Effect of testosterone and bovine follicular fluid on concentrations of luteinizing hormone and follicle-stimulating hormone in plasma of castrated rams that are homozygous carriers or non-carriers of the Booroola fecundity gene

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Summary. Castrated adult $Fec^B Fec^B$ and $Fec^+ Fec^+$ Booroola rams were injected with charcoal-treated bovine follicular fluid (bFF) (a source of inhibin-like activity) or given testosterone implants to examine whether the fecundity gene (Fec^B) influences sensitivity to negative feedback hormones in males. Mean concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) did not differ between genotypes before treatment. In Expt 1, injections of 5 ml bFF, but not of 1 ml (each given four times at intervals of 8 h), significantly ($P < 0.05$) depressed concentrations of LH and FSH, but there was no effect of genotype. After treatment, gonadotrophin concentrations returned to pretreatment values and for 2–2.5 days scaled (divided by pretreatment mean) LH values (235 ± 49 for $Fec^B Fec^B$ and $96 \pm 26\%$ for $Fec^+ Fec^+$ rams; $P < 0.05$) and scaled FSH values (106 ± 5 for $Fec^B Fec^B$ and $85 \pm 5\%$ for $Fec^+ Fec^+$ rams; $P < 0.05$) were significantly higher in $Fec^B Fec^B$ than in $Fec^+ Fec^+$ rams in the group that received 5 ml bFF. Irrespective of genotype, treatment with 5 ml bFF did not reduce mean FSH to concentrations observed in testis-intact rams.

In Expt 2, Silastic envelopes were implanted subdermally to give physiological or supraphysiological circulating concentrations of testosterone. Both doses significantly reduced scaled LH values in a biphasic manner, such that there was an initial suppression followed by a short-lived increase. During the initial period of suppression in the lower dose group, mean scaled LH values were significantly higher in $Fec^B Fec^B$ than in $Fec^+ Fec^+$ rams (48.3 ± 7.5 versus $23.1 \pm 5.5\%$; $P < 0.05$). Low doses of testosterone decreased LH pulse frequency in both genotypes but decreased ($P < 0.05$) pulse amplitude and mean concentrations in the $Fec^+ Fec^+$ animals only. In nonimplanted control rams, mean LH concentrations (in samples taken every 10 min for 12 h) were significantly lower in $Fec^B Fec^B$ than in $Fec^+ Fec^+$ rams (0.6 ± 0.2 versus 1.3 ± 0.1 ng ml⁻¹; $P < 0.05$). The mean FSH response to testosterone was not related to genotype.

These data suggest that expression of the Fec^B gene results in an altered sensitivity of the pituitary gland to changes in negative feedback from testicular hormones and that, irrespective of genotype, neither testosterone nor inhibin-like activity alone can fully control FSH secretion in castrated rams.

Keywords: Booroola; follicular fluid; testosterone; LH; FSH; ram

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Introduction

The Booroola fecundity gene (*Fec^B*) influences ovulation rate and litter size in sheep (reviewed by Bindon, 1984). Despite many studies over the last decade, the principal site of gene expression remains unknown. In adult ewes, plasma gonadotrophin (particularly follicle-stimulating hormone (FSH)) concentrations were higher in homozygous gene carriers (*Fec^BFec^B*) than in non-carriers (*Fec⁺Fec⁺*), although ovarian steroid secretion did not differ among genotypes (McNatty *et al.*, 1987, 1989a, 1990). The main factors that control FSH secretion in ewes are gonadotrophin-releasing hormone, oestradiol and inhibin, and the effects of *Fec^B* do not appear to involve differences in sensitivity to steroid negative feedback (McNatty *et al.*, 1989b).

In adult males, the evidence for an effect of *Fec^B* is weaker. No differences in testis size or in spermatogenic function have been described (Bindon *et al.*, 1982; Walker *et al.*, 1985; Hochereau-de Reviers & Seck, 1991; Price *et al.*, 1991a). There were no differences in testosterone secretion after injection with pregnant mares' serum gonadotrophin (Hochereau-De Reviers *et al.*, 1990; Price *et al.*, 1991a), although testosterone pulse amplitude was higher in *Fec^BFec^B* than in *Fec⁺Fec⁺* rams during seasonal testicular development in the absence of differences in LH pulse amplitude (Price *et al.*, 1991a). Mean luteinizing hormone (LH) and FSH concentrations do not differ among genotypes (Bindon *et al.*, 1985; Martin *et al.*, 1987a; Price *et al.*, 1991a), although the rise in FSH but not LH secretion after castration was significantly higher in *Fec^BFec^B* than in *Fec⁺Fec⁺* rams (Price *et al.*, 1991b), but no significant differences were noted between Booroola and non-Booroola control rams of undefined genotype (Bindon *et al.*, 1985). The factors that control FSH secretion in males are not clearly understood (Price, 1991) and it is not known whether *Fec^B* has an effect on the sensitivity of the hypothalamus or pituitary gland to testicular negative feedback in rams.

The present studies examined the effects of different doses of bovine follicular fluid (bFF) (as a source of inhibin-like activity) or of testosterone on LH and FSH secretion in castrated adult *Fec^BFec^B* and *Fec⁺Fec⁺* Booroola Merino rams.

Materials and Methods

Animals

The Booroola Merino rams were born as a result of controlled matings between *Fec^BFec^B* ewes and rams, or between *Fec⁺Fec⁺* ewes and rams. These dams and sires had been assigned their respective genotypes according to the criteria of Davis *et al.* (1982). The *Fec^BFec^B* rams were derived from seven sires and 11 dams, and the *Fec⁺Fec⁺* rams from three sires and 13 dams.

Thirteen animals of each genotype were used when 2.5–3 years of age, and were castrated in March (during the breeding season), 3–5 months before the start of the present studies. The animals were maintained outdoors at the Wallaceville Animal Research Centre (latitude 41°S), but were housed indoors during the intensive sampling periods. Blood samples were collected through indwelling jugular catheters, which were inserted at least one day before sampling. These studies were approved by the Wallaceville Animal Ethics Committee, in accordance with the 1987 Animals Protection (Codes of Ethical Conduct) Regulations of New Zealand. The same animals were used for the three studies, and were taken from the same group of animals that were subjects of previous studies (Price *et al.*, 1991a, b).

Experiment 1: effect of bFF on LH and FSH secretion

The bFF was aspirated from antral follicles in ovaries collected from a local slaughterhouse, and was pooled to form a single batch. The fluid was incubated with activated charcoal to reduce the steroid content greatly, as previously described (Henderson *et al.*, 1986).

Rams received injections of 1 or 5 ml bFF, or 1 ml sterile saline ($n = 5, 4$ and 4 per genotype, for each dose group, respectively), which were given subcutaneously four times at intervals of 8 h. Blood samples were collected every 2 h, starting 8 h before the first injection of bFF and continuing for 8 h after the fourth (last) injection, and then collected twice a day for a further 9 days.

Experiment 2: effect of testosterone on LH and FSH secretion

This experiment started 2 weeks after the last injection of bFF in Expt 1. Envelopes were prepared from Silastic sheeting (55 mm × 75 mm; Cat. No. 500-1; Dow Corning Corp., Midland, MI, USA) and were filled with crystalline testosterone (Sigma Chemical Co., St Louis, MO, USA) as described by Karsch *et al.* (1977). The filled envelopes

were soaked in water overnight to prevent a peak of steroid release on insertion. The implants were placed into the subcutaneous fascia of the medial aspect of the upper hind legs while the animals were sedated and given local anaesthetic with Lopaine (Ethical Agents Ltd, Auckland, New Zealand). The rams were randomly assigned to receive 0, 1 or 3 implants ($n = 4, 5$ and 4 per genotype for each dose, respectively); the controls were sham operated, but received no implants.

Blood samples were collected once an hour for 24 h before steroid implantation and until 48 h after implantation. Seven days after receiving implants, the rams were sampled every 10 min for 12 h to assess pulsatile LH secretion. At the end of the study, all implants were removed, and none showed signs of leakage.

Experiment 3: pituitary LH and FSH content

Five weeks after the end of Expt 2, all rams were killed by cervical dislocation followed by decapitation. The pituitary glands were removed, weighed, and frozen on dry ice. Tissues were later processed for the extraction of LH and FSH for radioimmunoassay, as described by McNatty *et al.* (1987).

Hormone assays

LH was measured by radioimmunoassay (McNatty *et al.*, 1987) using NIDDK-oLH-I-3 as tracer and NIAMDD-oLH-S23 as the reference preparation. All samples were analysed in duplicate. The minimum concentration that could be detected was 0.2 ng ml^{-1} , and the intra- and interassay coefficients of variation (CV) were 9 and 12%, respectively.

FSH was assayed with an homologous radioimmunoassay kit (McNatty *et al.*, 1987) using NIAMDD-oFSH-II as tracer, NIAMDD-oFSH-RP1 as standard, and NIAMDD-anti oFSH-I as antibody. The minimum detectable concentration was 0.2 ng ml^{-1} , and the intra- and interassay CV were 7 and 8%, respectively.

Testosterone concentrations were estimated after extraction with freshly distilled diethyl ether (McNatty *et al.*, 1982), with a mean extraction efficiency of 91%. This assay had a detection limit of 10 pg ml^{-1} , and the intra- and interassay CVs, were both 10%.

Statistical analysis

Owing to differences between dose groups in pretreatment values, the data were expressed as a percentage of the pretreatment mean. These scaled data were then transformed to square roots before analysis by two-way ANOVA to test for main effects of gene and dose. Duncan's multiple range test was used to identify differences between groups.

Pulsatility of LH was assessed using the algorithm programme Cluster (Veldhuis & Johnson, 1986). A pulse was specified as being composed of at least three data points and being preceded by a nadir of at least two data points. The minimum t statistics specified for the pulse upstroke and downstroke were 4.0 and 2.0, respectively. False-positives (as judged visually) resulting from baseline fluctuations were reduced by specifying a constant coefficient of variation of 15% and a minimum pulse height of 0.4 ng ml^{-1} . Pulse parameters were analysed by t tests for effects of dose and genotype.

Results

Experiment 1: effect of bFF on LH and FSH secretion

The mean hormone concentrations before treatment were 2.4 ± 0.8 and $2.6 \pm 0.4 \text{ ng LH ml}^{-1}$ and 15.3 ± 1.8 and $14.5 \pm 1.4 \text{ ng FSH ml}^{-1}$, for $Fec^B Fec^B$ and $Fec^+ Fec^+$ rams, respectively. There were no differences between genotypes, and no effect of sire for either gonadotrophin.

Analysis of variance indicated a significant ($P < 0.001$) effect of dose of bFF on scaled LH values, but there was neither an effect of genotype nor a gene $\times$ dose interaction (Fig. 1). Further analysis of the effect of dose revealed that 5 ml, but not 1 ml, bFF significantly ($P < 0.05$) reduced scaled LH values. Eight to 10 days after the last bFF injection, mean scaled LH values were significantly higher ($P < 0.05$) in the $Fec^B Fec^B$ ($235 \pm 49\%$) than in the $Fec^+ Fec^+$ rams ($96 \pm 26\%$) treated with 5 ml bFF, but for no other time or dose studied (Fig. 2).

For FSH as for LH, there was a significant effect ($P < 0.001$) of 5 ml bFF, but not of 1 ml bFF (Fig. 3). There was no significant effect of genotype. Scaled FSH values were significantly ($P < 0.05$) higher in $Fec^B Fec^B$ than in $Fec^+ Fec^+$ rams over days 4–6 after the last injection (Fig. 4). No such effects were evident in the 1 ml bFF dose group (data not shown).

Experiment 2: effect of testosterone on LH and FSH secretion

Mean concentrations of testosterone in plasma were 2.7 ± 0.5 and $1.9 \pm 0.2 \text{ ng ml}^{-1}$ in $Fec^B Fec^B$ and $Fec^+ Fec^+$ rams with one implant, and were 16.3 ± 3.7 and $11.6 \pm 1.6 \text{ ng ml}^{-1}$

Fig. 1. Effect of four injections of (a) 1 ml saline, (b) 1 ml bovine follicular fluid (bFF) and (c) 5 ml bFF on mean ($\pm$ SEM) scaled (divided by pretreatment mean) luteinizing hormone (LH) values in castrated *Fec^BFec^B* ($\bullet$) and *Fec⁺Fec⁺* ($\circ$) Booroola Merino rams. Injections were given at time 0 and at times marked by arrows.

in *Fec^BFec^B* and *Fec⁺Fec⁺* rams carrying three implants. There were no differences between the genotypes, and no dose by gene interaction. Testosterone concentrations could not be detected in any animal before implant insertion, or in the control rams throughout Expt 2.

A dose-dependent decrease in LH concentration occurred extremely rapidly and reached basal values within the first 20 h after testosterone implantation (Fig. 5). Thereafter, LH values began to increase. Both treatment doses significantly ($P < 0.001$) reduced scaled LH values; there was a gene $\times$ dose interaction ($P < 0.05$), but no main effect of genotype. One-way ANOVA within each dose group revealed that scaled LH values in the *Fec^BFec^B* group were significantly higher than

Fig. 2. Mean ($\pm$ SEM) scaled (divided by pretreatment mean) luteinizing hormone (LH) values after the cessation of bovine follicular fluid treatment of castrated *Fec^BFec^B* (●) and *Fec⁺Fec⁺* (○) Booroola Merino rams, for (a) control group and (b) group given 5 ml bovine follicular fluid.

in the *Fec⁺Fec⁺* group (48.3 ± 7.5 and $23.1 \pm 5.5\%$; $P < 0.05$) during the period of maximal suppression (12–24 h after implantation) in the group receiving one implant. There was no difference between genotypes after maximal suppression ($P = 0.08$), or at any time in the group receiving three implants or in the controls.

The FSH response to testosterone was less variable than that of LH, and showed a slow decrease in scaled values over time without evidence of any escape from suppression. The mean scaled values over the final 12 h of the samples taken once an hour (37–48 h after implantation) were significantly affected by dose but not by genotype (Table 1).

There was a significant effect ($P < 0.001$) of dose of testosterone but not of genotype on LH pulse frequency, and a difference ($P < 0.05$) in mean LH concentrations and pulse amplitude between groups receiving no and one implant for the *Fec⁺Fec⁺* but not the *Fec^BFec^B* genotype (Table 2). The three implants reduced all LH parameters to minimum detectable values. Within the control (non-implanted) rams, mean LH concentrations were higher ($P < 0.05$) in *Fec⁺Fec⁺* than in *Fec^BFec^B* rams. There was no effect of dose of testosterone on mean FSH concentrations.

Experiment 3: pituitary LH and FSH content

The mean pituitary gland wet weights were 0.8 ± 0.03 g for both *Fec^BFec^B* and *Fec⁺Fec⁺* rams. The geometric mean (and 95% confidence limits) gonadotrophin contents were 272.6 (213.4, 348.3) and 301.3 (226.8, 400.2) μ g LH per gland, and 33.6 (27.1, 42.7) and 32.9 (25.9, 41.8) μ g FSH per

Fig. 3. Effect of four injections of (a) 1 ml saline, (b) 1 ml bovine follicular fluid (bFF) and (c) 5 ml bFF on mean ($\pm$ SEM) scaled (divided by pretreatment mean) follicle-stimulating hormone (FSH) values in castrated *Fec^BFec^B* (●) and *Fec⁺Fec⁺* (○) Booroola Merino rams. Injections were given at time 0 and at times marked by arrows.

gland, for *Fec^BFec^B* and *Fec⁺Fec⁺* rams, respectively. There were no differences between genotypes, nor a main effect of sire.

Discussion

The strongest evidence that supports the expression of *Fec^B* in rams is the difference between genotypes in the rate of increase of FSH concentrations in plasma observed after castration (Price *et al.*,

Fig. 4. Mean ($\pm$ SEM) scaled (divided by pretreatment mean) follicle-stimulating hormone (FSH) values after the cessation of bovine follicular fluid treatment of castrated *Fec^BFec^B* (●) and *Fec⁺Fec⁺* (○) Booroola Merino rams, for (a) control group and (b) group given 5 ml bovine follicular fluid.

1991b). The present study supports these data by providing evidence for a gene effect on gonadotrophin concentrations after the withdrawal of testosterone or bFF treatment in castrated rams.

Although there were no gene-specific differences in gonadotrophin concentrations during bFF-induced suppression, gonadotrophin secretion was higher in *Fec^BFec^B* than in *Fec⁺Fec⁺* rams after the withdrawal of bFF. Scaled LH values were different from days 4 to 6 after the last injection of bFF, and scaled FSH values were different from days 7 to 9 after bFF. There was no gene-specific difference in gonadotrophin secretion during bFF treatment in ewes (McNatty *et al.*, 1989b), although Cummins *et al.* (1983) reported that FSH concentrations were more readily suppressed by bFF in Booroola ewes than in control Merino ewes. The mechanism of action of these effects is unknown, but may involve differences in sensitivity of the pituitary cells to inhibin or in the clearance of inhibin from the circulation. The differences in gonadotrophin concentrations in the present study were not sustained, and thus suggest a gene-related effect on the pituitary response to a change in the inhibitory signal from the testes. This pattern is similar to that observed after castration (Price *et al.*, 1991b). The physiological relevance is unclear as major changes in the plasma concentrations of gonadal hormones are not usually observed in Merino rams (Lincoln *et al.*, 1990; Price *et al.*, 1991a).

The 5 ml dose of bFF suppressed both LH and FSH, whereas 1 ml bFF was without significant effect on either gonadotrophin. These data differ from those for ovariectomized ewes (Martin *et al.*, 1987b; McNatty *et al.*, 1989b) and for castrated rams (Findlay *et al.*, 1985) for which low doses of follicular fluid (0.5–1 ml every 8 or 12 h) selectively suppressed FSH concentrations. The data for

Fig. 5. Hourly changes in the mean scaled (divided by pretreatment mean) luteinizing hormone (LH) values after (a) 0, (b) 1 or (c) 3 Silastic envelopes containing testosterone were given to castrated *Fec^BFec^B* (●) and *Fec⁺Fec⁺* (○) Booroola Merino rams. For clarity, the hourly SEM values have been omitted; mean least squares standard error bars are shown to the right of the panels.

ovariectomized ewes also indicate that moderate doses of bFF alone (<2 ml) maintained mean FSH concentrations within the range observed in gonad-intact ewes (Martin *et al.*, 1987b, 1988). In the present study, 5 ml bFF reduced LH concentrations to values close to the expected range for Merino testis-intact rams (0.7 ± 0.1 versus 0.4 ± 0.1 ng ml⁻¹ in intact Merino rams, Price *et al.*, 1991a), whereas FSH concentrations remained above the physiological range (7.5 ± 1.5 versus 0.3 ± 0.1 ng ml⁻¹ in intact rams, Price *et al.*, 1991a).

The effect of testosterone on LH secretion was extremely rapid, with mean values suppressed below 50% of the pretreatment values within 2 h of implant insertion, irrespective of the number of

Table 1. Effect of testosterone implants on mean ($\pm$ SEM) scaled follicle-stimulating hormone (FSH) values (% of pretreatment mean) in castrated *Fec^BFec^B* and *Fec⁺Fec⁺* rams, and mean FSH concentrations irrespective of genotype, from 37 to 48 h after implantation

Number of implants	Number of rams per genotype	Scaled FSH (%)		Mean FSH concentration
		<i>Fec^BFec^B</i>	<i>Fec⁺Fec⁺</i>	(Pooled genotype) (ng ml ⁻¹)
0	4	96 $\pm$ 4 ^a	97 $\pm$ 10 ^a	9.9 $\pm$ 0.9 ^a
1	5	78 $\pm$ 6 ^{a,b}	77 $\pm$ 4 ^{a,b}	7.8 $\pm$ 0.8 ^{a,b}
3	4	74 $\pm$ 2 ^b	62 $\pm$ 6 ^b	6.5 $\pm$ 0.8 ^b

Values with different superscripts are significantly different ($P < 0.05$).

Table 2. Effect of testosterone on mean concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and on LH pulse frequency and amplitude, assessed in samples taken every 10 min for 12 h, in castrated *Fec^BFec^B* and *Fec⁺Fec⁺* rams seven days after insertion of the steroid implants

Number of implants	Genotype	LH mean (ng ml ⁻¹)	LH pulse frequency (peaks in 12 h)	LH pulse amplitude (ng ml ⁻¹)	FSH mean (ng ml ⁻¹)
0	<i>Fec^BFec^B</i>	0.6 $\pm$ 0.2 ^a	5.0 $\pm$ 1.5	1.2 $\pm$ 0.5	4.8 $\pm$ 0.9
	<i>Fec⁺Fec⁺</i>	1.3 $\pm$ 0.1 ^{b,x}	6.8 $\pm$ 1.0	1.9 $\pm$ 0.1 ^a	5.0 $\pm$ 0.5
1	<i>Fec^BFec^B</i>	0.2 $\pm$ 0.1	1.2 $\pm$ 0.5	1.0 $\pm$ 0.3	3.9 $\pm$ 1.0
	<i>Fec⁺Fec⁺</i>	0.1 $\pm$ 0.1 ^y	0.8 $\pm$ 0.6	0.6 $\pm$ 0.1 ^y	2.6 $\pm$ 0.3
3	<i>Fec^BFec^B</i>	0.1 $\pm$ 0.1	0	—	4.3 $\pm$ 0.6
	<i>Fec⁺Fec⁺</i>	0.1 $\pm$ 0.1	0	—	4.3 $\pm$ 1.0

^{a,b}Significant difference between genotypes ($P < 0.05$).

^{x,y}Significant differences between one and no implant dose groups within the *Fec⁺Fec⁺* genotype ($P < 0.05$).

implants. During the second 24 h after implant insertion, LH values started to increase and, in the low dose group, occasionally reached pretreatment values. During the period of maximal suppression (12–24 h after implantation), scaled LH values were significantly higher in *Fec^BFec^B* than in *Fec⁺Fec⁺* rams in the low testosterone dose group, and there was a tendency ($P = 0.08$) for the same pattern to occur in the period of higher LH values 24–48 h after implant insertion. The effects of testosterone were exerted mainly on pulse frequency as previously described (D'Occhio *et al.*, 1982a, 1983). Mean LH concentrations were lower in *Fec^BFec^B* than in *Fec⁺Fec⁺* rams receiving no testosterone, and the effect of one implant compared to no implant was significant for the *Fec⁺Fec⁺* but not the *Fec^BFec^B* rams. Similarly, the effect of one implant compared with no implant on LH pulse amplitude was significant for the *Fec⁺Fec⁺* but not the *Fec^BFec^B* rams. Thus, in terms of pituitary LH secretion, the *Fec^B* gene may impart an altered sensitivity to testosterone negative feedback.

In contrast, there was no consistent effect of testosterone on mean FSH concentrations. These data do not agree with those of D'Occhio *et al.* (1982b), who suggested that physiological concentrations of testosterone administered in the form of implants fully restored physiological concentrations of FSH in castrated rams. The two key differences between these studies are that the present rams were used after a greater time lag after castration than those used by D'Occhio *et al.* (1982b), and were deprived of testosterone before the study, whereas those used by D'Occhio and colleagues were given testosterone at the time of castration. It has been suggested that complete control over FSH secretion in ewes is accomplished by an interaction between oestradiol and

inhibin (Martin *et al.*, 1988). It seems reasonable to propose a similar interaction between testosterone and inhibin for castrated rams, although this remains to be tested.

The size of the treatment groups in the present study precluded an examination of possible sire effects on gonadotrophin secretion. An effect of sire on plasma concentrations of FSH has been reported in ram lambs (Seck *et al.*, 1988; Purvis *et al.*, 1989) and in the present group of rams after castration (Price *et al.*, 1991b), but sire had no significant effect on LH secretion (Price *et al.*, 1991a, b). The analysis of scaled (to pretreatment means) hormone data in the present study offset any effects of sire on plasma concentrations *per se*, but it is possible that sire also modifies relative change in hormone secretion.

Finally, we report that there were no gene- or sire-related differences in pituitary LH or FSH concentrations in castrated rams, and mean FSH content was significantly lower than that of LH. These data are in agreement with those for testes-intact Booroola and control Merino rams (Robertson *et al.*, 1984).

In summary, the present studies provide further evidence to suggest that the *Fec^B* gene is expressed in Booroola rams. Decreases in plasma LH concentrations induced by replacement of testosterone to castrated rams, and the increase in LH and FSH secretion after cessation of bFF treatment, were significantly influenced by genotype.

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