter-assay CV were 10.1% and 10.7% respectively for plasma pools which displaced radiolabelled T_3 to 24% and 62% of the total bound. Serially diluted deer plasma produced a binding curve which was parallel to that of the standard curve in both assays (Figure 3.1).

3.4.4 Progesterone ELISA

Plasma progesterone concentrations were determined in triplicate 50 µl aliquots of plasma extract by ELISA, similar to that which has been described by Elder *et al.* (1987). The assay was performed in 96-well microtitre plates (Falcon 3912 Microtest III, Becton Dickinson Co, Oxnard, CA, USA) and utilised an automatic ELISA processor (Behring ELISA Processor II, Behring, Marburg, Germany) for absorbance reading. Addition of reagents to wells was performed with a 12-channel pipette (Titertek, Eflab, Finland) or an 8-channel repeating pipette (Eppindorf Multipette 4780 with Plus/8 adaptor, Eppindorf, Hamburg, Germany) and plate washing was by spraying wash buffer from a watering can over all plates after first emptying the contents into a sink. Plates were washed in this way three times after each incubation period and then inverted to dry on absorbent paper. All 96 wells were used, since any 'edge effect' was found to be negligible.

Antibodies and antigens

The primary antibody was progesterone monoclonal mouse antiserum (3D10 DEC in-house preparation), and the secondary antibody was horseradish peroxide-conjugated sheep anti-mouse Ig antiserum (Amersham, Auckland, NZ). Progesterone conjugate

solution was prepared by adding 5 µl of progesterone-3-*O*-carboxymethyloxime thyroglobulin conjugate (prepared using the method described by Elder *et al.* (1987), and stored at -20 °C) to 10 ml of 6 M guanidine hydrochloride (Sigma Chemical Co, St Louis, MO, USA). Primary antibody and progesterone conjugate were prepared by and purchased from Dr J.G. Lewis, Steroid Unit, Christchurch Public Hospital. A stock solution (3180 nmol.l^{-1}) of progesterone (Sigma Chemical Co, St Louis, MO, USA) was prepared in ethanol biannually and stored at -20 °C. This stock solution was diluted in assay buffer to make up a series of standards, ranging in concentration from 0.5 to 16 nmol.l^{-1} (for results calculation, these standard concentrations were multiplied by 4 to account for the 4-fold dilution of samples after reconstitution).

Buffer solutions

PBS (assay buffer) consisted of 0.03 M Na_2HPO_4 and 0.15 M NaCl, with 0.1% w/v gelatine, 0.1% v/v Tween 20 (BDH Chemicals Ltd, Poole, UK) and 0.01% w/v thiomersal (pH 7.4). Wash buffer solution contained 0.03 M NaH_2PO_4 and 0.1% v/v Tween 20 (pH 7.4). Substrate buffer solution consisted of 0.05 M Na_2HPO_4 and 0.025 M citric acid (pH 5.0).

Extraction

Redistilled hexane (4 ml) was added to 100 µl of sample or control plasma and vortex mixed for 2 minutes. The plasma was frozen in an ethanol-dry ice bath before decanting off the solvent organic phase containing extracted hormone into 15 x 100 mm glass tubes containing 50 µl of 10% v/v glycerol solution with 0.1% w/v sodium azide. After evaporating the solvent under air in a 37°C water bath, extracted progesterone was reconstituted in 400 µl of assay buffer containing 0.0003% w/v bromocresol green dye by vortex mixing for 10 minutes. Evaporated sample extracts were stored at 4°C for up to 5 days; reconstitution was performed on the day of assay.

ELISA

The ELISA microtitre plates were precoated with 100 µl per well of progesterone conjugate solution at 4°C overnight. The conjugate solution was discarded and the plates washed as described above. Any further active binding sites were then blocked by the addition of 150 µl/well assay buffer for at least 1 hour at room temperature.

Plates were emptied before dispensing (in triplicate) 50 µl of progesterone standards, or reconstituted sample or control extracts into the appropriate well. This was

followed by 50 µl of progesterone monoclonal antiserum at a working dilution of 1:100 in assay buffer per well. After incubation at room temperature for 1 hour the plates were washed and 100 µl peroxide-labelled secondary antibody at a working dilution of 1:1000 in assay buffer was added to each well. The plates were incubated for 30 minutes at room temperature, washed, and 100 µl of freshly prepared substrate solution (containing 40 mg *o*-phenylenediamine dihydrochloride (Sigma Chemical Co, St Louis, MO, USA) and 60 µl of 30% v/v hydrogen peroxide in 100 ml of substrate buffer) added per well to start the colour-forming reaction, which was allowed to proceed in the dark for 10-20 minutes before termination with 100 µl per well of 1.25 M H_2SO_4. Absorbance was read at 492 nm with a reference wavelength of 650 nm, and the progesterone concentration of each sample calculated using a computer spreadsheet (Quattro Pro Version 6.02, Novell Inc, USA) from the equation of the standard curve, which was generated using a computer graph plotting software package (SigmaPlot, Version 5.01 (1994), Jandel Corporation, San Rafael, CA, USA).

Validation of the assay

The sensitivity (95% confidence limit at 0 $nmol.l^{-1}$) averaged 0.86 $nmol.l^{-1}$ (15 separate assays involving 177 ELISA microtitre plates). Intra-assay CV averaged 11.9% and 6.6% respectively for plasma pools displacing progesterone-3-*O*-carboxymethyloxime thyroglobulin conjugate to approximately 91% and 59% of the total bound, and inter-assay CV were 12.8% and 20.7% for the same plasma pools. Cervine plasma serially diluted in assay buffer produced a binding curve which was parallel to that of the progesterone standard (Figure 3.2).

3.4.5 Prolactin ELISA

Plasma prolactin concentrations were determined in triplicate 50 µl aliquots of plasma by ELISA (Lewis *et al.* (1992), using the same techniques and equipment as described for the progesterone ELISA (section 3.3.4).

Antibodies and antigens

Rabbit anti-ovine prolactin primary antiserum was provided by Dr D.F.M. van de Wiele, Research Institute For Animal Husbandry, Schoonoord, Netherlands. Peroxidase-labelled goat anti-rabbit gamma globulin (Tago Immunodiagnostics Inc., Burlingame, CA, USA) was the secondary antibody. Ovine prolactin (NIDDK-oPRL-19, AFP-9221A, biopotency 31 I.U. per mg), in terms of which values are expressed, was provided by NIDDK.

Prolactin conjugate was prepared by the method described by Lewis *et al.* (1992) as follows. Ten mg of NIADDK-oPRL-19 was dissolved in 1 ml distilled water and mixed with a solution containing 10 mg bovine thyroglobulin (Sigma Chemical Co, St Louis, MO, USA) in 1 ml distilled water. Conjugation was achieved by adding N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co, St Louis, MO, USA) (5 mg in 0.5 ml distilled water) to the stirred prolactin and thyroglobulin solution. After 16 hours at room temperature, the mixture was dialysed for 24 hours against distilled water at 4°C and the dialysate freeze dried for storage. When required, this was reconstituted (10 mg in 1 ml distilled water) and a working prolactin conjugate solution prepared by adding 7 µl to 10 ml of 6 M guanidine hydrochloride (Sigma Chemical Co, St Louis, MO, USA). NIADDK-oPRL-19 was also used to prepare a series of standards in horse plasma, ranging in concentration from 8 to 1024 $ng.ml^{-1}$.

Buffer solutions

All buffers were identical to those used in the progesterone assay (Section 3.4.4).

ELISA

The ELISA microtitre plates were precoated with 100 µl per well of prolactin conjugate solution at 4°C overnight. The conjugate solution was discarded and the plates washed as described in Section 3.3.4. Any further active binding sites were then blocked by the addition of 150 µl/well assay buffer for at least 1 hour at room temperature.

Plates were emptied before dispensing (in triplicate) 50 µl of prolactin standard, sample or control plasma into the appropriate well. This was followed by 50 µl of rabbit anti-prolactin antiserum at a working dilution of 1:50,000 in assay buffer per well. After incubation at room temperature for 2 hours the plates were washed and 100 µl peroxide-labelled secondary antibody at a working dilution of 1:2000 in assay buffer was added to each well. The plates were incubated for 2 hours at room temperature, washed, and 100 µl of freshly prepared substrate solution (containing 40 mg *o*-phenylenediamine dihydrochloride (Sigma Chemical Co, St Louis, MO, USA) and 60 µl of 30% v/v hydrogen peroxide in 100 ml of substrate buffer) added per well to start the colour-forming reaction, which was allowed to proceed in the dark for 10-20 minutes before termination with 100 µl per well of 1.25 M H_2SO_4. Absorbance

was read at 492 nm with a reference wavelength of 650 nm, and the progesterone concentration of each sample calculated using a computer spreadsheet (Quattro Pro Version 6.02, Novell Inc, USA) from the equation of the standard curve, which was generated using a computer graph plotting software package (SigmaPlot Version 5.01, Jandel Co, San Rafael, CA, USA).

Validation of the assay

Specificity of the primary antiserum, expressed in terms of cross-reactivity with pituitary hormones other than prolactin, was reported by Lewis *et al.*, (1992) to be 0.7% for ovine growth hormone and negligible for ovine TSH, ovine LH and ovine FSH.

The sensitivity (95% confidence limit at 0 $nmol.l^{-1}$) averaged 7.2 $nmol.l^{-1}$ (3 separate assays involving 21 ELISA microtitre plates), and intra-assay CV averaged 14.1% plasma pools displacing thyroglobulin conjugated prolactin to approximately 27%, 46% and 65% of the total bound. Inter-assay CV were 9.9% for a plasma pool displacing thyroglobulin conjugated prolactin to approximately 27% of the total bound. Serially diluted deer plasma produced a binding curve which was parallel to that of the prolactin standard (Figure 3.2).

3.5 Data analysis and presentation

The terms breeding season and non-breeding season in this thesis indicate the periods of oestrous cyclicity and anoestrus respectively in adult red deer hinds in New Zealand, based on those reported by Meikle and Fisher (1996).

For determination of oestrous cyclicity, significant episodes of progesterone (taken as indicative of a luteal phase) were defined as 2 consecutive sample concentrations exceeding 2 $nmol.l^{-1}$, since this concentration divides typical follicular and luteal phase concentrations in red deer (Jopson *et al.*, 1990; Meikle and Fisher, 1996). Plasma concentrations of progesterone measured while CIDRs were in place were not included when determining oestrous cyclicity.

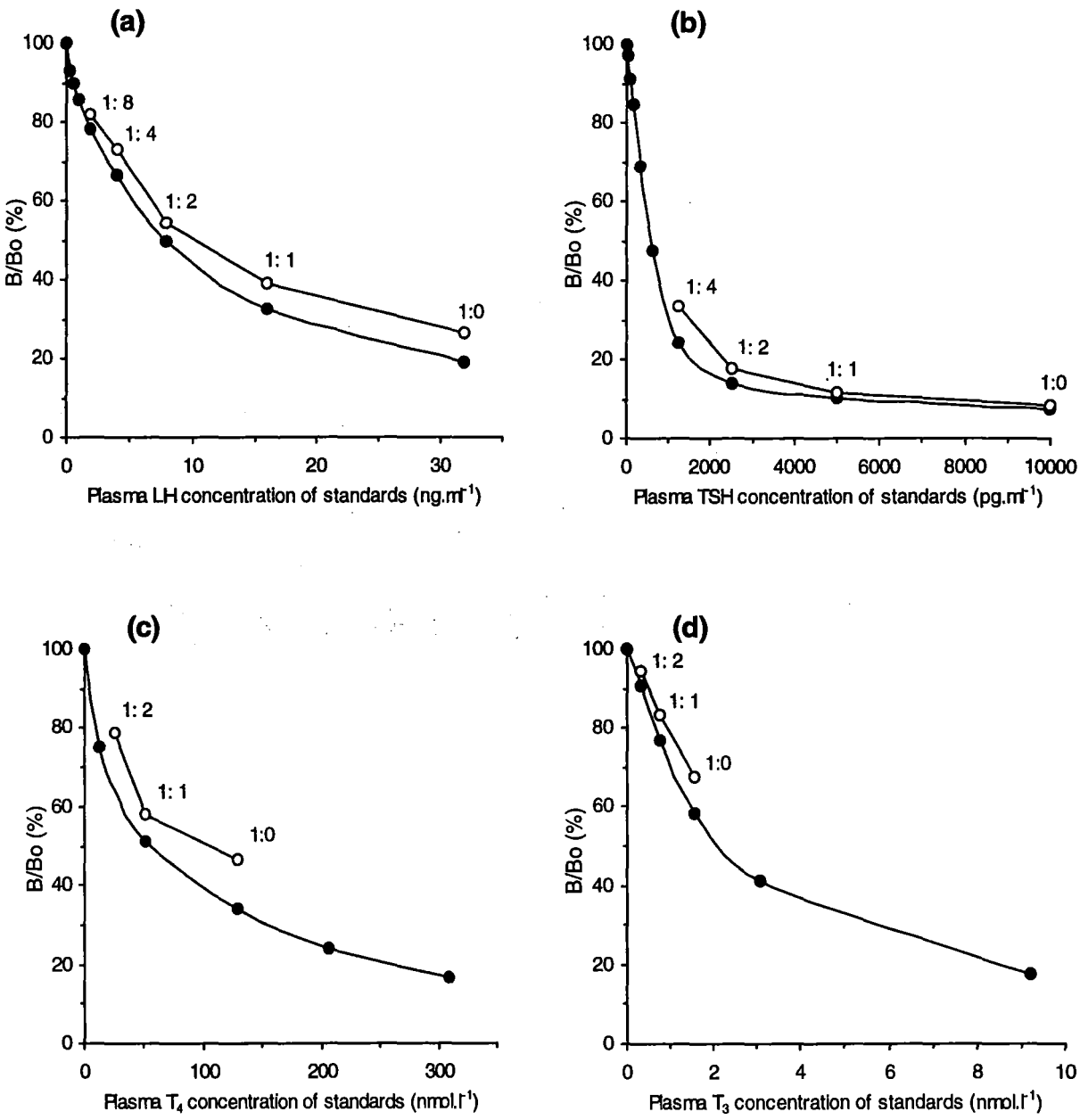

Figure 3.1 RIA binding inhibition curves for cervine plasma samples and (a) ovine LH standards (NIAMDD-oLH-S20), (b) bovine TSH standards (NIADDK-bTSH-I-1), (b) T_3 standards or (d) T_4 standards. Dilution rate of samples in standard diluent is given by each point. ●, standard; o, cervine sample; B, amount of tracer bound in the presence of unlabelled hormone; Bo, amount of tracer bound in the absence of unlabelled hormone.

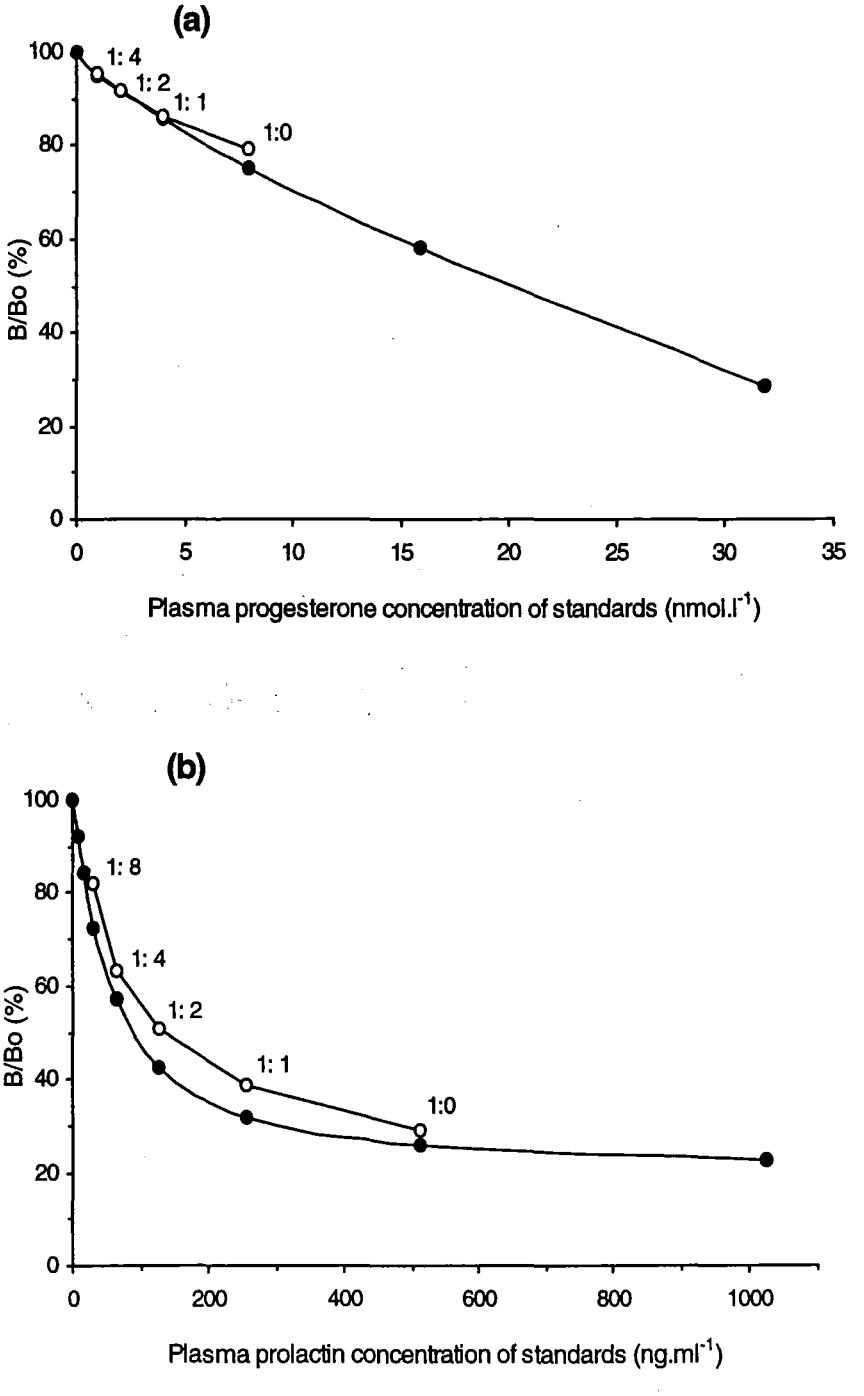

Figure 3.2 ELISA binding inhibition curves for cervine plasma samples and (a) progesterone standards or (b) ovine prolactin standards (NIDDK-oPRL-19). Dilution rate of samples in standard diluent is given by each point. ●, standard; o, cervine sample; B, amount of tracer bound in the presence of unlabelled hormone; Bo, amount of tracer bound in the absence of unlabelled hormone.

In data obtained during intensive sampling periods, LH pulse parameters were defined similarly to the method described by Goodman and Karsch (1980). LH pulse amplitude was calculated as the peak plasma LH concentration minus that of the preceding nadir. A pulse of LH was defined as any increase in plasma concentration where (1) the concentration was elevated relative to pre- and post-nadirs for at least 2 consecutive samples, (2) the pulse peaked within 2 sampling intervals, (3) the increment between peak and nadir concentrations exceeded by at least 2 standard deviations the pre- and post-nadir values and (4) the peak amplitude exceeded the sensitivity of the assay. Frequency of pulses is expressed as the number of pulses per 4 hours; where intensive sampling periods were longer than 4 hours mean pulse frequency was weighted to correct for the difference. To minimize the effect of between-assay variation, all samples obtained during intensive sampling periods from a given animal were measured in a single assay.

Hormone concentrations below the average assay sensitivity were assigned a value equal to the sensitivity for statistical analysis and data presentation. To avoid correlations between means and variance, hormonal data were log-transformed (base 10) when necessary, as described in each chapter. Unless described otherwise, means are presented ± SEM.

In all cases except in Chapter 7, profiles of plasma LH concentration over time or seasons from infrequent samples were analysed by multivariate ANOVA for repeated measurements (with time as the repeated measures factor) using the Systat Version 5.0 statistical software package (Systat Inc, Illinios, USA). Repeated measures analysis, which identified effects of treatment, time and treatment x time interaction, eliminated correlations due to multiple measurements taken over time on the same subject. Where significant treatment x time interactions occurred, data from each sampling date were subjected to Student's *t*-test to identify the times at which the effect occurred. All other statistical analysis including ANOVA and General Linear Model, linear regression, chi-square and paired Student's *t*-test were analysed using the Minitab Version 10.1 statistical software package (Minitab Inc, State College, PA, USA), and described separately for each chapter. Initial data handling and grouping was performed using either of Quattro Pro Version 6.02 (Novell Inc, USA) or Microsoft Excel Version 5.0 (Microsoft Corporation, USA) spreadsheet packages. All graphics were prepared using Microsoft Excel.

Chapter 4

Effects of timing and dose of thyroid hormone replacement in ovariectomized oestradiol-implanted red deer hinds

4.1 Introduction

Seasonal shifts in reproductive activity are manifest at the neuroendocrine level as changes in the pulsatile secretion of LH. In the red deer hind (Duckworth and Barrell, 1992) and ewe (Goodman *et al.*, 1982), an increase in pulsatile LH secretion is associated with the transition into the breeding season in autumn, and a decrease in pulsatile secretion is characteristic of the transition to anoestrus in late winter. This change in pulsatile secretion reflects a seasonal alteration in responsiveness to the negative feedback action of oestradiol (Karsch *et al.*, 1980; Goodman and Karsch, 1981).

Recent observations have led to the concept that the thyroid glands are required for at least one stage of the circannual rhythm of reproduction, the transition from the breeding to the non-breeding state, in many species of birds (Goldsmith and Nicholls, 1984; Follett and Nicholls, 1985) and mammals (Vriend, 1985; Jacquet *et al.*, 1986; Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Shi and Barrell, 1992; Parkinson and Follett, 1994). In the ovariectomized, oestradiol-treated ewe, pulse frequencies of LH (Moenter *et al.*, 1991) and GnRH (Webster *et al.*, 1991b) were maintained at high levels throughout the non-breeding season if the animals had been thyroidectomized during the preceding breeding season. In the red deer stag, thyroidectomy prevented antler casting in spring and the associated decline in testis diameter, testosterone concentration and responsiveness of the pituitary gland and testes to exogenous GnRH (Shi and Barrell, 1992). Treatment with exogenous T_4 overcame the effects of thyroidectomy in ewes (Webster *et al.*, 1991a) and stags (Shi and Barrell, 1994).

There have been no published reports of the effects of thyroidectomy on reproduction in female deer. In the two experiments described in this chapter, ovariectomized

oestradiol-implanted hinds were used to determine if thyroid hormones play a similar role in the red deer hind as has been reported for the ewe, and to characterise the timing of exogenous thyroid hormone treatment required for the seasonal increase in oestradiol negative feedback to occur. The second experiment also tested the hypothesis that a threshold dose-response relationship exists between exogenous T_3 and termination of reproductive activity in red deer hinds.

4.2 Materials and Methods

Animals and Management

The 2 experiments were conducted in 1993 and 1994, using thyroidectomized, ovariectomized, oestradiol-implanted red deer hinds (mean live weight at the beginning of each experiment 96.5 ± 1.7 and 109.1 ± 1.6 kg respectively). Surgery was performed at the start of Experiment 1. For Experiment 2, a further 8 hinds were thyroidectomized and ovariectomized at the start of the experiment in addition to 12 animals used from the previous experiment. Hinds were maintained outdoors on pasture for the duration of both experiments. Blood samples were collected twice weekly in Experiment 1 and weekly in Experiment 2 for measurement of plasma LH concentration. Once every month, the pelage of hinds was visually scored for the relative proportions of summer and winter coats as described in Chapter 3.

Experiment 1

Fifteen hinds received 2 x 20 mg T_4 tablets subcutaneously in the base of the ear either from April to late December (controls), from April to 27 July (breeding season), or from 15 October to late December (non-breeding season) (n = 5). T_4 tablets were replaced in control hinds and hinds receiving T_4 tablets during the breeding season in mid-June and again in control hinds in early September. The dose of T_4 tablets used was based on the results of Shi and Barrell (1992), in which a single 20 mg T_4 tablet restored plasma T_4 concentration in thyroidectomized stags (mean live weight 120 kg) to approximately half that seen in euthyroid control stags for 2-3 months. In November, oestradiol implants were removed for 2 weeks to facilitate measurement of LH in the absence of steroidal inhibition. Plasma T_3 concentration was measured at approximately 3 week intervals.

Experiment 2

Twenty hinds each received a series of three injections of T_3 (sodium salt, Sigma Chemical Co., St Louis, MO, USA) over a 6 day period at one of the following doses: 0, 0.025, 1.0 or 40 mg per hind s.c. (n = 5 per dose) in late October. The timing of this treatment was based on the observation that a single 1.5 mg T_3 injection at this time brings about antler casting in thyroidectomized stags (G.M. Anderson, unpublished). In addition, hinds were treated with the same doses of T_3 in mid-July, to test if high doses of T_3 are able to cause premature termination of reproductive activity. T_3 was injected in 2 ml of a 1:1 emulsion of water and heat-treated soybean oil (a stock solution of T_3 was prepared in water within 4 days of use, with a few drops of 5 M NaOH added to improve solubility). The doses of T_3 were designed to achieve zero, very low, approximately physiological (2-5 $nmol.l^{-1}$) and very high plasma T_3 concentrations, based around that used by Shi (1992), who reported that 1 mg T_3 s.c. restored physiological T_3 concentrations for 2-3 days in thyroidectomized stags, with peak concentrations occurring within 3 hours of injection.

Hinds were blood sampled intensively for 4 hours in July and December, and again in December after the oestradiol implants had been withdrawn for 2 weeks, to characterise the pulsatile secretion of LH. The pituitary LH response to 10 μg i.v. GnRH at 10 minutes post injection was measured weekly for 6 weeks after each series of T_3 injections. Plasma T_3 and TSH concentrations were measured prior to and 3 hours after each series of T_3 injections, and plasma T_3 concentration was measured again 4 days later. Heart rate was measured 3 hours after each series of T_3 injections.

Data analysis

Treatment effects on seasonal profiles of plasma LH concentration over time (including GnRH-induced LH concentration) were analysed by multivariate ANOVA for repeated measurements. Effects of graded doses of T_3 injections on plasma TSH concentration and heart rate in Experiment 2 were analysed by linear regression analysis to identify dose-related responses. All other treatment differences were analysed by one-way ANOVA or paired Student's t-test as appropriate. Plasma LH and TSH concentrations were transformed to their logarithms (base 10) for statistical analysis to normalise the variances.

4.3 Results

Experiment 1

In the absence of T_4 implants, plasma T_3 concentration was generally below the detection limit of the assay. Plasma T_3 concentration during T_4 implantation in all hinds was only slightly elevated (0.2 ± 0.06 nmol.l^{-1}, $P < 0.01$) and was at times below the detection limit of the assay.

There was no effect of treatment on seasonal profiles of plasma LH concentration ($P > 0.05$). Mean plasma LH concentration measured within the normal breeding season was 2.2 ± 0.3 ng.ml^{-1}, and declined during September to 1.0 ± 0.2 ng.ml^{-1} in all hinds ($P < 0.001$). When oestradiol implants were removed in November plasma LH concentration was increased, reaching 5.0 ± 1.0 ng.ml^{-1} after 2 weeks ($P < 0.001$), in all hinds except one control hind (Hind 1-7). In this hind, which had the second highest mean plasma T_3 concentration of all hinds in the experiment (0.52 ± 0.1 ng.ml^{-1}), plasma LH concentration remained undetectable throughout the period of oestradiol implant removal. A further increase in plasma LH concentration was observed 3 days after oestradiol implant replacement (21.9 ± 3.8 ng.ml^{-1} in 14 hinds, $P < 0.001$, and 5.4 ng.ml^{-1} in Hind 1-7), following which mean plasma LH concentrations returned to low (generally < 1.5 ng.ml^{-1}) values.

Pelage score averaged 3.1 ± 0.4 at the beginning of the experiment (April) and declined over the following two months, reaching 1 in all hinds by early July. No further changes were recorded throughout late winter and spring, but growth of the summer coat (as evidenced by pelage scores greater than 1) was significantly delayed (relative to control hinds) in hinds receiving T_4 during the breeding season (4 November $\pm$ 12.5 days and 10 December $\pm$ 5.1 days respectively, $P < 0.05$). Onset of summer coat growth was intermediate for hinds receiving T_4 during the breeding season (27 November $\pm$ 6.0 days). By the end of the experiment (late December), pelage score averaged 4.1 ± 0.3 in all hinds.

Experiment 2

One hind in the group receiving 1.0 mg T_3 died during September (cause of death was not identified). Data from this hind were removed from the experiment.

Plasma T_3 concentrations at the times of T_3 injections are provided in Table 4.1. Mean plasma T_3 concentrations three hours after the third T_3 injection in July and October were 5-10 time higher than anticipated, and there was evidence of T_3 contamination in blood samples and/or animals from the 0 mg T_3 group at this time.

There was no effect of dose of T_3 on seasonal profiles of mean plasma LH concentrations ($P > 0.05$) (Figure 4.1). In all hinds, mean plasma LH concentration measured within the normal breeding season was 2.0 ± 0.3 ng.ml^{-1} and declined during late September to 1.2 ± 0.1 ng.ml^{-1} during the non-breeding season ($P < 0.001$).

Table 4.1 Mean ± SEM plasma T_3 concentrations (nmol.l^{-1}) of hinds in Experiment 2 in response to graded doses of T_3 injections in July and October.

	July			October		
T_3 dose (mg)	Pre T_3	3 hours post T_3	4 days post T_3	Pre T_3	3 hours post T_3	4 days post T_3
0 (n=5)	0.1 ± 0.0	2.3 ± 0.6	0.1 ± 0.0	0.0 ± 0.0	1.7 ± 0.9	0.4 ± 0.2
0.025 (n=5)	0.0 ± 0.0	2.4 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	3.8 ± 1.2	0.4 ± 0.1
1.0 (n=4)	0.2 ± 0.1	26.8 ± 9.0	0.7 ± 0.6	0.0 ± 0.0	27.9 ± 6.7	0.5 ± 0.1
40 (n=5)	0.0 ± 0.0	255.3 ± 22.1	18.7 ± 1.1	0.1 ± 0.0	188.5 ± 32.2	18.2 ± 1.4

Between 11 July and 16 August, there was a marked decline in plasma LH response to an injection of GnRH in all hinds (July: 40.0 ± 3.5 ng.ml^{-1}, August: 7.3 ± 1.4 ng.ml^{-1}; $P < 0.001$). Plasma LH response to GnRH remained low (5.7 ± 0.9 ng.ml^{-1}) throughout November and early December (Figure 4.2). There was no effect of dose of T_3 on plasma LH response to GnRH ($P > 0.05$). Mean plasma LH concentration, pulse frequency and pulse amplitude during intensive sampling periods were also not affected by dose of T_3 ($P > 0.05$). Mean plasma LH concentration and pulse

frequency were lower in December in the presence of oestradiol (1.3 ± 0.1 ng.ml^{-1} and 1.6 ± 0.3 pulses/4 h) than in July (3.3 ± 0.3 ng.ml^{-1} and 3.6 ± 0.3 pulses/4 h) ($P <$ 0.05), but pulse amplitude was not significantly different in December (1.2 ± 0.3 ng.ml^{-1}) and July (2.2 ± 0.5 ng.ml^{-1}) ($P > 0.05$). Mean LH pulse frequency and amplitude during December in the absence of oestradiol implants were similar to those obtained during the breeding season (3.3 ± 0.8 pulses/4 h and 3.2 ± 0.3 ng.ml^{-1}), however in 3, 3, 1 and 2 hinds of groups receiving 0, 0.025, 1.0 and 40 mg T_3 injections respectively, high amplitude pulses (> 5 ng.ml^{-1}) were observed which were usually associated with high mean plasma LH concentrations (> 5 ng.ml^{-1}) and pulse frequencies (≥ 3 pulses/4 h). All other hinds exhibited generally low amplitude pulses (> 2 ng.ml^{-1}) which were usually associated with low mean concentrations (< 1.5 ng.ml^{-1}) and pulse frequencies (≤ 2 pulses/4 h).

Mean plasma TSH concentration prior to T_3 injections in July and October was 1489 ± 45 pg.ml^{-1}. There was an inverse relationship between dose of T_3 injected and plasma TSH concentration in both seasons ($P < 0.001$). Three hours after the third injection, plasma TSH concentrations in groups receiving 0, 0.025, 1.0 and 40 mg T_3 injections averaged 1610 ± 365, 1217 ± 186, 114 ± 32 and 44 ± 10 pg.ml^{-1} respectively. In both seasons, mean heart rate following the third T_3 injection tended to increase with increasing dose of T_3 (48.5 ± 4.1, 59.4 ± 2.3, 68.4 ± 3.9 and 70.0 ± 4.5 beats per minute in groups receiving 0, 0.025, 1.0 and 40 mg T_3 injections), but this correlation did not reach significance ($P < 0.05$).

Growth of the summer coat (as evidenced by pelage scores greater than 1) was significantly advanced (relative to hinds receiving 0 mg T_3) in hinds receiving the highest dose of T_3 (29 November ± 9.4 days and 7 November ± 3.0 days respectively, $P < 0.05$). Onset of summer coat growth was intermediate for hinds receiving 0.025 or 1.0 mg T_3 (22 November ± 7.2 days and 10 November ± 0.0 days, $P > 0.05$). By the end of the experiment (20 January), pelage score averaged 3.8 ± 0.7, 4.2 ± 0.6, 5.0 ± 0.0 and 4.4 ± 0.3 in groups receiving 0, 0.025, 1.0 and 40 mg T_3 injections.

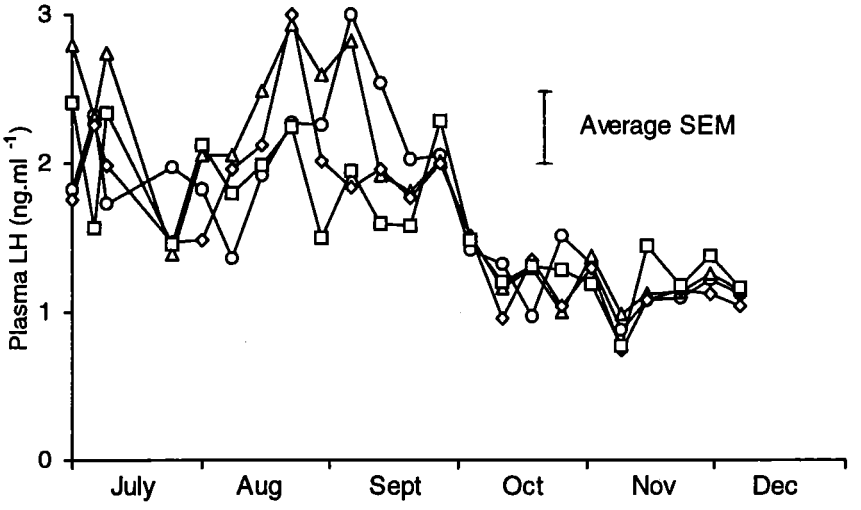

Figure 4.1 Mean plasma LH concentrations in thyroidectomized hinds in Experiment 2 recieving 0 mg (○), 0.025 mg (△), 1.0 mg (◇) and 40 mg (□) injections of T_3 (n = 5) in July and October. Average SEM is shown at the top of the graph.

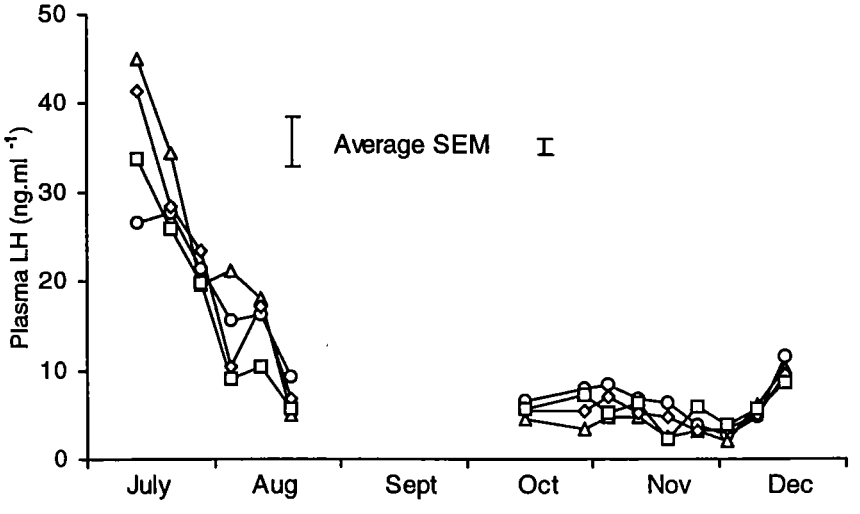

Figure 4.2 Mean plasma LH response to 10 μg GnRH i.v., measured at 10 minutes after injection, in thyroidectomized hinds in Experiment 2 recieving 0 mg (○), 0.025 mg (△), 1.0 mg (◇) and 40 mg (□) injections of T_3 (n = 5) in July and October. Average SEM during the breeding and non-breeding season is shown at the top of the graph.

4.4 Discussion

These results strongly suggest that the ovariectomized oestradiol treated red deer hind does not require the presence of thyroid hormones at the end of the breeding season for the seasonal suppression neuroendocrine reproductive activity to occur, since hinds in Experiment 1 which had undetectable plasma T_3 concentrations from late July onwards showed an identical decline in plasma concentrations of LH to those with detectable plasma T_3 concentrations throughout the experiment. Similarly in Experiment 2, basal and GnRH-induced plasma LH profiles were identical in all hinds regardless of T_3 treatment. These data are in contrast to those of Webster *et al.* (1991a) who showed that the increase in oestradiol negative feedback which is primarily responsible for causing seasonal neuroendocrine suppression in ewes is dependent on the presence of thyroid hormones.

Because problems were experienced with thyroid hormone delivery in both experiments, the results are somewhat equivocal. Due to poor performance of the T_4 tablets in Experiment 1, the validity of hinds which were implanted throughout the experiment as positive controls for seasonal neuroendocrine suppression is doubtful. While the earlier onset of summer pelage growth in control hinds compared to all other hinds in this experiment provides some evidence of the biological effectiveness of the T_4 tablets, the concentration of thyroid hormones required to stimulate this process may be less than that required for activation of reproductive neuroendocrine suppression. It is possible that the uptake of T_4 is influenced by site of implantation, since Shi (1992) showed that the same T_4 tablets produced effective thyroid hormone replacement when implanted subcutaneously in the shoulder of stags. In Experiment 2 thyroidectomized hinds which received 0 mg T_3 injections were intended to serve as negative controls for the effects of thyroid hormones on seasonal neuroendocrine reproductive suppression, but the possiblity of T_3 contamination casts some doubt on the validity of these animals as controls. However, the absence of suppression of plasma TSH concentration and the low mean heart rate in this group after the third T_3 injection, as well as the delayed of onset of summer pelage growth, provide evidence of hypothyroidism. It is therefore likely that the high T_3 concentrations measured in plasma from these hinds represent post-collection contamination of samples rather

than of animals, and they highlight the need for careful handling of thyroid hormone preparations in these experiments.

Despite limitations discussed above, it is interesting to note that in both experiments individual hinds showed marked differences in plasma LH concentrations during periods of oestradiol implant removal in November and December. There is recent evidence that plasma LH concentrations in ovariectomized euthyroid hinds are profoundly suppressed at this time by steroid-independent mechanisms (Meikle and Fisher, 1996); yet this was the case for only 1 hind in Experiment 1 and approximately half of hinds in Experiment 2, with all other hinds exhibiting high mean plasma LH concentrations and pulsatile LH secretion in the absence of oestradiol. In both experiments there was limited evidence of a positive relationship between suppression of mean plasma LH concentration or pulsatile LH secretion in the absence of oestradiol and plasma T_3 concentration, suggesting that the steroid-independent suppression of LH secretion may be dependent on the presence of a threshold concentration of thyroid hormones. The variable response in plasma LH concentration to high doses of T_3 in Experiment 2, a treatment which was shown to be capable of inducing appropriate biological effects in terms suppression of plasma TSH concentration and stimulation of heart rate (Saleh *et al.*, 1997), may indicate that the duration of exposure to T_3 was at the margin of that required for reproductive neuroendocrine suppression.

In conclusion, it was not possible to define the critical period when thyroid hormone replacement would enable the seasonal transition to anoestrus to occur; instead the results suggest that the steroid dependent suppression of LH secretion during the non-breeding season may not require the presence of thyroid hormones in red der hinds. In the following chapter, the role of the thyroid glands in steroid-dependent and steroid-independent suppression of LH secretion is examined in thyroidectomized and euthyroid animals. The timing of the period of neuroendocrine responsiveness to thyroid hormones is re-examined in Chapter 7.

Chapter 5

Effects of thyroidectomy and thyroxine replacement on seasonal reproduction in the red deer hind

5.1 Introduction

Red deer (*Cervus elaphus*) exhibit a pronounced annual breeding cycle that is regulated by photoperiod (Simpson *et al.*, 1983; Webster and Barrell, 1985). As with the majority of temperate ungulate species (Lincoln and Short, 1980), oestrous cyclicity in female hinds begins each year during decreasing photoperiods and in the absence of pregnancy recurrent cycles of approximately 18 days duration occur throughout the winter (Adam *et al.*, 1985; Jopson *et al.*, 1990; Meikle and Fisher, 1996). However recent evidence has shown that the physiological mechanisms underlying this annual rhythm in deer may differ from those acting in sheep, from which our understanding of seasonal reproduction in farm animals is largely derived. For example, red deer hinds exhibit dramatic seasonal fluctuations of plasma LH concentrations (Meikle and Fisher, 1996) and responsiveness of LH secretion to exogenous GnRH (Baker *et al.*, 1995; Meikle and Fisher, 1996) in the absence of gonadal steroids. In gonadectomized sheep the seasonal oscillations in these parameters are considerably smaller (Karsch *et al.*, 1980; Goodman and Karsch, 1981; Pau and Jackson, 1985; Robinson *et al.*, 1985; McLeod *et al.*, 1996; Brewer *et al.*, 1995) and sometimes undetectable (Jenkin *et al.*, 1977; Karsch *et al.*, 1987; Karsch *et al.*, 1993).

Recent observations have led to the concept that the presence of thyroid hormones is required for at least one stage of the circannual rhythm of reproduction, the transition from the breeding to the non-breeding state, in many species of birds (Goldsmith and Nicholls, 1984; Follett and Nicholls, 1985) and mammals (Vriend, 1985; Jacquet *et al.*, 1986; Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Shi and Barrell, 1992; Parkinson and Follett, 1994). In the ovariectomized, oestradiol-treated ewe, pulse frequencies of LH (Moenter *et al.*, 1991) and GnRH (Webster *et al.*, 1991b) were

maintained at high levels throughout the non-breeding season if the animals had been thyroidectomized during the preceding breeding season. In the red deer stag, thyroidectomy prevented antler casting in spring and the associated decline in testis diameter, testosterone concentration and responsiveness of the pituitary gland and testes to exogenous GnRH (Shi and Barrell, 1992). Treatment with exogenous thyroxine (T_4) overcomes the effects of thyroidectomy in ewes (Webster *et al.*, 1991a) and stags (Shi and Barrell, 1994).

There have been no published reports of the effects of thyroidectomy on reproduction in female deer. Because seasonal fluctuations in LH concentrations are much more marked in the ovariectomized red deer hind than in the ovariectomized ewe, the former species presents a unique experimental model for studying the role of thyroid hormones on steroid-independent processes controlling LH concentrations. The objectives for the current experiments were to test if thyroid hormones are required in female red deer for the cessation of oestrous cyclicity in spring, and to investigate whether this requirement applied to steroid-dependent or steroid-independent components of seasonal breeding regulation.

5.2 Materials and Methods

Animals and management

Two experiments were conducted using 25 mature red deer hinds (mean live weight at the start of the experiments 85.8 ± 1.7 kg). Hinds were maintained outdoors at all times of the year.

Experiment 1

To test if the thyroid gland is required for the annual cessation of oestrous cyclicity in red deer, 16 mature hinds were thyroidectomized (THX, $n = 7$), thyroidectomized and treated subcutaneously in the anterior neck region with 4 x 25 mg sodium L-thyroxine tablets (Glaxo Laboratories Ltd, Middlesex, England) in May, August and November (THX+T_4, $n = 4$) or untreated (euthyroid control, $n = 5$) early in the breeding season (early May). Blood samples were collected twice weekly until the end of December for measurement of plasma progesterone concentration. Plasma total tri-

iodothyronine (T_3) was measured approximately monthly. Plasma total T_4 was measured in THX+T_4 hinds immediately before and on four occasions during the 3 months following implantation of sodium L-thyroxine tablets in August to monitor the effectiveness of this treatment. Plasma TSH concentration was measured in June and December. In early November the ovarian status of all hinds was determined by laparoscopy as described in Section 3.2.

Experiment 2

To test if the thyroid gland is required for steroid-dependent and/or steroid-independent inhibition of reproductive activity in the non-breeding season, nine hinds were ovariectomized and thyroidectomized (THX, $n = 4$) or ovariectomized only (control, $n = 5$) in early May. All hinds were treated with slow-release silicone rubber oestradiol-impregnated implants (Compudose 200, Elanco Animal Health, Auckland, New Zealand), cut transversely so that each hind received one third of an implant containing 8 mg oestradiol 17β subcutaneously in the right ear from June until April the following year, except for three periods of approximately 1 month each, beginning on 1 August, 31 October and 9 January when the implants were removed to facilitate measurement of LH concentrations in the absence of steroidal inhibition. Blood samples were collected on a weekly basis for measurement of plasma LH concentration. Once every month, 5 µg of GnRH (LH-RH acetate salt, Sigma Chemical Co., St Louis, MO, USA) in 1 ml of sterile physiological saline solution was administered i.v. immediately following the weekly blood sample and a further sample obtained exactly 10 minutes later to assess the pituitary responsiveness to GnRH (calculated as the plasma LH concentration at 10 minutes minus the concentration prior to injection). In early July (breeding season) and late October (non-breeding season), blood samples were collected at 10 minute intervals for 4 h in the presence of the oestradiol implants to determine episodic LH secretion. Plasma total T_3 concentration was measured approximately monthly.

Data analysis

A significant episode of progesterone, taken as indicative of a luteal phase, was defined as 2 consecutive sample concentrations exceeding 2 nmol.l^{-1}, since this concentration divides typical follicular and luteal phase progesterone concentrations in

red deer (Jopson *et al.*, 1990; Meikle and Fisher, 1996). Mean number of cycles per group during the breeding season and non-breeding season was calculated assuming 5 September as the date of transition from the breeding season to the non-breeding season in New Zealand, based on that reported by Meikle and Fisher (1996).

Effects of treatments on plasma T_3 concentrations over time were analysed by linear regression analysis to identify increases in concentrations. Treatments effects on plasma LH concentrations over time were analysed by multivariate analysis of variance (ANOVA) for repeated measurements (basal LH profiles were subdivided into periods of time corresponding to removal and replacement of oestradiol implants for repeated measures ANOVA). To determine at which point the treatment effects occurred, data from each sampling time were analysed by one-way ANOVA. All other treatment differences were analysed by one-way ANOVA or paired Students *t*-test as appropriate. Hormone concentrations were log transformed (base 10) to equalize variances before statistical analysis. Mean results are presented ± SEM.

5.3 Results

One THX hind (Hind 26) in Experiment 1 and one THX ovariectomized hind in Experiment 2 (Hind 8) died early in the winter. Hind 26 had developed an abscess in one hoof which did not respond to antibiotic treatment and resulted in a decline in body condition. Cause of death was not identified in Hind 8. Both animals were replaced within a few days with a recently THX hind and a long-term (< 1 year) THX ovariectomized hind. Breeding season data from Hind 26 and its replacement were excluded from the analysis.

Experiment 1 and 2: Plasma T_3 and TSH concentrations

Since there was no effect of ovariectomy on mean plasma T_3 concentration, these data are presented collectively for Experiments 1 and 2 (Figure 5.1). Euthyroid control hinds exhibited a seasonal increase in plasma T_3 concentration ($P < 0.001$), peaking around November (early summer). A similar pattern was observed in THX+T_4 hinds in Experiment 1, with plasma T_3 concentration gradually increasing with each administration of T_4 ($P < 0.001$). Mean plasma total T_4 concentration in these hinds was 23.3 ± 4.4 nmol.l^{-1} immediately prior to implantation of T_4 in August, and

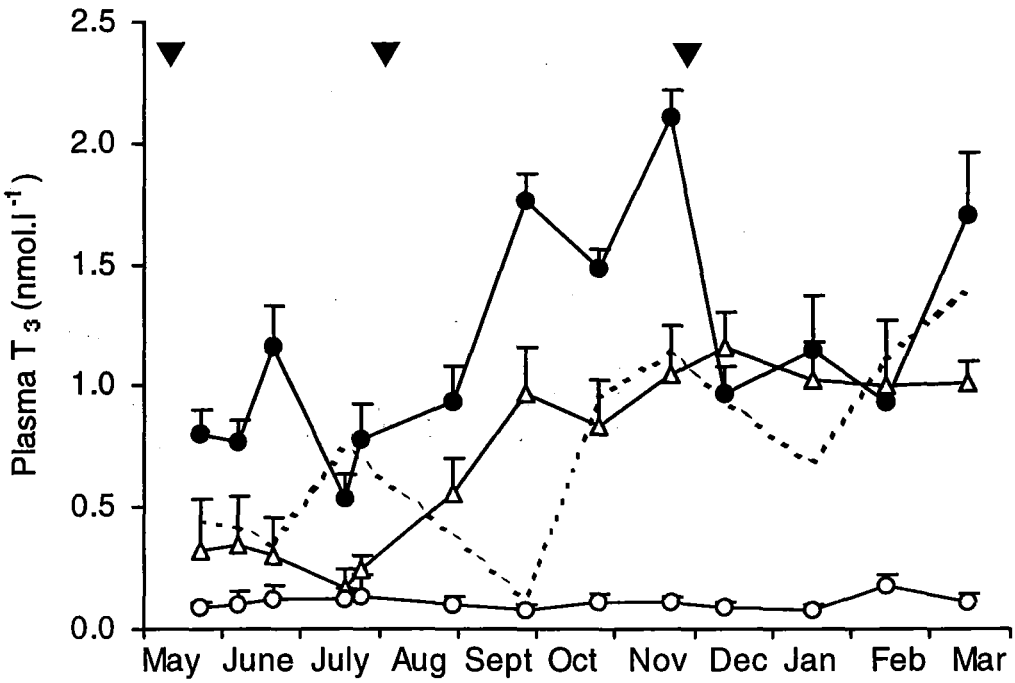

Figure 5.1 Mean (± SEM) plasma concentrations of total T_3 in euthyroid control (●, n = 10), THX+T_4 (△, n = 4) and THX (○, n = 10) hinds in Experiments 1 and 2. Data for Hind 29 are plotted separately (dotted line, see text for details). Arrowheads indicate times of T_4 treatment (4 x 25 mg T_4 tablets subcutaneously) for THX+T_4 hinds.

following this implantation plasma T_4 increased to peak at 82.1 ± 17.5 nmol.l^{-1} one month later. Thereafter concentrations gradually declined, reaching 53.6 ± 14.1 nmol.l^{-1} by mid November. In THX hinds, mean plasma T_3 concentration was generally undetectable throughout the year. One THX hind (Hind 29) had T_3 concentrations in excess of 1.0 nmol.l^{-1}; data from this hind were excluded from the analysis and are plotted separately. Mean plasma TSH concentration was significantly elevated in THX hinds (1633.2 ± 350.3 pg.ml^{-1}) compared with euthyroid hinds (130.3 ± 38.3 pg.ml^{-1}) ($P < 0.001$). In euthyroid and THX hinds in Experiment 1, mean plasma TSH concentrations were similar in June and December ($P > 0.05$), while in THX+T_4 hinds the progressive increase in plasma T_3 concentration was associated with a decline in mean plasma TSH concentration (June: 1353.0 ± 377.9, December: 435.8 ± 318.4; $P < 0.05$).

Experiment 1

Mean numbers of progesterone episodes detected in the breeding and non-breeding seasons are shown in Table 5.1. Between the beginning of the experiment and 5 September (4 months), all hinds exhibited episodes of progesterone indicative of oestrous cycles. THX+T_4 hinds exhibited fewer progesterone episodes than control hinds ($P < 0.05$). Between 5 September and 29 December, there was significantly less evidence of reproductive activity in control and THX+T_4 hinds ($P < 0.001$); although all hinds appeared to have a single oestrous cycle during October. When ovaries of hinds in these two groups were examined by laparoscopy in early November, no corpora lutea or large (> 5 mm diameter) follicles were evident. One animal in the THX group which had high T_3 levels (Hind 29) exhibited a progesterone concentration profile and ovarian status similar to that of control hinds. In marked contrast, THX hinds (excluding Hind 29) had similar numbers of progesterone episodes during the non-breeding season and breeding seasons ($P > 0.05$); reproductive activity continued until the end of December when sampling ceased. Upon laparoscopic examination of ovaries, all THX hinds (excluding Hind 29) had either a single large (> 5 mm diameter) follicle (3/6 hinds) or a single corpus luteum (3/6 hinds) present. Examples of plasma progesterone concentration profiles from hinds representative of each group are shown in Figure 5.2.

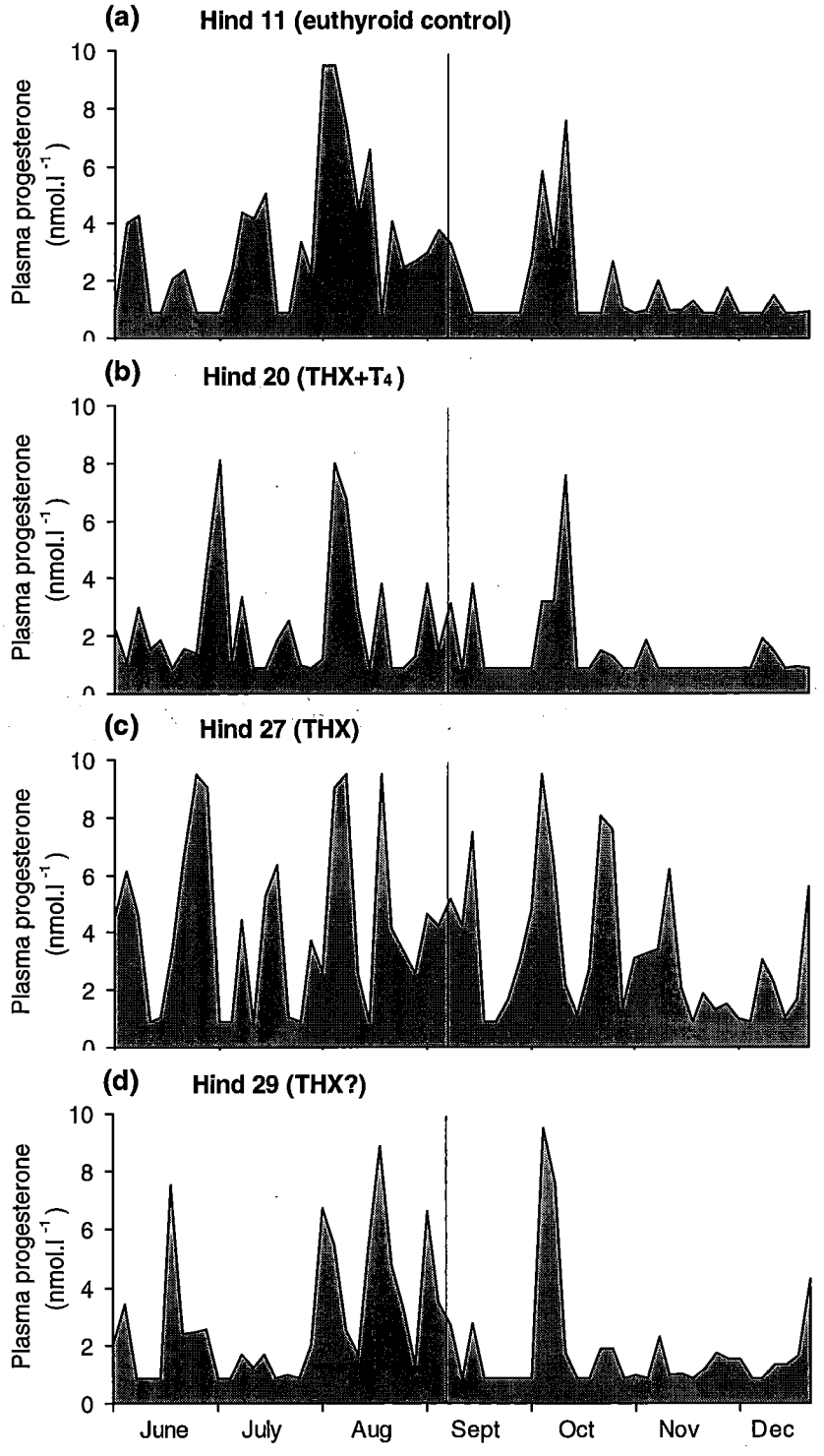

Figure 5.2 Representative individual profiles of plasma progesterone concentration for (a) euthyroid control, (b) THX+T_4 and (c) THX hinds in Experiment 1. Panel (d) contains progesterone concentrations from Hind 29 (THX) which had plasma T_3 concentrations in excess of 1 nmol.l^{-1}. Vertical bars denote the time of the end of the breeding season for red deer hinds in New Zealand.

Table 5.1 Mean numbers of progesterone episodes during the breeding and non-breeding seasons (prior to and after 5 September)

Group	Breeding season	Non-breeding season
Control ($n = 5$)	4.6 ± 0.2^{a}	1.6 ± 0.2^{c}
	(4 - 5)	(1 - 2)
THX+T_4 ($n = 4$)	2.5 ± 0.3^{b}	1.3 ± 0.3^{c}
	(2 - 3)	(1 - 2)
THX ($n = 6$)	4.0 ± 0.5^{ab}	4.3 ± 0.8^{ab}
	(3 - 6)	(2 - 5)

Numbers within brackets refer to ranges. Values not assigned common letters are significantly different ($P < 005$).

Experiment 2

In control and THX hinds, plasma LH concentrations averaged 2.4 ± 0.4 ng.ml^{-1} while oestradiol implants were present during the breeding season and declined ($P < 0.05$) to 0.9 ± 0.1 ng.ml^{-1} between September and March (non-breeding season); there was no effect of thyroidectomy ($P > 0.05$ at all times). In the absence of oestradiol, plasma concentrations of LH were high (2.6 ± 0.3 ng.ml^{-1}) in both control and THX hinds ($P > 0.05$) except during November, when mean concentrations were significantly lower in control than THX hinds (1.3 ± 0.8 *vs* 3.1 ± 0.8 ng.ml^{-1}, $P < 0.001$) (Figure 5.3). There was a seasonal pattern of LH response to GnRH injections in all hinds, with peak LH responses in July (17.3 ± 8.0 ng.ml^{-1}) and in April in the following year (23.2 ± 5.7 ng.ml^{-1}) and nadir LH response in December (4.6 ± 1.6 ng.ml^{-1}). LH response was similar for the two groups at all times ($P > 0.05$) except at the time oestradiol implants were withdrawn in November, when the response was lower for control than THX hinds (4.7 ± 0.4 *vs* 25.9 ± 3.6 ng.ml^{-1}, $P < 0.001$) (Figure 5.4).

Mean number of LH pulses and pulse amplitude in the presence of oestradiol were similar in July (2.6 ± 0.7 *vs* 1.8 ± 0.6 pulses/4 h, $P < 0.05$ and 3.0 ± 2.2 *vs* 1.3 ± 0.4 ng.ml^{-1}, $P < 0.05$) and October (0.8 ± 0.4 *vs* no pulses/4 h, $P < 0.05$ and 0.6 ± 0.03 ng.ml^{-1}) for euthyroid and THX hinds respectively. Between July and October there was a significant decline in mean LH pulse amplitude and number of LH pulses in all hinds ($P < 0.05$; see Figure 5.5 for representative examples).

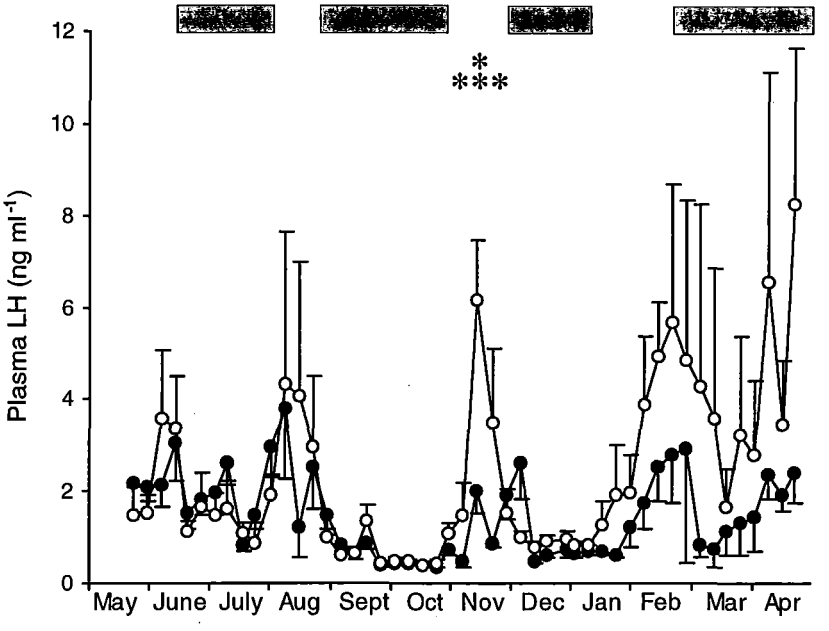

Figure 5.3 Mean (± SEM) plasma concentrations of LH in euthyroid control (●, $n = 5$) and THX (○, $n = 4$) hinds in Experiment 2. Periods of oestradiol treatment are indicated by shaded blocks. Vertically aligned asterisks indicate significant (*, $P < 0.05$; **, $P < 0.001$) differences between means.

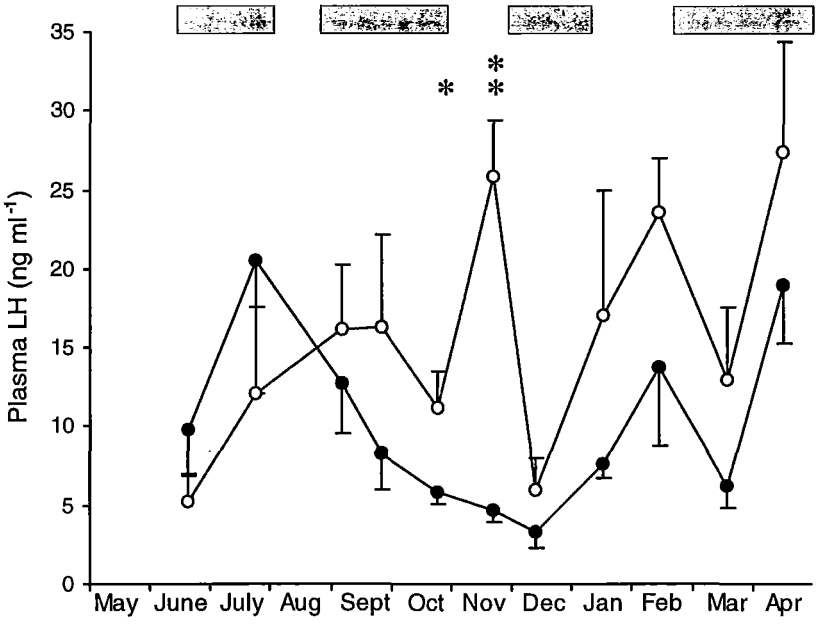

Figure 5.4 Mean (± SEM) plasma LH response (at 10 minutes) to 5 μg GnRH i.v. in euthyroid control (●, $n = 5$) and THX (○, $n = 4$) hinds in Experiment 2. Periods of oestradiol treatment are indicated by shaded blocks. Vertically aligned asterisks indicate significant (*, $P < 0.05$; **, $P < 0.001$) differences between means.

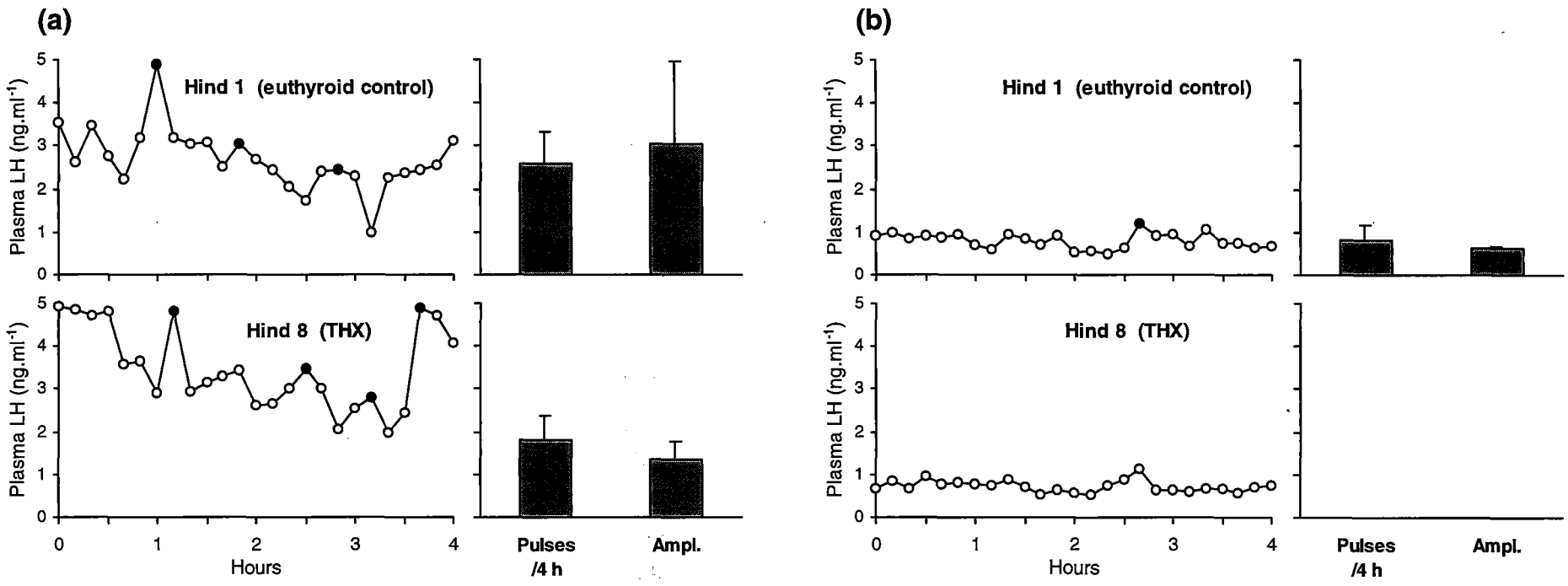

Figure 5.5 Representative individual plasma LH profiles from intensive sampling periods during the breeding season (a) and non-breeding season (b). Profiles from euthyroid control hinds are shown in upper panels and profiles from THX hinds in lower panels. Closed circles denote the peak of each pulse. Histograms show group mean (± SEM) number of pulses and pulse amplitude during the 4 hour period.

5.4 Discussion

The results of Experiment 1 form the first published report to show that the cessation of oestrous cyclicity during the non-breeding season is dependent on the presence of thyroid hormones. This is supported by the findings of Nicholls *et al.* (1988b), which showed that thyroidectomy prevented the cessation of oestrous cyclicity in ewes induced by long photoperiods or by development of refractoriness to equatorial photoperiods, and of other researchers who have used thyroid hormone replacement experiments to demonstrate a requirement for thyroid hormones for the seasonal onset of steroid-dependent suppression of LH concentrations in ewes (Webster *et al.*, 1991a; Thrun *et al.*, 1996) and testicular regression and associated changes in red deer stags (Shi and Barrell, 1994). Experiment 2 extended this finding by showing that in the red deer hind the effects of thyroid hormones are specifically directed at the steroid-independent component of the mechanism regulating seasonal breeding. This is unlike the case of the ewe in which thyroid hormones appear to be required for initiating the negative feedback influence of oestradiol on reproductive neuroendocrine activity during anoestrus (Webster *et al.*, 1991a), i.e. their effect is directed at the steroid-dependent component.

There are relatively few published reports of the effects of thyroidectomy on oestrous cyclicity in any species. Thyroidectomy has been shown not to affect the steroid feedback responses required for generation of the pre-ovulatory LH surge or for suppression of ovulation during the luteal phase in breeding season ewes (Webster *et al.*, 1991a). Nicholls *et al.* (1988b) showed that ewes thyroidectomized just prior to the breeding season initiated cycles at the same time as control animals, but thereafter continued to cycle for more than one year and were still cycling when general debilitation necessitated termination of the experiment. There may be species differences in the requirement of thyroid hormones for oestrous cyclicity, since in two separate studies, thyroidectomy or suppression of thyroid function in female goats has been shown to impair or even abolish normal ovarian function (Walkden-Brown *et al.*, 1996; Reddy *et al.*, 1996). Although the plasma progesterone concentration and ovarian status of THX hinds during the non-breeding season in Experiment 1 indicate this is not the case for red deer, it should be noted that many of the progesterone episodes seen in THX and THX+T_4 hinds were shorter in duration than those of

euthyroid hinds and irregular in occurrence, suggesting a possible role for thyroid gland secretions other than T_4 or its metabolite T_3 for normal ovarian function. Nevertheless, normal mating, conception and pregnancy have been recorded in at least 5 thyroidectomized hinds (see Chapter 8), indicating that if such a role exists it is not an absolute requirement for successful breeding in this species.

Replacement of thyroid hormones to physiological levels in Experiment 1 restored the seasonal pattern of oestrous cyclicity in thyroidectomized hinds to that of euthyroid control hinds. This confirms that the effects of thyroidectomy are due to the absence of thyroid hormones. It could be argued that the cessation of oestrous cycles in the thyroid hormone-replaced hinds was actively driven by increasing concentrations of the exogenously administered T_4, rather than being permitted by the mere presence of thyroid hormones. The premature termination of the breeding season in ewes injected with large doses of T_4 (O'Callaghan *et al.*, 1993) and its extension in ewes with suppressed thyroid secretion (Follett and Potts, 1990) lend some support to this possibility. However recent studies in which thyroid hormone levels have been manipulated approximately within the normal physiological range have failed to alter the timing of the non-breeding season in either ewes (Dahl *et al.*, 1995) or red deer stags (Shi and Barrell, 1994). Since the T_4 implants in Experiment 1 produced very similar circulating T_4 and T_3 concentrations to those seen in euthyroid animals, it is unlikely in the light of the aforementioned studies that the onset of anoestrus was due to the gradual increase in concentrations of exogenously administered thyroid hormones.

An intriguing finding from Experiment 2 is the striking similarity of LH profiles in ovariectomized control and thyroidectomized hinds in the presence of oestradiol. LH concentrations in plasma of thyroidectomized hinds in Experiment 2 were profoundly suppressed by oestradiol during the period of the year corresponding to the non-breeding season of entire, non-pregnant hinds (Figure 5.2; also see Meikle and Fisher, 1996). This is in marked contrast to the growing body of evidence for the ewe (e.g. Moenter *et al.*, 1991; Webster *et al.*, 1991a), in which it has repeatedly been demonstrated that the thyroid gland is required for oestradiol-induced suppression of LH concentrations. It is possible that the low plasma LH concentrations in THX hinds merely reflect profound suppression by oestradiol at the level of the pituitary gland

(see Baker *et al.*, 1995; Meikle and Fisher, 1996), even though pulsatile GnRH secretion remains high as is known to occur in the thyroidectomized ewe (Webster *et al.*, 1991b). However this is unlikely for two reasons. Firstly, exogenous GnRH-induced LH concentrations in thyroidectomized hinds did not decline until October whereas basal LH concentrations declined in August around the time of the onset of the non-breeding season (compare Figures 5.3 and 5.4), so a loss of pituitary responsiveness to GnRH cannot account for the low basal LH concentrations in the intervening period. Secondly, the number of LH pulses detected over the four hour intensive sampling period in late October was reduced in thyroidectomized oestradiol-treated hinds; indicating that the effect was occurring at the level of GnRH pulse generation since GnRH and LH pulses are temporally (and presumably causally) coupled (Clarke and Cummins, 1982; Levine *et al.*, 1982; Karsch *et al.*, 1987; Barrell *et al.*, 1992; Karsch *et al.*, 1993).

The elevated plasma LH concentrations in thyroidectomized hinds relative to control animals which were recorded in the absence of oestradiol during November indicate that there is a major species difference between sheep and deer in the mode of action of thyroid hormones on regulation of seasonal breeding cycles. From this result it can be argued that thyroid hormones are required specifically for the steroid-independent component of seasonal reproductive regulation which transiently but profoundly suppresses plasma LH concentrations around the time of the summer solstice in this species (Meikle and Fisher, 1996).

There was a pronounced seasonal pattern of LH concentrations released in response to exogenous GnRH in control hinds in Experiment 2. During the breeding season and while oestradiol implants were present during the non-breeding season, there was no effect of thyroidectomy on LH response to exogenous GnRH; however in thyroidectomized hinds responsiveness was significantly elevated compared with control animals during the non-breeding season (November) when oestradiol was absent. This suggests that the steroid-independent seasonal variations in pituitary responsiveness to GnRH which have recently been described for red deer hinds (Meikle and Fisher, 1996) may also be dependent on the presence of thyroid hormones. An effect of thyroid hormones in deer at the level of the anterior pituitary gland may represent another difference in the mode of thyroid hormone action

between red deer and sheep, since in the latter species the responsiveness of the anterior pituitary gland to GnRH exhibits very little or no seasonal variation (e.g. Jenkin *et al.* 1977; Brewer *et al.* 1995).

In the thyroidectomized hind, oestradiol appears to retain the ability to suppress reproductive neuroendocrine function during the non-breeding season at both the hypothalamic and anterior pituitary levels. It is possible that the effects of thyroidectomy on steroid-independent pathways were masked by the potent steroid-dependent suppression of LH concentrations while oestradiol was present in Experiment 2, but that in the ovary-entire hinds in Experiment 1 endogenous oestrogen secretion was low enough to allow the steroid-independent effects to be manifest as a continuation of oestrous cyclicity throughout the non-breeding season.

Differences in the degree of gonadotrophin suppression exerted by steroid-independent mechanisms are known to exist between various of species and breeds of birds and mammals (e.g. Gibson *et al.*, 1975; Garcia and Ginther, 1976; Lincoln and Kay, 1979; Lincoln and Short, 1980; McLeod *et al.*, 1996). It has been suggested that these differences may reflect the degree of domestication (Goodman and Karsch, 1981), since seasonal gonadotrophin fluctuations in the absence of gonadal steroids are greater in the Soay ram, a relatively undomesticated breed (Lincoln and Short, 1980), compared with the domestic Suffolk ewe (e.g. Robinson *et al.*, 1985). As red deer have been introduced to the farm environment only within the last 30 years (Fisher and Bryant, 1993), their reproductive neuroendocrine function may be more akin to that of relatively undomesticated animals. The present results indicate that the thyroidectomized, ovariectomized red deer hind offers an unique and interesting animal model for studying the relative roles of steroid-dependent and steroid-independent mechanisms of gonadotrophin suppression.

In conclusion, the results presented here do not fit into the commonly proposed model for regulation of seasonal breeding in the domestic ewe, whereby steroid-dependant regulation is considered to be the major determinant of seasonality (e.g. Karsch *et al.*, 1993). Rather, the overall finding that the transition to anoestrus in red deer hinds can be prevented by thyroidectomy, a treatment which appears to disrupt steroid-independent but not steroid-dependent processes, strongly suggests that the former

mechanisms may be at least as important in regulating seasonal breeding as the latter in this species.

Chapter 6

Pulsatile LH secretion in the ovariectomized, thyroidectomized red deer hind following treatment with dopaminergic, opioidergic and serotonergic agonists and antagonists

6.1 Introduction

Little is known about the neuronal control of seasonal fluctuations in GnRH secretion in deer. Since the vast majority of the GnRH neurons do not possess oestradiol receptors in the ewe (Karsch and Lehmen, 1988; Lehmen and Karsch, 1993) and other species (Shivers *et al.*, 1983; Sullivan *et al.*, 1990; Watson *et al.*, 1992), it is generally considered that the effects of this steroid in suppressing tonic LH secretion during anoestrus must be relayed via at least one type of neural system. Convincing evidence has been obtained over the last 15 years to show that dopaminergic neurons fulfil this role in the ewe (Meyer and Goodman, 1985; Halvern *et al.*, 1991; Viguié *et al.*, 1996) and ram (Tortonese and Lincoln, 1994a; 1994b). This has been most clearly demonstrated by measuring changes in LH secretion in response to injection of receptor agonists and antagonists. In such experiments, dopaminergic D_2 receptor agonists are able to transiently suppress circulating LH concentrations during the breeding season, while in the non-breeding season D_2 receptor antagonists overcome LH suppression (e.g. Tortonese and Lincoln, 1994a). Recently it has been shown that the dopaminergic neurons themselves reflect afferent input from an adrenergic system (Goodman, 1989).

Several reports have indicated that opioid peptides are also able to suppress circulating LH concentrations in ewes during the non-breeding season in the absence of gonadal steroids (Shillo *et al.* 1985; Yang *et al.*; 1988; Schall *et al.*, 1991), while others disagree with this finding (Brooks *et al.* 1986b; Meyer and Goodman, 1986; Whisnant and Goodman, 1988). Steroid-independent seasonal suppression of LH secretion probably also involves a serotonergic neural system, since serotonin reduces (Riggs and Malven, 1974) and cyproheptadine (a serotonergic antagonist) increases

(Meyer and Goodman, 1986) pulsatile LH secretions in castrate sheep. As seen in the previous chapter, the ovariectomized red deer hind presents a unique model for studying such steroid-independent mechanisms of reproductive suppression due to the large seasonal fluctuations in plasma LH concentration exhibited by this species.

The seasonal transition to the state of reproductive quiescence in the ewe (Webster *et al.*, 1991a; Thrun *et al.*, 1996, 1997) and red deer stag (Shi and Barrell, 1994) has been shown within the last decade to be dependent on the presence of thyroid hormones. In the ewe, this role is specifically manifest as a disruption of onset of oestradiol-induced GnRH suppression in animals thyroidectomized during the breeding season (Webster *et al.*, 1991b), so that LH concentrations remain elevated throughout the non-breeding season (Moenter *et al.*, 1991; Webster *et al.*, 1991a; Dahl *et al.*, 1994; 1995; Thrun *et al.*, 1996). Since the mechanism by which thyroid hormones elicit seasonal transitions is not yet understood, the current experiment was set up to test if the thyroid glands are required for the development of inhibitory neuronal pathways at the end of the breeding season in red deer hinds.

In the first experiment, reported here a receptor agonist and antagonist were used to test the hypothesis that the thyroid glands are required for a dopaminergic neural system to suppress LH secretion in ovariectomized oestradiol-treated red deer hinds during anoestrus. The second experiment tested whether the thyroid glands are required for dopaminergic, opioidergic or serotonergic neurons to suppress LH secretion during anoestrus in the absence of gonadal steroids. As it is well established that prolactin secretion is modified by these neurotransmitter pathways (Schillo *et al.*, 1985; Thomas *et al.*, 1988; Johnson *et al.*, 1990, Parrott and Goode, 1992; Lipmam *et al.*, 1992; Aurich *et al.*, 1996), prolactin responses to these drugs were measured in addition to LH as an indicator of their biological effectiveness.

6.2 Materials and Methods

Animals and Management

Ten mature red deer hinds (mean live weight at the beginning of the study 86.7 ± 4.4 kg) were ovariectomized or ovariectomized and thyroidectomized (THX) (n = 5) early in the breeding season (May 1995).

Experiment 1

To test if the thyroid gland is required for a dopaminergic neuronal pathway to mediate oestradiol-induced suppression of LH in red deer during the non-breeding season, hinds were treated in early June 1995 with slow-release silicone rubber implants containing oestradiol 17-β and challenged with a dopaminergic agonist and antagonist during the breeding and non-breeding season as outlined below. Oestradiol implants were removed between 1 August and 25 August as part of another experiment.

In July (mid-breeding season), all hinds received an i.m. vehicle (3.0 ml of a 1:1 mixture of 15% ethanol and 0.1 M tartaric acid) injection followed 4 hours later by either a single i.m. injection of the dopamine-D_2 receptor agonist 2-bromo-α-ergocriptine methanesulfonate (bromocriptine, 0.06 $mg.kg^{-1}$) or the dopamine-D_2 receptor antagonist S(-)-sulpiride (0.60 $mg.kg^{-1}$). The ethanol/tartaric acid mixture was the vehicle for both drugs; both vehicle components have been shown separately not to affect LH concentrations in sheep (Meyer and Goodman, 1985; 1986). Blood samples were taken every 10 minutes for plasma LH analysis from the time of vehicle injection until 5 hours after drug injection. Plasma prolactin concentrations were measured at -240, -120, 0, 40, 80, 160 and 300 minutes relative to drug injections. Four days later hinds received the same treatment but were injected with the other drug, so that all animals received both agonist and antagonist. This procedure was repeated during late October (non-breeding season). Drugs and dosages were selected for their ability to elicit changes in LH and prolactin concentrations and for absence of clinical side effects (McNeilly and Land, 1979; Curlewis *et al.*, 1988; Milne *et al.*, 1990; Ssewannyana and Lincoln, 1990; Tortonese and Lincoln, 1994) in similar

studies in sheep and red deer. All drugs were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Experiment 2

To test if the thyroid gland is required for inhibitory neural pathways to suppress LH concentrations in red deer during the non-breeding season in the absence of gonadal steroids, hinds were treated at least 3 months after oestradiol implant removal with receptor agonists during the breeding season and receptor antagonists during the non-breeding season. In July 1996 all hinds were treated with 0.06 $mg.kg^{-1}$ bromocriptine i.m. and blood sampled for plasma LH and prolactin concentrations as for Experiment 1, except that the vehicle was 3 ml of 15% ethanol. Four days later, hinds were treated with a saline vehicle injection followed 4 hours later by a single injection of 0.12 $mg.kg^{-1}$ serotonin creatine sulphate complex (Sigma Chemical Co., St Louis, MO, USA) i.v. in 1.5 ml of 0.9% saline solution. After a further 3 days, hinds were treated with a saline vehicle injection followed 4 hours later by a single injection of 1 $mg.kg^{-1}$ morphine sulphate i.v. in 3.0 ml of 0.9% saline solution (David Bull Laboratories, Melbourne, Australia). Blood samples were taken at 10 minute intervals from the time of vehicle injection until 4 hours after serotonin or morphine injection for measurement of plasma LH concentrations and for measurement of plasma prolactin concentrations at -240, -120, 0, 10, 20, 40, 80 and 160 minutes relative to morphine injection. Serotonin was administered as described by Thomas *et al.* (1988), who demonstrated a central site of action for peripherally injected serotonin in ewes. Morphine was administered as described in other published experiments (Ebling and Lincoln, 1985; Lincoln *et al.*, 1987), none of which reported any side-effects at this dose.

In early November receptor antagonists were injected following exactly the same protocol and sampling regime as for the agonists during the breeding season. Antagonists for dopamine, serotonin and opioid peptides were sulpiride (0.60 $mg.kg^{-1}$ i.m.), cyproheptadine (3.0 $mg.kg^{-1}$ i.v.) and naloxone (2.5 $mg.kg^{-1}$ i.v.) (Sigma Chemical Co., St Louis, MO, USA). The vehicle for sulpiride was 3 ml 0.1 M tartaric acid, for cyproheptadine 1.5 ml of 50% ethanol and for naloxone 2 ml 0.9% saline solution. The doses of cyproheptadine and naloxone were based on previous studies in sheep (Ebling and Lincoln, 1985; Meyer and Goodman, 1985; 1986; Lincoln *et al.*,

1987; Schall *et al.*, 1991; Kao *et al.*, 1992), none of which reported any adverse behavioural side-effects. However in another study (Le Corre and Chemineau, 1993) respiratory depression in 40% of animals was reported following injection of cyproheptadine at 3.0 $mg.kg^{-1}$ i.v.

Data analysis

One THX hind became debilitated and died during 1995 and was not replaced until June the next year; data from this hind are excluded from the non-breeding season of Experiment 1.

Responses to drug injections were identified using paired Student's *t*-tests to compare average post-drug LH values with average pre-drug values, or in the case of plasma prolactin concentrations, with the concentration immediately before drug injection since a large diurnal variation was observed for this hormone. As thyroidectomy did not affect concentrations of prolactin or the prolactin response to drug challenges, these data were pooled for euthyroid and thyroidectomized hinds. To examine the effects of thyroidectomy and season on mean plasma LH concentration, LH pulse frequency, LH pulse amplitude and prolactin concentration, pre-drug injection data from the 2 intensive sampling dates in each season were pooled prior to analysis of variance or paired *t*-tests as appropriate. Hormone concentrations were transformed to their logarithms (base 10) before statistical analysis. Mean values are presented ± SEM.

6.3 Results

Experiments 1 and 2: Plasma T_3 Concentrations

Plasma concentrations of total T_3 were similar in Experiments 1 and 2. In euthyroid hinds, a seasonal pattern of plasma total T_3 concentration was observed with nadir concentrations of 0.9 ± 0.1 $nmol.l^{-1}$ in winter (June) and peak concentrations of 1.9 ± 0.2 $nmol.l^{-1}$ in late summer ($P < 0.001$). In contrast, mean concentrations of plasma total T_3 in THX hinds were low (< 0.2 $nmol.l^{-1}$) at all times and often undetectable (average concentration over both experiments was 0.1 ± 0.1 $nmol.l^{-1}$).

Experiment 1

Mean plasma prolactin concentrations prior to drug injections in all hinds were lower during July (breeding season) than during October (early non-breeding season) (73.1 ± 17.5 $ng.ml^{-1}$ and 146.9 ± 32.8 $ng.ml^{-1}$ respectively; $P < 0.01$). Sulpiride caused an increase ($P < 0.01$) in plasma prolactin concentrations in both seasons. Bromocriptine decreased plasma prolactin concentrations in October ($P < 0.001$) but the decrease was not significant in July ($P > 0.05$) when concentrations were already low (Figure 6.1).

In contrast to prolactin, mean plasma LH concentration, pulse frequency and amplitude in all hinds were unaffected by bromocriptine or sulpiride in either season ($P > 0.05$) (Figure 6.2). Mean plasma LH concentration and pulse frequency during the period prior to drug injections were lower in October than in July in both euthyroid and THX hinds ($P < 0.05$) but pulse amplitude declined significantly ($P < 0.05$) in euthyroid hinds only (Table 1).

Experiment 2

In contrast to Experiment 1, mean plasma prolactin concentration in all hinds prior to drug injections was not significantly lower during the breeding season than during the non-breeding season (277.8 ± 57.1 $ng.ml^{-1}$ and 228.9 ± 27.2 $ng.ml^{-1}$ respectively; $P > 0.05$). Bromocriptine and morphine both caused a decrease ($P < 0.01$) whereas sulpiride, cyproheptadine and naloxone caused an increase ($P < 0.01$) in plasma prolactin concentration (Figure 6.3). Serotonin did not affect plasma prolactin concentration ($P > 0.05$).

Mean plasma LH concentration, pulse frequency and amplitude in all hinds were unaffected by any of the drug treatments in July or by sulpiride and naloxone in November ($P > 0.05$), except for a small increase in pulse amplitude following sulpiride ($P < 0.05$) (Figure 6.4). Cyproheptadine caused all three LH parameters to decline significantly ($P < 0.05$) in euthyroid and THX hinds in November (representative examples shown in Figure 6.5). Mean plasma LH concentration, pulse frequency and pulse amplitude during the period prior to drug injections were lower in November than July in euthyroid hinds ($P > 0.05$). In THX hinds mean pulse

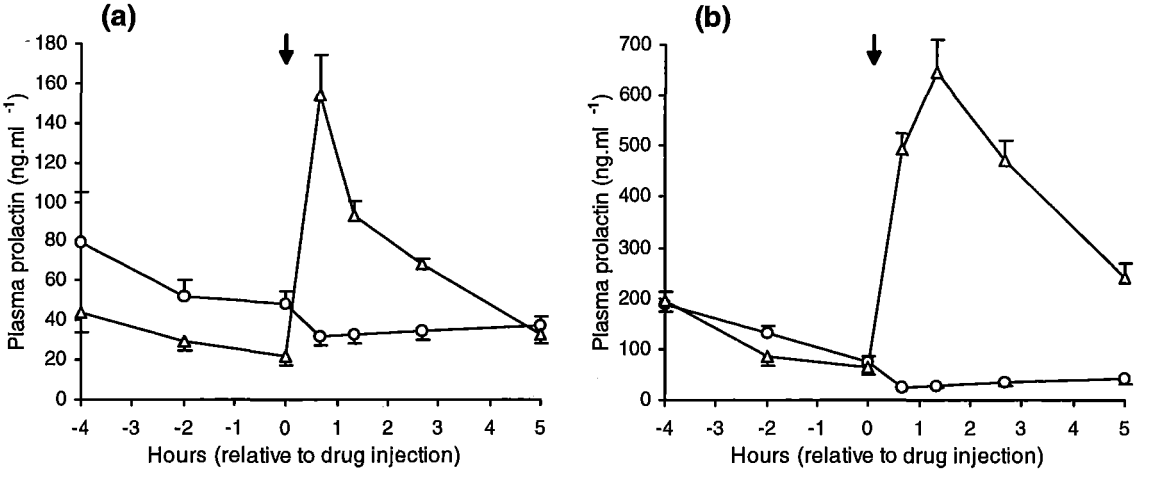

Figure 6.1 Mean (± SEM) concentrations of plasma prolactin in response to bromocriptine (○) or sulpiride (△) during July (breeding season; panel a) or October (non-breeding season; panel b) in ovariectomized estradiol-implanted hinds in Experiment 1. Arrows denote drug injection times. Note that the scale differs for the two graphs. Data are pooled from euthyroid (n = 5) and THX (n = 4) hinds.

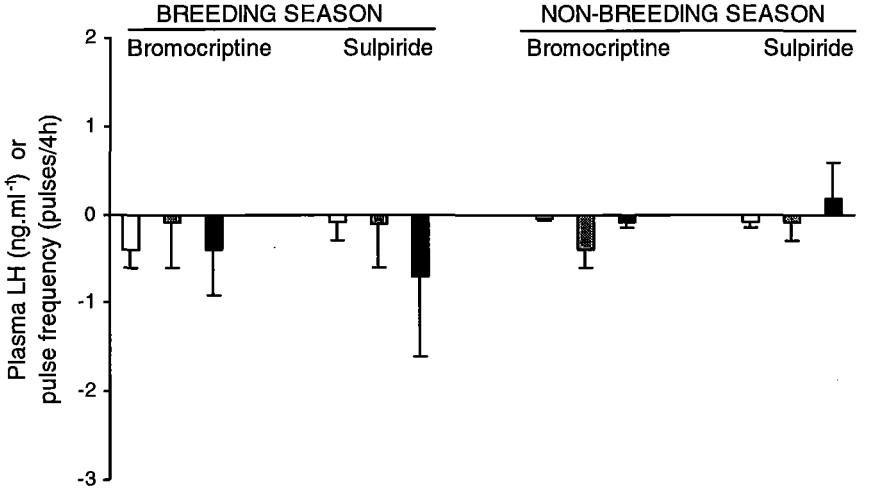

Figure 6.2 Mean (± SEM) change in plasma LH concentrations (open bars), LH pulse frequency (shaded bars) and LH pulse amplitude (solid filled bars) in ovariectomized estradiol-implanted hinds in Experiment 1 following bromocriptine or sulpiride during the breeding season or non-breeding season as indicated at the top of the graph. There were no significant responses ($P > 0.05$) (paired t-tests). Data are pooled from euthyroid (n = 5) and THX (n = 4) hinds.

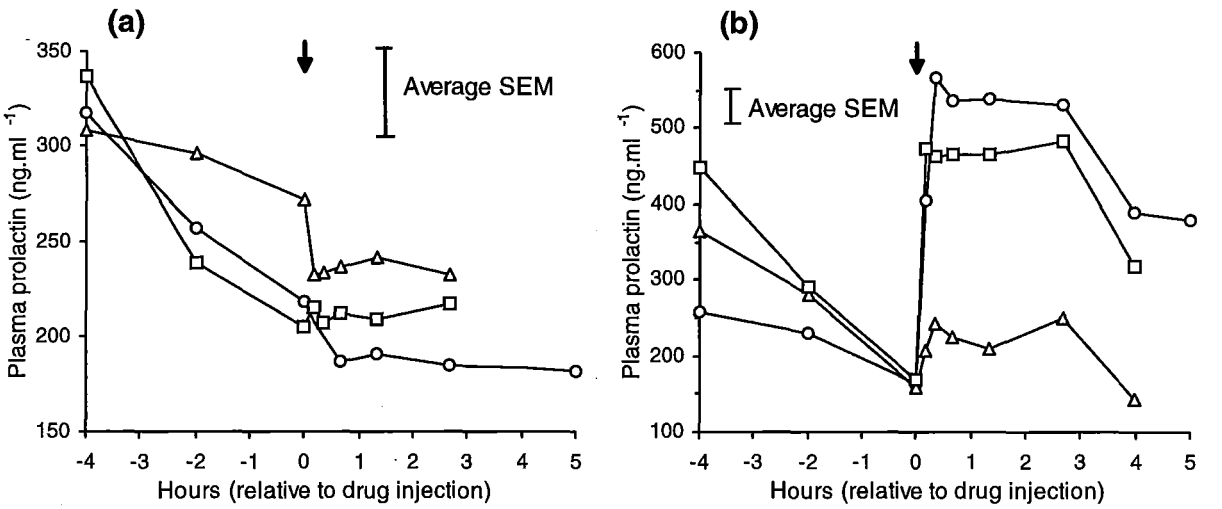

Figure 6.3 Mean concentrations of plasma prolactin in response to bromocriptine (○), morphine (△) and serotonin (□) injections during July (breeding season; panel a) and sulpiride (○), naloxone (△) and cyproheptadine (□) injections during November (non-breeding season; panel b) in ovariectomized hinds in Experiment 2. Arrows denote drug injection times. Note that the scale differs for the two graphs. Data are pooled from euthyroid and THX hinds (n = 5). SEM is shown as an average for clarity.

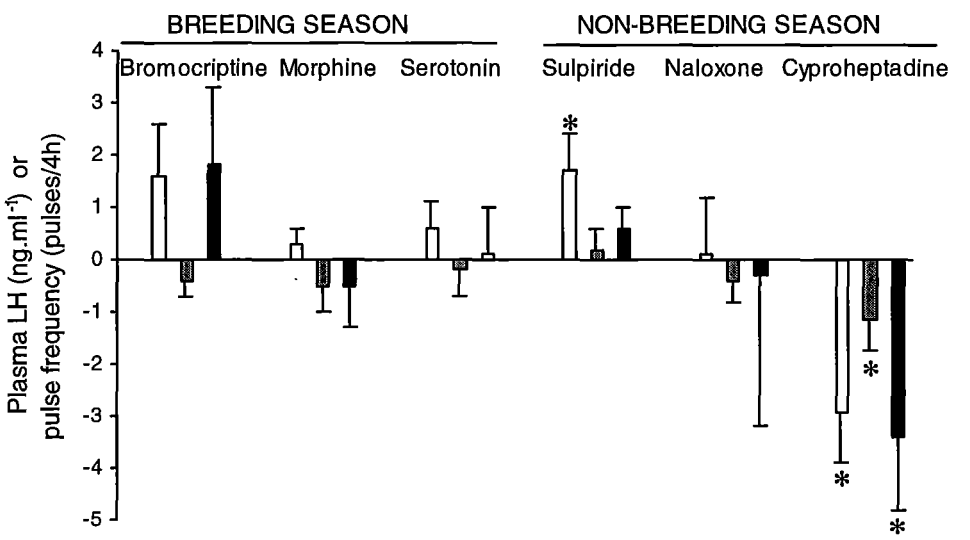

Figure 6.4 Mean (± SEM) change in plasma LH concentrations (open bars), LH pulse frequency (shaded bars) and LH pulse amplitude (solid filled bars) in ovariectomized hinds in Experiment 2 following drug challenges during the breeding season or non-breeding season as indicated at the top of the graph. Asterisks denote significant responses ($P < 0.05$) (paired t-tests). Data are pooled from euthyroid and THX hinds (n = 5).

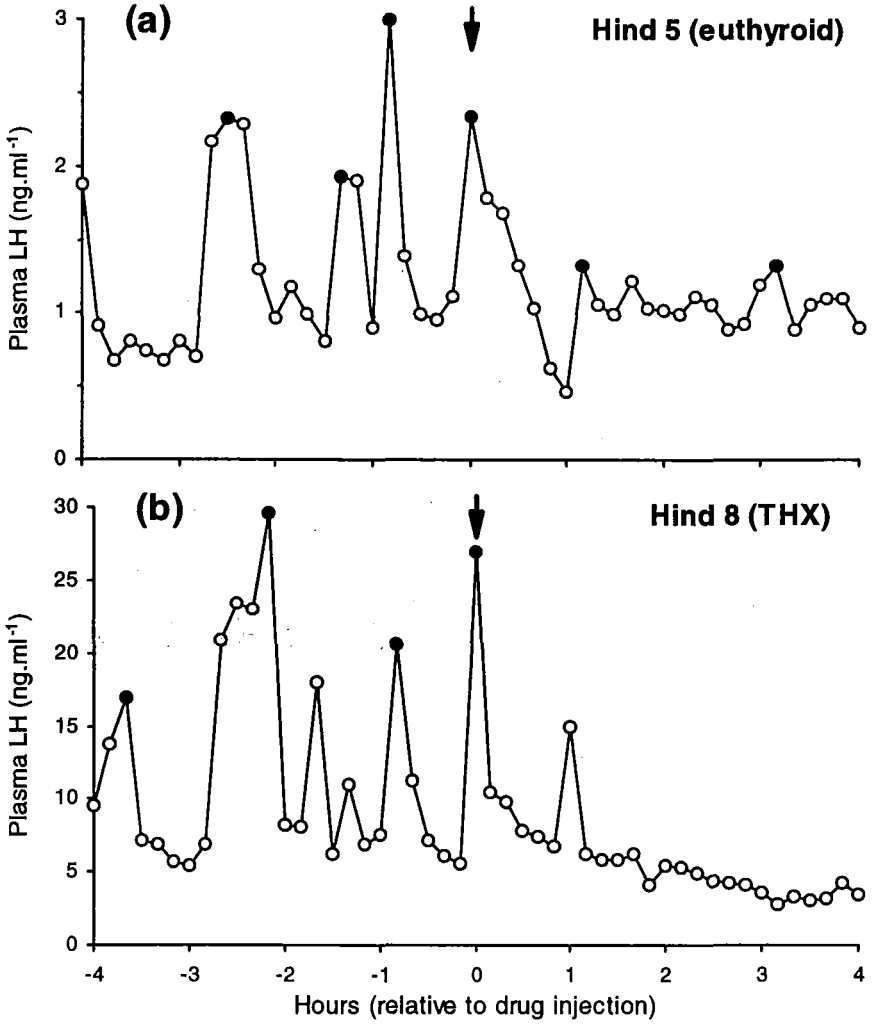

Figure 6.5 Individual plasma LH profiles representative of ovariectomized hinds treated with cyproheptadine during the non-breeding season in Experiment 2. Panel (a) is representative of euthyroid hinds; panel (b) is representative of thyroidectomized hinds. Note that the scale differs for the two graphs. Closed circles denote the peak of each pulse.

frequency did not decline ($P > 0.05$) and mean plasma LH concentration and pulse amplitude increased ($P < 0.05$) in November (Table 6.2).

Table 6.1 LH pulsatility in ovariectomized oestradiol-implanted hinds in Experiment 1.

Pulse parameter	Season	Euthyroid	Thyroidectomized
Concentration	Breeding season	4.2±1.8^a	2.0±0.4^a
	Non-breeding season	0.8±0.1^b	0.7±0.1^b
Number / 4 h	Breeding season	1.9±0.4^a	1.8±0.7^a
	Non-breeding season	0.7±0.3^b	0.8±0.3^b
Amplitude	Breeding season	1.8±0.9^a	0.9±0.3^b
	Non-breeding season	0.6±0.03^b	0.7±0.04^b

Means not assigned common superscript letters within a pulse parameter are significantly different ($P < 0.05$).

Table 6.2 LH pulsatility in ovariectomized hinds in Experiment 2

Pulse parameter	Season	Euthyroid	Thyroidectomized
Concentration	Breeding season	4.3±1.0^a	4.7±1.1^a
	Non-breeding season	1.5±0.2^b	9.3±1.8^c
Number / 4 h	Breeding season	4.2±0.1^a	4.2±0.6^a
	Non-breeding season	2.0±0.3^b	4.3±0.3^a
Amplitude	Breeding season	3.7±1.2^a	4.3±0.8^a
	Non-breeding season	1.9±0.5^b	8.8±1.8^c

Means not assigned common superscript letters within a pulse parameter are significantly different ($P < 0.05$).

Morphine caused panting and signs of anxiety (e.g. pacing behaviour) in 3 of the 10 hinds, beginning approximately 30 minutes after injection and lasting up to 4 hours. Cyproheptadine caused haemolysis, occasional vocalisation, disorientation and agitation when handled in all hinds. These effects, which were particularly severe in 2 of the 10 hinds, lasted for 2-2.5 hours.

6.4 Discussion

This study is the first of its kind to be reported for deer. Most of our present understanding of the neural pathways regulating seasonal reproductive cycles comes from experiments using the ewe (e.g. Meyer and Goodman, 1985). The current results strongly suggest that the neural mechanisms which modify GnRH secretion may differ between the two species, since treatment with dopaminergic and opioidergic agonists and antagonists which have previously been shown to evoke clear LH responses in sheep failed to do so in deer, and treatment with a serotonin antagonist which increases LH secretion in ovariectomized sheep caused suppression of pulsatile LH secretion in ovariectomized deer.

In Experiments 1 and 2, the dopamine agonist bromocriptine and antagonist sulpiride were administered at doses which have been shown to be effective in evoking LH responses lasting for over 4 hours in rams (Tortonese and Lincoln, 1994a). Since bromocriptine suppressed LH concentrations and pulse frequency only under short days and sulpiride increased LH concentrations and pulse frequency only under long days, it was concluded in that study as in most similar studies using ovariectomized oestradiol-treated ewes (Meyer and Goodman, 1985; Thiéry *et al.*, 1989; Whisnant and Goodman, 1994) or entire ewes (Meyer and Goodman, 1985; Curlewis *et al.*, 1991; Tilbrook and Clarke, 1992), that an inhibitory DA neural system is activated during the non-breeding season to suppress LH and bring about the sexually inactive state. In two studies using ovariectomized oestradiol-treated ewes (Kao *et al.*, 1992; Le Corre and Chemineau; 1993b) and one using entire or testosterone-treated castrate rams (Tilbrook and Clarke, 1992), there was no LH response to the dopamine antagonist pimozide. Although the results of these studies may initially appear to be comparable to the results of Experiment 1, it must be noted that whereas sulpiride is a specific DA-D_2 antagonist (Kebabian and Calne, 1979; Niznik, 1987), pimozide is less specific for these receptors and binds also to DA-D_1 receptors in the brain (Pinder *et al.*, 1976). In a comparative study of the two drugs, sulpiride elicited an LH response under long days in entire rams while pimozide was ineffective in this regard (Tortonese and Lincoln, 1994), and it was surmised that activation of DA-D_1 receptor-mediated responses may possibly negate effects on GnRH/LH secretion.

Since the lack of LH responses in red deer hinds in Experiment 1 is not in agreement with most comparable studies in entire or castrated steroid-treated sheep, the current

results indicate that the neuromodulation of GnRH pulse generation differs between the two species. Although a mere quantitative species difference in the dose of bromocriptine or sulpiride required to elicit an LH response in red deer hinds cannot be ruled out, it is suggested rather that DA-D_2 receptor-mediated neural pathways are not important modulators of steroid-dependent suppression of LH in hinds, based on 3 observations. Firstly, the doses of bromocriptine and sulpiride were generally sufficient to generate robust changes in prolactin secretion, a response known to be activated by DA-D_2 receptor agonists and antagonists in sheep (Ssewannyana and Lincoln, 1990) and red deer hinds (Curlewis *et al.*, 1988; Milne *et al.*, 1990). Although prolactin responses do not necessarily prove that the dose of drug used was sufficient to affect DA-D_2 receptor mediated pathways controlling GnRH secretion, they do at least show that the drug treatments were effective in binding to DA-D_2 receptors and evoking an appropriate biological response. Secondly, administration of bromocriptine at five times the dose used in the current experiment to ovariectomized oestradiol-implanted hinds during the non-breeding season caused similar prolactin responses to those shown here, while LH secretion remained unchanged (G.M. Anderson and G.K. Barrell, unpublished). This indicates that the lack of an effect of bromocriptine in Experiment 1 on LH secretion was unlikely to be due to an inadequate dose. Thirdly, differences in neuromodulation of seasonal reproduction between sheep and other species are known to exist; for example DA agonists and antagonists do not evoke LH responses in the anoestrous mare (Besognet *et al.*, 1996).

Most studies of DA neural pathways using the ovariectomized sheep without steroid replacement have concluded that steroid-independent seasonal reproductive changes are not mediated by dopamine (Meyer and Goodman, 1986; Thiéry *et al.*, 1989; Tilbrook and Clarke, 1992). This is in agreement with results from Experiment 2 in which bromocriptine and sulpiride evoked prolactin but not LH responses in ovariectomized hinds without oestradiol. Steroid-independent pathways are of particular significance to red deer reproduction (Meikle and Fisher, 1996) since they are appear to make a much larger contribution to seasonal breeding in this species than in the ewe, where they have received relatively little attention. Therefore the current experiment also examined the role of endogenous opioid peptides, which appear to play an inhibitory role that is independent of oestradiol in some (Shillo *et al.* 1985; Yang *et al.*; 1988; Schall *et al.*, 1991) but not all (Brooks *et al.* 1986b; Meyer and Goodman, 1986; Whisnant and Goodman, 1988) studies of seasonal LH secretion in ovariectomized sheep, and serotonin, which has also been reported to be inhibitory to pulsatile LH secretion in ovariectomized ewes in several studies (Riggs and

Malven, 1974; Meyer and Goodman, 1986; Whisnant and Goodman, 1990). As was the case for bromocriptine and sulpiride, there was no LH response to morphine and serotonin in the breeding season or to naloxone in the non-breeding season, although plasma prolactin concentrations were significantly increased by morphine and decreased by naloxone. One possible explanation for the lack of LH response to these drugs that endogenous opioid peptides suppress LH secretion during puberty in red deer hinds but non-opioidergic systems progressively take over this role during post-pubertal maturation, as has been demonstrated in some experiments in the ewe (Brooks *et al.* 1986a; Schall *et al.*, 1991), cow (Wolfe *et al.*, 1991) and human (Genazzani *et al.*, 1993). The current results also do not preclude a role for opioids in inhibiting GnRH pulse size as has been demonstrated by Goodman *et al.* (1995) in ovariectomized ewes irrespective of oestradiol treatment, since the effects of naloxone on episodic GnRH secretion in that study were not clearly reflected in episodic LH secretion and would be unlikely to be detected in studies such as the current experiment where GnRH was not measured directly. Notwithstanding these considerations, the present results indicate that endogenous opioid peptides do not play a major role in steroid-independent seasonal suppression of LH concentrations in red deer hinds.

The suppression of pulsatile LH secretion by cyproheptadine during the non-breeding season may indicate that a stimulatory serotonergic system operates in red deer hinds. This finding represents a further contrast between neuroendocrine control of seasonality in sheep and deer, since cyproheptadine suppresses LH secretion in ovariectomized ewes (Meyer and Goodman, 1988; Whisnant and Goodman, 1990). Cyproheptadine appeared to suppress plasma LH concentrations and pulses to a greater degree in THX hinds than in euthyroid hinds (Figure 6.5). During the breeding season, intravenous injection of serotonin was without effect. A possible explanation for these results is that the degree of serotonergic stimulation is greatest during the breeding season resulting in high plasma LH concentrations, and declines progressively via thyroid hormone-dependent mechanisms during the non-breeding season resulting in decreasing plasma LH concentrations. In November, when serotonergic stimulation would be lowest in euthyroid hinds, blockade of these pathways with cyproheptadine would result in a very moderate decline in LH secretion. In THX hinds, serotonergic stimulation would be maintained throughout the breeding season, so that antagonism with cyproheptadine would result in a large decline in LH secretion. The lack of an LH response to serotonin during the breeding season may have been due to maximal stimulation of pulsatile LH secretion by

serotonergic pathways at this time of the year, so that further stimulation with serotonin or serotonergic antagonists was not possible. However, the lack of a prolactin response to serotonin may indicate that the dose employed in this experiment, which was able to elicit a two-fold increase in plasma prolactin concentrations lasting for over a hour in ewes (Thomas *et al.*, 1988), was insufficient to alter LH and prolactin secretion in deer.

In one study in ovariectomized ewes (Deaver and Dailey, 1982), low-doses of serotonin slightly decreased, while a higher dose elevated mean LH concentration. The authors suggested that different classes of neurotransmitter receptors may exist with different affinities for agonists and antagonists, so that activation or inhibition of these receptors might affect secretion of LH differently. It is therefore possible that the absence of an LH response to serotonin in the current study can be explained by the dosage, as well as the method of administration, since in the study of Deaver an Dailey continuous infusions of serotonin were used.

As was noted previously in relation to bromocriptine and sulpiride, the significant responses of prolactin to morphine, naloxone and cyproheptadine recorded in the current experiment confirm that the dosages used were sufficient to elicit biological responses in this species. However the response to morphine observed in hinds in Experiment 2 was not the same as has been reported in studies in the sheep (Schillo *et al.*, 1985 Parrott and Goode, 1992), where this opiate drug exhibits stimulatory effects on circulating prolactin concentrations. Opioids are thought to stimulate prolactin secretion by reducing dopamine release into the hypothalamo-pituitary portal circulation (Gudalsky and Porter, 1979; Van Loon *et al.*, 1980a; 1980b; Wilkes and Yen, 1980). Serotonin is also thought to stimulate prolactin by inhibiting hypothalamic dopamine release (Pilotte and Porter, 1981) as well as by stimulating the release of prolactin-releasing factors into hypophyseal portal blood (Kaji *et al.*, 1980); however the increase in prolactin following cyproheptadine in the current study suggests against serotonergic prolactin-stimulating pathways in deer.

On all intensive sampling occasions a decline in mean plasma prolactin concentration was observed during the first 4 hours of sampling. As the mean plasma prolactin concentrations were considerably higher in these experiments than in many other reports of plasma prolactin concentration in red deer (e.g. Curlewis *et al.*, 1988; Milne *et al.*, 1990), it is possible that this progressive decline is reflective of an initial stress period at the start of each sampling period. Although such artificially elevated

concentrations could affect the interpretation of hormone profiles in plasma, the administration of all drugs in the current experiments except for serotonin produced immediate and profound changes in plasma prolactin concentration which unequivocally indicate a biological response to the drug injections. The lower mean concentration of plasma prolactin in hinds in Experiment 1 in the presence of oestradiol compared with hinds in Experiment 2, and the absence of a seasonal decrease in plasma prolactin concentration during the breeding season in ovariectomized hinds in Experiment 2 may indicate a seasonal suppressive effect of oestradiol on prolactin secretion; a possibility which warrants further investigation.

In this study neither dopamine or endogenous opioid peptides were implicated in seasonal LH suppression in either euthyroid or thyroidectomized red deer hinds; thus the pathways on which thyroid hormones act to bring about the anoestrous state remain to be described. Further experimentation is required to clarify the role of serotonergic neurons in this process. As was the case in Chapter 5, thyroidectomy during the breeding season was unable to overcome the steroid-dependent effects on LH secretion, since LH pulse frequency and amplitude were as much suppressed in thyroidectomized hinds as in euthyroid hinds during the non-breeding season. Taken together with the absence of LH responses to DA-D_2 receptor agonists and antagonists in Experiment 1 in this chapter, the present results indicate that the steroid-dependent mechanisms which contribute to seasonal breeding in the red deer hind operate differently to those in the ewe.

The results for pulsatile LH secretion in euthyroid ovariectomized hinds (Experiment 2) during the breeding season are similar to those of Limsirichaikul (1992), but during the non-breeding season pulse frequency and amplitude were much lower in the current experiment. This difference can be explained by the time of year in which sampling was conducted, since pulses were measured close to the breeding season in February and March in the study of Limsirichaikul. More recently it has been shown that LH concentrations in ovariectomized hinds at that time of year are similar to those during the breeding season (Meikle and Fisher, 1996). The present results describe episodic LH secretion in ovariectomized hinds during the breeding season and during the non-breeding season at the time of maximal reproductive neuroendocrine suppression (i.e. just prior to the summer solstice). In euthyroid hinds pulse frequency was low at this time compared with the breeding season. This finding is in agreement with results from ovariectomized ewes (Karsch *et al.*, 1980; Goodman *et al.*, 1982; Robinson *et al.*, 1985; Moenter *et al.*, 1991), although the magnitude of

the seasonal change appears to be much greater in hinds. Ovariectomized ewes show an increase in pulse amplitude during the non-breeding season (Karsch *et al.*, 1980; Goodman *et al.*, 1982; Moenter *et al.*, 1991), but in contrast pulse amplitude declined in the current experiment. The role of thyroid hormones in steroid-independent seasonal LH secretion in the ewe has received no direct attention; however the results of Moenter *et al.* (1991) suggest that pulse frequency and amplitude may remain at breeding season levels in thyroidectomized ovariectomized ewes during the non-breeding season. Thyroidectomy prevented the seasonal decline in LH pulse frequency in the ovariectomized hinds in Experiment 2, but in addition caused pulse amplitude to increase to more than twice the breeding season amplitudes. One possible explanation for this is that high amplitude pulses of GnRH occur in hinds during the non-breeding season as in ewes (Barrell *et al.*, 1992), but correspondingly large LH pulses are prevented due to the seasonal decline in responsiveness of the anterior pituitary gland to GnRH (Meikle and Fisher, 1996). Since this seasonal decline in pituitary responsiveness is greatly diminished in the absence of the thyroid gland (see Chapter 5), high amplitude LH pulses are able to be expressed in thyroidectomized hinds. Since methods for collecting portal blood and measuring GnRH have not been developed in our laboratory it is not currently possible to test this hypothesis directly.

In conclusion, the absence of LH responses to bromocriptine or sulpiride in the presence or absence of oestradiol, despite the fact that these drugs appeared to bind effectively to DA-D_2 receptors, as evidenced by appropriate changes in prolactin secretion, indicates that DA-D_2 receptor-mediated neural pathways are not important modulators of LH regulation in this species. Furthermore, endogenous opioid peptides also do not appear to modulate steroid-independent LH suppression in this species, as morphine and naloxone elicited prolactin but not LH responses in the absence of oestradiol. However the serotonergic antagonist cyproheptadine significantly suppressed pulsatile LH secretion in the absence of oestradiol, suggesting that serotonin may be stimulatory in the ovariectomized red deer hind. Since the effects of thyroidectomy in these experiments was manifest only in steroid-independent conditions, the role of serotonergic neural pathways deserves further research to determine if thyroid hormones influence seasonal reproduction in red deer hinds by modifying these pathways.

Chapter 7

Thyroxine treatment during the non-breeding season allows steroid-independent suppression of reproductive neuroendocrine activity in the thyroidectomized red deer hind

7.1 Introduction

It has been suggested that the neuroendocrine mechanisms which bring about seasonal anoestrus may be responsive to thyroid hormones only during a short 'window' of time within the circannual reproductive cycle (Karsch *et al.*, 1995). If such a window of responsiveness was shown to exist, blockade of thyroid function during the critical period might enable completely aseasonal reproduction in animals that are traditionally seasonal breeders. Support for the existence of a window of responsiveness to thyroid hormones comes from recent experiments in which a brief (60 - 90 days) period of exposure of thyroidectomized ewes to T_4 just prior to the non-breeding season was sufficient to bring about neuroendocrine anoestrus at the appropriate time (Thrun *et al.*, 1997). However it remains to be tested if the period of responsiveness continues during the non-breeding season, when inhibitory photoperiods and the endogenous reproductive rhythm may both be signalling that anoestrus should be occurring.

The purpose of the present study was to delineate a period of thyroid hormone responsiveness in ovariectomized red deer hinds. This model animal is specifically concerned with steroid-independent mechanisms of gonadotrophin suppression, since only these appear to be thyroid hormone-dependent in the red deer hind.

7.2 Materials and Methods

Animals and management

The experiment used 25 mature red deer hinds (mean live weight at the start of the experiment 94.2 ± 2.3 kg) which were ovariectomized and thyroidectomized during the breeding season (June, $n = 16$) or sourced from a previous experiment.

Experimental Design

Ovariectomized hinds were allocated in June 1996 to the following treatment groups ($n = 5$ per group): (a) thyroidectomized (THX negative controls); (b) thyroidectomized and treated subcutaneously in the anterior neck region with 4 x 25 mg sodium L-thyroxine tablets (Glaxo Laboratories Ltd, Middlesex, England) until 2 August, about 40 days prior to the normal time of onset of anoestrus in cycling hinds (THX+early T_4); (c) thyroidectomized and treated with T_4 as above from 2 August to 21 October, about 40 days after the normal onset of anoestrus (THX+mid T_4); (d) thyroidectomized and treated with T_4 from 21 October to 10 February the following year (THX+late T_4), and (e) untreated (euthyroid positive controls). Blood samples were collected weekly until 27 January (euthyroid and THX control hinds) or 10 February (THX+T_4 hinds) 1997 for measurement of plasma LH concentration. Once every month, a 5 μg dose of GnRH (LH-RH acetate salt, Sigma Chemical Co., St Louis, MO, USA) was administered i.v. immediately following the weekly blood sample and a further sample obtained exactly 13 minutes later to assess the pituitary responsiveness to GnRH (calculated as the LH concentration at 13 minutes minus the concentration prior to injection). In July (breeding season) and November (non-breeding season, at the time when steroid-independent suppression is maximal), blood samples were collected at 10 minute intervals for 4 hours to facilitate measurement of episodic LH secretion. Plasma total tri-iodothyronine (T_3) was measured approximately monthly.

Data analysis

Hormone concentrations below the average assay sensitivity were assigned a value equal to the sensitivity. Suppression of LH happens gradually during the non-breeding season in the ovariectomized hind (see Meikle and Fisher; 1996); therefore

the transition from reproductive neuroendocrine activity to quiescence in individual animals was arbitrarily taken as the date of the first sample that fell below 1 $ng.ml^{-1}$ for 2 consecutive samples Similarly, the date of the first of 2 consecutive samples where LH concentrations rose above 1 $ng.ml^{-1}$ was taken to indicate the return to a state of reproductive neuroendocrine activity. Treatment effects on the date of transition to reproductive quiescence, LH pulse frequency and amplitude, LH released in response to exogenous GnRH, and the increase in mean plasma T_3 concentration during periods of T_4 implantation were identified using analysis of variance followed by mean comparisons among groups using Student's *t*-test. Changes in plasma T_3 concentration over time in euthyroid and THX control hinds were identified by linear regression analysis. All hormone concentrations were log transformed (base 10) to equalize variances before statistical analysis. Mean results are presented ± SEM.

7.3 Results

A seasonal increase in mean plasma concentration of total T_3 was observed throughout the summer in euthyroid positive control hinds ($P < 0.01$), with a mean nadir concentration of 1.06 ± 0.19 $nmol.l^{-1}$ in spring (September) and a peak concentration of 1.83 ± 0.21 $nmol.l^{-1}$ in late summer (February). In contrast, mean plasma T_3 concentration remained low (< 0.2 $nmol.l^{-1}$) and was often undetectable throughout the year in THX control hinds. In the remaining three groups, mean T_3 concentrations were low or undetectable in the absence of T_4 tablets (0.05 ± 0.01 $nmol.l^{-1}$) and significantly elevated, albeit slightly, during periods of implantation with T_4 tablets (0.45. ± 0.03 $nmol.l^{-1}$; $P < 0.05$ for THX+early T_4 hinds and $P < 0.001$ for THX+mid T_4 and THX+late T_4 hinds) (Figure 7.1).

Mean plasma LH concentrations and periods of reproductive neuroendocrine activity for individual hinds are shown in Figure 7.2. As expected, mean LH concentrations in euthyroid control hinds were high during winter and spring (June - mid-October) and low during early summer (mid-October - December). The mean date for onset of reproductive neuroendocrine quiescence was 19 October ± 6.6 days; with reproductive activity resuming again on 20 December ± 1.9 days. In all THX negative control hinds however, LH concentrations remained high throughout the summer so that reproductive quiescence could not be detected. A similar pattern was observed for

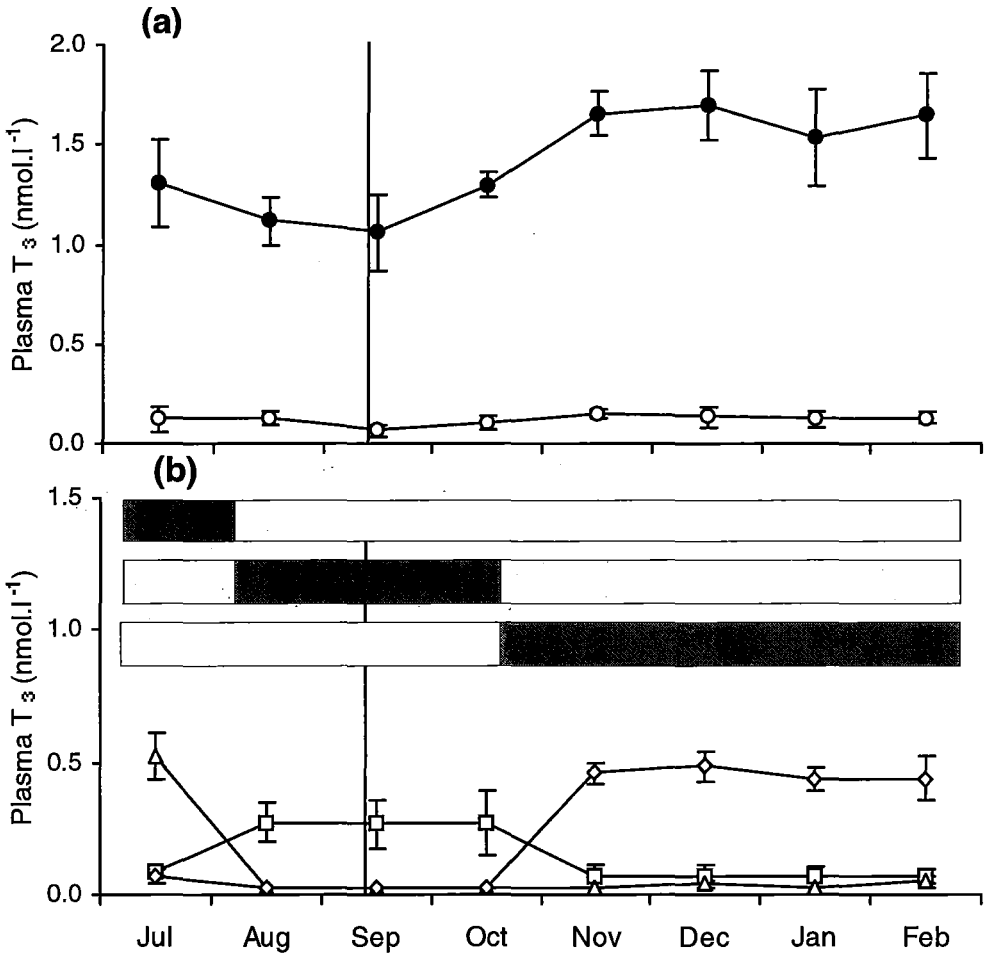

Figure 7.1 Mean (± SEM) plasma concentrations of total T3 in (a) euthyroid control (●) and THX control (○) hinds, and (b) THX+early T4 (△), THX+mid T4 (□) and THX+late T4 hinds (◇) (n = 5). Shaded horizontal bars indicate times of T4 treatment (4 x 25 mg T4 tablets subcutaneously) for the three THX+ T4 groups respectively. Vertical bars denote the time of the end of the breeding season for red deer hinds in New Zealand.

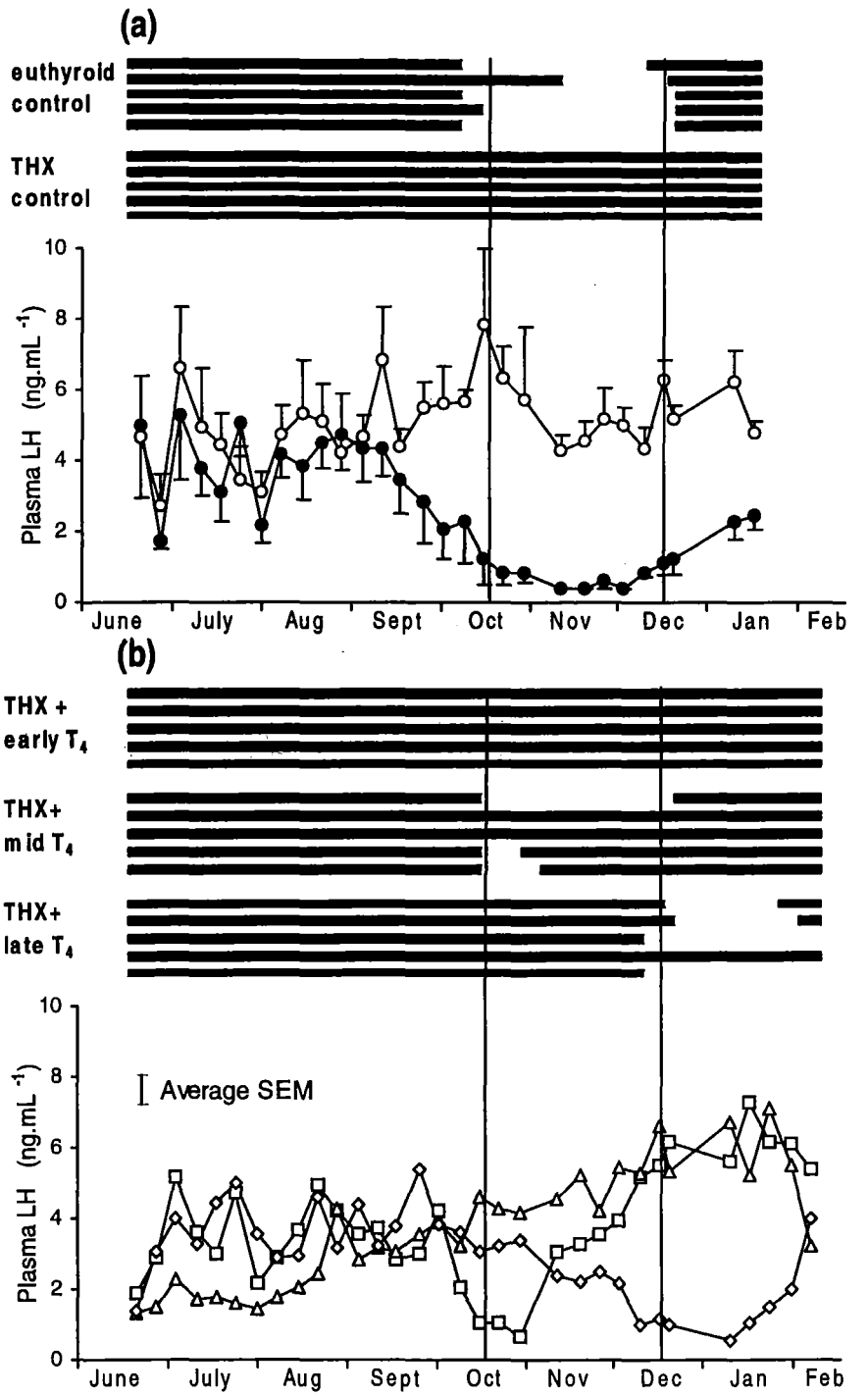

Figure 7.2 Mean (± SEM) plasma concentrations of LH in (a) euthyroid control (●) and THX control (○) hinds, and (b) THX+early T4 (△), THX+mid T4 (□) and THX+late T4 (◇) hinds (n = 5). Horizontal lines indicate times when individual hinds were defined as being reproductively active. Vertical bars delineate the mean period of reproductive quiescence in euthyroid positive control hinds.

THX+early T_4 hinds. In 3 of the 5 THX+mid T_4 hinds, the dates of reproductive quiescence onset were not different to those of euthyroid controls (18 October for all 3 hinds; $P > 0.05$), however LH concentrations returned to > 1 $ng.ml^{-1}$ within 2 - 3 weeks in 2 of these hinds (1 and 8 November) while remaining low until 23 December in the third hind (which had plasma T_3 concentrations 2-3 times higher than others in this group). In the remaining 2 THX+mid T_4 hinds (in which plasma T_3 concentrations averaged 0.1 $nmol.l^{-1}$; the lowest of all T_4-treated hinds), reproductive quiescence was not recorded despite a decline in LH concentrations around this time. Reproductive quiescence occurred significantly later than for euthyroid controls in 4 THX+late T_4 hinds (17 December ± 2.5 days; $P < 0.001$); in the remaining hind reproductive quiescence was not detected although a decline in LH concentrations occurred. Reproductive activity resumed on 27 January and 3 February for 2 of the 4 hinds in which quiescence occurred, but LH was still suppressed in the other 2 hinds at the end of the experiment (10 February).

Data for pulsatile LH secretion during July and November are shown in Table 1. The mean number of LH pulses was lower in November than July in euthyroid control hinds ($P < 0.001$) and in the THX+mid T_4 hind which had low LH concentrations from October until December (no pulses were detected over the 4 hour sampling period in November in this hind). In all other hinds, number of pulses did not differ significantly between July and November ($P > 0.05$) while pulse amplitude increased in November ($P < 0.05$). Individual LH profiles representative of hinds which became reproductively quiescent or remained reproductively active are shown in Figure 7.3.

In all hinds, there was a seasonal change in pituitary responsiveness to exogenous GnRH, with maximal responsiveness (38.3 ± 2.8 $ng.ml^{-1}$) occurring during late July (breeding season) and minimal responsiveness in November and December (non-breeding season), although responsiveness was also lowered during T_4 implantation in February in THX+late T_4 hinds (9.2 ± 2.6) compared to THX+early T_4 (19.6 ± 3.3) and THX+mid T_4 (24.6 ± 3.4) hinds ($P < 0.05$) (Figure 7.4). Pituitary LH response to GnRH during November and December was lower in euthyroid control hinds (average of both months: 6.6 ± 1.1 $ng.ml^{-1}$) than in all other treatment groups (average: 15.4 ± 1.2 $ng.ml^{-1}$) ($P < 0.05$). At all other times of the year, LH responses to GnRH were not significantly different between groups.

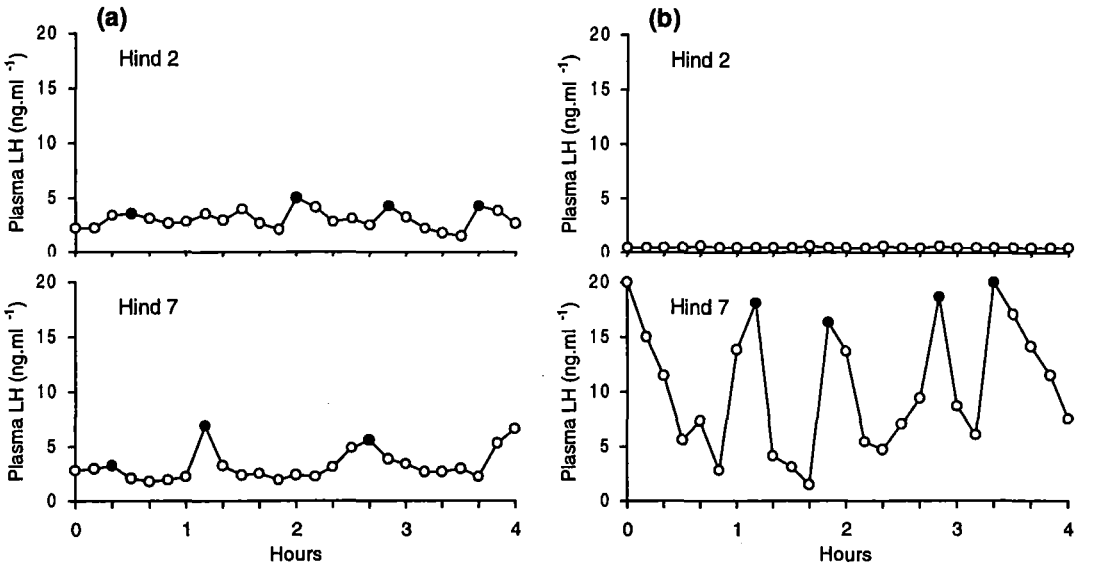

Figure 7.3 Representative individual plasma LH profiles from intensive sampling periods during the breeding season (a) and non-breeding season (b). Upper panels are representative of euthyroid positive control hinds and a single THX+mid T4 hind; lower panels are representative of all other hinds (see text for further details). Closed circles denote the peak of each pulse.

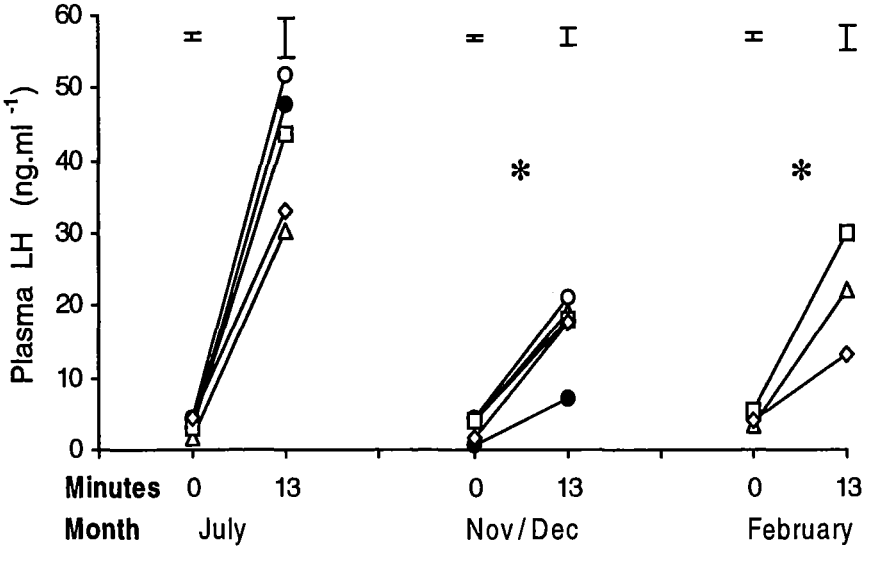

Figure 7.4 Mean (± SEM) plasma LH concentrations, measured at 0 and 13 minutes, in response to 5 µg GnRH i.v. in euthyroid control (●), THX control (○),THX+early T4 (△), THX+mid T4 (□) and THX+late T4 (◇) hinds (n = 5) during July (maximal response), December and November (minimal response) and February (latter 3 groups only). Average SEM at each time is shown at the top of the graph. Asterisks indicate significant ($P < 0.05$) differences in response of all groups compared to euthyroid control hinds during November/December or to THX+mid T_4 hinds during February.

Table 7.1 LH pulse characteristics in deer during 4 hour intensive sampling periods in July (breeding season) and November (non-breeding season)

Group	Number / 4 hours		Amplitude	
	July	November	July	November
Euthyroid control	4.0 ± 0.3	1.0 ± 0.4**	3.3 ± 1.3	1.1 ± 0.4 ^{NS}
THX control	3.0 ± 0.7	3.0 ± 0.4^{NS}	3.3 ± 0.8	14.5 ± 1.9**
THX+early T_4	2.4 ± 0.4	2.5 ± 0.5^{NS}	4.9 ± 1.7	8.3 ± 2.0*
THX+mid T_4	2.4 ± 0.6	2.8 ± 0.7^{NS†}	1.5 ± 0.5	11.1 ± 2.1**
THX+late T_4	2.4 ± 0.2	3.8 ± 0.8^{NS}	2.7 ± 0.6	7.7 ± 1.6*

^{NS} = non-significant; * $P < 0.05$; ** $P < 0.001$

† Data from one hind with no detectable pulses are excluded from this mean (see text for details).

7.4 Discussion

The present results demonstrate that thyroid hormones have a season-specific role in mediating the steroid-independent suppression of plasma LH concentrations in red deer hinds since T_4 replacement in thyroidectomized hinds during the breeding season did not initiate this suppression whereas T_4 replacement applied around and after the end of the breeding season did. The results confirm recent findings in the ewe which demonstrated that thyroid hormones need only be present for a short time prior to the end of the breeding season to permit the seasonal decline in LH concentrations (Thrun *et al.*, 1996; 1997); they also extend these findings by demonstrating that the red deer hind remains capable of responding to thyroid hormones for at least 40 days after the breeding season. Because T_4 replacement at the latest occasion attempted in the present study was generally effective in bringing about neuroendocrine reproductive quiescence, the offset of the period of thyroid hormone responsiveness cannot be identified, as was set out in the objectives for this experiment. Our results suggest that if such a window of neuroendocrine responsiveness exists, it begins less than 40 days prior to the transition to the non-breeding season and extends beyond this transition for an as yet unknown period of time.

Only 3/5 hinds which received T_4 replacement around the time of the end of the breeding season (August to October) entered reproductive quiescence, and the

duration of quiescence was brief (2 - 3 weeks) in 2 of these hinds, indicating that the transition to the reproductively inactive state had not been totally achieved. However the thyroid hormone concentrations achieved by this mid-T_4 treatment were probably at the margin of those required to restore normal seasonal reproductive patterns to those seen in euthyroid control hinds. This is corroborated by the observation that the one hind in which duration of suppression of LH concentrations and pulsatile secretion of LH in November was similar to that of euthyroid control hinds had the highest T_3 concentrations for the group (mean 0.5 $nmol.l^{-1}$), and the 2 hinds in which LH was not completely suppressed had barely detectable plasma concentrations of T_3 (mean 0.1 $nmol.l^{-1}$); these were the lowest concentrations recorded from any of the T_4 treated hinds while the implants were in place. Interestingly, these low T_3 concentrations equate approximately to 5 $nmol.l^{-1}$ T_4 (assuming a 50:1 total T_4:total T_3 ratio based on previous studies (see Chapter 5) using the same implants) which is close to the minimum threshold concentration of 2 $nmol.l^{-1}$ T_4 estimated to be required for achieving anoestrus in sheep (Thrun *et al.*, 1997). It should be noted that all 5 hinds in the THX+mid T_4 group showed a decline in plasma LH concentrations during October. It is likely that much of the variation in response to T_4 treatment prior to October can be explained by circulating thyroid hormone concentrations achieved by the implants being near to threshold in some animals.

The results of this experiment strongly suggest that the mechanisms which suppress plasma LH concentration in ovariectomized hinds are responsive to thyroid hormones only when the prevailing photoperiods and/or the endogenous reproductive rhythm are inhibitory to reproductive activity, since T_4 replacement during inductive short photoperiods (i.e. THX+early T_4 group) caused no reproductive suppression whereas T_4 replacement during inhibitory long or increasing photoperiods (i.e. THX+late T_4 and THX+mid T_4 groups) did. If this is the case, the 'window' of responsiveness to the inhibitory influence of thyroid hormones under natural conditions would be expected to continue throughout the summer until the endogenous signalling mechanisms and decreasing photoperiods signal the next breeding season. In support of this concept, treatment of thyroidectomized stags with T_4 implants or even a single injection of T_3 during summer caused cessation of rutting behaviour and antler casting in red deer stags (G.M. Anderson, unpublished observations). The recent finding that T_4 replacement of thyroidectomized ewes about 1 month prior to the *onset* of the

breeding season in autumn (when photoperiods are decreasing) did not cause serum LH concentrations to fall (Thrun *et al.*, 1997) lends further support to the argument that thyroid hormones exert their action only during inhibitory photoperiods and the appropriate phase of the endogenous rhythm. An important underlying assumption here is that thyroidectomy does not prevent neuroendocrine perception of photoperiodic changes or generation of the endogenous reproductive rhythm, but it is the normal *response* of the hypothalamo-hypophysial axis to these stimuli which is disrupted. That thyroidectomy does not disrupt sensitivity to photoperiodic stimuli has been repeatedly demonstrated by appropriate prolactin and melatonin responses to fluctuating photoperiods in thyroidectomized animals (Jacquet *et al.*, 1986; Nicholls *et al.*, 1988b; Moenter *et al.*, 1991; Shi and Barrell, 1992; Dahl *et al.*, 1994a). To confirm unequivocally the existence of an endogenous rhythm of reproductive function in thyroidectomized animals would presumably require that they be maintained under long-term fixed photoperiods (or pinealectomized) and treated with thyroid hormones at different times of the year. To date this has not been demonstrated.

As was observed in ovariectomized hinds in the previous chapter, pulse frequency in euthyroid hinds was low during the non-breeding season (November) compared with the breeding season, and there was also a decline in pulse amplitude (albeit non-significant) during the non-breeding season. Thyroidectomy prevented the seasonal decline in LH pulse frequency, and in addition caused pulse amplitude to increase approximately four-fold compared with breeding season amplitudes. The results for episodic LH secretion confirm that all hinds except euthyroid controls and a single THX+mid T_4 treated hind were equivalent to being reproductively active in November, since pulse frequency remained elevated at this time. Presumably if sampling for pulsatile LH secretion had been conducted earlier in November while most of the THX+mid T_4 group were still reproductively suppressed, this may have provided further confirmation of their responsiveness to thyroid hormones around the end of the breeding season. Similarly, it may have been of value to have monitored pulsatile LH secretion in January following the decline in mean plasma LH concentration in THX+late T_4 treated hinds.

The stimulatory effect of thyroidectomy on pulsatile secretion of LH in hinds during November indicates that thyroid hormones may modify the output of the hypothalamic GnRH neurons, since each individual LH episode has been shown to be temporally related to (and presumably the direct consequence of) an episode of GnRH (Clarke and Cummins, 1982; Levine *et al.*, 1982; Karsch *et al.*, 1987; Barrell *et al.*, 1992; Karsch *et al.*, 1993). Therefore, the maintenance of high LH concentrations throughout the summer following thyroidectomy in this experiment probably reflects a continuation of elevated episodic GnRH secretion during this period as has been shown in the ewe (Webster *et al.*, 1991b). The observation that thyroid hormone receptors are present in neuroendocrine tissues including GnRH neurons (Janson *et al.*, 1994) and the demonstration that thyroid hormones can act centrally to alter hypothalamic secretions (Dahl *et al.*, 1994b), support this view. In the present experiment and others involving red deer (Shi and Barrell, 1992, see also Chapter 5), evidence was also obtained for an effect of thyroidectomy at the level of the pituitary gland, since in all THX hinds the seasonal decline in LH release following a GnRH challenge during November was markedly reduced (although not completely abolished). It is interesting that T_4 treatment around the end of the breeding season (see THX+mid T_4 hinds, Figure 7.4) did not entirely overcome this effect; as is also evident in a previous study in red deer stags (Shi and Barrell, 1994). One possible explanation for this is that the action of thyroid hormones at the level of the pituitary gland might be dose-responsive, since T_4 treatment in both studies achieved plasma T_3 concentrations considerably lower than those seen in euthyroid deer. In THX+late T_4 hinds pituitary responsiveness to GnRH was also lowered in February relative to THX+early T_4 and THX+mid T_4 hinds. This may reflect a delayed response to T_4 treatment as was observed for basal LH concentrations, although the time taken to respond to T_4 was much greater for pituitary responsiveness than for basal LH. Collectively, our observations imply that thyroid hormones act in a different way and have a different response time at the pituitary level than at a central level.

In conclusion, this study has shown that thyroid hormones are required for the steroid-independent mechanisms which suppress LH in this species during inhibitory photoperiods and the appropriate phase of the endogenous rhythm, and it is hypothesised that thyroid hormones remain effective in this regard throughout most of the non-breeding season. The results do not support the existence of a brief window

of neuroendocrine responsiveness to thyroid hormones which acts only around the end of the breeding season in red deer hinds, since exposure to T_4 starting about 6 weeks after this time brought about suppression of plasma LH concentration.

Chapter 8

Feasibility of out-of-season breeding in thyroidectomized red deer hinds

8.1 Introduction

Considerable research effort has been devoted to advancing the timing of conception in red deer (e.g. Adam *et al.*, 1986; Moore and Cowie, 1986; Fennessy *et al.*, 1986; Duckworth and Barrell, 1988; 1991;. Wilson, 1992). This is due primarily to the economic costs of meeting the feed requirements of lactating hinds following calving in December, when pasture in many parts of New Zealand undergoes a seasonal decline in growth rate and quality. Other advantages of advancing the breeding season of deer include being able to grow the young to the desired carcase weight at the time of optimum venison schedules in Northern Hemisphere markets (mid August to late December), reduced weaning stress due to heavier (earlier born) calves and better returns on weaner stags and hinds due to increased live weights (Wilson, 1989).

The hormonal treatments which have been evaluated to date for advancement of breeding in female deer are based on those already employed successfully for other species of livestock such as sheep and cattle (Barrell, 1985; Wilson, 1989). These involve either parenteral administration of PMSG or GnRH in conjunction with a period of intravaginal progesterone treatment, or administration of melatonin (orally or parenterally) in a manner designed to mimic the effects of inductive photoperiods during the breeding season. While all these techniques will induce ovulation prior to the normal breeding season, only the latter has proved successful in advancing the date of calving with any degree of reliability (Asher *et al.*, 1994b). For example, the use of subcutaneous melatonin implants has led to calving advancements of up to 6 weeks (Asher, 1990). This treatment is contra-indicated during pregnancy however as it may impair lactogenesis (Asher *et al.*, 1994) and is therefore limited in its potential for changing the breeding season of red deer.

The low pregnancy rates achieved following treatment with progesterone and GnRH or GnRH analogues (Moore and Cowie, 1986; Fisher *et al.*, 1986; Duckworth and Barrell, 1988), even when given in conjunction with oestradiol to induce overt oestrus and mating (Duckworth and Barrell, 1991) suggest that insufficient GnRH release is not the only factor limiting ovulatory activity in anoestrous red deer hinds. It is possible that the reduction in pituitary responsiveness to GnRH during the non-breeding season (Meikle and Fisher, 1996; see also results in Chapters 4, 5 and 7) renders such treatments ineffective in stimulating the hormonal changes required for synchronised oestrus and ovulation. Another possibility is that a functional corpus luteum is required prior to ovulation to secrete some essential steroid for successful conception. In natural mating systems, a 'silent' ovulation at the start of each breeding season (Webster and Barrell, 1985, Asher *et al.*, 1991) could fulfil this role.

Thyroidectomy was shown in previous experiments (Chapters 5 and 7) partly to overcome the seasonal decline in pituitary responsiveness to GnRH and permit oestrous cyclicity during the non-breeding season. Manipulation of thyroid gland function may therefore provide a means for artificial control of the breeding season in female deer. The current experiment describes an attempt to induce mating, ovulation and conception in thyroidectomized red deer hinds. If successful out-of-season breeding could be demonstrated in thyroidectomized hinds, treatments might then be developed for on-farm use involving suppression of thyroid gland secretions or blocking thyroid hormones at their site of action during the non-breeding season. Because many of the progesterone episodes recorded in thyroidectomized hinds in Chapter 5 appeared to be shortened in duration and irregular in occurrence compared with those recorded in euthyroid hinds, the efficacy of intravaginal progesterone treatment for synchronising oestrus and ovulation in thyroidectomized hinds was examined initially during the breeding season.

8.2 Materials and Methods

Animals and Management

The experiment used 15 mature red deer hinds (mean live weight at the start of the experiment 102.3 ± 2.3 kg) which were thyroidectomized at the start of the breeding

season (March and April) (THX, n = 10) or remained euthyroid as controls. A further control hind was added in August. Two stags (mean live weight in December 154.3 ± 4.3 kg) were used to enable detection of oestrus. One stag was vasectomized in April and used to enable detection of oestrus during the breeding season and non-breeding season, the other remained fertile and was used for out-of-season breeding. Both stags were thyroidectomized in late June to prevent the seasonal decline in reproductive traits such as testosterone concentration, testis diameter and responsiveness of the pituitary gland and testes to exogenous GnRH (Shi and Barrell, 1992).

Experimental Design

To test if thyroidectomized hinds exhibit overt oestrus and develop a functional corpus luteum following oestrus synchronization in the breeding season, ovulation was synchronized in all hinds in May by treating with a controlled internal drug releasing device (CIDR Type G, InterAg, Hamilton, NZ) containing 0.3 g progesterone intravaginally from 15 days previously, renewed after 10 days (Fennessy *et al.*, 1990). CIDRs were inserted while hinds were restrained in a crush using a modified CIDR applicator with a 10 cm barrel. A vasectomized stag was run with the hinds from 2 days before until 6 days after CIDR withdrawal. Hinds were continuously observed for oestrus and mating from 15 to 100 hours after CIDR withdrawal, and the occurrence of ovulation was determined by laparoscopy 8 days after CIDR withdrawal, as described in Chapter 3, section 3.2. Heart rate was measured 12 days after CIDR withdrawal. To test if the synchronization treatment described above was equally effective during the non-breeding season, hinds were again synchronized for ovulation in late October, and oestrus, ovulation and heart rate were recorded as before.

To test if out-of-season conception can be induced in thyroidectomized hinds following mating by a thyroidectomized stag, hinds were synchronized as above in early December, except that 200 I.U. PMSG (Folligon, Intervet International, Boxmeer, Holland) was administered intramuscularly at the time of CIDR withdrawal. A presumably fertile (non-vasectomized) stag was substituted for the vasectomized stag, and hinds were monitored for oestrus and mating from 10 to 80 hours after CIDR withdrawal and for ovulation at 14 days after CIDR withdrawal. Any hinds not mated on this occasion were immediately re-synchronized with an 11 day CIDR treatment

(CIDRs renewed after 9 days) in conjunction with 750 μg i.m. cloprostenol (Estrumate, Pitman-Moore, Upper Hutt, NZ) at the time of CIDR renewal and 400 I.U. i.m. PMSG at the time of CIDR withdrawal. Hinds were observed for oestrus from 10 to 100 hours after CIDR withdrawal. Ovulation was not checked for on this occasion.

Plasma progesterone concentration was measured in twice-weekly blood samples, except for periods during and for 1 month following CIDR treatment, when hinds were blood sampled thrice-weekly for plasma progesterone concentration. Pelage score was assessed each month.

Data analysis

For calculation of the number of oestrous cycles during the breeding season, the date used for the transition from the breeding season to the non-breeding season was 5 September (based on results of Meikle and Fisher, 1996). Treatment effects on the number of hinds in each group which showed oestrus or ovulated were determined by a chi-squared test for differences between proportions. All other effects were identified by one-way ANOVA.

8.3 Results

Two THX hinds died during cold weather in June and September. A decline in body condition had been noted in both hinds, and they were preferentially fed lush pasture throughout the winter. The hind which died in June was replaced immediately with a newly thyroidectomized hind; the hind which died in September was not replaced. All data from these hinds were included in the statistical analysis.

Mean plasma T_3 concentrations were 1.5 ± 0.2 nmol.l^{-1} in euthyroid control hinds and low or undetectable (0.1 ± 0.1 nmol.l^{-1}) in THX hinds ($P < 0.001$). In control hinds, mean plasma T_3 concentrations were lower in July (1.1 ± 0.1 nmol.l^{-1}) than in January (1.8 ± 0.2 nmol.l^{-1}) ($P < 0.01$). In control hinds, growth of summer pelage (as evidenced by pelage scores greater than 1) began on 16 November ± 5.7 days. Summer pelage growth was delayed in 6/9 THX hinds (11 December ± 4.0 days, $P <$

0.05), and in 3/9 THX hinds there was no evidence of summer pelage growth by the end of the experiment (February) (Plate 8.1). Mean pelage scores at the end of the experiment were 5.0 ± 0.0 in control hinds and 3.3 ± 0.6 in THX hinds.

Between early May and early September, THX hinds a similar number of progesterone episodes to the control hinds (3.6 ± 0.7 and 4.6 ± 0.4 respectively; $P > 0.05$), but in 3 THX hinds progesterone episodes were evident only following CIDR treatment (see example in Figure 8.1, panel d). Overt oestrus was recorded in fewer THX hinds than control hinds in the period following CIDR withdrawal in May ($P < 0.05$). A similar trend was observed for the occurrence of ovulation, but the difference was not significant ($P > 0.05$). In control hinds, the occurrence of oestrus and ovulation was lower in the period following CIDR withdrawal in October compared with July ($P < 0.05$), however in THX hinds there was no significant difference between the two seasons in the proportion of hinds exhibiting oestrus and ovulation ($P > 0.05$) (Table 8.1). The interval from CIDR withdrawal until the onset of oestrus was not affected by thyroidectomy or season (64.7 ± 4.5 h; $P > 0.05$) and the duration of oestrus were not affected by thyroidectomy during May (10.7 ± 2.4 hours; $P > 0.05$). In the 3 THX hinds which exhibited overt oestrus during October, the duration of oestrus tended to be much shorter (0.5 ± 0.4 h) than in May, but this effect did not reach statistical significance due to low numbers ($P < 0.07$).

Mean heart rates in THX hinds were lower in May than in October (45.4 ± 2.3 and 54.2 ± 2.6 respectively, $P < 0.05$) but were not significantly lower than heart rates in control hinds at either of these times (50.8 ± 3.1 and 55.5 ± 2.5 respectively) ($P > 0.05$).

Table 8.1 Percentages of hinds in each group showing oestrus or ovulating following CIDR withdrawal in May and October. Within a parameter, values not assigned common superscript letters are significantly different ($P < 0.05$).

Group	Oestrus		Ovulation	
	May	October	May	October
Control ($n = 6$)	100.0^{a}	0.0^{b}	100.0^{a}	0.0^{c}
THX ($n = 9$)	44.0^{b}	33.3^{b}	55.0^{ab}	44.4^{bc}

Plate 8.1 Representative THX (left) and euthyroid control (right) hinds during summer (December). Note that the THX hind has not moulted the pale winter coat.

In early December, the occurrence of oestrus and ovulation was low in both groups following CIDR withdrawal and injection of 200 I.U. PMSG; only 2 matings (both with THX hinds) were recorded. Upon ultrasonic pregnancy diagnosis, one of these 2 hinds was found to be pregnant. However when all other hinds were resynchronized with cloprostenol and progesterone CIDRs in conjunction with 400 I.U. PMSG, the occurrence of oestrus increased in both groups ($P > 0.05$) (Table 8.2). Time to onset and duration of oestrus (48.6 ± 3.2 h and 3.2 ± 1.1 h respectively) were not affected by thyroidectomy or dose of PMSG ($P < 0.05$). In 3/3 control and 2/5 THX hinds which were mated following 400 I.U. PMSG, successful pregnancies were recorded as indicated by the presence of foetuses and placentomes upon ultrasonic examination. Foetal crown to rump measurement averaged 26.0 ± 0.6 mm on day 45 and 56.0 ± 0.0 mm on day 58 after mating; these values were within the normal range reported by Bingham *et al.*, (1990) indicating normal foetal development. Establishment of pregnancy was associated with a sustained increase in plasma progesterone concentration beginning soon after mating and continuing until sampling

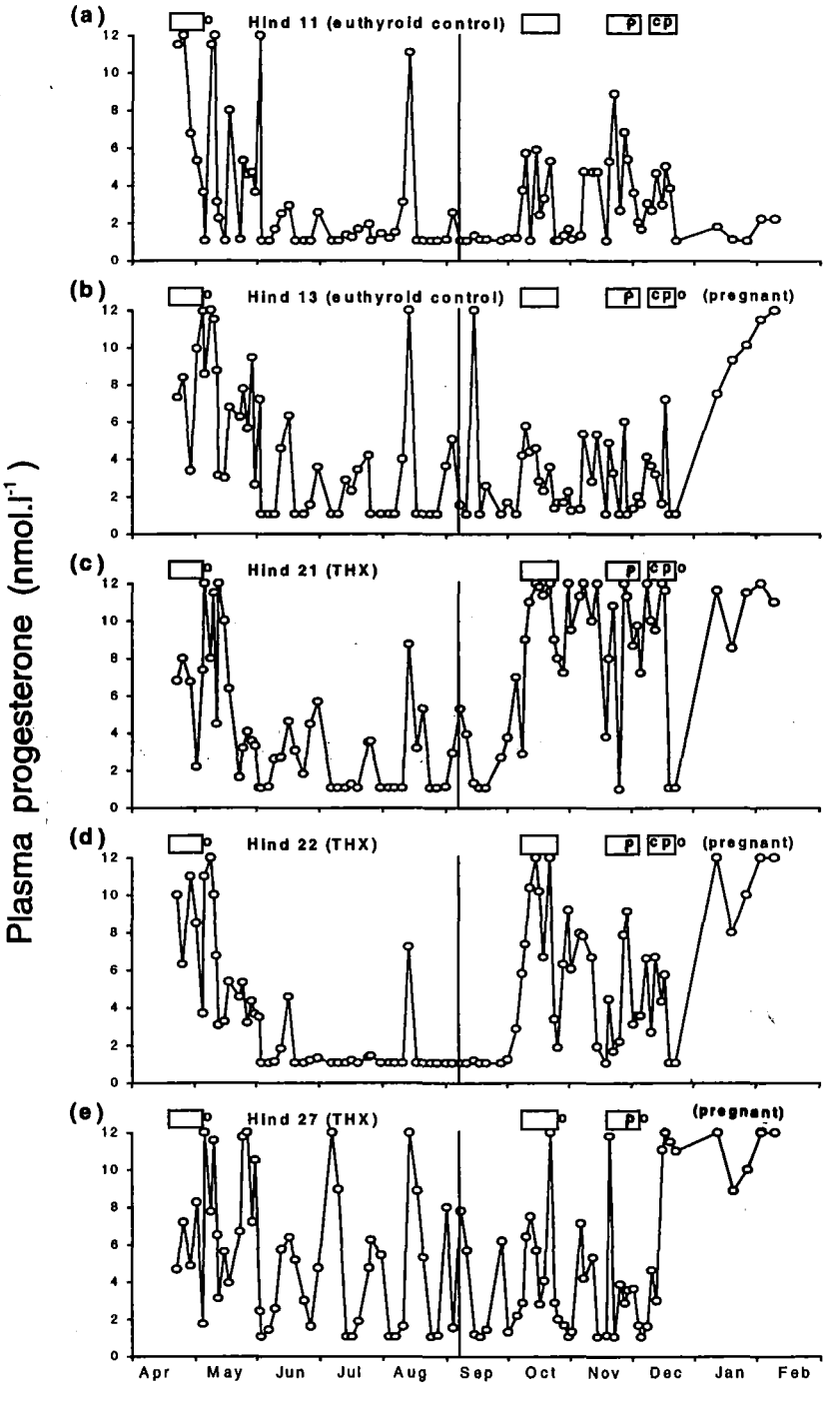

Figure 8.1 Representative individual profiles of plasma progesterone concentration for euthyroid control (panels a and b) and THX (panels c, d and e) hinds. Vertical bars denote the end of the breeding season for red deer in New Zealand. Open boxes denote periods of CIDR insertion. c, injection of 750 μg cloprostenol; p, injection of 200 or 400 I.U. PMSG; o, oestrus and ovulation recorded; (pregnant), conceptus identified by ultrasound.

ceased in early February (see examples in Figure 8.1, panels b, d and e). This pattern was also evident in all 4 THX hinds which were mated by the fertile stag during December but were not diagnosed as being pregnant by ultrasonic examination (see example in Figure 8.1, panels c), suggesting that conception occurred but the resultant embryos or foetuses were aborted prior to ultrasonic examination.

Table 8.2 Percentages of hinds in each group exhibiting oestrus or ovulating following CIDR withdrawal in December and injection of 200 I.U. or 400 I.U. PMSG. Within a parameter, values not assigned common superscript letters are significantly different ($P < 0.05$).

Group	% Oestrus		% Ovulation	
	200 I.U.	400 I.U.	200 I.U.	400 I.U.
Control ($n = 6$)	0.0^{a}	50.0^{bc}	33.3^{a}	not measured
THX ($n = 9$)	22.2^{ab}	71.4^{c}	22.2^{a}	not measured

8.4 Discussion

The results of this experiment indicate that thyroidectomy impairs ovarian function in female red deer, since fewer THX hinds exhibited oestrus following synchronization with progesterone during the breeding season and there was a non-significant decline in ovulation rate compared with control hinds. In the non-breeding season the occurrence of oestrus and ovulation was maintained at a similar level in THX hinds, while in control animals oestrus and ovulation ceased to occur following progesterone withdrawal. Collectively these results imply that although thyroid gland secretions inhibit reproduction during anoestrus (as was also shown in Chapter 5), they also have a supportive effect on the hypothalamo-pituitary-gonadal axis for the occurrence of ovulation and overt oestrus behaviour during the breeding season.

It is possible that changes in metabolic rate may trigger changes in reproductive function (Kennedy and Mitra, 1963). Thyroid hormones are involved in a wide variety of metabolic functions, and a reduced metabolic rate could have contributed to

the poor reproductive performance of THX hinds in the current experiment However because heart rate was not significantly lowered by thyroidectomy in either season, it cannot be determined from these results whether lowered metabolic would have had a major influence on reproductive performance of THX hinds.

The published literature on the thyroid hormone requirements for successful reproduction suggest considerable variation exists between species. In female cattle, the incidence of overt oestrus following progesterone and oestradiol treatment was higher in thyroidectomized cows than euthyroid cows, although measures of the intensity of oestrus showed no effect of thyroidectomy (Stewart *et al.*, 1993). In contrast, behavioural signs of oestrus were reduced in thyroidectomized mares (Lowe *et al.*, 1987). Similarly, Reddy *et al.* (1996) observed a suppression of behavioural oestrus in female goats induced to hypothyroidism with thiourea and Walkden-Brown *et al.* (1996) reported cessation of oestrous cyclicity in this species following thyroidectomy. Silent ovulations in Egyptian water buffalo were associated with significantly reduced serum T_3 and T_4 concentrations (Boradady *et al.*, 1985). In rats, ovulation rate was reduced following ^{131}I-radiothyroidectomy (Mattheij *et al.*, 1995). Data from several studies in ewes suggest that oestrous cyclicity is not dependent on the thyroid gland (Falconer, 1963; Brooks *et al.*, 1965; Nicholls *et al.*, 1988b; Peeters *et al.*, 1989), although Brooks *et al.* noted a reduction in twinning and lower lamb birth weights in thyroidectomized ewes.

Since preovulatory plasma LH concentration was not measured in this experiment, it is not possible to determine if thyroid gland secretions are required at the hypothalamo-hypophyseal level for generation of the increase in gonadotrophin secretion which culminates in the LH surge (Caraty *et al.*, 1995), or at the ovarian level for follicular steroid production. The administration of a high dose of PMSG to stimulate follicle development increased the incidence of overt oestrus and ovulation in hinds in the current experiment. While initially this might imply that the effects of thyroidectomy on oestrous cyclicity were due to inadequate secretion of pituitary hormones during the follicular phase, it should be noted that the dose of PMSG

required to stimulate ovulation in the current study was higher than that normally employed for successful induction of oestrus and ovulatory responses 2-3 weeks before the onset of natural mating activity (Asher *et al.*, 1994b). This may indicate a reduction in ovarian responsiveness to gonadotrophin stimulation in THX hinds. The results of other studies also strongly suggest an ovarian site of action of thyroid hormones. For example, induction of hypothyroidism was associated with reduced oestradiol and progesterone concentrations and impaired oestrous cyclicity in goats (Reddy *et al.*, 1996) and lowered steroid metabolism in growing follicles and corpora lutea in rats (Mattheij, 1995). *In vitro*, T_3 has been shown to stimulate progesterone formation in ovarian follicles (Bhattacharya *et al.*, 1996), and T_3 and T_3 augment FSH-stimulated processes such as LH receptor induction by granulosa cells (Maruo *et al.*, 1987).

A surprising finding in this experiment is the efficacy of a high dose of PMSG in inducing reasonably successful (50%) out-of season pregnancies in euthyroid control hinds. There are no published reports of attempts to achieve breeding of red deer this far out-of-season using progesterone and PMSG, however when administered in February or March this treatment typically results in 10-20% of treated hinds calving despite induced ovulation rates of 70-85% (e.g. Fisher *et al.*, 1986; Moore and Cowie, 1986; Fennessy *et al.*, 1986; Fennessy and Fisher, 1988). These low calving percentages have been attributed in part to low fertility of stags in February and early March (Fennessy and Fisher, 1988), since in one study fertility was improved (59% of progesterone/PMSG-treated hinds calving) when melatonin-treated stags were used (Moore, 1987), and in another study 79% of progesterone/PMSG-treated hinds conceived following artificial insemination in March (Fennessy *et al.*, 1991). The current results lend some support to this argument, since the stag used for mating in December exhibited rutting behaviour characteristic of the breeding state, and previous studies have shown that thyroidectomized stags do not experience a seasonal decline in reproductive traits such as testosterone concentration, testis diameter and responsiveness of the pituitary gland and testes to exogenous GnRH (Shi and Barrell, 1992).

Another possible reason for successful out-of-season pregnancy in these euthyroid hinds may be social stimulation of ovarian activity. For example, oestrus and ovulation was induced in anovulatory ewes (Zarco *et al.*, 1995) and goats (Restall *et al.*, 1995) by exposure to females induced to oestrus. Such a social cue may have been provided by cycling THX females in the current experiment. Furthermore, the presence of a stag in which rutting behaviour was advanced by melatonin treatment has been shown to advance the breeding season in adult red deer hinds (Moore and Cowie, 1986; Wilson, 1992), and in yearling hinds the timing of puberty was slightly advanced if they were reared with a stag in the same paddock (Fisher *et al.*, 1995). In the current experiment, the hinds which were mated in late December had been kept in one paddock with either a vasectomized or fertile rutting stag for over 2 months. Both stags showed typical herding behaviour during this time and interacted closely with the hinds. This contact would presumably have provided powerful olfactory, visual and auditory stimuli for reproductive activity in the hinds. Such stimulatory effects of male animals on reproduction in anovulatory females have been described for sheep (Martin *et al.*, 1986). The argument that successful out-of-season breeding in euthyroid hinds in the current experiment may have been achieved at least in part by social stimulation and mating with a fertile stag implies that the current inability to alter markedly the breeding season of deer on farms is not primarily due to a lack of responsivenss of the hind to hormonal treatments. The use of thyroidectomized stags to provide stimulatory social cues to the hinds and for out-of-season mating could prove to be a useful tool for further investigating this possibility and for future out-of-season breeding studies.

In summary, while thyroidectomized hinds did not display a seasonal decline in ability to ovulate or display oestrus behaviour following synchronization with progesterone, the incidence of ovulation and overt oestrus was low in both seasons, suggesting that a side-effect of thyroidectomy may be impaired fertility. Following gonadal stimulation with a high dose of PMSG and joining with a fertile, rutting stag, six out-of-season pregnancies were obtained from eight matings. However because three of these

pregnancies occurred in euthyroid control hinds no improvement in out-of-season reproductive performance could be attributed to thyroidectomy. An important implication of these results is that before the effects of the thyroid glands on seasonal reproduction can be exploited to achieve practical out-of-season breeding, techniques must first be developed for blocking their specific effects on the reproductive neuroendocrine centres without necessitating the induction of hypothyroidism.

Chapter 9

General Discussion

The primary objective of the studies described in this thesis was to improve our understanding of the ways in which the thyroid gland influences seasonal cycles of reproduction in the red deer hind. Collectively the experiments have shown that thyroid hormones have a specific role in permitting steroid-independent suppression of seasonal reproductive activity, and they suggest that the steroid-independent pathways play a relatively important role in the control of seasonal breeding in female red deer. As a focus for the discussion in this chapter, a conceptual model of the role of the thyroid glands in modulating steroid-dependent and steroid-independent suppression of reproduction during the non-breeding season is presented for red deer hinds (Figure 9.1). Each numbered site on this model is discussed below in relation to the results of the studies in this thesis.

Several important species differences in neuroendocrine control of seasonality between the red deer hind and the domestic ewe (a species which has been used much more extensively than the hind as an animal model for experiments on seasonal reproduction) have become apparent from these experiments. Since techniques used for advancing the onset of seasonal breeding in sheep are relatively ineffective in deer, an understanding of how control of seasonal reproduction differs between these species will aid in the development of manipulative techniques that are appropriate to red deer. Therefore, comparisons between deer and sheep are made in relation to the conceptual model.

Chapter 4 describes two experiments which were conducted using ovariectomized oestradiol-implanted females as the animal model to study the effects of thyroidectomy and timing of thyroid hormone replacement on seasonal neuroendocrine reproduction. This model is commonly used in similar studies in ewes (e.g. Moenter *et al.*, 1991; Thrun *et al.*, 1997), since seasonal reproductive quiescence is usually attributed primarily to the dramatic increase in negative feedback of oestradiol on the hypothalamic GnRH pulse generator (e.g. Karsch *et al.*,

1993). A decrease in mean plasma concentrations of LH were recorded in all hinds regardless of timing or dose of thyroid hormone treatment. This provided initial evidence that thyroid hormones are not involved in the steroid-dependent suppression of LH (Site 1 in Figure 9.1) in female red deer, and this finding was confirmed in two following experiments using euthyroid and thyroidectomized ovariectomized oestradiol-treated hinds without thyroid hormone replacement (Chapters 5 and 7). Oestradiol retained the ability to suppress GnRH pulse frequency (as indicated by LH pulse frequency measurements) in thyroidectomized hinds during the non-breeding season. Oestradiol also decreased LH pulse amplitude; but since GnRH was not measured it is not possible to say if this reflects a seasonal loss of pituitary responsiveness to GnRH (Meikle and Fisher, 1996) or a direct effect of oestradiol on GnRH pulse amplitude. A reduction in GnRH pulse amplitude by oestradiol during the non-breeding season would be inconsistent with the effects of this steroid in the ovariectomized oestradiol-treated ewe, which exhibits high amplitude, low frequency pulses of GnRH during the non-breeding season in response to oestradiol (Karsch *et al.*, 1993).

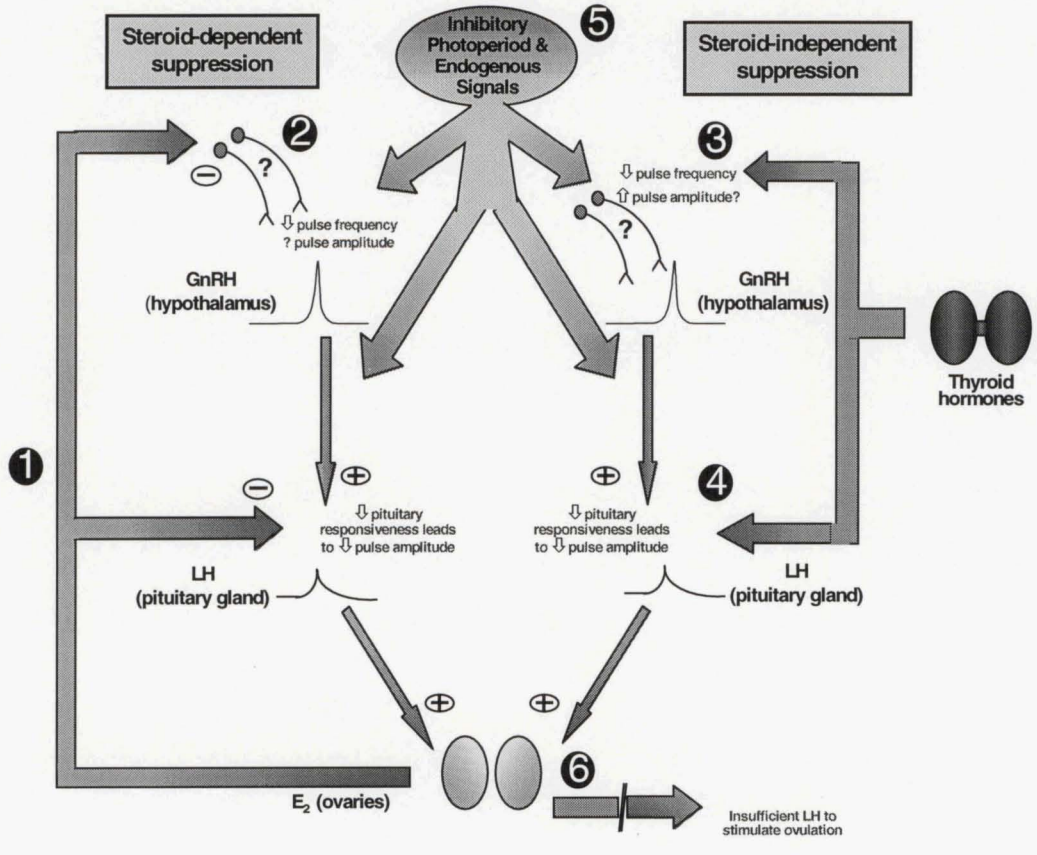

Figure 9.1 A conceptual model of the role of thyroid hormones in steroid-dependent and steroid-independent suppression of reproduction during the non-breeding season in red deer hinds. Numbered points are discussed in the text.

The lack of effect of thyroidectomy on seasonal fluctuations in oestradiol negative feedback contrasts with a growing body of literature from Karsch and his coworkers (e.g. Moenter *et al.*, 1991; Webster *et al.*, 1991a; Dahl *et al.*, 1995b) which clearly demonstrate a permissive role for thyroid hormones in oestradiol-induced suppression of LH concentrations. It has been hypothesized that seasonal changes in output of the GnRH neurosecretory system in the ovariectomized oestradiol-treated ewe could result at least in part from a thyroid hormone dependent seasonal rearrangement of synaptic inputs onto GnRH neurons (Karsch *et al.*, 1995). In the red deer hind, thyroid hormones may target different neural pathways which do not mediate oestradiol feedback, or alternatively an entirely different set of neurons may convey oestradiol feedback in deer, which are not responsive to thyroid hormones. The absence of an LH response to a dopaminergic-D_2 receptor agonist or antagonist in hinds (Chapter 6) is evidence that the latter possibility may be the case, since dopaminergic neurons are known to mediate oestradiol negative feedback in sheep (Meyer and Goodman, 1985; Halvern *et al.*, 1991; Tortonese and Lincoln, 1994a; 1994b; Viguié *et al.*, 1991). Further evidence that dopamine may not be involved in LH suppression in deer comes from the demonstration that suppression of circulating plasma prolactin concentrations in red deer hinds by long-acting bromocriptine treatment causes a delay of the onset of anoestrus (Curlewis *et al.*, 1988), a result which would appear paradoxical if this DA-D_2 agonist also exhibited a direct inhibitory action on the GnRH neurosecretory system. The possibility that oestradiol does not require mediation by interneurons (indicated at Site 2 in Figure 9.1) to influence GnRH secretion in deer cannot be ruled out; however the consistent finding of an absence of oestradiol receptors on GnRH neurons across a diverse range of species (Herbison, 1995) suggests that such a direct action is unlikely.

In contrast to the lack of an effect of thyroidectomy in the presence of oestradiol implants, it was shown that the dramatic decrease in circulating plasma LH concentrations that occurs during the middle of the non-breeding season in ovariectomized red deer hinds in the absence of oestradiol did not occur in thyroidectomized hinds (Chapter 5), and this steroid-independent LH suppression was able to be restored by an appropriately timed thyroxine replacement treatment (Chapter 7). Plasma LH pulse frequency remained high during the non-breeding

season in thyroidectomized hinds, indicating that the thyroid gland is required for the seasonal decline in frequency of GnRH pulses (Site 3, Figure 9.1). Neither dopamine or endogenous peptides appear to play a major role in this suppression (see Chapter 6), however the inhibitory effects of the serotonin antagonist cyproheptadine provided preliminary evidence for a stimulatory role of serotonergic neural pathways in the control of LH secretion the in ovariectomized red deer hind, as has also suggested for the ewe (Deaver and Dailey; 1982). Three pivotal questions to be answered in relation to a role for serotonin are: does serotonin stimulate LH secretion; can a seasonal increase in serotonergic stimulation explain any or all of the increase in plasma LH concentrations and pulse frequency during the breeding season, and are the steroid-independent effects of thyroidectomy attributable to a blockade of a seasonal decline in serotonergic stimulation? Experiments which will address these questions (not a part of this thesis) are currently being conducted.

In Chapter 7, it was shown that thyroidectomy reduced but did not completely abolish the seasonal decline in pituitary responsiveness to GnRH, indicating a participatory role for thyroid hormones in this process (Site 4, Figure 9.1). Low-dose thyroxine replacement (Chapter 7) did not entirely overcome this effect of thyroidectomy, suggesting that the action of thyroid hormones on pituitary responsiveness to GnRH may be dose responsive. Measurements of pulsatile LH concentration in ovariectomized hinds (described in Chapters 6 and 7) suggested that the low-amplitude of LH pulses during the non-breeding season may be a result of reduced pituitary GnRH responsiveness, since thyroidectomized hinds in which pituitary responsiveness was partially restored had very large amplitude LH pulses, similar to those that occur in ovariectomized anoestrous ewes (Karsch *et al.*, 1980; Goodman and Karsch, 1982; Moenter *et al.*, 1991). A low releasability of LH from the pituitary gonadotrophs could partly explain the failure of attempts to advance the breeding season of red deer hinds using GnRH infusions (Fisher and Fennessy, 1985; Fisher *et al.*, 1986; Moore and Cowie, 1986) or injections of a GnRH analogue (Duckworth and Barrell, 1988; 1991).

The study described in Chapter 7 sought to identify the period during which the steroid-independent mechanisms of gonadotrophin suppression were responsive to thyroid hormones. The results of that experiment suggest that thyroid hormones exert

their action only during inhibitory photoperiods and the inhibitory phase of the endogenous rhythm (Site 5, Figure 9.1). This conclusion has practical implications in relation to the possible exploitation of the thyroid gland to achieve out-of-season reproduction. For example, any treatment which suppressed or blocked the effect of thyroid hormones would presumably have to begin before the transition to the non-breeding season and continue throughout the inhibitory photoperiods of summer until the desired time of conception.

The degree of steroid-independent suppression is directly correlated with the length of both natural and artificially manipulated photoperiods in the red deer hind (Fisher and Meikle, 1995; Meikle and Fisher, 1996) and the ewe (Robinson *et al.*, 1985), although seasonal changes in LH pulse frequency were still observed in ovariectomized ewes held on constant long photoperiods (Robinson *et al.*, 1985). Because the experiment described in Chapter 7 was conducted outdoors under natural lighting, it was not possible to determine if the 'window' of steroid-independent responsiveness to thyroid hormones is timed by inhibitory photoperiods or the inhibitory phase of the endogenous rhythm, or both. If thyroxine could be shown to exert its action only during the non-breeding season in hinds that were devoid of photoperiodic cues, this would provide strong evidence for the existence of an extant endogenous rhythm in thyroidectomized animals, the expression of which was constituitively dependent on the presence of thyroid hormones.

The finding that the transition to anoestrus in cycling red deer hinds (Chapter 5) can be prevented by thyroidectomy, which disrupts steroid-independent but not steroid-dependent processes, strongly suggests that the former mechanisms are at least as important in regulating the seasonal occurrence of ovarian cyclicity and ovulation (Site 6, Figure 9.1) as the former in this species. This is in contrast to the commonly proposed model for the ewe, whereby steroid-dependent regulation is considered to be the major determinant of seasonality (e.g. Karsch *et al.*, 1993). However thyroidectomy had little effect on the occurrence of mating behavior or ovulation following progesterone priming in the non-breeding season (Chapter 8), and did not improve the number of out-of-season pregnancies when compared with euthyroid control hinds. During the breeding season, mating behavior and ovulation following progesterone treatment tended to be reduced by thyroidectomy. These results suggest

that while thyroidectomy can overcome steroid-independent seasonal reproductive inhibition, a reduction in fertility may be a side-effect of hypothyroidism. Therefore, development of technologies to induce out-of-season breeding in red deer hinds by exploiting the role of thyroid hormones should be focused on identifying and blocking their proximal effects on the hypothalamic GnRH pulse generator and anterior pituitary gland, rather than simply reducing their concentrations in general circulation.

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Appendix

Many of the results of the experiments in this thesis have been presented at conferences and been published as conference abstracts, and several full papers have, at the time of writing, been submitted to journals for publication. These are listed below.

Anderson, G.M. and Barrell, G.K. (1995). LH secretion in thyroidectomized, ovariectomized red deer hinds given oestradiol, GnRH and thyroid hormones. *Endocrine Society of Australia Proceedings* **38:** 159.

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