Gonadotrophin-induced follicle development in red deer hinds during the breeding and non-breeding seasons

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The effect of exogenous equine chorionic gonadotrophin (eCG) and endogenous (GnRH-treatment) gonadotrophins in promoting antral follicle development in red deer hinds was assessed during the breeding and non-breeding seasons. After progesterone pretreatment, hinds received no further treatment, a single injection of 300 iu equine chorionic gonadotrophin or infusion of GnRH ($1.0 \mu\text{g h}^{-1}$) for up to 4 days. Ovaries were recovered ($n = 5$ per group) at the time of, or 36 h after, progesterone removal. All follicles ≥ 2.0 mm in diameter were dissected out, their health status assessed and follicular fluid oestradiol content measured. Granulosa cells were counted and their capacity for oestradiol and cAMP production assessed *in vitro*. Oestrus, the preovulatory LH surge and ovulation rates were monitored in additional groups of hinds that had been treated identically ($n = 5$ per group). During the breeding season, all monitored animals ovulated, but five

of 15 (one eCG, three GnRH, one progesterone alone) did not exhibit oestrus, and three (all eCG) had luteinized follicles. During seasonal anoestrus, four hinds (all eCG-treated) displayed oestrus, but only two ovulated. Two non-ovulating hinds (one eCG, one progesterone alone) had luteinized follicles. The total number of follicles, or of large (≥ 4 mm) follicles, did not differ significantly between seasons. There were proportionately more healthy follicles during seasonal anoestrus ($P < 0.05$). Treatment with GnRH, and to a lesser extent eCG, increased the number of oestrogenic follicles and their follicular fluid oestradiol content. In summary, exogenous and endogenous gonadotrophins affected antral follicle development similarly in both the breeding and non-breeding seasons, although the response was variable among animals and often associated with failure of oestrous expression, ovulation and with follicle luteinization.

Introduction

Red deer (*Cervus elaphus*) are adapted to mixed browsing and grazing (Hoffman, 1985) and have evolved a breeding strategy that copes with seasonal extremes in food supply and quality (Lincoln and Short, 1980). Under farmed conditions in managed pastoral environments, there is considerable misalignment between seasonal breeding in red deer and the seasonal peak of pasture production and quality. In New Zealand, for example, the spring flush of pasture growth occurs in August–October, but red deer hinds calve naturally in November–December (Asher *et al.*, 1996). Thus, peak demands for lactation and the requirements of the young calf for high quality feed occur in late summer when pasture growth and quality is limited and senescence is occurring in pasture plants.

A number of strategies have been used to control breeding in deer with the aim of better aligning calving and the onset of lactation with pasture growth, by inducing ovulation and mating during seasonal anoestrus. These

treatments, which typically involve the administration of exogenous gonadotrophic hormones, have also been used to synchronize oestrus during the breeding season or to induce superovulation. However, the responses have been variable and unpredictable in deer, with some hinds failing to ovulate and with widely varying ovulation rates in those that did ovulate (Fennessey *et al.*, 1994; Asher *et al.*, 1995).

In other ruminants that, like red deer, ovulate a single follicle at each oestrous cycle (for example, cows and some breeds of sheep), a number of growing antral follicles enter a growth phase early in the follicular phase of the oestrous cycle. However, only a single follicle completes pre-ovulatory development and ovulates, with the remainder undergoing atretic degeneration (Hirschfield, 1991). The development of this single dominant follicle suppresses the growth and results in atresia of the other large antral follicles present (McNatty *et al.*, 1982; McNatty and Henderson, 1987). The inclusion of antral follicles into the cohort of rapidly growing follicles early in the follicular phase (termed 'selection' or 'cyclic recruitment') is known to be under the influence of FSH (for review, see McGee and Hseuh, 2000). The administration of exogenous FSH or FSH-like gonadotrophins during the follicular phase will induce superovulation in monovular animals, presumably

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by increasing the number of antral follicles recruited into the growing pool or preventing their degeneration. Equine chorionic gonadotrophin (eCG) is used widely as a source of exogenous gonadotrophin to stimulate superovulation in monovular species. In sheep, eCG increases the numbers of antral follicles ≥ 3 mm in diameter (Dott *et al.*, 1979) and, when administered at the time of luteolysis, eCG prevents the occurrence of atresia in large follicles and enhances their capacity to produce oestradiol (McNatty *et al.*, 1982).

Equine chorionic gonadotrophin has long been used to induce ovulation in seasonally anoestrous ruminants, including deer, but as occurs in the breeding season, ovulation rates in response to this treatment are sometimes extremely variable (red deer: Adams *et al.*, 1985; fallow deer: Asher and Smith, 1987). An alternative approach has been to treat seasonally anoestrous animals with GnRH administered as either a series of injections or as a continuous infusion to promote endogenous gonadotrophin secretion. In sheep, this approach has proved consistently reliable and has induced ovulation rates comparable to those occurring in the same flock during the breeding season (McLeod *et al.*, 1982, 1983). Similar treatment regimens have been used in Père David's deer (McLeod *et al.*, 1991) and in red deer (Fisher *et al.*, 1989a). However, in both studies, the percentage of treated animals that ovulated was inconsistent and appeared to be related to the stage of the anoestrous season.

Recently, we reported that the number of antral follicles present in red deer hinds during the breeding season differed widely among individuals (4–81 follicles ≥ 2 mm diameter per hind), and that the proportion of follicles that were healthy increased progressively over the follicular phase of the oestrous cycle (McLeod *et al.*, 2001). Therefore, the reported variability in response to induction–superovulation treatments may simply reflect between-animal differences in the numbers of antral follicles present that are capable of responding to the exogenous hormones. The objective of the present study was to compare the responses to exogenous gonadotrophin (eCG) and to GnRH-induced secretion of endogenous gonadotrophins, in promoting antral follicle development in the breeding and non-breeding seasons, to determine the underlying basis of seasonal differences in ovarian function in red deer. Some of these data (hinds treated with progesterone alone during the breeding season) were included in the earlier report of follicle populations in cyclic red deer hinds (McLeod *et al.*, 2001).

Materials and Methods

Animals and management

A total of 55 parous adult red deer (*Cervus elaphus*) hinds, aged between 3 and 9 years, were used in this study. Ovaries were recovered from 40 of these animals during either the breeding season (March, $n = 20$) or seasonal

anoestrus (January, $n = 20$). The remaining 15 hinds (control groups) were monitored on both of these occasions to determine the times of onset of oestrus and the preovulatory LH surge, so that the time of ovary recovery in their herdmates could be related to these endocrine events.

All hinds were maintained on pasture throughout the treatment period, with the exception that those animals from which ovaries were recovered were fasted overnight before ovariectomy or slaughter. All experimental procedures performed had been given prior approval by the AgResearch Invermay Animals Ethics Committee according to the Animals Protection (Codes of Ethical Conduct) Regulations, 1987.

Treatment and blood sampling

Oestrus was synchronized during the breeding season in the animals studied so that ovaries could be recovered at a known stage of the oestrous cycle. All hinds were subjected to exogenous progesterone treatment for 12 days, administered via controlled intravaginal drug releasing devices (EAZI-BREED CIDR, 0.3 g progesterone; InterAg, Hamilton). A single CIDR, left for 8 days *in situ*, was replaced with a fresh CIDR for the following 4 days to ensure that increased plasma progesterone concentrations were maintained throughout. All hinds treated during seasonal anoestrus were given an identical progesterone treatment, to allow unbiased comparison of follicle populations between the two reproductive states.

At the time of withdrawal of the second CIDR, hinds were assigned randomly to one of four treatment groups ($n = 5$ animals group). Animals in groups 1 and 2 (breeding season) and groups 1a and 2a (anoestrus) received no further treatment. Hinds in groups 3 (breeding season) and 3a (anoestrus) were given a single i.m. injection of 300 iu eCG (Folligon; Intervet, Lane Cove, NSW) and those in groups 4 (breeding season) and 4a (anoestrus) were treated with synthetic GnRH (Sigma, St Louis, MO) administered via osmotic minipumps (ALZET 2ML4; Alza Corp, Palo Alto, CA) at the rate of $1.0 \mu\text{g h}^{-1}$ for 4 days or until the time of ovariectomy. These treatment regimens are typical of those used in oestrus synchronization–superovulation programmes in red deer. Both the insertion and removal of minipumps was carried out under local anaesthetic (2% (w/v) xylocaine hydrochloride), injected s.c. at the site of implantation in the neck region. Hinds that were to be blood sampled and monitored for oestrus and ovulation were included in all three treatment regimens (note that groups 1 and 2 received the same treatment). The same blood-sampled animals were assigned to the same treatment protocol in both the breeding and non-breeding seasons.

Ovaries were recovered from hinds ($n = 5$ per group) either at the time of (groups 1 and 1a), or approximately 36 h after the time of (groups 2, 2a, 3, 3a, 4 and 4a; Table 2) CIDR removal and follicle development within them was assessed. Ovaries were recovered either by ovariectomy

carried out under barbiturate-induced and halothane-maintained anaesthesia or within minutes of death at slaughter at a registered deer slaughter facility (Otago Venison Ltd, Mosgiel).

Control animals were monitored for behavioural signs of oestrus and were blood sampled via an indwelling jugular vein catheter to determine the time of onset of the preovulatory LH surge. Blood samples were collected and observations of oestrus were made at 2 h intervals from 4 h before until 72 h after CIDR withdrawal as described by McLeod *et al.* (2001). The incidence of ovulation was determined in all these animals at laparoscopy, carried out 7 days after CIDR withdrawal under xylazine (Rompun; Bayer NZ Ltd, Petone, NZ) and fentanyl citrate plus azaperone (Fentaz; Smith, Kline and French (NZ) Ltd, Auckland) anaesthesia.

Ovarian tissue collection and preparation

Techniques used in the collection of ovarian tissue and analysis of follicle populations and their health status were as described by McLeod *et al.* (2001). Briefly, excised ovaries were placed immediately on ice in Dulbecco's PBS solution (KC Biological Inc., Lenexa, KS), and transferred to an adjacent laboratory where they were weighed and all follicles ≥ 2.0 mm in diameter were dissected out under a stereomicroscope. After being dissected free of extraneous tissue, the diameter of each follicle was recorded (to the nearest 0.1 mm) and follicle health was assessed. Follicular fluid was aspirated from each follicle, its volume measured and a sample snap-frozen for subsequent analysis of oestradiol content. Granulosa cells were harvested, washed and counted as described by McLeod *et al.* (2001).

Granulosa cell aromatase activity and cAMP production

Aromatase activity and cAMP production of the harvested granulosa cells was determined *in vitro* using the methods for deer follicles described by McLeod *et al.* (2001). Cells collected from each follicle were re-suspended in ice-cold Dulbecco's PBS solution containing 0.1% BSA (Sigma Chemical Company), 20 mmol Hepes buffer l⁻¹ and 0.2 mmol 3-isobutyl-1-methylxanthine l⁻¹ (Sigma; Medium A, pH 7.4, 4.0°C), with a final cell concentration of $2\text{--}4 \times 10^5$ cells ml⁻¹. Aliquants (0.5 ml, in duplicate or triplicate depending on the total number of granulosa cells recovered) of these cell suspensions were incubated to assess oestradiol (aromatase activity) or cAMP production.

For the determination of aromatase activity, cells were incubated in either medium alone (time zero incubations), or in medium containing 2.0 µg testosterone ml⁻¹ (testosterone incubations), both of which were made up to a final incubation volume of 1.0 ml. Time zero incubations were snap-frozen immediately at -70°C and testosterone incubations were snap-frozen after incubation for 3 h at 37°C in a shaking water bath. All tubes were then stored at -20°C until the media were assayed for oestradiol content.

Cell suspensions were incubated in either the media alone (time zero incubations), or in media containing 200 ng LH (NIH-LH-S17) ml⁻¹ (LH incubations), again made up to a final volume of 1.0 ml, and granulosa cell cAMP production was assessed. The LH incubations were placed in a shaking water bath at 37°C for 45 min, after which time the reaction was stopped by immersion in a water bath at 80°C for 15 min. Time zero incubations were immediately incubated at 80°C for 15 min. All tubes were stored at -20°C until the incubation media were assayed for cAMP.

Aromatase activity (expressed as ng oestradiol per 10⁶ cells h⁻¹) and production rates of cAMP (mmol per 10⁶ cells h⁻¹) in granulosa cells from individual follicles was calculated by subtracting the oestradiol or cAMP content of supernatant from time zero incubations from the values for the same follicle after the testosterone or LH incubations.

Follicle classification

Follicles were classified as healthy or atretic on the basis of the criteria described by McLeod *et al.* (2001), which had been adapted from the methods of McNatty *et al.* (1985) used for sheep follicles. The factors considered included the presence or absence of blood capillaries within the thecal tissue and of debris in the follicular fluid, the condition of the oocyte (healthy or degenerating, on the basis of the presence of a cumulus cell matrix, signs of cytolysis, necrosis or loss of spherical shape) and the total granulosa cell complement of the follicle.

Follicle health was classified as one of four categories (classes 1, 2a, 2b and 3) as described by McLeod *et al.* (2001). Only class 1 follicles are regarded as healthy (non-atretic), with those classed as classes 2a, 2b and 3 representing follicles in progressive stages of degeneration and atresia. Non-atretic follicles were arbitrarily classified as 'oestrogenic', or 'non-oestrogenic' on the basis of their granulosa cell population, follicular fluid oestradiol content and aromatase activity and cAMP production (McLeod *et al.*, 2001).

Follicles were described as having luteinized if they had a thickened, solid and opaque wall, with or without a fluid-filled cavity, and when there was no evidence of a rupture site.

Hormone assays

Plasma LH concentrations were determined using the heterologous double-antibody radioimmunoassay method described for deer plasma by Meikle and Fisher (1996). All samples were completed within one assay in which the intra-assay coefficient of variation was 11.7%. The limit of detection was 0.08 ng LH ml⁻¹ plasma.

Concentrations of oestradiol in follicular fluid and in granulosa cell incubation media were measured using the tritiated oestradiol radioimmunoassay described for sheep by McNatty *et al.* (1981) and subsequently validated for deer (McLeod *et al.*, 2001). Results were expressed as ng

oestradiol per 10^6 cells. Follicular fluid oestradiol was measured directly without extraction, in diluted (10–100 fold dilution with $0.1 \text{ mol PBS l}^{-1}$) aliquants of follicular fluid. The limit of detection was $1.0 \text{ ng oestradiol ml}^{-1}$.

Granulosa cell cAMP production was determined using the method described by McNatty *et al.* (1985), and cAMP production was expressed as pmol per 10^6 cells. Within this study, the inter- and intra-assay coefficients of variation were both $< 10\%$ and the limit of detection was $0.02 \text{ pmol per } 10^6 \text{ cells}$.

Statistical analyses

The definition used to characterize a preovulatory LH surge was that there was a sustained ($> 6 \text{ h}$) increase of plasma LH concentrations above 2 ng LH ml^{-1} and that peak concentrations attained during this time exceeded 5 ng ml^{-1} .

Comparison of follicle populations among treatment groups was made by analysis of variance, performed in conjunction with the Neuman and Keuls test, and of follicular fluid oestradiol content and granulosa cell hormone production rates by Student's *t* test. The incidence of oestrus and ovulation was compared by chi-squared analysis, and changes in follicle health distribution, by cluster analysis. Unless otherwise stated, all values are presented as the mean $\pm \text{SEM}$.

Results

Liveweights, oestrus, the preovulatory LH surge and ovulation rates

There was no significant difference in mean liveweight between hinds treated during the breeding season ($n = 35$, mean liveweight $100.1 \pm 2.0 \text{ kg}$) and those treated in mid-anoestrus ($n = 35$, mean liveweight $97.1 \pm 2.2 \text{ kg}$). Both within and between seasons, there were no significant differences in liveweight among the treatment groups.

The mean incidence and the time of onset of oestrus and the preovulatory LH surge, and ovulation rates are shown for each of the groups of the blood-sampled hinds (Table 1). During the breeding season, four of five hinds treated with CIDRs alone and four of five eCG-treated hinds, but only two of five GnRH-treated hinds, exhibited oestrus during the 76 h period of observation. A preovulatory LH surge was recorded in all except one hind (treated with CIDR alone), and all animals ovulated. When ovaries were observed by laparoscopy 7 days after CIDR withdrawal, two eCG-treated hinds had a double ovulation, and the remaining 13 animals each had ovulated a single follicle. Luteinized follicles were present in three hinds (all eCG-treated). There were no significant differences among treatment groups in the timing of oestrus or the preovulatory LH surge, or in ovulation rates.

When treated during seasonal anoestrus, none of the control animals subjected to CIDR alone or to GnRH exhibited oestrus, had a preovulatory LH surge or ovulated.

In contrast, four of five eCG-treated hinds were observed in oestrus and all these animals had a preovulatory surge. However, only two of these animals ovulated (single ovulation) and there was a luteinized follicle present on the ovaries of two hinds (one eCG-treated, one GnRH-treated) that had failed to ovulate.

Antral follicle populations

The mean times of ovary recovery are shown for each of the treatment groups (Table 2). In those animals in which the time of collection was nominally set at 36 h, the mean time of recovery was $38.6 \pm 0.34 \text{ h}$ after CIDR withdrawal. There were no significant differences in the mean recovery time among groups.

When all treatment groups are combined, the mean number of antral follicles $\geq 2.0 \text{ mm}$ in diameter present did not differ significantly between the breeding (mean 19.6 ± 2.81 , range 4–49 follicles per hind) and non-breeding seasons (mean 18.6 ± 2.2 , range 10–39 follicles per hind). Similarly, the mean number of follicles present was not significantly different among groups in the breeding season (Table 2). However, hinds treated with GnRH during seasonal anoestrus had significantly more ($P < 0.05$) antral follicles than animals treated with CIDR alone in the same season or animals receiving the same treatment (GnRH) in the breeding season. The numbers of large ($\geq 4.0 \text{ mm}$ in diameter) follicles did not differ among treatment groups or between seasons.

Follicle health status

The mean number of healthy and oestrogenic follicles, and the mean diameter of the largest follicle, are shown (Table 3). With a single exception, the number of healthy follicles did not differ among treatments or between seasons. The exception was that the number of healthy follicles in animals treated with GnRH during the non-breeding season was significantly higher than it was in the other treatment groups at both reproductive states, and higher than it was in animals subjected to the same treatment during the breeding season. A similar trend was seen in the total number of oestrogenic follicles, but this was significant only between the GnRH-treated group and those treated with CIDRs alone in anoestrus. The number of hinds with at least one oestrogenic follicle present was not significantly different in either the breeding or non-breeding seasons (three of five versus two of five, three of five versus four of five, four of five versus five of five, four of five versus five of five for groups 1–4, respectively). There were no significant differences in the mean diameters of the largest healthy, or largest oestrogenic follicles present.

When animals from all treatment groups were combined and the health status of their follicles expressed as a percentage of all follicles present, there was a significantly higher proportion of healthy follicles present during seasonal anoestrus than during the breeding season (72.2 and 56.6% healthy, respectively, $P = 0.019$, pooled SD =

Table 1. Mean ($\pm$ SEM) times of onset of oestrus and the preovulatory LH surge and ovulation rates in red deer (blood-sampled hinds) treated with progesterone (CIDR) alone, or in conjunction with equine chorionic gonadotrophin (eCG) or GnRH during the breeding or non-breeding seasons

Treatment	Time of onset (h after CIDR removal)		
	Oestrus*	LH surge*	Ovulation rate*
Breeding season			
CIDR alone	45.0 $\pm$ 5.8 ^a (4/5)	43.5 $\pm$ 6.2 ^a (4/5)	1.0 $\pm$ 0.0 ^a (5/5)
CIDR plus eCG	41.5 $\pm$ 3.9 ^a (4/5)	39.2 $\pm$ 4.1 ^a (5/5)	1.4 $\pm$ 0.3 ^a (5/5)
CIDR plus GnRH	52.0 $\pm$ 6.0 ^a (2/5)	41.6 $\pm$ 5.5 ^a (5/5)	1.0 $\pm$ 0.0 ^a (5/5)
Seasonal anoestrus			
CIDR alone	– (0/5) ^b	– (0/5) ^b	– (0/5) ^b
CIDR plus eCG	47.0 $\pm$ 1.3 ^a (4/5)	45.5 $\pm$ 1.0 ^a (4/5)	1.0 $\pm$ 0.0 ^a (2/5)
CIDR plus GnRH	– (0/5) ^b	– (0/5) ^b	– (0/5) ^b

*Numbers in parentheses show the numbers of hinds in each group in which oestrus, an LH surge and ovulation were recorded.

Within columns, values with different superscripts are significantly different ($P < 0.05$).

Table 2. Mean times of ovary recovery and total number of antral follicles in red deer hinds ($n = 5$ per group) treated with progesterone (CIDR) alone, or in conjunction with equine chorionic gonadotrophin (eCG) or GnRH during the breeding or non-breeding seasons

Group	Treatment	Ovary recovery time (h from CIDR withdrawal)	Total number of follicles*	
			≥ 2.0 mm	≥ 4.0 mm
Breeding season				
1	CIDR alone (0 h)	0 ± 0.0 ^a	22.8 ± 8.22 ^{ab} (4–49)	2.6 ± 0.40 ^a (2–4)
2	CIDR alone (36 h)	38.9 ± 0.36 ^b	23.0 ± 6.86 ^{ab} (6–39)	3.8 ± 0.97 ^a (1–7)
3	CIDR/eCG	37.6 ± 0.76 ^b	17.6 ± 4.24 ^{ab} (5–31)	4.2 ± 1.53 ^a (1–10)
4	CIDR/GnRH	38.8 ± 0.61 ^b	14.2 ± 4.82 ^a (4–32)	3.4 ± 0.51 ^a (3–5)
Seasonal anoestrus				
1a	CIDR alone (0 h)	0 ± 0.0 ^a	11.2 ± 0.58 ^a (10–13)	3.2 ± 1.32 ^a (1–8)
2a	CIDR alone (36 h)	38.5 ± 0.56 ^b	13.8 ± 1.28 ^a (10–17)	3.0 ± 0.55 ^a (1–4)
3a	CIDR/eCG	38.1 ± 0.64 ^b	20.2 ± 4.38 ^{ab} (10–36)	3.0 ± 0.55 ^a (1–4)
4a	CIDR/GnRH	38.7 ± 0.52 ^b	29.2 ± 4.80 ^b (12–39)	6.2 ± 1.66 ^a (1–10)

*Numbers in parentheses represent the range in numbers of follicles per hind.

Within columns, values with different superscripts are significantly different ($P < 0.05$).

3.51). The percentage of follicles in each health class is shown for individual treatment groups (Fig. 1). The distribution of atretic follicles across the three classes was significantly different ($P < 0.05$) between seasons (anoestrus: 19.4, 9.6 and 0.1%; breeding season: 11.2, 25.5 and 6.8% for classes 2a, 2b and 3, respectively), but not among treatment groups.

Oestradiol and cAMP production

Mean concentrations of oestradiol in the follicular fluid of oestrogenic follicles, and *in vitro* oestradiol (aromatase activity) and cAMP production by their granulosa cells, are shown (Table 4). During both seasons, oestradiol content of follicular fluid was significantly higher ($P < 0.05$) in GnRH-treated animals than in hinds treated with CIDRs alone.

Follicular fluid concentrations in eCG-treated hinds were midway between these two groups. Neither aromatase activity nor cAMP production differed significantly among groups or between seasons.

Discussion

Treatment with exogenous gonadotrophin (eCG) or inducing endogenous gonadotrophin secretion (with GnRH) promoted antral follicle development in red deer hinds, increasing the number of oestrogenic follicles present and their follicular fluid oestradiol content in both the breeding and non-breeding seasons. However, the response was variable and it is likely that few of these follicles would have successfully completed preovulatory development and ovulated. These

Table 3. Mean ($\pm$ SEM) number of healthy and oestrogenic follicles, and the mean diameter of the largest follicle present, in red deer hinds treated with progesterone (CIDR) alone, or in conjunction with equine chorionic gonadotrophin (eCG) or GnRH during the breeding or non-breeding seasons

Group	Treatment	Number of healthy follicles*	Number of oestrogenic follicles*	Mean diameter (mm) of largest follicle	
				Healthy	Oestrogenic
Breeding season					
1	CIDR alone (0 h)	8.0 ± 1.64 ^a (4–13)	0.8 ± 0.37 ^a (0–2)	7.5 ± 0.33 ^a	7.7 ± 0.33 ^a
2	CIDR alone (36 h)	9.0 ± 2.47 ^a (3–18)	1.0 ± 0.45 ^a (0–2)	8.0 ± 0.63 ^a	6.3 ± 0.76 ^a
3	CIDR/eCG	8.4 ± 2.46 ^a (3–16)	2.0 ± 0.71 ^{ac} (0–4)	8.0 ± 1.04 ^a	9.0 ± 0.33 ^a
4	CIDR/GnRH	8.0 ± 2.35 ^a (1–14)	1.8 ± 0.58 ^{ac} (0–3)	7.8 ± 0.88 ^a	8.2 ± 0.94 ^a
Seasonal anoestrus					
1a	CIDR alone (0 h)	8.6 ± 0.75 ^a (7–11)	0.4 ± 0.24 ^a (0–1)	7.4 ± 0.42 ^a	7.3 ± 0.35 ^a
2a	CIDR alone (36 h)	9.0 ± 1.05 ^a (7–12)	0.8 ± 0.20 ^{ab} (0–1)	7.9 ± 0.25 ^a	7.9 ± 0.27 ^a
3a	CIDR/eCG	11.0 ± 0.84 ^a (9–14)	1.4 ± 0.24 ^{bc} (1–2)	9.3 ± 0.64 ^a	9.0 ± 0.87 ^a
4a	CIDR/GnRH	23.6 ± 4.38 ^b (7–33)	2.6 ± 0.68 ^c (1–4)	7.7 ± 0.77 ^a	7.7 ± 0.77 ^a

*Numbers in parentheses represent the range in numbers of follicles per hind. Within columns, values with different superscripts are significantly different ($P < 0.05$).

hormonal treatments resulted in a double ovulation in only two of the blood-sampled animals in the breeding season and only two of ten animals had an induced ovulation when treated in seasonal anoestrus. There were surprisingly few between-season differences in the parameters of follicle development monitored.

The ovulation rates observed in the hinds in the present study are not dissimilar to those reported previously for a variety of cervid species subjected to these treatments. Comparable doses of eCG administered during or within a few weeks of the start of the breeding season have resulted in double ovulations in 10–50% of treated red and fallow deer (Fisher *et al.*, 1989a; Asher *et al.*, 1992). Multiple ovulations (> 2) can be induced in deer with higher doses of eCG (Kelly *et al.*, 1982; Asher and Smith 1987). At least 50% of treated animals ovulated a single follicle when GnRH was administered 3–6 weeks before the start of the breeding season (red deer: Fisher *et al.*, 1989a,b; fallow deer: Asher and Macmillan, 1986; Père David’s deer: McLeod *et al.*, 1991). However, few if any hinds ovulated when treated in mid-anoestrus (McLeod *et al.*, 1991). There are also reports of seasonal variation in the ovulatory response to treatment with exogenous gonadotrophins in other species. For example, when sheep were subjected to repeated injections of low doses of LH for an extended period, 100% ovulated when treated in early anoestrus, but only 25% ovulated when treated in mid-anoestrus (Oussaid *et al.*, 1998).

The times of onset of oestrus and of the preovulatory LH surge recorded in the blood-sampled hinds in the present study are similar to those reported earlier in red deer (Asher *et al.*, 1992). They also indicate that follicle collection from the experimental animals would have occurred at a stage equivalent to late follicular phase before occurrence of the LH surge. The time of onset of oestrus and the preovulatory

LH surge, both events that are initiated by increasing plasma oestradiol concentrations, was not influenced by treatment with exogenous gonadotrophins. This was in contrast to previous reports in sheep in which there was an inverse relationship between the time of onset of oestrus and ovulation and dose of eCG (Boshoff *et al.*, 1973).

The mean number of large antral follicles recorded in hinds in this study (range 1–10 per hind) was similar to that recorded by ultrasonography in red deer hinds during the breeding season (Asher *et al.*, 1997). In both the present study and that of Asher *et al.* (1997), there were no significant differences in the numbers of antral follicles present in the breeding season compared with seasonal anoestrus. This finding is in contrast to the findings in sheep reported by McNatty *et al.* (1984) in which significantly more antral follicles ≥ 1 mm in diameter were present during seasonal anoestrus than during the breeding season. In the present study, there were seasonal changes in the proportion of healthy follicles in untreated hinds (CIDR-alone groups), with follicle populations being skewed towards class 1 (non-atretic) in anoestrus and towards classes 2b and 3 (advanced atresia) in the breeding season. Treatment with gonadotrophins during seasonal anoestrus tended to alter follicle health status towards that seen in the breeding season. Similar seasonal changes in follicle health distribution have previously been reported in sheep (McNatty *et al.*, 1984).

The increase in follicular fluid oestradiol concentrations observed in the gonadotrophin-treated hinds concurs with the pattern reported in both sheep (McNatty *et al.*, 1982) and pigs (Miller *et al.*, 1999) treated with eCG. The increased oestradiol concentrations did not promote the time of onset of oestrus or the onset of the preovulatory LH surge but rather there appeared to be adverse effects on oestrous behaviour, with four of ten of the gonadotrophin-

Fig. 1. Percentage of antral follicles ≥ 2 mm in diameter in each follicle health class (class 1: healthy (■); classes 2a (▨), 2b (▩) and 3 (□): progressive stages of atresia) in ovaries recovered from red deer hinds either at the time of (0 h), or 36 h after, removal of progesterone during the (a) anoestrus or (b) breeding seasons.

treated control hinds failing to exhibit oestrus in the breeding season, although they all ovulated. In addition, despite the significant increase in the number of oestrogenic follicles present, and in their oestradiol content, evident in their experimental counterparts, none of the GnRH-treated hinds that were blood sampled displayed oestrus, produced a preovulatory LH surge or ovulated when treated during

seasonal anoestrus. Meikle and Fisher (1991) showed that a single injection of oestradiol after pretreatment with progesterone induced both an LH surge and oestrus in long-term ovariectomized hinds during anoestrus, except when the treatment was given in mid-anoestrus (January). This is the same time of year in which the seasonal anoestrus experiment was carried out in the present study, in which only four of ten gonadotrophin-treated hinds displayed oestrus and had a preovulatory LH surge, and only two of ten ovulated. It remains to be determined whether the same treatment regimens would promote follicle development and induce oestrus and ovulation at other stages of seasonal anoestrus.

A further abnormality in ovarian function observed in gonadotrophin-treated hinds, was the luteinization of large antral follicles in the blood-sampled animals. Luteinized follicles were observed in five hinds (three breeding season, two anoestrus) in which gonadotrophins had been manipulated. Asher and Smith (1987) reported the occurrence of luteinized follicles in over 50% of fallow deer treated with eCG, and this phenomenon has also been described in sheep treated with GnRH (Gilbert *et al.*, 1990). Luteinization of large antral follicles occurs typically at a very low rate in both red and fallow deer treated with CIDRs alone ($< 1\%$, $n > 300$, G. W. Asher and I. C. Scott, unpublished) but does appear to be a consequence of the administration of exogenous gonadotrophins. In rats, a single injection of a low dose of exogenous LH given some hours before the expected time of the preovulatory LH surge results in luteinization without ovulation in virtually all of the Graafian follicles present (Mattheij and Swarts, 1995). A cytokine (vascular endothelial growth factor) that induces angiogenesis, which is a feature of follicle luteinization, has been proposed as the mediator of luteinization in women (Anasti *et al.*, 1998), and its production in primate preovulatory follicles is induced by LH (Hazzard *et al.*, 1999).

Despite the incidence of follicle luteinization in the blood-sampled hinds, no luteinized follicles were present in ovaries recovered from the experimental animals. These hinds would have been exposed to exogenous gonadotrophins for a period of < 40 h and the ovaries were recovered before the onset of the preovulatory LH surge, which presumably triggers luteinization.

In summary, manipulating plasma gonadotrophin concentrations in hinds during either the breeding or non-breeding season influences follicle development, but does not change the number of antral follicles that enter the growing phase or the size distribution of the antral follicle population. Nevertheless, the extent to which this promotion of antral follicle growth results in the development of healthy, oestrogenic follicles varies widely among individual animals in both the breeding and non-breeding seasons. This variability is probably a reflection of the numbers of healthy antral follicles present at the time of treatment. As a consequence of this variability, the number of follicles that ovulate will be variable and unpredictable.

Table 4. Mean ($\pm$ SEM) diameter, follicular fluid oestradiol concentration and granulosa cell aromatase activity and cAMP production of oestrogenic follicles recovered from red deer hinds treated with progesterone (CIDR) alone, or in conjunction with equine chorionic gonadotrophin (eCG) or GnRH during the breeding or non-breeding seasons

Group	Treatment	n	Follicle diameter (mm)	Follicle fluid oestradiol (ng ml ⁻¹)	Granulosa cell	
					Aromatase (ng per 10 ⁶ cells h ⁻¹)	cAMP (pmol per 10 ⁶ cells h ⁻¹)
Breeding season						
1	CIDR alone (0 h)	4	7.4 ± 0.4	79.7 ± 14.5 ^a	10.8 ± 0.1 ^a	5.8 ± 4.2 ^a
2	CIDR alone (36 h)	5	5.8 ± 0.6	85.1 ± 18.9 ^a	7.1 ± 1.9 ^a	4.8 ± 3.2 ^a
3	CIDR/eCG	10	5.6 ± 0.9	120.1 ± 18.3 ^{ab}	7.5 ± 1.5 ^a	10.2 ± 4.7 ^a
4	CIDR/GnRH	9	5.6 ± 0.9	158.8 ± 31.4 ^b	5.8 ± 1.2 ^a	6.5 ± 1.5 ^a
Seasonal anoestrus						
1a	CIDR alone (0 h)	2	7.3 ± 0.2	89.4 ± 16.4 ^a	7.1 ± 2.3 ^a	0.4 ± 0.24 ^a
2a	CIDR alone (36 h)	4	7.9 ± 0.3	167.9 ± 29.5 ^a	8.9 ± 2.1 ^a	0.8 ± 0.20 ^a
3a	CIDR/eCG	7	7.6 ± 1.1	264.8 ± 49.3 ^{ab}	8.6 ± 1.8 ^a	1.4 ± 0.24 ^a
4a	CIDR/GnRH	13	5.8 ± 0.5	293.5 ± 37.6 ^b	8.7 ± 1.9 ^a	2.6 ± 0.68 ^a

Within columns, values with different superscripts are significantly different ($P < 0.05$).

Most importantly, these treatments are often associated with abnormalities in follicle development that may result in luteinization without ovulation.

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