

Reduced LH sensitivity *in vivo* and *in vitro* of corpora lutea induced during anoestrus by GnRH, and during the late breeding season, in Scottish Blackface ewes

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Abstract

Scottish Blackface ewes were synchronised in mid-breeding (November; group 1; $n=12$ ewes) or late-breeding season (March; group 2; $n=16$). Anoestrous ewes (May) were treated with progestagen sponges for 7 days and then given 250 ng GnRH 3-hourly for 24 h, 2-hourly for 24 h and hourly for a further 24 h (group 3; $n=12$). A second group of anoestrous ewes (group 4, $n=19$) received three bolus injections (30 μg) of GnRH at 90-min intervals without progestagen pretreatment. After ovulation, ewes were bled twice daily until slaughter (day 4 or day 12: oestrus=day 0). Mid-breeding season (group 1) and anoestrous ewes in group 3 formed 'adequate' corpora lutea (CL) with high plasma progesterone levels (3–4 ng/ml) maintained for at least 12 days, and responded *in vivo* to ovine LH (oLH) (10 μg) with a rise in plasma progesterone on day 11 (group 3, but not group 1, ewes also responded on day 3). CL minces from these ewes responded to human chorionic gonadotrophin (hCG) *in vitro* with a dose-dependent increase in progesterone secretion. Ewes in group 4 had a foreshortened luteal phase (8–10 days) and low plasma progesterone levels

(~ 1 ng/ml), consistent with formation of inadequate CL. LH injection failed to induce a significant plasma progesterone increase. Furthermore, although progesterone secretion *in vitro* in response to maximally stimulating doses of hCG or dibutyryl cAMP (dbcAMP) was similar to that in adequate CL, the sensitivity of these CL to hCG (EC (effective concentration)₅₀, 1 IU hCG/ml) was reduced 10-fold compared with adequate CL (EC₅₀, 0.1 IU hCG/ml; $P<0.01$). Ewes that ovulated in the late breeding season (group 2) had high plasma progesterone, although levels began to decrease after day 10. Injection of oLH *in vivo* increased plasma progesterone. However, sensitivity to hCG *in vitro* (EC₅₀, 0.5 IU hCG/ml) was intermediate between that of adequate luteal tissue (groups 1 and 3; EC₅₀, 0.1 IU/ml) and that of group 4 ewes (EC₅₀, 1 IU hCG/ml). Our data demonstrate a markedly reduced luteal sensitivity to LH *in vivo* and hCG *in vitro* in Scottish Blackface ewes with inadequate CL, and suggest that a similar loss of sensitivity to LH may occur in the late breeding season.

Journal of Endocrinology (2004) **183**, 517–526

Introduction

Corpora lutea (CL) formed in a variety of physiological situations (as during the post-partum interval (Wright *et al.* 1984, Braden *et al.* 1989), during puberty, during the transition from anoestrus to breeding season (Yuthasastrakosol *et al.* 1975, Legan *et al.* 1985) or in response to introduction of a ram during anoestrus (Martin *et al.* 1986)) have shortened lifespans and/or diminished peripheral progesterone levels. The conditions that give rise physiologically to 'inadequate' CL are transient, making them difficult to control and replicate. However, injection of an ovulatory stimulus (luteinising hormone

(LH), human chorionic gonadotrophin (hCG) or gonadotrophin-releasing hormone (GnRH)) to ewes during anoestrus or post-partum induces ovulation in the majority of animals (Garverick & Smith 1986, Hunter 1991, Garverick *et al.* 1992). In the absence of progesterone priming, the CL formed during anoestrus or post-partum were frequently inadequate. However, progestagen pretreatment for a few days or hours before, but not after (Keisler & Keisler 1989), ovulation induction significantly increased the proportion of adequate CL formed (McLeod *et al.* 1982a,b, McLeod & Haresign 1984, Hunter *et al.* 1986, Southee *et al.* 1988a,b). Thus, if we vary the treatment regimens (for example, with or without

progesterone pretreatment and/or with different patterns of GnRH injection), anoestrous ewes ovulate in response to GnRH to form CL that are predominantly adequate (with (+) progesterone priming; luteal phase plasma progesterone concentrations of >1 ng/ml on 3 consecutive days) (Hunter 1991) or inadequate (without (-) progesterone priming; luteal phase plasma progesterone concentrations failed to achieve 1 ng/ml on 3 consecutive days). Interestingly, we showed in an earlier study in Welsh Mountain ewes that CL likely to become inadequate could be distinguished from those likely to become adequate as early as day 3 after ovulation, because of their failure to respond to a bolus of 10 µg oLH *in vivo* with a rise in plasma progesterone and their reduced sensitivity to hCG *in vitro* (Bramley *et al.*, 2005). However, we found no effect of progesterone pretreatment alone on the frequency of adequate luteal phases. We have therefore extended our previous studies to investigate the responsiveness of CL induced by GnRH treatment protocols in anoestrous ewes of a different breed (Scottish Blackface), and examined the responses of CL formed spontaneously during the mid- and late-breeding season.

Materials and Methods

Materials

All fine chemicals and reagents were purchased from Aldrich (Gillingham, UK), BDH (Poole, UK) or Amersham. Radiolabelled [1,2,6,7-³H]progesterone (100 Ci/mmol) was from Amersham. ¹²⁵I-Labelled pregn-4-ene-3, 20-dione was purchased from Sigma. Highly purified hCG (Profasi; 15 000 IU/vial) for radioiodination was purchased from Serono (Welwyn Garden City, UK). hCG for the measurement of non-specific binding (Chorulon, 5000 IU/vial) was from Intervet Laboratories (Cambridge, UK). hCG was radioiodinated (Na¹²⁵I], 100 mCi/ml; Amersham, UK) by the lactoperoxidase method to a specific binding activity of 30 Ci/g (measured by self-displacement assay) (Bramley *et al.* 1987). Antibodies for the assay of progesterone (S-361) and ovine LH (R29) were the generous gifts of Prof. Alan McNeilly, MRC Human Reproductive Sciences Unit, Edinburgh, UK. Normal rabbit serum and donkey antirabbit serum were purchased from the Scottish Antibody Production Unit, Carlisle, UK.

Animal treatments and experimental protocols

In vivo studies Sixty-seven Scottish Blackface ewes (a breed with a mean ovulation rate of 1.2 that experiences a profound suppression of ovarian activity during seasonal anoestrus) were maintained outdoors under natural lighting conditions at the Dryden Field Laboratory, Roslin, UK. All ewes were of proven reproductive history, having lambed at least once, but were kept unmated over the

preceding breeding season. Ewes were weighed, ranked for condition and allocated randomly to the appropriate groups. The study was performed in three parts:

In November (mid-breeding season), 12 ewes (group 1) were synchronised with progestagen pessaries (Chronogest; Intervet Laboratories, Cambridge, UK). Plasma progesterone concentrations after pessary treatment were in the physiological range (3.9 ± 0.4 ng/ml; mean ± S.E.M.). Ewes were left untreated in the ensuing cycle. Then, after detection of oestrus of the second cycle with a vasectomised ram, venous blood samples were collected twice daily until slaughter on day 4 or 12 (oestrus = day 0).

In March (late-breeding season; group 2), 16 ewes were synchronised with progestagen pessaries and treated in an identical manner to ewes in group 1.

In May (seasonal anoestrus), 31 ewes were randomly allocated to group 3 (12 ewes) or group 4 (19 ewes). Ewes in group 3 (ramp-treated) were treated with progestagen sponges for 7 days before receiving injections of 250 ng GnRH intravenously at increasing frequency (3-hourly for 24 h, 2-hourly for 24 h and hourly for a further 24 h). Ewes in group 4 received no progestagen treatment, but were given three bolus injections of 30 µg GnRH at 90-min intervals. All injections were given in 2 ml sterile saline via indwelling jugular vein catheters.

Blood sampling Ewes in groups 1 and 2 were sampled twice daily from oestrus until slaughter on either day 4 or 12. Ewes in groups 3 and 4 were sampled before injection of GnRH, every 4 h for a subsequent 24 h and then twice daily until slaughter on day 4 or 12. All ewes were subjected to a period of intense (15-min) blood sampling on day 3 or 11. After the first hour, each ewe was given 10 µg ovine LH (oLH) (NIH-LH-S15) to assess the *in vivo* response to a pulse of LH. Blood samples (3 ml) were taken via an indwelling venous catheter at intervals of 4 h or less. Twice-daily blood samples (7 ml) were collected by jugular venepuncture.

In vitro studies

Tissue processing Ovaries were collected from ewes at slaughter on day 4 or 12 of the luteal phase, and transported to the laboratory on ice within 1 h. CL were excised, trimmed free of fat and connective tissue, and weighed, before being divided into portions for tissue incubations, receptor assays and morphology.

Progesterone secretion in vitro Duplicate aliquots of minced tissue from each CL were incubated at 37 °C in either 1 ml M199 alone (Flow Laboratories, Irvine, UK) or M199 supplemented with hCG (Chorulon, Intervet Laboratories; 10⁻⁴-10² IU/ml in 10-fold increments) or N⁶,2'-O-dibutyryl cyclic 3',5'-monophosphate (DBcAMP; 0.3 mM), as described in the figure legends.

After incubation, tubes were centrifuged (5000 *g* for 10 min), and tissue pellets and media were stored separately at -20°C . Tissue pellets were homogenised and assayed for protein (Lowry *et al.* 1951) to correct steroid secretion for differing amounts of tissue.

Assays oLH was measured by radioimmunoassay by the method of McNeilly *et al.* (1985). Assay sensitivity was 0.2 ng/ml, and intra- and interassay coefficients of variation were 5.2% and 12.1% respectively.

Serum progesterone concentrations were measured by radioimmunoassay (Scaramuzzi & Baird 1974), as described previously (Bramley *et al.*, 2005). Incubation medium did not interfere with the assay; therefore, the progesterone content of media was measured without solvent extraction. Assay sensitivity was 0.1 ng/ml, and intra- and interassay coefficients of variation were 7.2% and 10.9% respectively.

Assay of occupied and unoccupied LH receptors

Unoccupied LH receptor levels were measured by specific binding of ^{125}I -hCG (specific binding activity, 30 Ci/g) to triplicate aliquots (20–100 μl) of ovine luteal homogenates in the presence or absence of 10 IU unlabelled hCG. Values of binding affinity (K_d) were calculated from Scatchard plots constructed from triplicate measurements of specific binding of ^{125}I -hCG (0.5–30 pM) for a number of representative CL for each treatment group.

LH receptor occupancy was measured by acid dissociation of bound oLH from triplicate aliquots (100 μl) of luteal homogenate with ice-cold 0.1 M citrate buffer, pH 3.0, as described previously (Bramley *et al.*, 2005). Receptor concentrations were adjusted for DNA content (Burton 1956), using calf thymus DNA as standard.

Analysis of data

Preliminary experiments comparing cell number after collagenase dispersion and DNA content indicated a mean DNA content of 6 pg/cell. This factor was used to convert DNA content to total cell number.

Luteal function was defined as adequate if plasma progesterone concentrations were elevated for at least 8 days, starting within 4 days of the LH surge, and maximal concentrations of progesterone exceeded 1.5 ng/ml for at least 2 consecutive days (Hunter 1991). Adequacy of luteal function was compared between groups by the chi-square test or Fisher's exact test. Hormone profiles were compared by two-way analysis of variance with repeated measures, followed by Duncan's multiple-range test where appropriate. Other luteal parameters were compared between groups by Student's *t*-test, with the Bessel correction for small numbers.

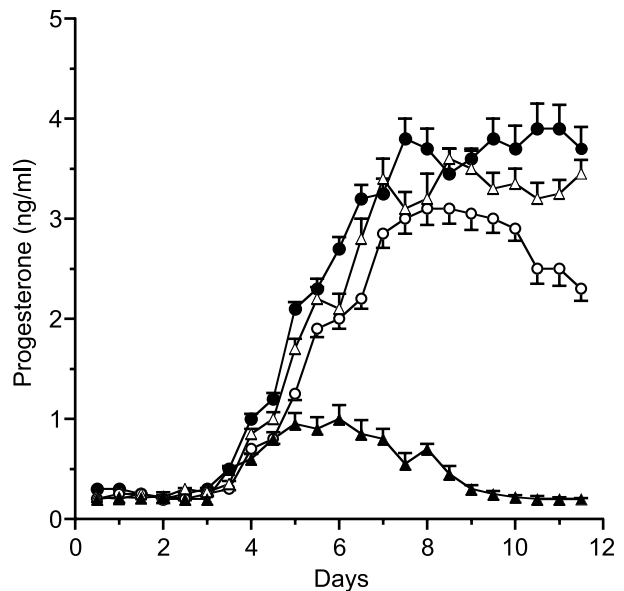


Figure 1 Plasma progesterone levels in ewes during the mid- or late-breeding season, or after induction of ovulation by GnRH injection during anoestrus. Plasma progesterone levels were measured in Scottish Blackface ewes during the mid-breeding season (group 1, ●), the late-breeding season (group 2, ○), or during anoestrus after pretreatment with progesterone followed by injection of GnRH at increasing frequency (group 3, △) or without progesterone treatment, with three bolus injections of GnRH (group 4, ▲). Day 0 = day of oestrus. Points are means \pm S.E.M.

Results

All group 1 and 2 ewes ovulated at both stages of the breeding season. However, the ovulation rate was significantly higher for ewes during the mid-breeding season (group 1; 1.5 ± 0.2 CL per ewe) than during the late-breeding season (group 2; 1.0 ± 0.1 CL per ewe; $P < 0.05$). The proportion of ewes that ovulated during anoestrus was significantly higher for group 3 than group 4 (10/12 vs 8/18; $P < 0.01$).

In vivo studies

Plasma progesterone levels in ewes from treatment groups 1, 2 and 3 began to increase on day 4 and continued to rise to a plateau (3–4 ng/ml) at around day 8 (Fig. 1). There was no significant difference between groups 1–3 until days 10–11, when plasma progesterone levels began to fall in animals from the late-breeding season (group 2). In contrast, mean progesterone levels were significantly lower in ewes from group 4 than in groups 1, 2 and 3 by day 5, peaking at ≥ 1 ng/ml, and then declining prematurely to reach basal levels by days 9–10 (Fig. 1). All CL in group 4 had undergone luteolysis by day 11.

Mean plasma LH levels were consistently higher on day 3 than on day 11 in all groups, but were lowest in ewes in

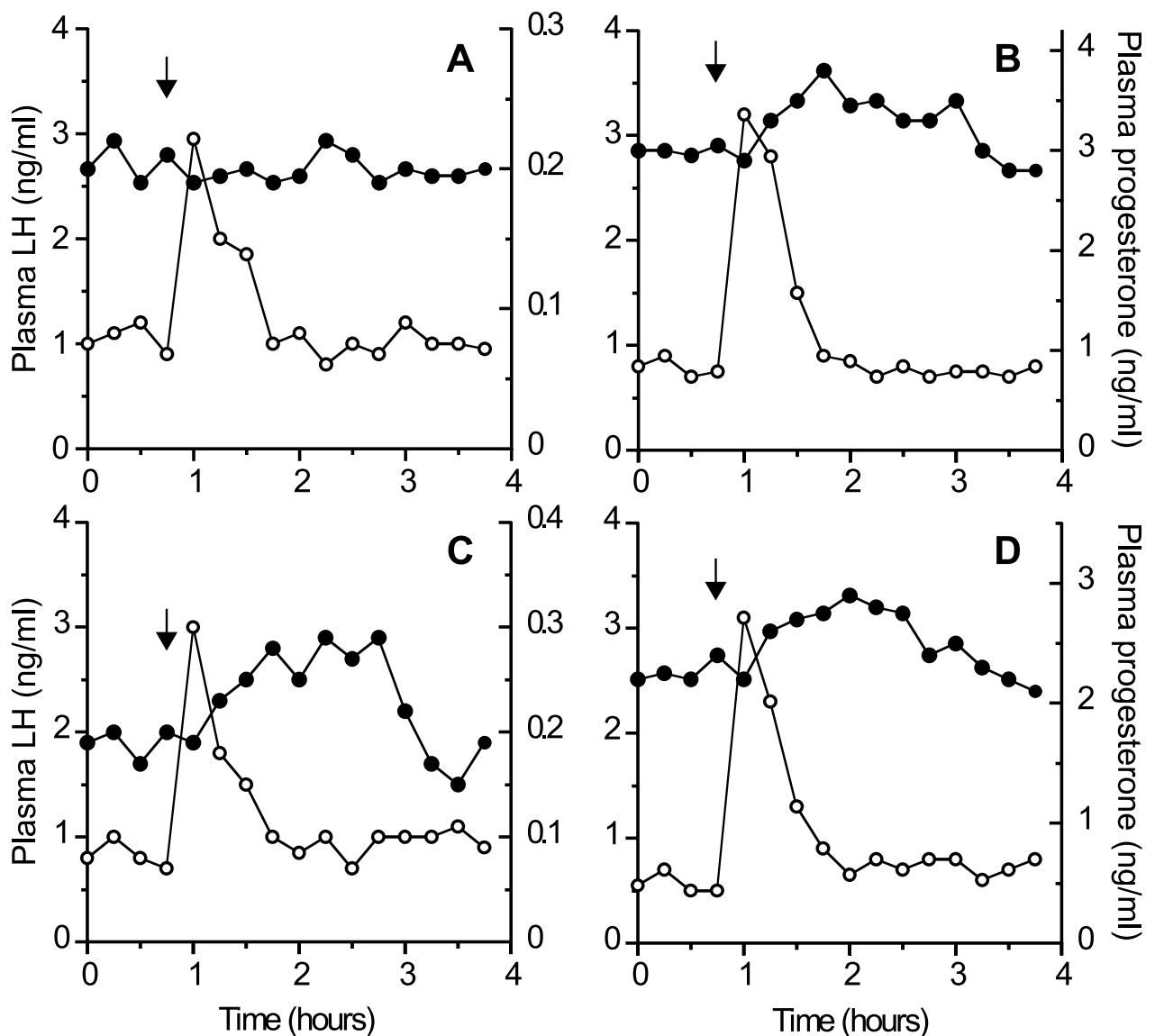
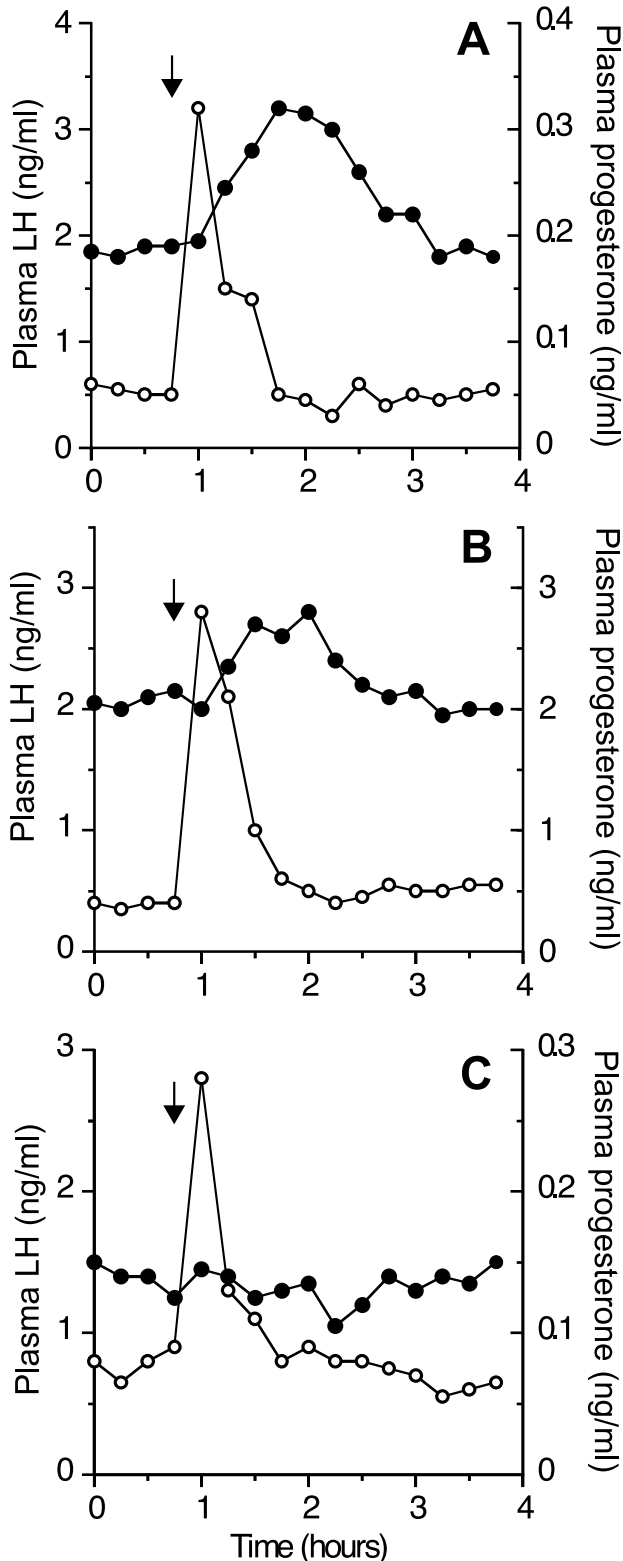


Figure 2 Plasma progesterone concentrations in response to oLH injection *in vivo*. Scottish Blackface ewes were treated during the mid- (group 1) or late-breeding season (group 2), as described in the Materials and Methods section, and injected with 10 μ g oLH (arrows) on either day 3 (A and C) or 11 (B and D) of the luteal phase. Blood samples were collected every 15 min for 4 h, and plasma levels of oLH (○) and progesterone (●) were measured by immunoassay. Points are means for 4–8 ewes per group. ↓ indicates point of oLH injection.

group 3 at both stages of the luteal phase (Table 2). When ewes were challenged with 10 μ g oLH on day 3 of the luteal phase in the mid- or late-breeding season (Fig. 2), ewes in groups 2 (Fig. 2C) and 3 (Fig. 3A and B) responded with a marked rise in progesterone; however, no significant response was apparent in ewes in group 1 (Fig. 2A). When ewes were challenged with oLH on day 11, groups 1–3 responded with an increase in progesterone (Fig. 2B and D and Fig. 3B). However, group 4 ewes failed to respond on day 3 (Fig. 3C).

Characteristics of CL

Luteal weights and estimated total cell number (DNA content) of CL recovered on day 4 were not significantly different for CL formed during the mid- or late breeding season (groups 1 and 2; Table 1). Progesterone content was significantly lower during the late breeding season than during the mid-breeding season ($P < 0.05$). However, both luteal weight and progesterone content were lower in group 3 ewes than in both groups of breeding season ewes,



and progesterone content was reduced further in group 4 ewes on day 4 (Table 1). In contrast, there were no significant differences in estimated total cell numbers per CL (DNA content) on day 4.

Luteal weight and DNA content (cell number) were significantly higher in CL recovered on day 12 than in CL from the same treatment group recovered on day 4 (Table 1), and luteal weight, total cell number and progesterone content on day 12 were significantly lower in CL from the late breeding season than in CL from the mid-breeding season. Luteal progesterone content was significantly lower on day 12 for group 1 than on day 4, but was no different for groups 2 and 3.

Levels of unoccupied and occupied LH receptors were similar in CL from all treatment groups on day 4 (Table 2), with a mean occupancy ratio (occupied/unoccupied receptors) of 7–8%. Luteal levels of occupied and unoccupied LH receptor increased dramatically between days 4 and 12 (Table 2), with unoccupied LH receptor levels increasing to a greater extent than occupied receptors, and leading to a fall in mean LH receptor occupancy from ~8% on day 4 to 2–3% on day 12. Values of K_a derived from Scatchard plots of 125 I-hCG binding to homogenates of CL from the three groups on day 12 did not differ significantly (K_a , $0.5 \pm 0.2 \times 10^{10} M^{-1}$, mean \pm S.E.M.; data not shown).

In vitro responsiveness

Basal progesterone secretion *in vitro* was significantly greater in CL recovered on day 4 than on day 12 for all treatment groups (Fig. 4). In contrast, progesterone secretion in the presence of a maximally stimulating dose of hCG or dbcAMP was not significantly different between treatment groups on either day 4 or day 12 (Fig. 4; $P > 0.1$). However, despite similar responses to maximal doses of hCG, dose–response curves for progesterone secretion with increasing levels of hCG (Fig. 5) revealed marked differences in hCG sensitivity between treatment groups. Luteal tissue from mid-breeding season (group 1) and ‘adequate’ GnRH-induced anoestrous ewes (group 3) was significantly more sensitive to hCG stimulation (EC_{50} , 0.1 IU hCG/ml) than luteal tissue from group 4 ewes (EC_{50} , 1.07 IU hCG/ml; Table 2) on day 4 (Fig. 5A). The hCG sensitivity of luteal tissue from

Figure 3 Plasma progesterone concentrations in response to oLH injection *in vivo*. Anoestrous Scottish Blackface ewes were treated as described in the Materials and Methods section and injected with 10 µg oLH (arrows) on either day 3 (A and C) or 11 (B) of the luteal phase. No ewes in group 4 had CL that persisted until day 11. Blood samples were collected every 15 min for 4 h, and plasma levels of oLH (○) and progesterone (●) were measured by immunoassay. (A and C) Group 3 ewes; (B) group 4 ewes. Points are means for 4–8 ewes per group. ↓ indicates point of oLH injection.

Table 1 Comparison of some characteristics of corpora lutea (CL) formed during the breeding season with GnRH-induced CL formed during anoestrous in Scottish Blackface ewes

	Group	Luteal weight (mg)	DNA content (mg)	Estimated cell number ($\times 10^{-6}$)	Progesterone content (ng)
Day					
4	1	168 \pm 19	0.85 \pm 0.11	104 \pm 13	78.5 \pm 1.8
4	2	219 \pm 25	0.92 \pm 0.13	113 \pm 16	62.0 \pm 2.6 ^a
4	3	101 \pm 21 ^a	0.76 \pm 0.09	93 \pm 11	35.5 \pm 1.1 ^b
4	4	140 \pm 27	0.81 \pm 0.10	99 \pm 12	17.3 \pm 0.6 ^c
12	1	792 \pm 63*	2.61 \pm 0.18*	320 \pm 22*	155.8 \pm 14.1*
12	2	622 \pm 44 ^{a,*}	1.92 \pm 0.14 ^{a,*}	235 \pm 17 ^{a,*}	58.0 \pm 5.6 ^b
12	3	439 \pm 10 ^b	1.51 \pm 0.11 ^{b,*}	185 \pm 13 ^{b,*}	31.9 \pm 3.9 ^c
12	4	—	—	—	—

No CL in group 4 persisted up to day 12. Figures are means \pm S.E.M. for 6–8 ewes.

Values within columns with different letters are significantly different ($P < 0.05$).

*Significantly different from value for that group on day 4 ($P < 0.01$).

late-breeding season ewes on days 4 and 12 (EC_{50} , 0.5 IU/ml hCG) was intermediate between that of groups 1 and 3, and group 2 ewes (Fig. 5A and B; Table 2).

Discussion

Mean ovulation rate (as judged by number of fresh CL at autopsy on day 4) was significantly higher during the mid-breeding season (group 1, 1.5 \pm 0.2) than toward the end of the season (group 2, 1.0 \pm 0.1; $P < 0.05$). Treatment of anoestrous Scottish Blackface ewes with progestagen priming and with increasing GnRH frequency (group 3) induced ovulation in 10/12 ewes. In contrast, three bolus injections of 30 μ g GnRH at 90-min intervals in the absence of progesterone priming (group 4) induced ovulation in only 8 of 18 ewes ($P < 0.01$ compared with groups 1–3). These ovulation rates are similar to data from

previous reports in a variety of different sheep breeds (Bramley *et al.*, 2005).

CL induced in anoestrous Scottish Blackface ewes that were pretreated with progestagen and given GnRH injections of increasing frequency (group 3) had a similar lifespan to ewes that ovulated spontaneously in the mid-breeding season, secreted similar levels of progesterone *in vivo*, and showed a similar response to oLH injection *in vivo* (day 12; Figs 2B and 3B). Moreover, although these CL tended to be smaller than CL formed during the mid- or late-breeding season (Table 1), and had a significantly lower luteal progesterone content (Table 1), luteal tissue from these ewes had a similar sensitivity to hCG *in vitro* to mid-breeding season ewes (Fig. 5A and B). These observations confirm those made previously with an identical injection protocol in Welsh Mountain ewes at a similar stage of anoestrus (Bramley *et al.*, 2005).

Table 2 hCG binding and sensitivity of progesterone secretion *in vitro* to hCG of corpora lutea (CL) formed during the breeding season and GnRH-induced CL formed in anoestrous Scottish Blackface ewes

Day	Group	LH receptor binding (pg/ μ g DNA)		Serum LH (ng/ml)	EC_{50} for hCG (IU/ml)
		Unoccupied	Occupied		
4	1	6.24 \pm 2.1	0.52 \pm 0.06	1.04 \pm 0.07	0.09 \pm 0.01
4	2	5.31 \pm 2.8	0.41 \pm 0.07	0.80 \pm 0.07 ^a	0.49 \pm 0.02 ^a
4	3	4.80 \pm 1.4	0.43 \pm 0.05	0.59 \pm 0.04 ^b	0.10 \pm 0.01 ^b
4	4	4.81 \pm 0.8	0.39 \pm 0.07	0.74 \pm 0.04 ^a	1.07 \pm 0.01 ^c
12	1	31.7 \pm 5.4*	0.89 \pm 0.08*	0.77 \pm 0.07*	0.11 \pm 0.21
12	2	28.3 \pm 2.1*	0.76 \pm 0.06*	0.60 \pm 0.06*	0.52 \pm 0.32
12	3	24.0 \pm 7.6*	0.77 \pm 0.07*	0.39 \pm 0.02 ^{a,*}	0.11 \pm 0.14
12	4	—	—	—	—

EC_{50} values (dose of hCG required to increase progesterone secretion to half-maximum) were calculated from dose–response curves for luteal minces of individual CL from each group performed in duplicate. No CL in group 4 persisted up to day 12.

Figures are means \pm S.E.M. Values within columns with different letters are significantly different ($P < 0.05$).

*Significantly different from value for that group on day 4 ($P < 0.01$).

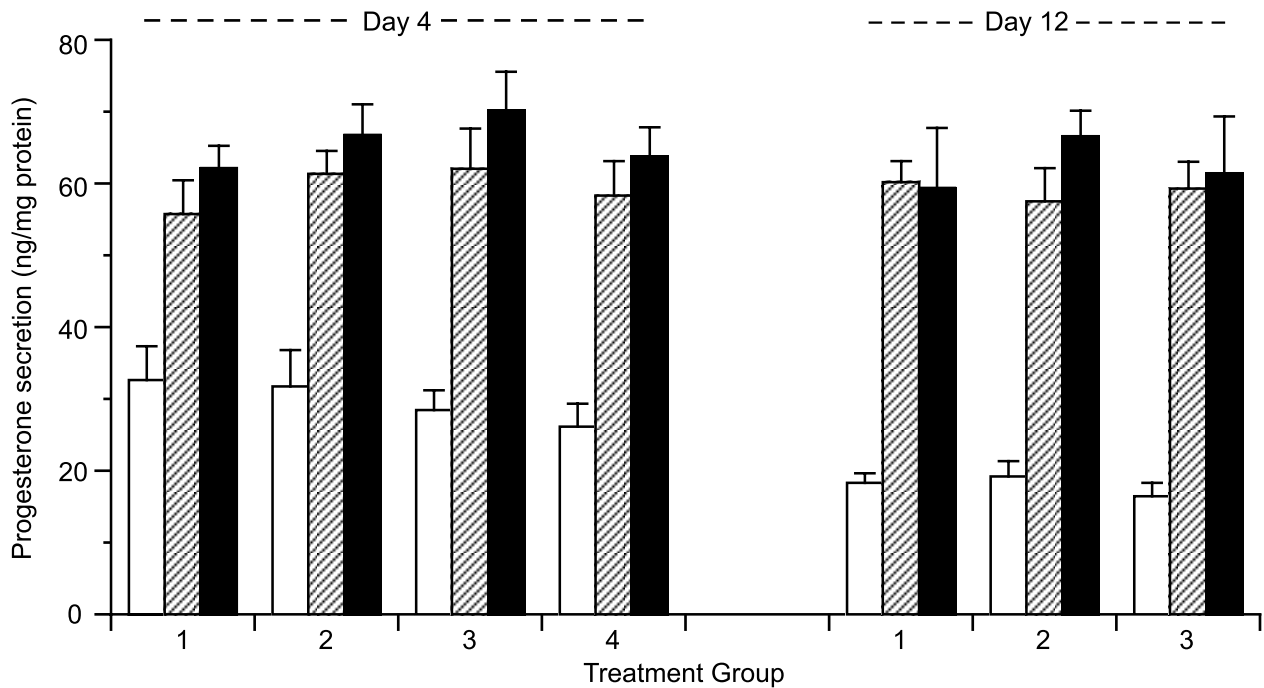


Figure 4 Progesterone secretion by luteal tissue from the different experimental groups on day 4 or 12 of the luteal phase, incubated with or without 10 IU/ml hCG or 0.5 mM dbcAMP *in vitro*. Luteal tissue was recovered from the different treatment groups on either day 4 or 12 and minced finely with new razor blades, and triplicate aliquots were incubated for 2 h in M199 at 37 °C in the absence (open columns) or presence of a maximal dose (100 IU/ml) of hCG (diagonal hatched columns) or dbcAMP (0.5 mM; solid columns). Column height represents means and vertical bars S.E.M. for $n=4-8$ animals per group.

In contrast, Scottish Blackface ewes induced to ovulate without progestagen priming with three bolus injections of GnRH (group 4) had a greatly reduced luteal lifespan; indeed, no CL persisted *in vivo* to day 12. Serum progesterone concentrations *in vivo* were similar in all groups up to day 5, but thereafter were dramatically reduced in group 4 ewes compared with ewes in groups 1–3 (Fig. 1). Furthermore, luteal wet weights in these animals were similar to those of CL from groups 1–3 (day 4), but they had a greatly reduced luteal progesterone content ($P<0.01$; Table 1), confirming the earlier study of McNeilly *et al.* (1981), and these ewes failed to respond to a bolus injection of LH on day 4 with a rise in serum progesterone (Fig. 3C). Furthermore, the sensitivity of $-P/GnRH$ bolus CL to hCG *in vitro* was reduced 10-fold compared with mid-breeding season or $+P/GnRH$ ramp-treated (group 3) animals (Fig. 5A and B), although steroidogenic responses to maximally stimulating doses of hCG or DbcAMP were unchanged (Figs 4 and 5).

The marked shortening of the luteal phase in anoestrous Scottish Blackface ewes treated with three bolus injections of GnRH contrasted with our data from Welsh Mountain ewes given an identical treatment (Bramley *et al.*, 2005). In fact, 4 out of 9 Welsh Mountain ewes continued to secrete progesterone up to day 12, although plasma levels of progesterone bordered on the inadequate range

(~ 1 ng/ml). Maintenance of the inadequate luteal phase in Welsh Mountain ewes appeared to correlate with higher serum concentrations of prolactin in these animals (Bramley *et al.*, 2005). There were no significant differences in the properties of CL induced by the $-P/GnRH$ bolus regimen between Welsh Mountain and Scottish Blackface ewes that might predict such a difference in lifespan between these breeds. Indeed, early CL (day 4) of Scottish Blackface animals had more total cells per CL (99 ± 12 vs 56 ± 11 million cells respectively; $P<0.05$), and higher levels of unoccupied LH receptor (4.8 ± 0.8 vs 2.1 ± 0.7 pg LH/ μ g DNA respectively) than Welsh Mountain ewes treated in an identical fashion ($P<0.05$; Table 3), suggesting that shortened lifespan in $-P/GnRH$ bolus Scottish Blackface ewes was not due to an LH receptor deficit. Levels of occupied LH receptors were lower in Welsh Mountain ewes than Scottish Blackface ewes on day 4 of treatment in both breeding season and progestagen-primed GnRH ramp-treated ewes (Table 3; $P<0.05$). However, these differences were no longer apparent by day 12, the only significant difference in day 12 CL being lower luteal wet weights for Welsh Mountain than Scottish Blackface ewes during the mid-breeding season (792 ± 63 vs 569 ± 50 mg; $P<0.05$). Moreover, LH receptor percentage of occupancy was similar for both breeds on days 4 (6–13%) and 12 (2.8–3.9%). The decline

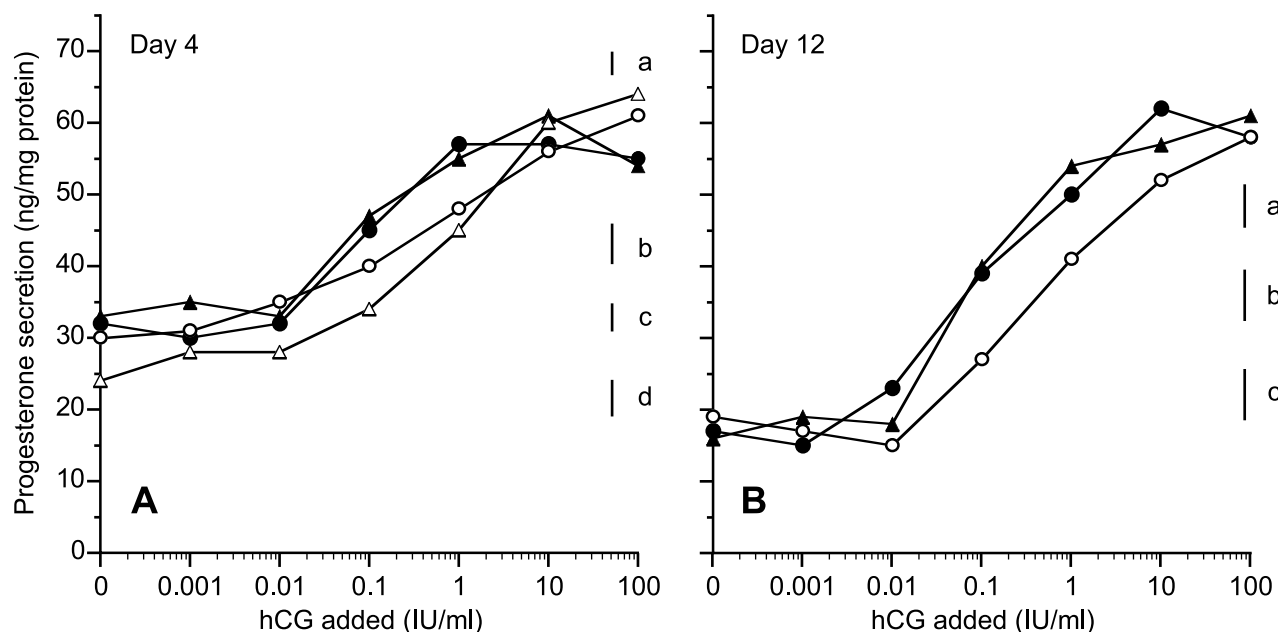


Figure 5 Progesterone secretion of luteal tissue from the different treatment groups in response to increasing doses of hCG *in vitro*. Luteal tissue recovered from the different treatment groups on either day 4 (A) or 12 (B) was minced, and triplicate aliquots were incubated for 2 h in M199 at 37 °C in the presence of increasing doses (0.1 mIU/ml to 100 IU/ml) of hCG. Tubes were spun at 5000 g for 10 min, and conditioned medium was aspirated and assayed for progesterone. Group 1 (●); group 2 (○); group 3 (▲); group 4 (△). Points are means for 4–7 animals in each group. Vertical lines indicate overall s.e.m. values for group 1 (a), group 2 (b), group 3 (c) or group 4 (d).

in percentage of occupancy from days 4 to 12 probably largely reflects residual bound LH from the ovulatory surge present in day 4 CL. Indeed, this was suggested by the observation that 'basal' secretion of progesterone *in vitro* by CL from day 4 ewes was significantly greater than for day 12 CL in all treatment groups ($P < 0.01$; Figs 4 and 5). However, increasing total LH receptor levels (Table 2) coupled with reduced LH secretion (Table 2) in response

to feedback from rising luteal progesterone levels between days 4 and 12 would also contribute to a decrease in receptor occupancy.

There were some interesting differences between CL formed in Scottish Blackface ewes in the mid- and late-breeding season. Luteal lifespan was shortened in ewes during the late breeding season compared with mid-season, as judged by the premature fall in progesterone

Table 3 Comparison of some characteristics of corpora lutea (CL) formed during the mid-breeding season with GnRH-induced CL formed during anoestrus in Scottish Blackface (SB) and Welsh Mountain (WM) ewes

Group	Luteal weight (mg)		Estimated total cell number ($\times 10^{-6}$)		LH-Receptor binding (pg/ μ g DNA)			
	SB	WM	SB	WM	Unoccupied		Occupied	
					SB	WM	SB	WM
<i>Day 4</i>								
Mid-breeding	168 \pm 19	203 \pm 29	104 \pm 13	87 \pm 13	6.2 \pm 2.1	5.7 \pm 1.2	0.52 \pm 0.06	0.34 \pm 0.04*
+P/GnRH ramp	101 \pm 21	113 \pm 18	93 \pm 11	63 \pm 10	4.8 \pm 1.4	4.7 \pm 1.0	0.43 \pm 0.05	0.28 \pm 0.03*
-P/GnRH bolus	140 \pm 27	127 \pm 12	99 \pm 12	56 \pm 11*	4.8 \pm 0.8	2.1 \pm 0.7*	0.39 \pm 0.07	0.27 \pm 0.05
<i>Day 12</i>								
Mid-breeding	792 \pm 63	569 \pm 50*	320 \pm 22	274 \pm 22	31.7 \pm 5.4	19.5 \pm 4.3	0.89 \pm 0.08	0.65 \pm 0.08
+P/GnRH ramp	439 \pm 10	397 \pm 34	185 \pm 13	194 \pm 13	24.0 \pm 7.6	14.8 \pm 2.1	0.77 \pm 0.07	0.58 \pm 0.07
-P/GnRH bolus	—	338 \pm 91	—	135 \pm 16	—	7.9 \pm 3.2	—	0.31 \pm 0.04

No CL in Scottish Blackface–P/GnRH bolus group persisted up to day 12. Figures are means \pm s.e.m. for 5–8 ewes.

*Data for Welsh Mountain (from Bramley *et al.*, 2005) were significantly different from Scottish Blackface ewes ($P < 0.05$).

after day 10 in group 2 ewes versus group 1 ewes (Fig. 1). Furthermore, luteal sensitivity to hCG *in vitro* was significantly depressed on day 4 (Fig. 5A; Table 2), although the difference in sensitivity to hCG was not significant on day 12 because of the large between-animal variability (Table 2). This variability may reflect the difference in responsiveness between ewes in which the CL is beginning to fail and ewes with a CL that continues to function up to day 12. Alternatively, the defect present on day 4 may be corrected in a proportion of animals as the CL matures.

Although ewes in groups 2 and 3 responded to oLH with a pulse of progesterone secretion on days 3 and 11 (Fig. 2C and D; Fig. 3A and B), ewes in group 1 (mid-breeding season) did not respond on day 3 (Fig. 2A), although they did respond on day 11 (Fig. 2B). The failure to observe a response at this time may be related to the higher circulating levels of progesterone in group 1 ewes than group 2 and 3 ewes (2.7–2.9 vs 1.9–2.4 ng/ml respectively), making detection of a small increase more difficult. Serum LH was also higher in this group of ewes on day 4 (Table 2), suggesting that LH receptor occupancy was higher, leading in turn to maximal steroid secretion. However, measurements of receptor occupancy were not significantly higher in these ewes (Table 2), and basal steroid secretion *in vitro* was not significantly different from that by CL from group 2 or group 3 animals (Fig. 4). Moreover, steroid secretion *in vitro* could still be stimulated by both hCG and dbcAMP (Figs 4 and 5a).

Our data indicate that luteal function in Scottish Blackface ewes was severely compromised in CL-induced pharmacologically during anoestrus by the –P/GnRH bolus (but not the +P/GnRH ramp-treatment) regimen. However, luteal function in Scottish Blackface ewes was also subtly compromised in spontaneously ovulating ewes as they approached the spring breeding season–anoestrus transition. These changes in luteal function at different stages of the season did not appear to be due to inadequate levels of LH receptors or receptor occupancy (Table 2). Moreover, sensitivity to oLH *in vivo* (Figs 2 and 3) was unaffected (Fig. 2). Furthermore, LH receptor affinity in the CL from these groups subjected to Scatchard analysis was unchanged (K_a , $0.5 \pm 0.2 \times 10^{10}$ /M; data not shown). However, steroidogenic sensitivity to hCG *in vitro* was clearly compromised in late-breeding season ewes relative to mid-breeding season ewes (Table 2; Fig. 5). Despite this, responsiveness to maximal doses of hCG or to dbcAMP was unaffected (Figs 4 and 5). This suggests that the defect in inadequate CL (induced pharmacologically by –P/GnRH bolus treatment or physiologically after ovulation late in the season) lies downstream of the LH receptor, but upstream of cAMP, and probably affects coupling of hormone binding to the LH receptor to the appropriate intracellular signalling system. Luteal LH/hCG receptors are known to activate a number of different stimulatory and inhibitory cell signalling pathways

(Davis & Rueda 2002, Niswender 2002), and it will be of great interest to compare the effects of LH on these different signalling pathways in both ‘adequate’ and ‘inadequate’ ovine CL.

Acknowledgements

We thank the staff of the Large Animal Unit of the Roslin Institute for their assistance with animal handling and blood sampling, and Prof. Alan McNeilly for provision of antisera for radioimmunoassays. This work was supported in part by grants (D.S., T.A.B. and D.T.B.) from the Medical Research Council, UK. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 19 July 2004

Accepted 9 September 2004