Regulation of reproductive seasonality in the red deer hind: oestradiol-dependent and -independent influences on the patterns of LH concentrations

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The control of reproductive seasonality was studied in farmed adult red deer hinds that had been either ovariectomized or ovariectomized and oestradiol-treated (s.c. implants). The breeding season, delineated by progesterone secretion in intact hind herdmates, was characterized by high (mean 0.6, range 0.1–2.5 ng ml⁻¹ plasma) LH concentrations in ovariectomized oestradiol-treated hinds. In contrast, during the non-breeding season plasma LH concentrations in these animals were significantly lower (mean 0.1, range 0–0.9 ng ml⁻¹ plasma). LH secretion in ovariectomized untreated hinds also displayed a marked seasonal pattern, approximately the inverse of daily photoperiod (that is, a winter peak and summer trough). The pituitary LH response to 10 µg exogenous GnRH was also maximal during the breeding season in ovariectomized (mean 7.4, range 1.2–14.6 ng ml⁻¹) and ovariectomized, oestradiol-treated (mean 16.4, range 1.4–32.3 ng ml⁻¹) hinds. These results indicate that LH secretion in the hind is regulated by both steroid-dependent and -independent mechanisms.

Introduction

Our understanding of the mechanisms that control reproductive seasonality in female farm animals has largely been derived from studies in ewes (see Karsch *et al.*, 1984). However, domestication may alter some reproductive parameters, including seasonality (see Setchell, 1992). Thus the underlying physiological mechanisms in sheep may differ from those in other species. For example, throughout seasonal anoestrus, the ovariectomized ewe retains the ability to produce a preovulatory-like LH surge in response to exogenous oestradiol (Land *et al.*, 1976; Howland *et al.*, 1978; Goodman *et al.*, 1981; Haresign and Friman, 1983), but it has been demonstrated that this response is absent during some stages of anoestrus in ovariectomized red (Meikle and Fisher, 1990) and fallow (Jabbour *et al.*, 1992) deer. Furthermore, there is limited evidence of a marked seasonal pattern of LH secretion in ovariectomized red (Meikle and Fisher, 1990) and fallow (G. W. Asher, unpublished data) deer. These patterns are not as clearly evident in ovariectomized ewes (Legan *et al.*, 1977; Goodman *et al.*, 1982; Webster and Haresign, 1983; Robinson *et al.*, 1985). Although there are some important breed differences in both the negative and positive effects of oestradiol on LH secretion during anoestrus in ewes (Land *et al.*, 1976; McNeilly *et al.*, 1985; Thomas *et al.*, 1988), Karsch *et al.* (1984) suggested that seasonal anoestrus in ewes is a relatively uniform physiological state. In contrast, in Père David's hinds pituitary LH responsiveness to GnRH and its efficacy in inducing ovarian activity vary

significantly with season (Curlewes *et al.*, 1991; McLeod *et al.*, 1991).

In ewes, reproductive seasonality is related to a change in the sensitivity of LH secretion to the negative feedback action of oestradiol and this is concomitant with the onset and cessation of the breeding season (Legan *et al.*, 1977; Karsch *et al.*, 1984). However, the possible contribution of steroid-independent mechanisms to the control of reproductive seasonality is not well understood.

The present study was undertaken to test the hypothesis that in ovariectomized red deer hinds, the seasonal patterns of LH concentrations are indicative of both steroid-dependent and -independent neuroendocrine control mechanisms. Furthermore, the possible contribution of pituitary responsiveness to seasonal LH concentrations was assessed with exogenous GnRH.

Materials and Methods

Experimental summary

The experiment used 24 adult, farmed red deer (*Cervus elaphus*) hinds, allocated to the following treatment groups (eight per group): (1) ovariectomized; (2) ovariectomized and treated with oestradiol administered via subcutaneous implants, and (3) intact hinds. In sheep, gonadotrophin secretion in ovariectomized animals is influenced by time from ovariectomy (Montgomery *et al.*, 1985; Joseph *et al.*, 1992), as well as by season (Goodman *et al.*, 1982; Robinson *et al.*, 1985); therefore, hinds with two different histories of ovarian removal were used. Blood samples were collected once a week over the

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following 15 months (February 1990 to April 1991) and plasma concentrations of LH determined. In addition, the pituitary LH responsiveness to exogenous GnRH was assessed every 4 weeks, while the timing and duration of the breeding season in intact herd mates was determined by monitoring ovarian progesterone secretion.

Animal management, ovariectomy and oestradiol treatment

The hinds were run together as a single group, outdoors (latitude 45°53'S) under normal farm management (except that access to stags was denied) on predominantly ryegrass-white clover pasture, supplemented with meadow hay in winter. At the beginning of the experiment mean ($\pm$ SEM) ages and masses were 12 ± 0.4 years and 114 ± 2.2 kg, respectively. Ovariectomies were performed via midline laparotomy under general anaesthesia induced i.v. with 1.5 mg fentanyl citrate and 12 mg azaperone (Fentaz: Smith, Kline and French (NZ) Ltd, Auckland) and 77 mg xyaline hydrochloride (Rompun: Bayer NZ Ltd, Petone) and reversed i.v. with 25 mg yohimbine (Mackintosh and Van Reenen, 1984) and 0.2 mg naloxone hydrochloride (Narcan: Endo Laboratories, Artarmon, NSW). Half of the ovariectomized hinds had undergone ovariectomy 2.3 years earlier (May, 1987); these animals had previously been exposed for short periods to progestagens, oestrogen or ACTH treatment (see Jopson *et al.*, 1990; Meikle and Fisher, 1990). The other half underwent ovariectomy at the beginning of the experiment (February, 1990). Silastic implants were prepared from 0.335 cm inner $\times$ 0.465 cm outer diameter silastic tubing (Dow Corning Corp., Midland, MI) 5 cm in length (of which 0.5 cm was plugged at each end) containing oestradiol (Sigma Chemical Co., St Louis, MO) as described by Karsch *et al.* (1973). Ovariectomized hinds that did not receive oestradiol treatment received empty implants. Implants were soaked separately in water overnight, and were immersed in 70% ethanol for at least 30 min before insertion. Implants were inserted s.c. into the inguinal region under general anaesthesia, on 27 February 1990 (long-term ovariectomized hinds) or during ovariectomy (short-term ovariectomized animals) on 20 February 1990.

Blood sampling

Blood samples (10 ml) were taken by jugular venepuncture, with the hinds physically restrained in a compressed air operated deer crush. Blood was collected into heparinized tubes and the plasma removed and stored at -20°C until analysed. Blood samples were taken once a week from all hinds for the duration of the experiment (22 February 1990 to 24 April 1991) and twice a week from entire hinds at about the expected time of the breeding season (March to November). In addition, at intervals of approximately 4 weeks, all animals were treated with GnRH (10 μg ; Sigma Chemical Co.; made up at the start of the study and frozen in a concentrated form) administered i.v. in saline, immediately after sampling and a further sample was taken 15 min later. This limited sampling protocol was used because of the relatively intractable nature of some farmed animals and was considered appropriate since other studies have revealed that after GnRH treatment, LH concentrations

peak at 15 min (red hinds: M. W. Fisher and B. J. McLeod, unpublished data; Père David's hinds: Curlewis *et al.*, 1991).

Hormone analyses

Plasma LH concentrations were measured using a heterologous double-antibody radioimmunoassay developed in our laboratory. The antiserum (R2), raised in a rabbit against highly purified ovine LH (NIAMDD-oLH-24), was used at an initial dilution of 1:60 000. The antiserum exhibited low ($< 1\%$) crossreactivity with ovine and cervine growth hormone and bovine thyroid stimulating hormone and little reactivity ($< 0.1\%$) with ovine FSH or ovine prolactin. The reference preparation and iodinated tracer were the highly purified ovine LH CY1085 (biopotency $3.45 \times \text{NIH-LH-S1}$). Standards were made up in 0.1% (w/v) BSA phosphate buffer to which 100 μl of cervine plasma containing low LH (obtained from medroxyprogesterone acetate-treated hinds; Sigma Chemical Co.) was added. Interassay coefficients of variation for plasma pools containing 0.2, 1.5 and 4.5 ng ml^{-1} were 16.6%, 6.7% and 4.6%, respectively. The intra-assay coefficient of variation calculated on 30 duplicate pairs per assay ($n = 6$) was 9.1%. The limit of assay sensitivity, defined as the apparent concentration at two standard deviations above the zero standard, averaged 0.05 ng ml^{-1} . Parallelism was demonstrated by serial dilutions of hind plasma in assay buffer. Progesterone concentrations were determined, by solid phase ^{125}I radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA) as described by Jopson *et al.* (1990). All samples were completed within one assay and the coefficient of variation on a plasma pool containing 3.50 ng ml^{-1} measured 18 times was 13.4%. The limit of assay sensitivity was 0.08 ng ml^{-1} .

Statistical analyses

The onset of the breeding season for an individual hind was defined as the date when plasma progesterone concentrations first exceeded 0.5 ng ml^{-1} and remained above that value for at least 7 days. The end of the breeding season was determined as the last date on which progesterone concentrations fell, and remained below 0.5 ng ml^{-1} for at least 7 days. Furthermore, there had to be evidence of a regular cyclic pattern (approximately 17–21 day ovarian cycles) in individual hinds.

The variances were equalized by log transforming LH results and the mean concentrations for breeding and anoestrous season were analysed by analysis of variance, fitting terms for oestradiol treatment, history of ovarian removal (long-term or short-term ovariectomized), and their interaction. In addition, a comparison of the patterns of basal LH secretion between ovariectomized and ovariectomized oestradiol-treated hinds was made by analysis of variance of the concentrations obtained on each sampling occasion, and by comparing the areas under the curve between the peak in the first breeding season and the nadir during anoestrus and between the nadir and second breeding season. Patterns of LH concentrations during anoestrus were further analysed with regard to the time that peripheral plasma concentrations first fell below, or increased above, 0.2 ng ml^{-1} and remained at these values for two samples or more. In the analysis of the response to GnRH,

Fig. 1. Plasma progesterone profiles for four individual, intact, red deer hinds sampled once a week (non-breeding season) or twice a week (breeding season). (a) and (d) are representative of most (6 of 8) animals with the remaining two hinds (b) and (c) having patterns in which it was not possible to distinguish between breeding and non-breeding seasons.

baseline LH concentrations (time 0) were subtracted from the sample taken 15 min after GnRH treatment for each hind. All results are expressed as the mean and range or as the mean $\pm$ SEM.

Results

Breeding and non-breeding seasons

The 1990 breeding season, during which six of eight hinds had seven to nine ovarian cycles, began at a mean date of

29 March $\pm$ 1.4 days and ended on 4 September $\pm$ 8.4 days (Fig. 1). Seasonal anoestrus had a mean duration of 202 ± 16.8 days. The 1991 breeding season began on 3 April $\pm$ 9.0 days (although two hinds had not become cyclic before the end of sampling on 19 April).

Seasonal plasma concentrations of LH

One short-term ovariectomized, oestradiol-treated hind lost its implant during the experiment and was subsequently

removed from analysis. Profiles of mean LH concentrations in ovariectomized hinds are shown (Fig. 2).

During the time of the breeding season (determined from the intact hinds) mean LH concentrations in ovariectomized oestradiol-treated hinds were 0.6 (range: 0.1–2.5) ng ml⁻¹ plasma, gradually increasing from 0.3 ± 0.09 ng ml⁻¹ on 2 April to 1.1 ± 0.16 ng ml⁻¹ on 29 May and then decreasing to 0.2 ± 0.07 ng ml⁻¹ at the end of the breeding season on 3 September. In contrast, the non-breeding season was characterized by significantly ($P < 0.001$) lower mean LH concentrations (mean 0.1, range: 0–0.9 ng ml⁻¹ plasma).

In ovariectomized hinds that did not receive oestradiol treatment, mean LH concentrations also displayed a seasonal pattern, being highest during the breeding season, peaking at 1.1 ng ml⁻¹ on 18 June, and reaching lowest values during the non-breeding season, 0.1 ng ml⁻¹ on 26 November (Fig. 2). Concentrations recorded at these seasonal peaks and nadirs were similar to those recorded in the ovariectomized oestradiol-treated hinds and the nadir was also similar to concentrations recorded in the intact hinds. However, although peak and trough values were similar, LH concentrations in the ovariectomized hinds decreased from peak values more slowly and increased earlier compared with ovariectomized oestradiol-treated hinds. This was evident in the significantly higher LH concentrations recorded in the ovariectomized hinds on nearly all occasions except for periods during the breeding season, and around November when concentrations were at their lowest in all animals, and was also apparent when analysed as the area under the curve between the first breeding season peak and the anoestrous nadir ($P < 0.01$) and the nadir to the second breeding season ($P < 0.001$). This gradual change in LH concentrations in ovariectomized hinds occurred throughout the study (Fig. 2), and was inversely proportional to and about 4 weeks in advance of the prevailing photoperiod (LH concentration = 1.84–0.0991 daily photoperiod; $r^2 = 0.62$; $P < 0.001$).

Ovariectomized hinds had higher mean LH concentrations than did ovariectomized, oestradiol-treated hinds during both the breeding (0.9, range 0.3–2.8 versus 0.6, range 0.1–2.5 ng ml⁻¹, respectively; $P < 0.01$) and non-breeding (0.5, range 0.1–2.3 versus 0.1, range 0–0.9 ng ml⁻¹, respectively; $P < 0.001$) seasons.

Plasma LH concentrations in intact hinds generally remained below 0.2 ng ml⁻¹. However, concentrations of 1.5–5.0 ng ml⁻¹ were recorded on seven occasions within the breeding season in four animals, and were accompanied by behavioural oestrus on three occasions. Apart from this, there was no evidence of any seasonal patterns in LH secretion.

Seasonal pituitary LH response to GnRH

The LH response to exogenous GnRH given at intervals of 4 weeks are presented (Fig. 2). Between April and August (breeding season) the mean LH concentration for ovariectomized hinds treated with oestradiol averaged 16.4 (range: 1.4–32.3) ng ml⁻¹, significantly greater than that recorded in ovariectomized hinds without oestradiol treatment (mean 7.4, range: 1.2–14.6 ng ml⁻¹; $P < 0.001$). Mean LH concentrations in ovariectomized, oestradiol-treated hinds decreased rapidly

over September to November, averaging 3.1 (range 0.2–14.0) ng ml⁻¹ over the non-breeding season. In contrast, the decrease in mean LH response at this time in untreated ovariectomized hinds was of a much lower magnitude, with concentrations averaging 4.2 (range 0.8–12.8) ng ml⁻¹ during the non-breeding season, significantly ($P < 0.001$) greater than that of oestradiol-treated hinds.

The LH response to GnRH treatment in the intact hinds was of a much lower magnitude than that in either group of ovariectomized hinds, averaging just 1.0 (range: 0.2–2.3) and 1.3 (range: 0–5.7) ng ml⁻¹ during the breeding and non-breeding season, respectively. A marked decline in responsiveness was noted (Fig. 2) during part of anoestrus and was at a minimum in November (mean 0.2, range: 0–0.3 ng ml⁻¹).

Effect of history of ovarian removal

Essentially similar patterns of LH secretion were measured in those hinds ovariectomized at the beginning of the experiment, compared with those ovariectomized 2.3 years earlier. However, LH concentrations were slightly greater in those animals ovariectomized for the longer period (Table 1). Similarly, the nadir (mean concentrations < 0.2 ng ml⁻¹ plasma) in LH concentrations recorded during anoestrus was reached significantly earlier in the short-term compared with long-term ovariectomized hinds (29.2 days earlier in ovariectomized, oestradiol-treated hinds, $P < 0.05$; and 21.3 days earlier in ovariectomized, untreated hinds, $P < 0.05$). The subsequent increase in LH concentrations occurred at similar times in both long-term and short-term ovariectomized hinds. The interaction between oestradiol treatment and history of ovarian removal was significant ($P < 0.05$) in only one instance, that of basal LH secretion during the breeding season.

Discussion

An important finding of the study reported here was the marked seasonal variation in the ability of exogenous oestradiol to suppress tonic LH concentrations in ovariectomized hinds. The breeding season was characterized by minimal suppression and anoestrus by maximal suppression; these changes were approximately aligned with the onset and cessation of ovarian activity recorded in intact herd mates. This is in agreement with previous work in sheep (Legan *et al.*, 1977; Goodman *et al.*, 1982; Webster and Haresign, 1983) and goats (Henniaiwati *et al.*, 1995), indicating that a change in the steroid-feedback mechanism determines, or at least accompanies, changes in reproductive status. However, the present study also revealed that two other mechanisms may be important in regulating seasonality. The first, a steroid-independent mechanism, is suggested by the marked circannual pattern in LH secretion in ovariectomized hinds that did not receive oestradiol. This was even apparent 2.3–3.5 years after ovariectomy. This pattern has also been noted in ovariectomized hares (Davis and Meyer, 1973; Calliol *et al.*, 1990) and mares (Garcia and Ginther, 1976; Freedman *et al.*, 1979) as well as in ewes, in which it is evident as a change in the frequency of episodic LH secretion (Goodman *et al.*, 1982; Robinson *et al.*, 1985). A similar pattern, although somewhat equivocal, has

Fig. 2. Mean ($\pm$ SEM) concentrations of (a) seasonal plasma LH and (b) GnRH-induced LH, in ($\circ$) ovariectomized and ($\bullet$) ovariectomized and oestradiol-treated hinds, and (c) GnRH-induced LH concentrations in intact hinds. LH concentrations were determined from blood samples taken approximately once a week, while the response to GnRH, determined by subtracting the time zero concentration from that obtained 15 min after i.v. administration of 10 μ g GnRH, was determined at intervals of approximately 4 weeks. The horizontal bars represent the mean breeding season determined by monitoring progesterone secretion in the intact hinds. The asterisks indicate significant differences (at least $P < 0.05$) between ovariectomized and ovariectomized oestradiol-treated hinds at each sampling occasion.

Table 1. The effect of time from ovariectomy until the beginning of the experiment on plasma LH concentrations in oestradiol-treated and untreated ovariectomized red deer hinds during the breeding and non-breeding seasons.

Group	Mean (range) LH secretion (ng ml ⁻¹ plasma)		
	Short-term ovariectomized (1 week)	Long-term ovariectomized (2.3 years)	Significance of contrast between short- and long-term ovariectomized [†]
Basal LH secretion			
Ovariectomized			
Breeding season	0.8 (0.3–1.6)	0.9 (0.4–2.8)	NS
Anoestrus	0.4 (0.1–1.2)	0.5 (0.1–2.3)	NS
Ovariectomized + oestradiol treated			
Breeding season	0.4 (0.1–1.7)	0.7 (0.1–2.5)	***
Anoestrus	0.1 (0.1–0.6)	0.1 (0.0–0.9)	NS
GnRH-induced LH secretion			
Ovariectomized			
Breeding season	6.6 (2.9–12.5)	8.2 (1.2–14.6)	NS
Anoestrus	3.3 (0.8–9.2)	5.1 (2.1–12.8)	*
Ovariectomized + oestradiol treated			
Breeding season	16.8 (7.3–32.3)	16.1 (1.4–27.6)	NS
Anoestrus	2.0 (0.2–11.0)	4.0 (0.8–14.0)	***

[†]NS = not significant; **P* < 0.05; ****P* < 0.001.

been reported in castrated male deer (red, Lincoln and Kay, 1979; McMahon, 1994; white-tailed, Bubenik *et al.*, 1982). The second mechanism is suggested by the seasonal variation in pituitary responsiveness to exogenous GnRH, seen in both untreated and oestradiol-treated ovariectomized hinds, and in intact hinds. This has also been reported in red deer stags (Suttie *et al.*, 1984; Fennessy *et al.*, 1988). Although this variation is also steroid independent, its magnitude is clearly modified by oestradiol. Separate studies in ovariectomized deer have shown a period of seasonal quiescence in the positive feedback effects of oestradiol (Meikle and Fisher, 1990; Jabbour *et al.*, 1992). Collectively, these results would suggest that seasonal influences act on several components of the hypothalamic–pituitary–ovarian axis and, although likely to be driven by a central neuroendocrine mechanism, the contribution of all mechanisms to seasonality, albeit in a passive or subordinate manner, should not be overlooked. For instance, the change in pituitary responsiveness to exogenous GnRH, thought to be of relatively little importance in regulating ovarian function in ewes (McLeod *et al.*, 1982), is a function of releasable LH which in turn is inversely related to the frequency of endogenous GnRH secretion (Clarke and Cummins, 1985; Clarke *et al.*, 1987). However, in hinds, the greatest response to exogenous GnRH occurs during the breeding season when the frequency of endogenous GnRH would be expected to be maximum, at least as determined by more frequent pulsatile LH secretion in these animals (L. M. Meikle and M. W. Fisher, unpublished data). This probably reflects differences between physiological amounts of GnRH and the 10 µg pharmacological dose used in the present study (chosen so that the possible confounding influences of coincident endogenous GnRH would be minimized).

Since both steroid-dependent and -independent mechanisms are evident and since present experimental techniques cannot differentiate between the mechanisms, the contention that steroid-dependent regulation is the major determinant of seasonality (Karsch *et al.*, 1993) remains unconvincing. As both mechanisms may be mediated via different neural systems (Pau and Jackson, 1985; Meyer and Goodman, 1986; Goodman, 1988), it is possible that they both interact in the regulation of seasonality (Robinson *et al.*, 1985). Furthermore, the effects of season on basal and GnRH-induced LH secretion in the present study suggest that the real central physiological control mechanism is steroid independent, its effects merely amplified, both in magnitude and temporally, by the presence of oestradiol (steroid-dependent), as Lincoln and Short (1980) suggested. Animals with quiescent or regressed gonads during anoestrus do not immediately show a gonadotrophin response to castration (Lincoln and Short, 1980) indicating that a steroid-dependent mechanism is not fully competent at all times, and that some other mechanism inhibits or prevents LH secretion. Further evidence for steroid-independent regulation may be the differing depths or stages of anoestrus noted in sheep (McNeilly *et al.*, 1985) and deer. For instance, in the present study the intact hinds displayed a marked reduction in the LH response to exogenous GnRH in the early compared with the later part of anoestrus, a pattern also noted in ovary-intact Père David's deer (Curlewis *et al.*, 1991; McLeod *et al.*, 1991). These results suggest that anoestrus is not a uniform state but is characterized by an early 'deep' period (Curlewis *et al.*, 1991) and a steroid-independent mechanism is the most likely explanation.

All the steroid-independent, as well as the steroid-dependent, seasonal alterations in LH concentrations observed

in hinds in the present study are also evident in at least some breeds of ewe, albeit at a significantly reduced magnitude. Since domestication can reduce the amplitude of seasonal reproductive parameters (see Setchell, 1992), the differences between the hind and ewe may merely indicate the extent to which they have been domesticated, or reflect the degree of gonadal regression that occurs during the non-breeding season (Goodman and Karsch, 1981). It will be interesting to see if farming and domestication modifies reproductive seasonality in deer, and indeed this animal provides an almost unique opportunity to monitor the effects of domestication in an ungulate (Fisher and Bryant, 1993).

The use of a relatively recently domesticated experimental animal, such as the deer, also imposes some other limitations upon interpretation of the data. These animals are not as docile as the domestic ewe, and the acts of yarding, handling and blood sampling may possibly affect hormone secretion. For instance, progesterone secretion throughout anoestrus recorded in two hinds may have been of adrenal origin (Jopson *et al.*, 1990) or alternatively might reflect persistent luteal activity (Curlewis *et al.*, 1988; Brinklow *et al.*, 1992). However, although these limitations should be taken into consideration it is unlikely that they would affect the major conclusions reached in this study.

The duration of the interval between ovariectomy and the start of the experiment had little effect on the results, apart from altering the magnitude of the seasonal alterations in LH secretion as might be expected given the longer steroid-free environment and probable differences in metabolic clearance rates (Montgomery *et al.*, 1984). Since the seasonal patterns of LH secretion were similar in both groups of hinds, the inclusion of animals of varied experimental histories in seasonality experiments ensures the interpretation of seasonal patterns is not unduly confounded by time after ovariectomy (Montgomery *et al.*, 1985).

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