Gonadotrophin-releasing hormone and the control of ovulation rate by the Fec^B gene in Booroola ewes

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Booroola sheep carry a Fec^B gene that confers high fecundity. The aims of the present studies were to determine in homozygous carriers (BB) and non-carriers (++) of the Booroola Fec^B gene whether there are Fec^B differences in the secretory characteristics of GnRH in hypophyseal–portal blood of ovariectomized ewes (Expt 1) and whether differences in ovulation rate would occur following the administration of PMSG and pulsatile GnRH to ovary-intact Booroola ewes with hypothalamic–pituitary disconnection (HPD) (Expt 2). In Expt 1, no Fec^B gene-specific differences were noted for GnRH with respect to pulse frequency, pulse amplitude or overall secretion rate. Irrespective of genotype there were 1.9 ± 0.2 GnRH pulses h^{-1} ($n = 20$ ewes; 10 BB and 10 ++ animals) and 1.9 ± 0.1 pulses of immunoreactive (i) LH h^{-1} . In Expt 2, ovulation was induced in the HPD ovary-intact animals on three occasions (e.g. 56, 393 and 423 days after HPD surgery) using a PMSG (200 or 400 iu)–GnRH pulse (250 ng i.v. for 96 h)–GnRH bolus (10 μ g i.v.) regimen after pretreating the animals with GnRH pulses (250 ng i.v. for 14 days). On all occasions the ovulation rates were significantly higher ($P < 0.05$) in BB ewes ($n = 6$ or 7) than in ++ animals ($n = 7$). No differences between the genotypes were noted with respect to the mean concentrations of progesterone in plasma notwithstanding the differences in ovulation rates. No ovulations occurred after treating the HPD ovary-intact animals with GnRH pulses alone, a GnRH bolus alone or PMSG followed 96 h later by a GnRH bolus without the intervening GnRH pulses. During the GnRH pretreatment or ovulation-induction period Fec^B differences were sometimes noted in the plasma concentrations of either i-FSH or bioactive FSH but not for i-LH (BB > ++; $P < 0.05$). These results suggest that there are unlikely to be Fec^B differences with respect to GnRH secretion in ovariectomized BB and ++ ewes. Moreover they show that the Fec^B differences in ovulation rate remain when an identical ovulation–induction regimen was given to HPD–ovary-intact BB and ++ ewes. However, it is uncertain whether the ovulation rate differences were due to Fec^B differences in plasma FSH or to intraovarian factors.

Introduction

Booroola ewes have a major gene(s) Fec^B which influences their ovulation rate (i.e. the number of ovulations per oestrous cycle; see Bindon, 1984 for review). Homozygotes (BB), heterozygotes (B+) and non-carriers (++) of the B gene(s) are segregated on the basis of ovulation rate recordings of ≥ 5 , 3 or 4 and 1 or 2, respectively (Davis *et al.*, 1982). Previous studies on ovary-intact, ovariectomized, hypothalamic–pituitary disconnected (HPD) and hypophysectomized Booroola ewes have established that there are differences between the Fec^B genotypes in ovarian morphology and function and in the plasma concentrations of pituitary gonadotrophins (see McNatty *et al.*, 1991a for review). With respect to pituitary function, ovary-intact, ovariectomized and GnRH treated HPD–ovariectomized animals all contain

higher plasma concentrations of immunoreactive (i)-FSH in BB than in ++ genotypes. Recent studies also show that ovariectomized BB ewes contain higher concentrations of bioactive (b)-FSH in plasma than ++ animals (Phillips *et al.*, 1993). However, it is not yet certain whether some of these gene-specific differences in plasma FSH are due to differences in the secretory patterns of GnRH.

Although ewes with the Fec^B gene contain higher concentrations of FSH than animals without the gene there is also evidence to suggest that the higher ovulation rate in BB ewes is due to intraovarian factors. Fry *et al.* (1988) induced ovulation in a proportion of hypophysectomized B+ and ++ ewes using a PMSG–hCG regimen; 12 of 27 B+ and 13 of 32 ++ ewes were observed to have ovulated as assessed by laparoscopy. In the ewes that had ovulated, there were significantly more corpora lutea in the B+ animals than in the ++ ewes ($P < 0.01$) but the plasma concentrations of gonadotrophins over the treatment

period and the functional status of the corpora lutea were not reported. In contrast to the difficulties in stimulating ovulation in hypophysectomized ewes (Fry *et al.*, 1988; Sawyer *et al.*, 1991), ovulation induction in HPD ewes has been achieved using exogenous PMSG/hCG or GnRH pulse regimens (Phillips *et al.*, 1989; Sawyer *et al.*, 1991). HPD-ovary-intact ewes contain chronically low or undetectable concentrations of i-FSH and i-LH (McNatty *et al.*, 1992a) and as such are a useful model in which to examine the effects of exogenous GnRH on endogenous gonadotrophin concentrations and ovulation rate in Booroola ewes.

The aims of the present studies were to determine whether there are Fec^B differences among Fec^B genotypes in the GnRH secretory characteristics in hypophyseal portal blood of ovariectomized ewes (Expt 1) and whether exogenous GnRH regimens given to HPD ovary-intact Booroola ewes would induce the expected differences in ovulation rate (Expt 2). The effects of exogenous GnRH treatment on the plasma concentrations of i-FSH, b-FSH and i-LH were also examined.

Materials and Methods

Animals

Ewes that were homozygous carriers (BB) or non-carriers (++) of the Fec^B gene were classified as such by pedigree analysis and this was confirmed by ovulation rate measurement. Carriers (BB) of the gene were derived from mating BB rams with BB ewes and non-carriers from ++ rams with ++ ewes; the BB animals had been recorded to have ovulation rates ≥ 5 on three occasions and the ++ ewes had ovulation rates of one or two on three occasions, as assessed by laparoscopy (Davis *et al.*, 1982).

Experiments

All animal experimentation was done in accordance with the 1987 Animals Protection (Codes of Ethical Conduct) Regulations of New Zealand after approval was granted by the Animal Ethics Committee at the Wallaceville Animal Research Centre.

Experiment 1. Ovariectomized Booroola $\times$ Merino ($n = 7$ ++ and 7 BB) and Booroola $\times$ Romney ($n = 3$ ++ and 3 BB) ewes were studied. The mean $\pm$ SEM ages and bodyweights of the animals were 8.1 ± 0.5 years (++) and 8.2 ± 0.5 years (BB) and 43.5 ± 0.9 kg (++) and 41.2 ± 0.7 kg (BB), respectively; there were no genotypic differences in age or bodyweight. The mean $\pm$ SEM time from ovariectomy was 209 ± 36 days for the ++ genotype and 229 ± 40 days for the BB genotype. The ++ animals originated from five different sires and the BB from nine sires.

Surgical preparation and cannulation of the above animals for hypophyseal portal blood collection was performed 2 days before blood sampling as described by Clarke and Cummins (1982, 1985) and Li *et al.* (1989). A jugular venous catheter was also prepared on the day of surgery. On the day of blood sampling heparin (Fisons Pty Ltd, Thornleigh, NSW) was given as a bolus injection (i.v.) of 25 000 iu at 07:00, 08:00, 08:30 h,

and thereafter 12 500 iu were administered at approximately hourly or two hourly intervals until the end of the experiment. In addition to the bolus injections, a heparin drip of 7500 iu h^{-1} was run. At 07:15 h jugular blood samples were taken at 2.5 or 5 min intervals and at approximately 08:30 h the portal blood vessels were cut as described by Clarke and Cummins (1982, 1985). The portal blood was collected into tubes containing 0.2 ml of 5 mmol Bacitracin l^{-1} (Sigma Chemical Co., St Louis, MO) in 0.9% (w/v) saline under continuous suction. When the flow rate of portal blood was $\geq 1.5 \text{ ml in 10 min}$, sampling at 10 min (2 BB, 2 ++), 5 min (7 BB, 4 ++) or 2.5 min (1 BB, 4 ++) intervals began and continued for up to 8 h. When the portal blood samples were being collected, the jugular blood samples were taken immediately after changing each tube for portal blood.

The portal and jugular samples were stored in centrifuge tubes on ice until centrifugation at 4000 g for 8 min at 4°C within 10 min after collection. The volume of plasma was recorded and a haematocrit measurement was made once every hour. Additional samples of 5 ml jugular blood were collected every hour into Bacitracin to be used as peripheral plasma and Bacitracin 'blanks' in the GnRH assay. All plasma samples were stored at -20°C until assayed. At the end of the experiment the ewes were killed with an overdose of barbiturate (sodium pentobarbitone, $\approx 20 \text{ ml of } 500 \text{ mg ml}^{-1}$, w/v, i.v.) and the pituitary glands removed to verify the site at which the pituitary portal vessels had been cut.

Experiment 2. Booroola Merino (4 BB, 4 ++) and Booroola Romney (5 BB, 5 ++) ewes were studied. The mean $\pm$ SEM ages of the ++ and BB genotypes at HPD surgery were 6.0 ± 0.2 and 6.4 ± 0.2 years, respectively. The mean $\pm$ SEM bodyweights of the ++ and BB genotypes were 41.7 ± 2.1 and 37.4 ± 1.0 kg, respectively; these values were not significantly different. The ++ and BB animals each originated from four different sires with no sire contributing more than three animals to each genotype. The animals were subjected to HPD surgery as described by Clarke *et al.* (1983). The first of five studies with these animals began 6 weeks after HPD surgery. All the studies were conducted during October and February (the anoestrous period) over two years. An outline of the studies performed is given (Table 1). In all five studies with the ovary-intact HPD Booroola ewes, the animals were pretreated for 14 days with GnRH (250 ng i.v. via a jugular venous cannula over a 1 min interval once every 2 h; Peninsula Laboratories Inc., Belmont, CA). The GnRH infusion solution consisted of GnRH and sterile isotonic saline containing ovine serum albumin (0.1% w/v; Sigma Chemical Co). At the end of the pretreatment period (i.e. on the morning of the 15th day) the animals in studies 1, 2, 4 and 5 were given PMSG (i.m.; Folligon: Intervet, Lane Cove, NSW). All animals received 200 iu in PMSG except in study 5 where all animals were given 400 iu in an attempt to ensure that all animals produced corpora lutea. After the GnRH pretreatment and PMSG, the animals in studies 1, 4 and 5 were administered with GnRH (i.v.) once every hour for 72 h and then once every half hour for 24 h followed by a GnRH bolus of 10 μg . In studies 2 and 3 during the interval between the GnRH pretreatment and GnRH bolus, the animals were administered with a pulsatile regimen of the infusion

Table 1. Outline of studies performed for Expt 2

Study number (time)†	Booroola genotype	Animal number	GnRH pretreatment (250 ng GnRH i.v. once every 2 h for 14 days)	PMSG (i.m.)	Ovulation induction			GnRH maintenance (250 ng GnRH i.v. once every 2 h for 12 days)
					GnRH once per hour for 72 h (i.v.)	GnRH once per 0.5 h for 24 h (i.v.)	GnRH bolus 10 µg (i.v.)	
1	BB	1–7	Yes	200 iu	Yes	Yes	Yes	Yes
(42)	++	10–16	Yes	200 iu	Yes	Yes	Yes	Yes
2	BB	2, 3, 4, 6	Yes	200 iu	No	No	Yes	Yes
(98)	++	10–13	Yes	200 iu	No	No	Yes	Yes
3	BB	2, 4, 5, 6, 8, 9	Yes	No	No	No	Yes	Yes
(142)	++	12, 13, 17, 18	Yes	No	No	No	Yes	Yes
4	BB	2, 4, 5, 6, 8, 9	Yes	200 iu	Yes	Yes	Yes	Yes
(379)	++	10, 11, 12, 14, 15, 17, 18	Yes	200 iu	Yes	Yes	Yes	Yes
5	BB	2, 4, 5, 6, 8, 9	Yes	400 iu	Yes	Yes	Yes	Yes††
(409)	++	10, 12, 13, 14, 15, 17, 18	Yes	400 iu	Yes	Yes	Yes	Yes††

†Time (days) from hypothalamic–pituitary disconnection surgery when the GnRH pretreatment regimen began.

††Animals were killed 4–5 days after the GnRH bolus.

solution without GnRH to determine whether this interval of GnRH treatment was essential for ovulation induction. In a separate study (study b) not involving Booroola ewes five anoestrous Romney ewes were given the 10 µg i.v. GnRH bolus to confirm that the bolus alone was ineffective in inducing ovulation even to intact ewes. At the completion of the fifth study the ewes were killed by exsanguination and cervical dislocation and the pituitaries and ovaries recovered shortly thereafter. Before the pituitary was excised, the brain was carefully removed from the head and examined to confirm that the HPD procedure had disconnected the pituitary from the brain (Clarke *et al.*, 1983). At 4–8 days after each study, ovulation rates were determined by laparoscopy. Moreover, daily blood samples were collected via a jugular venous cannula from initiation of GnRH pulse treatment and before and after GnRH bolus treatment to examine plasma gonadotrophin and progesterone concentrations.

The pituitary glands were homogenized into 2 ml buffer (5 mmol potassium phosphate l⁻¹ + 0.1% BSA (w/v) + 1 mmol phenylmethylsulfonylfluoride l⁻¹, pH 7.4) with 2 × 25 s bursts with an Ultraturrax homogenizer (Janke & Kunkel, IKA-Labortechnik, Staufen) on ice. The homogenates were centrifuged at 400 g for 5 min and the supernatant was then centrifuged at 30 000 g for 30 min at 4°C. Thereafter the supernatants were decanted (extract 1) and the pellets homogenized and centrifuged once more under the same conditions to obtain a second supernatant (extract 2). The supernatants (extracts 1 and 2) were stored at -20°C until assayed. The second supernatant routinely contained ≤ 10% of the gonadotrophin content of the first supernatant. For assay, each supernatant was serially diluted and the results for both extracts 1 and 2 summed to obtain the total for each pituitary.

The ovaries were collected into chilled (4–6°C) medium (i.e. Minimum Essential Medium + Earle's salts + L-glutamine + 0.1% (w/v) BSA. All ovarian antral follicles (≥ 1 mm diameter) and corpora lutea were dissected free of extraneous tissue. The

weights of the ovaries and corpora lutea were measured as were the diameters of the individual ovarian follicles.

Blood samples

All blood samples were centrifuged at room temperature for 5–10 min at 400 g within 20 min of collection and the plasma stored at -20°C until assayed.

Hormone assays

GnRH. The GnRH assay was similar to that described by Jonas *et al.* (1978). All samples from each ewe were analysed in the same assay. The antiserum (No. EL 14: supplied by W. E. Ellinwood; Oregon Regional Primate Center, Beaverton, OR) specificity and dilution (e.g. 1:500 000) was as described by Ellinwood *et al.* (1985). The GnRH used for iodination and standard was the native hormone supplied by Peninsula Laboratories Inc. (Belmont, CA). The GnRH was measured in methanol extracts of 400 µl plasma. The mean ± SEM recovery of GnRH added to peripheral plasma (i.e. 2.5, 5 and 10 pg ml⁻¹) after extraction was 62 ± 3%. All values were corrected assuming a 60% recovery. Assay sensitivity was 0.2 pg per tube. The intra-assay coefficients of variability (CVs) for the low (2.5 pg ml⁻¹), medium (5.0 pg ml⁻¹) and high (10.0 pg ml⁻¹) internal standards were 8.6%, 16.1% and 10.1%, respectively, and the inter-assay CVs for the internal standards were 17.4%, 17.7% and 15.8%, respectively. The GnRH values in control jugular plasma were at or below the sensitivity of the assay. GnRH secretion rates were calculated from a knowledge of the portal plasma concentrations of GnRH, the portal blood volume and haematocrit.

i-FSH. The radioimmunoassay kit was that supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, MD. The ovine (o) FSH for

iodination was NIAMDD-oFSH-I-1, the oFSH reference preparation was NIAMDD-oFSH-RP-1 (biopotency $75 \times$ NIH-FSH-S1) and the oFSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113). The volume of plasma or pituitary extract used in the assay was 0.1 ml and each sample was assayed in duplicate (McNatty *et al.*, 1989a). The internal standards, standard curve samples and serially diluted extracts were prepared in FSH-free hypophysectomized ewe plasma and the minimum detectable concentration was 0.2 ng ml^{-1} . The intra- and interassay CVs for the internal standards were both $< 8\%$.

b-FSH. The plasma b-FSH concentrations were measured in some of the above samples as described by Padmanabhan *et al.* (1987); the only modification was the use of a 0.5 ml substrate incubation volume instead of 1 ml. The validation of this assay for sheep plasma has been reported by Phillips *et al.* (1992). Concentrations of b-FSH were assessed using the production of oestradiol *in vitro* from Sertoli cells isolated from immature male rats. The reference standard used was NIADDK-oFSH-RP-1. The sensitivity of the oestradiol radioimmunoassay (McNatty *et al.*, 1989b) was 5 pg ml^{-1} and the intra- and interassay CVs were both $< 10\%$. The sensitivity of the FSH bioassay was $< 1 \text{ ng ml}^{-1}$ and the intra- and interassay CVs were < 9 and 13% , respectively.

i-LH. The radioimmunoassay was identical to that described by McNatty *et al.* (1989a). Briefly the iodination standard was NIDDK-oLH-I-3 (AFP-9598B), the LH antiserum was raised at Wallaceville and is described by McNatty *et al.* (1987). The oLH reference preparation was NIAMDD-oLH-23 (biopotency $2.3 \times$ NIH-LH-S1). Pituitary extracts were assayed as described above. The volume of pituitary extract or plasma, which was assayed in duplicate, was 0.1 ml. The internal standard dilutions of pituitary extracts and standard curve samples were prepared in LH-free hypophysectomized ewe plasma. The overall intra- and interassay CVs for the internal standards which were included with every standard curve estimation were both $< 10\%$. The minimum detectable concentration of LH was 0.2 ng ml^{-1} .

Progesterone. The progesterone radioimmunoassay together with the dilution and specificity of the progesterone antiserum (WA 26) were as described by McNatty *et al.* (1981). The volume of plasma assayed was $200 \mu\text{l}$ and the detection limit was 20 pg progesterone per tube. The inter- and intra-assay CVs were $< 10\%$.

Statistical analysis

The pulsatile characteristics of GnRH in the hypophyseal portal circulation and that of LH in peripheral blood were analysed using a PC based Turbo Pulsar programme (Objective Software, 9/16 Wilga St, Bondi, NSW). Both the GnRH and LH peaks were identified using the values of 1.9, 1.3, 0.95, 0.75 and 0.60 for G(1), G(2), G(3), G(4) and G(5), respectively (Merriam and Wachter, 1982). The calculated constant and linear values for GnRH were 14.8 and 11.9, respectively, and for LH the constant value was 11 and the linear value 0; no quadratic term was needed for either hormone.

When variances were different between the groups (Bartlett's test), the data were log transformed. Untransformed data are

presented as arithmetic means $\pm$ SEM, and transformed data as geometric means (and 95% confidence limits). Preliminary analysis of the data in Expt 2 with respect to ovulation rate and progesterone for Booroola Romney and Booroola Merinos indicated that there were no obvious differences. The effects of genotype, breed and time (56 and 393 days) with respect to the overall concentrations of FSH during the PMSG (200 iu) GnRH ovulation induction period were examined by ANOVA. No differences with respect to breed or time or any interactions were found. The data from these animals were therefore pooled across breeds to test for the effects of genotype at each time. Differences between the genotypes were tested by Analysis of Variance, Student's *t* test or the Kruskal–Wallis test. The distribution of mean FSH and LH values for each genotype were compared using Box and Whisker plots (Velleman and Hoaglin, 1981).

Results

Secretory characteristics of GnRH and LH and plasma concentrations of FSH

No differences among *Fec*^B genotypes were noted for the secretory characteristics for either GnRH or i-LH. Irrespective of genotype, there were 1.9 ± 0.2 GnRH pulses h^{-1} and 1.9 ± 0.1 i-LH pulses h^{-1} (Table 2). Irrespective of genotype most (i.e. $\geq 70\%$) of the GnRH pulses were coincident with the i-LH pulses (Fig. 1). Coincidence was considered to occur when the GnRH peak preceded the i-LH peak by 0–10 min. The overall geometric mean (and 95% confidence limits) for the concentrations of i-FSH were 8.1 ($5.2, 12.5$) ng ml^{-1} and 8.1 ($5.7, 11.4$) ng ml^{-1} for the ++ and BB genotypes, respectively. For b-FSH the arithmetic means $\pm$ SEM were 10.9 ± 2.2 and $10.4 \pm 2.2 \text{ ng ml}^{-1}$ for the BB and ++ genotypes, respectively. The respective b:i ratios were 1.1 ± 0.1 and 1.2 ± 0.1 . For i-FSH, b-FSH and the b:i ratios, no genotype differences were noted.

Induction of ovulation in HPD ovary-intact ewes with respect to treatment, Booroola genotype and time after HPD surgery

Ovulation occurred only in the HPD ovary-intact animals using the PMSG–GnRH pulse–GnRH bolus regimen (Table 3); the other treatments namely the GnRH pulses at 250 ng every 2 h by themselves (study 3), the PMSG–GnRH bolus (without the intervening GnRH pulse treatment) (study 2) or GnRH bolus alone (study 6) were without effect. In the three instances when the PMSG–GnRH pulse–GnRH bolus regimen was given, the mean ovulation rate was higher in the BB than in the ++ group: in all three cases the differences were significant by the Kruskal–Wallis test ($P < 0.05$). Evidence that the PMSG–GnRH pulse–GnRH bolus (ovulation induction) treatments resulted in an increase in progesterone concentration is shown (Fig. 2). After the first and second ovulation induction experiments (i.e. at 56 and 393 days after HPD surgery), the mean plasma progesterone concentrations increased in both genotypes from $\leq 0.2 \text{ ng ml}^{-1}$ to $> 1 \text{ ng ml}^{-1}$ some 5–7 days after the GnRH bolus. After the first ovulation induction experiment (study 1; Table 1), 4 of 7 animals of both genotypes had

Table 2. GnRH and immunoreactive (i) LH secretion characteristics in ovariectomized Booroola ewes with respect to genotype

(a) GnRH				
Genotype (n)	Hypophyseal portal concentration (pg ml ⁻¹)	Number of pulses h ⁻¹	Peak amplitude (pg ml ⁻¹)	Hypophyseal portal secretion rate (pg min ⁻¹)
++ (10)	1.6 ± 0.2	2.1 ± 0.3	7.2 ± 1.4	1.2 ± 0.3
BB (10)	1.4 ± 0.1	1.8 ± 0.1	5.4 ± 0.8	0.8 ± 0.1
(b) LH				
Genotype (n)	Peripheral plasma concentration (ng ml ⁻¹)	Number of pulses h ⁻¹	Peak amplitude (ng ml ⁻¹)	
++ (10)	1.2 ± 0.3	2.0 ± 0.2	0.7 ± 0.1	
BB (10)	1.1 ± 0.1	1.7 ± 0.2	0.7 ± 0.1	

Values are means ± SEM.

n = number of ewes.

Fig. 1. Hypophyseal-portal plasma concentrations of GnRH (○—○) and peripheral plasma concentrations of i-LH (□—□) before and after lesioning of portal blood vessels (time 0 h) in an (a) BB and a (b) ++ ewe.

progesterone concentrations above 1 ng ml⁻¹, whereas the remaining 3 of 7 animals in each genotype had plasma concentrations between 0.3 and 1 ng ml⁻¹ suggesting that ovulation induction did not lead to normal corpus luteum formation (Fig. 2a), even though all animals appeared to have formed normal corpora lutea as judged through the laparoscope. After the second ovulation induction experiment (study 4; Table 1), 1 of 6 BB and 2 of 7 ++ animals had progesterone concentrations < 1 ng ml⁻¹, suggesting that these also did not produce normal corpora lutea; these three animals were found by laparoscopy to have no corpora lutea. In the last of the ovulation induction treatments (study 5; Table 1), the ovaries were removed 4–5 days after the GnRH bolus and the progesterone concentrations

were all < 1 ng ml⁻¹ (data not shown). The follicular and luteal characteristics of the excised ovaries show that there were no differences among the Fec^B genotypes in number of ovarian follicles (≥ 1 mm diameter) or in total weight of corpus luteum tissue, but that individual corpora lutea were significantly lighter in BB than in ++ animals (Student's *t* test; *P* < 0.01) (Table 4). All but one ++ animal in this study contained normal corpora lutea. Two HPD ovary intact ewes (1 BB, ewe 3; 1 ++, ewe 16; see Table 1) that were not subjected to the GnRH-PMSC regimen in study 4 or 5 also had their ovaries removed and none contained follicles > 2 mm thus supporting the long-term stability of the HPD model (McNatty *et al.*, 1992a).

Table 3. PMSG–GnRH ovulation induction in hypothalamic–pituitary disconnected (HPD) ovary-intact Booroola ewes with respect to time after HPD surgery and genotype

Genotype (n)	PMSG dose (iu)	Time of PMSG injection after HPD surgery	Ovulation rate	Number of ewes ovulating†
BB (7)	200	56	4.2 (3.1, 5.7)	7
++ (7)	200	56	1.7 (1.0, 3.0)*	7
BB (6)	200	393	2.7 (0.7, 6.7)	5
++ (7)	200	393	0.8 (0.2, 1.8)*	5
BB (6)	400	423	4.3 (2.3, 7.7)	6
++ (7)	400	423	1.2 (0.5, 2.4)*	6

n = Number of ewes.

Values for ovulation rate are geometric means (and 95% confidence limits).

* $P < 0.05$ compared with BB (Kruskal–Wallis test).

†As judged by laparoscopy.

Fig. 2. Peripheral plasma progesterone concentrations both before and after a GnRH bolus in hypothalamic–pituitary disconnected (HPD) ovary-intact Booroola BB (●—●) and ++ (○—○) ewes. The PMSG–GnRH pulse–GnRH bolus regimen was initiated (a) 56 days and (b) 393 days after HPD surgery. Values are geometric means and the vertical bars represent 95% confidence limits. In (a) there were seven BB and seven ++ animals, whereas in (b) there were six BB and seven ++ animals.

Effect of GnRH pulses and the ovulation-induction regimens on gonadotrophin concentrations in HPD ovary-intact Booroola ewes

For both Booroola genotypes, the mean weekly i-FSH and i-LH concentrations from 6 days after HPD surgery until just

before GnRH pulse treatment over five sampling periods were ≤ 0.4 and 0.3 ng ml^{-1} , respectively. After the initiation of GnRH treatment, the i-FSH concentrations rose in both genotypes to peak concentrations 2–3 days later and then declined by about 40% to relatively constant concentrations by day 6 (Fig. 3). Overall the mean i-FSH concentrations in BB ewes were significantly higher than those in ++ ewes after the treatment. Box-plot analysis showed that 75% of the individual overall means (i.e. from day –4 to day +5) for the BB ($n = 6$) exceeded 75% of the overall means for the ++ ewes ($n = 7$) ($P < 0.05$). In contrast to the pattern of change seen in plasma for i-FSH, the mean i-LH concentrations rose steadily in both genotypes until about day 11 of GnRH treatment (Fig. 3). No overall differences in plasma i-LH concentrations were noted between the genotypes.

The mean plasma concentrations of i-FSH before and after the 200 iu or 400 iu PMSG–GnRH pulse–GnRH bolus (ovulation induction) treatment with respect to Booroola genotype and the mean concentrations of b-FSH during the 400 iu PMSG ovulation induction treatment are shown (Fig. 4); samples from the 200 iu PMSG treatment regimen were not measured for b-FSH. For i-FSH, there were no differences between the ++ and BB genotypes during the first 5 days before the GnRH bolus or in the first 5 days afterwards or over the entire 10 sampling days irrespective of whether the treatment regimen included 200 iu or 400 iu of PMSG. However, the daily mean values were more frequently higher in the BB than in the ++ genotype. With respect to b-FSH, the daily mean values were always higher in the BB than in the ++ genotype. Box plot analysis of the overall means for the 10 sampling days showed that the mean b-FSH values in the BB were higher than those in the ++ animals ($P < 0.01$; Student's t test). No significant differences were noted overall in the b:i FSH ratios; the mean $\pm$ SEM b:i ratios in the BB and ++ genotypes were 2.6 ± 0.2 and 2.8 ± 0.4 , respectively. For i-LH no Fec^B differences were noted before or after the GnRH bolus or over all sampling days (data not shown).

The mean i-LH but not i-FSH content or pituitary weight was significantly lower ($P < 0.05$) in the BB than in the ++ genotype (Table 5).

Table 4. Follicular and luteal characteristics following PMSG–GnRH ovulation induction in hypothalamic–pituitary disconnected (HPD) ovary intact Booroola ewes with respect to genotype^a

Genotype (n)	Number of follicles (≥ 1 mm diam.)	Ovulation rate	Mean weight of each corpus luteum (g)	Total weight of corpora lutea (g)
BB (6)	24 (14, 42)	4.3 (2.3, 7.7)	0.14 (0.03, 0.25)	0.57 (0.06, 1.34)
++ (7)	21 (11, 40)	1.2* (0.5, 2.4)	0.57*** (0.40, 0.77)	0.94 (0.36, 1.76)

^aPMSG injection was given 423 days after HPD surgery.

Values are geometric means (and 95% confidence limits).

P* < 0.01, **P* < 0.001 compared with BB (Kruskal–Wallis test). **Fig. 3.** Peripheral plasma concentrations of (a) i-FSH and (b) i-LH in BB (●—●) and ++ (○—○) hypothalamic–pituitary disconnected (HPD) ovary-intact Booroola ewes before and during treatment with GnRH which was administered as 250 ng i.v. pulses over a 1 min interval once every 2 h. Values are means $\pm$ SEM. The box and whisker plots refer to the overall means of the i-FSH and i-LH values for individual ewes following GnRH treatment. The i-FSH and i-LH values refer to the ovulation induction experiment which began 379 days after HPD surgery (see Table 1). There were six BB and seven ++ animals. **P* < 0.05, NS = not significantly different between the BB and ++ genotypes.

Discussion

The major findings from these studies were that no differences among the Fec^B genotypes were observed in the secretory characteristics of GnRH (concentration, secretion rate, pulse frequency or amplitude) in ovariectomized Booroola ewes and that the administration of an identical PMSG/GnRH regimen to HPD ovary-intact BB and ++ ewes led to a repeatable and statistically significant difference in ovulation rate (BB > ++). By examining GnRH secretion in ovariectomized ewes it was

Fig. 4. Peripheral plasma concentrations of i- or b-FSH in BB (●—●) and ++ (○—○) hypothalamic–pituitary disconnected (HPD) ovary-intact Booroola ewes during a PMSG–GnRH pulse–GnRH bolus treatment programme. (a) The mean i-FSH values from a 200 iu PMSG i.m. treatment; (b) the mean i-FSH values from a 400 iu PMSG i.m. treatment; and (c) b-FSH values from a 400 iu PMSG i.m. treatment. The values in (a) are the means from two separate experiments namely those initiated at 56 and 393 days after HPD surgery, whereas in (b) and (c) the values are those from the experiment initiated at 423 days after HPD surgery (see Table 3). The box and whisker plots refer to the overall mean values for the 10 sampling days.

Table 5. Pituitary weights and immunoreactive (i)-FSH and i-LH contents after PMSG–GnRH induction of ovulation in hypothalamic–pituitary disconnected (HPD) Booroola ewes with respect to genotype

Genotype (n)	Pituitary weight (g)	Pituitary content (µg per pituitary)	
		i-FSH	i-LH
BB (6)	0.92 (0.64, 1.24)	3.8 (0.8, 11.8)	8.4 (2.7, 23.3)
++ (7)	1.15 (0.99, 1.31)	6.4 (1.1, 24.6)	26.9 (17.0, 42.3)*

PMSG was given 423 days after HPD surgery.

Values are geometric means (and 95% confidence limits). n = number of sheep.

*P < 0.05 relative to the BB genotype (Student's *t* test).

anticipated that the GnRH pulse frequency would be higher than in ovary-intact sheep so that if there were any underlying differences between the genotypes in GnRH secretion, these would have been more obvious than in ovary-intact ewes. In previous studies of intact or ovariectomized BB and ++ ewes no differences between the genotypes were noted in the GnRH contents in hypothalamic tissue or in the receptor binding characteristics in pituitary homogenates (Gale *et al.*, 1988; Fleming *et al.*, 1990). Moreover, the mean plasma concentrations of i- or b-FSH in BB animals are not always different from those in ++ ewes on individual sampling days (McNatty *et al.*, 1987, 1989a, b, 1992b; D. J. Phillips *et al.*, 1993). However, these previous studies have shown a significantly greater frequency of higher mean concentrations of i- or b-FSH and higher i-LH pulse amplitudes, but not frequency, in intact, ovariectomized and HPD–ovariectomized BB than in ++ animals. In the present study, therefore, it was anticipated that genotype differences might not be found for the mean i- or b-FSH concentrations or i-LH pulse frequency at the time of sampling for GnRH. In addition, as the portal blood vessels had been lesioned, the i-LH peak amplitudes were predictably 1–2 ng ml⁻¹ lower than in non-lesioned ovariectomized Booroola ewes (McNatty *et al.*, 1989b). Despite the reductions in i-LH pulse amplitude, both the mean GnRH and LH pulse frequencies were found to be about 1.9 pulses h⁻¹. A frequency of one i-LH pulse about every 32 min is within the range expected for ovariectomized ewes (Martin, 1984).

Ovaries of long-term HPD ewes are normally devoid of follicles > 2 mm diameter (McNatty *et al.*, 1992a). This is probably the result of low plasma concentrations of i-FSH (McNatty *et al.*, 1985) and presumably b-FSH. Before the ovulation induction experiments were initiated, all the HPD ovary-intact ewes had FSH concentrations ≤ 0.4 ng ml⁻¹. After treatment with the 250 ng GnRH (i.v.) pulse regimen, the mean plasma i-FSH concentrations increased from about 0.4 ng ml⁻¹ to between 1.0 and 1.4 ng ml⁻¹. At the end of the pretreatment period, the concentrations of i-FSH were similar to those found for intact Booroola ewes with the BB ewes having significantly higher overall means than the ++ animals. In HPD animals that had been ovariectomized, exogenous GnRH treatment also led to a gene-specific difference in i-FSH (McNatty *et al.*, 1991b). These findings suggest that in the present study the differences between the Booroola genotypes in plasma i-FSH

were at least in part due to a difference in the pituitary response to exogenous GnRH rather than to ovarian hormone feedback.

As stated above, the GnRH pretreatment resulted in an overall mean difference between the genotypes in plasma i-FSH but not i-LH concentrations before the onset of the ovulation induction regimen. The mean b-FSH, but not i-FSH, concentrations between the genotypes were also different over the 400 iu PMSG–GnRH pulse–GnRH bolus period. In this study, we cannot discount the possibility that the subsequent differences in ovulation rate were due to the ovaries of the BB and ++ genotypes being exposed to different concentrations of i- and b-FSH. Although the GnRH pretreatment and ovulation induction regimens did not influence the plasma concentrations of i-LH, significant differences between the Booroola genotypes in pituitary i-LH content were observed at the end of the experiment. Indeed both the mean contents of i-FSH and i-LH were lower in the BB than in the ++ genotypes. In intact Booroola ewes the pituitary contents of i-FSH have been found to vary from 20 to 82 µg per gland and those for i-LH to vary from 300 to 1400 µg per gland (Bindon, 1984; McNatty *et al.*, 1987). In the present study, the respective pituitary contents of i-FSH varied from 0.8 to 24.6 µg per gland and those of i-LH from 2.7 to 42.3 µg per gland notwithstanding the fact that the pituitary weights in the two genotypes were similar to those in control ewes. Clearly, in contrast to control animals, the pituitary gonadotrophin contents in the GnRH-treated HPD ovary-intact animals were very low. Collectively the results of the present study support the view that in Booroola ewes the response of the pituitary gland to GnRH is not the same between the BB and ++ genotypes (McNatty *et al.*, 1991a, b).

The ovulation induction regimen used in these studies caused a difference in ovulation rate between the genotypes similar to that observed between control BB and ++ ewes (McNatty *et al.*, 1991a). In addition, in the present study the difference in ovulation rate did not lead to any genotype difference in plasma progesterone concentration, or total weight of corpora lutea tissue. Thus, the HPD ovary-intact BB ewes produced consistently smaller corpora lutea but similar numbers of antral follicles (≥ 1 mm diameter) compared with the HPD ovary-intact ++ ewes, as is the case with control BB and ++ animals (McNatty and Henderson, 1987).

The ovulation induction regimen initiated with 200 iu PMSG and GnRH pulses at 1 h followed by 30 min intervals led to

most animals (e.g. 9 of 13 BB and 9 of 14 ++ animals) ovulating and producing normal functional corpora lutea consistent with a previous study using exogenous GnRH pulses to ovulate HPD-ovary-intact ewes (Phillips *et al.*, 1989). The failure of the remainder to produce normal amounts of progesterone suggests that the ovulation induction regimen was not ideal in all cases. Indeed inadequate luteal function is a common feature of the first ovulation at the onset of puberty, the breeding season or following other ovulation induction programmes (McLeod *et al.*, 1982; McNatty *et al.*, 1982). It was not possible to assess whether the regimen initiated with 400 iu instead of 200 iu PMSG was better because the 400 iu PMSG experiment was terminated before progesterone concentrations had begun to increase.

In conclusion, these studies show that there are unlikely to be Fec^B gene-specific differences with respect to GnRH secretion in ovariectomized ewes. Moreover, they show that the genotypic differences in ovulation rate remain when an identical PMSG-GnRH ovulation induction regimen was given to BB and ++ ewes. The ovulation induction regimen led to differences in plasma i- and b-FSH concentration consistent with the notion that the Fec^B gene influences the pituitary response to GnRH.

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