Relationships between the number of immunostaining gonadotropes and the plasma concentrations of gonadotrophins in ewes with and without the *FecB^B* gene

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Booroola ewes possess a major gene, *FecB^B*, that influences their ovulation rate (number of ovulations per oestrous cycle). Homozygous (BB) carriers of the *FecB^B* gene have higher plasma concentrations of FSH and sometimes LH relative to the non-carriers (++) . The aim of this study was to determine whether the plasma concentration differences in FSH or LH between the genotypes were due to a greater number of FSH β - or LH β -immunostaining cells in the anterior pituitary gland of BB ewes during the luteal phase of the oestrous cycle. No differences were found between the BB ($n = 7$) ewes and ++ ($n = 8$) ewes in total number of pituitary cells, pituitary volume, numbers or diameters of FSH β - or LH β -immunostaining cells, notwithstanding significantly higher concentrations of immunoreactive plasma FSH ($P < 0.001$) but not LH in BB compared with ++ animals. Significant linear relationships were found within each genotype between plasma FSH and number of FSH β -immunostaining cells. No such relationship was found for plasma LH and number of LH β cells. For the FSH relationship, the slopes of the regression lines were the same. It is hypothesized that the differences in plasma concentration of FSH between the genotypes is due to a greater output of FSH per pituitary cell in the BB animals.

Introduction

Booroola ewes possess a major gene, *FecB^B*, that influences their ovulation rate (for review see Montgomery *et al.*, 1992). Homozygous (BB), heterozygous (B+) and non-carriers (++) of the *FecB^B* gene have ovulation rates of ≥ 5 , 3–4 and 1–2, respectively. It is known that the immunoreactive (i) and bioactive (b) plasma concentrations of FSH (and on some occasions, i-LH concentrations) are higher in intact or ovariectomized ewes relative to ++ ewes (McNatty *et al.*, 1987, 1989; Phillips *et al.*, 1993). These differences between the BB and ++ genotypes can also be demonstrated in hypothalamic–pituitary disconnected (HPD), intact or ovariectomized ewes given identical amounts of exogenous GnRH (McNatty *et al.*, 1991, 1993). The implications from these findings of *FecB^B* differences in the plasma concentrations of FSH and LH are that they are inducible by GnRH action and that they are independent of factors secreted by the gonad. Moreover, the effects on pituitary hormone release are specific to GnRH action, since no gene differences are observed with respect to thyroid stimulating hormone (TSH) or growth hormone (GH) after treatment with exogenous thyroid hormone releasing hormone or growth hormone releasing hormone, respectively (McNatty *et al.*, 1994).

The underlying reason for this difference in pituitary responsiveness to GnRH is not known. The *FecB^B* gene has been assigned to chromosome 6 and therefore excluded from linkage to the FSH β region of chromosome 15 or the LH β region of chromosome 14 (Montgomery *et al.*, 1990, 1993; Penty *et al.*, 1995). The *FecB^B* gene does not influence the number or size of the FSH β , LH β or α gonadotrophin gene transcripts in pituitary mRNA preparations (Montgomery *et al.*, 1990, 1993; Fleming *et al.*, 1995). Moreover, it does not influence the median charge or half-life of FSH in intact or ovariectomized ewes (Phillips *et al.*, 1995), the hypothalamic GnRH contents or the pituitary GnRH-binding characteristics (Fleming *et al.*, 1990). In studies of fetal development in Booroola sheep, differences have been noted in the development of the mesonephros and germ cells in newly forming gonads (Smith *et al.*, 1993). Similar studies on the development of pituitary gonadotropes have not been carried out.

It is possible that the differences in plasma FSH (and LH) concentrations occur because the pituitary gland in BB ewes contains more cells that synthesize β -FSH or co-synthesize β -FSH and β -LH but not α -gonadotrophin when stimulated with GnRH. The aim of the present study was to determine whether the differences between the genotypes in plasma gonadotrophin concentrations were due to a greater number of FSH- and LH-immunostaining pituitary cells in the BB genotype.

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Materials and Methods

All experiments were performed 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.

Animals and blood sampling

Pituitaries were recovered from Booroola $\times$ Merino ewes (+ +, $n = 8$; BB, $n = 7$) after exsanguination. For the + + and BB animals, respectively, the mean $\pm$ SEM ages were 6.3 ± 0.3 and 6.5 ± 0.3 years, the mean body masses were 43.6 ± 1.4 and 40.9 ± 0.8 kg, and the mean days of the oestrous cycle when the pituitaries were recovered were 9.8 ± 0.6 and 9.2 ± 0.5 .

A blood sample (10 ml) was collected from each animal before slaughter by jugular venepuncture and the plasma was recovered and stored at -20°C until assayed for either FSH or LH. Frequent samples were not taken for LH pulse analysis.

Tissue preparation

Immediately after recovery, the pituitaries were immersion fixed in a solution consisting of PBS (0.1 mol l^{-1}), anhydrous disodium hydrogen orthophosphate (0.08 mol l^{-1}), sodium dihydrogen orthophosphate dihydrate (0.03 mol l^{-1}), NaCl (0.8 mol l^{-1}) containing 4% (w/v) paraformaldehyde and 15% (v/v) aqueous saturated picric acid ("supafix", pH 7.3) for 7 h at 4°C . With five sheep (+ +, $n = 3$; BB, $n = 2$), the pituitaries were fixed by perfusing 20 l of chilled 0.9% (w/v) saline through the carotid arteries followed by a further 20 l of "supafix". Subsequently, these pituitaries were sectioned longitudinally and immersed in "supafix" for an additional 24 h at 4°C and then transferred to Bouin-Hollandes fixative containing mercuric chloride, cupric acetate, (0.11 mol l^{-1}) picric acid (0.15 mol l^{-1}), mercuric chloride (0.18 mol l^{-1}) and 11.8% (v/v) formaldehyde for 24 h at room temperature. The pituitaries were then rinsed in running tap water for 24 h, processed through graded alcohols and xylene and embedded in paraffin wax. Serial sections ($5 \mu\text{m}$) were cut through the entire pituitary and after every fiftieth section, four consecutive pairs of sections were mounted on microscope slides coated with 3-aminopropyltriethoxysilane (Aldrich Chemical Co. Inc., WI).

Pituitary hormones

The pituitary gonadotrophin subunits, FSH β (Batch L128302) and LH β (Batch L1313022), were obtained from Bioscan International Inc., St Eustache, Quebec, whereas gonadotrophin α -subunit (AFP-13; NIAMDD and NICHD), NIADDK-oLH-26 (2.3 iu mg^{-1}), NIH-TSH-S8 (1.7 iu mg^{-1}), NIADDK-oTSH-12 (27 iu mg^{-1}), NIH-prolactin-S13 (30 iu mg^{-1}), NIH-GH-S11 (0.56 iu mg^{-1}) and USDA-oFSH-18 (1640 iu mg^{-1}) were supplied via the National Hormone and Pituitary Program, Bethesda, MD.

Antibodies

Antisera against highly purified ovine FSH β (Courot *et al.*, 1984; No. 178) and pig LH β (Dacheau and Dubois, 1978;

No. 19526) were raised in horse and rabbit, respectively. When tested in our laboratory by radioimmunoassay, the FSH β antibody relative to FSH β (i.e. 100%) showed significant crossreaction with intact ovine FSH (21%), but against the common α subunit of oFSH, oLH and oTSH or intact oGH, oTSH, oLH and oprolactin, all crossreactions were $< 0.02\%$. For the LH β antibody (No. 19526), there was a significant crossreaction with intact oLH (36%) but limited crossreactions were observed with other ovine pituitary hormones: prolactin and oFSH ($< 0.02\%$), gonadotrophin α subunit (0.3%), oGH (1.2%) and oTSH (3.8%).

Immunocytochemistry

An avidin-biotin immunoperoxidase technique, based on the method of Hsu *et al.* (1981), was used to localize FSH β and LH β subunits in separate sections of ovine pituitary tissue. No attempts were made to study the proportions of cells that localized both the FSH β and LH β subunits. Paraffin-embedded sections were deparaffinized with xylene, rehydrated through graded alcohols and endogenous peroxidase activity was quenched with exposure to 3% (v/v) hydrogen peroxidase in methanol for 30 min. The sections were washed ($3 \times 10 \text{ min}$) in Tris-HCl buffer (Tris (hydroxymethyl) methylamine, 0.5 mol l^{-1} ; 0.9% w/v NaCl; pH 7.6). Sections were then incubated with nonimmune blocking serum, diluted 1:15 in Tris-HCl buffer for 30 min to reduce nonspecific immunostaining (non-immune goat serum and nonimmune swine serum were used for the localization of FSH β and LH β , respectively). The appropriate primary antibody (horse anti-FSH β ; diluted 1:200 or rabbit anti-LH- β ; diluted 1:300 in Tris-HCl buffer + 0.1% BSA (w/v; reagent grade; Immuno-chemical Products Ltd, Auckland)) was applied to the sections and they were incubated overnight at 4°C in a humid chamber. All other incubations and washes were performed at room temperature. The sections were then washed three times for 10 min each in Tris-HCl buffer and then incubated for 30 min with a second antibody, namely biotinylated goat anti-horse IgG (Pierce, Rockford, IL) for the detection of FSH β and biotinylated swine anti-rabbit IgG (DAKOPATTS, DAKO Corporation, Carpinteria, CA) for the detection of LH β . Both secondary antibodies were diluted 1:500 in Tris-HCl buffer containing 0.1% (w/v) BSA and 1% (v/v) nonimmune sheep serum. After a further three 10-min washes in Tris-HCl buffer, an avidin-biotinylated horseradish peroxidase complex (DAKOPATTS) was applied to the sections and they were incubated for 30 min. The sections were given a further three 10-min washes in Tris-HCl buffer and the immunoperoxidase activity visualized with 3,3'-diaminobenzidine tetrahydrochloride (0.56 mmol l^{-1}) in Tris-HCl buffer containing ammonium nickel sulphate (13 mmol l^{-1}), 0.002% (v/v) hydrogen peroxidase in which the sections were incubated for 5 min. After a final 5 min wash in Tris-HCl buffer, the sections were counterstained in eosin, dehydrated through graded alcohols and xylene and mounted under coverslips using DePeX mounting medium.

A projection microscope (NeoPromar; Leitz) was used to observe and count nuclei in cells containing immuno-FSH β or immuno-LH β by the presence of a clear nonstaining portion within a cell surrounded by black immunostaining. The number

of total cells in each pituitary was estimated in the same manner, using sections stained with haematoxylin and eosin.

Hormone assays

The radioimmunoassays for FSH and LH were identical to those described by McNatty *et al.* (1989). Briefly, for FSH the radioimmunoassay kit was supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Bethesda, MD. The oFSH for iodination was NIAMDD-oFSH-I-1; the oFSH reference preparation was NIAMDD-oFSH-RP1 (biopotency $\times 75$ NIH-FSH-S1); and the oFSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113). For LH, the iodination standard was NIDDK-oLH-I-3 (AFP9598) and the LH antiserum was raised at Wallaceville and is described by McNatty *et al.* (1989). The oLH reference preparation was NIAMDD-oLH-23 (biopotency $\times 2.3$ NIH-LH-S1). The minimum detectable concentration for FSH and LH was 0.2 ng ml⁻¹. All samples for each hormone were measured in one assay, and the intra-assay coefficients of variation for the internal standards were < 8% for FSH and < 10% for LH.

Morphometric analysis

Pituitary volumes (V_0) were estimated by the Cavalieri principle using the formula $V_0 = \Sigma ah$, where a , determined by point counting, is the area in μm^2 of every 50th or 100th section (5 μm thickness) and h is the distance in μm between the sections used to determine a (Gundersen, 1986).

The number of cells, N , was determined using the nuclear dissector method (Gundersen, 1986) using the formula:

$$N = \frac{\Sigma C \times V_0}{\Sigma a_{(c)} \times h_{(c)}}$$

where $h_{(c)}$ is the thickness of adjacent sections in μm (in this case $h_{(c)} = 5 \mu\text{m}$) and C is the number of cells exclusive to one section in a known area, $a_{(c)}$.

Pituitary cell diameter

Cell diameters were determined by capturing images of cells and measuring their average diameter using the MICROSCALE version 2.1 programme (Digithurst Ltd, Royston, Herts). Briefly, the average diameter of each cell was calculated from the mean of four measurements. Each of the four diameters intersected the centre of the nucleus. Ten cells were measured per pituitary and an average cell diameter per animal was then calculated.

Statistical analyses

The data were subjected to analysis of variance to test for differences between genotypes. Linear relationships were examined between the total pituitary cell number, pituitary volume and the number of FSH β - or LH β -immunostaining cells. In addition, linear relationships were examined between the number of FSH β -immunostaining cells and the peripheral plasma concentrations of FSH. As LH concentrations were close to or at the detection limit of the assay, linear

relationships between the number of LH β -immunostaining cells and plasma LH were not examined.

Results

Antibody specificity

To test the specificity of the FSH β and LH β antibodies in the immunocytochemical procedure, increasing amounts of the pituitary hormone (LH, FSH, GH, prolactin and TSH; i.e. 0.08, 1.6, 3.1, 6.3, 12.5 ... 1600 $\mu\text{g ml}^{-1}$) were preincubated with the FSH β or LH β antibodies and applied to the tissue sections, and the amounts needed to block binding of the FSH β or LH β antibody were assessed. Relative to FSH β ligand, a 110-fold excess of oLH, oGH, oprolactin and oTSH was required to block immunostaining with the FSH β antibody. Relative to LH β ligand, a > 110-fold excess of oFSH, a 40-fold excess of oprolactin and oGH, and an 11-fold excess of oTSH were required to block immunostaining with the LH β antibody. The absence of nonspecific staining was confirmed by replacement of the antibodies with either Tris-HCl buffer + 0.1% (w/v) BSA or nonimmune rabbit serum.

Numbers of FSH β - and LH β -immunostaining pituitary cells

The plasma concentrations of FSH and LH, the total number of pituitary cells and the number of FSH β and LH β immunostaining pituitary cells with respect to Booroola genotype are shown in Table 1. There were gene-specific differences in the plasma concentrations of FSH ($P < 0.001$) but not LH. The plasma LH concentrations were not distinguishable from the detection limit (i.e. 0.2 ng ml⁻¹) in both genotypes. No gene-specific differences were noted with respect to the total number of pituitary cells, or in the numbers immunostaining for FSH β or LH β . There were no gene-specific differences noted for the estimated pituitary volumes (i.e., BB = 224 ± 35 and ++ = $209 \pm 47 \text{ mm}^3$). The ratio obtained after dividing the plasma FSH concentration by the number of FSH β cells to provide some estimate of cell output of FSH revealed a significant difference between the genotypes (Table 1).

Diameters of FSH β - and LH β -immunostaining pituitary cells

The mean $\pm$ SEM diameters (μm) of immunostaining cells with respect to Booroola genotype were 8.9 ± 0.2 (++) and 8.8 ± 0.2 (BB) for FSH β , and 9.3 ± 0.1 (++) and 9.2 ± 0.2 (BB) for LH β . The mean $\pm$ SEM diameters (μm) of haematoxylin and eosin staining cells with respect to genotype were 9.6 ± 0.2 and 9.5 ± 0.2 , respectively; these diameters were determined without knowledge of whether the cells were gonadotropes or some other cell type. No significant differences were found ($P > 0.05$) between genotypes for each of the cell types (FSH β , LH β or pituitary cells in general) or between cell types within genotypes.

Linear relationships among pituitary volumes, numbers of pituitary cells and plasma FSH

Some significant linear relationships were found among the number of pituitary cells, pituitary volume, numbers of

Table 1. Plasma concentrations of FSH and numbers of pituitary cells and FSH β - and LH β -immunostaining pituitary cells with respect to Booroola genotype

Booroola genotype (n)	Plasma concentrations (ng ml ⁻¹) FSH	Total number of pituitary cells ($\times 10^{-6}$)	Number of immunostaining pituitary cells ($\times 10^{-6}$)		Plasma FSH concentration/number of FSH β cells (pg ml ⁻¹ per cell)
			FSH β	LH β	
BB (7)	1.88 $\pm$ 0.07***	234 $\pm$ 44	12 $\pm$ 1	26 $\pm$ 4	166 $\pm$ 13**
++ (8)	0.70 $\pm$ 0.04	229 $\pm$ 52	10 $\pm$ 2	26 $\pm$ 7	90 $\pm$ 18

Values are means $\pm$ SEM

The plasma LH concentrations were not distinguishable from the detection limit of the assay (i.e. 0.2 ng ml⁻¹).

*** P < 0.001; ** P < 0.01 (Student's t test).

Table 2. Correlation coefficients among number of pituitary cells, pituitary volume and plasma concentrations of FSH (ng ml⁻¹) with respect to Booroola genotype or the genotypes combined

Dependent variable	Independent variable	Genotype		++/BB combined
		++	BB	
Pituitary volume	Total number of pituitary cells	1.00***	0.99***	1.0***
Pituitary volume	Number of LH β cells	0.96***	0.85***	0.93***
Total number of pituitary cells	Number of LH β cells	0.96***	0.82*	0.92***
Pituitary volume	Number of FSH β cells	0.47	0.52	0.48
Total number of pituitary cells	Number of FSH β cells	0.48	0.58	0.48
Plasma FSH	Number of FSH β cells	0.81**	0.83**	0.48

Significant linear relationship: *** P < 0.001, ** P < 0.01, * P < 0.05. The relationship between plasma LH and number of LH β cells could not be tested as most LH values were not detectable.

immunostaining LH β or FSH β cells and plasma FSH concentrations (Table 2). The relationship between the total number of pituitary cells and volume of the pituitary could be expressed by the equation $y = 2.5 + 0.912x$ (for both genotypes combined), where y = pituitary volume (mm³) and x = number of cells $\times 10^{-6}$. The number of LH β cells showed a significant linear relationship with the total number of pituitary cells, namely $y = 1.96 + 0.1x$ (for both genotypes combined) where y = number of LH β cells $\times 10^{-6}$ and x = total number of pituitary cells $\times 10^{-6}$. In addition, the number of LH β cells showed a significant linear relationship with the pituitary volume (mm), namely $y = 3.0 + 8x$ (for both genotypes combined), where y = pituitary volume (mm³) and x = number of LH β cells $\times 10^{-6}$. Comparison showed that, for any of the above relationships, the slopes of the lines between the genotypes were not significantly different (Student's t test). No significant linear relationship between the number of FSH β cells and either pituitary volume or the total number of pituitary cells was noted. However, a significant linear relationship was established between the number of FSH β cells and the plasma FSH concentrations within the genotypes, namely $y = 0.48 + 0.02x$ (++) and $y = 1.38 + 0.04x$ (BB), where y = plasma FSH (ng ml⁻¹) and x = number of FSH β cells $\times 10^{-6}$. The slopes of these lines were not significantly different between the genotypes. However, the linear relationship was not maintained when the data for both genotypes were combined.

Discussion

The key findings from this study are that the total numbers or mean diameters of FSH β - and LH β -immunostaining pituitary cells do not differ between the Booroola genotypes, notwithstanding a two-fold difference in plasma concentration of FSH. Moreover, no differences between the genotypes were noted for the total number of pituitary cells, mean diameter of pituitary cell or for the pituitary volume. Collectively, these findings, together with results showing no differences in pituitary FSH isoforms (Robertson *et al.*, 1984; Phillips *et al.*, 1995) or in the circulating half-lives ($t_{1/2}$) of FSH (Fry *et al.*, 1987; Phillips *et al.*, 1995) raise the possibility that the concentration difference in plasma FSH between the genotypes is due to a greater output of FSH from each pituitary cell in BB animals.

The hypothesis that BB animals have a greater FSH output per cell relative to ++ animals is supported by results in ovariectomized HPD ewes, in which the FSH release after an i.v. dose of GnRH (250 ng) in BB animals was about 1.6-fold higher (P < 0.05) than in the ++ genotype (McNatty *et al.*, 1991). Whether these differences in FSH output are related directly to differences in mRNA synthesis or degradation or the synthesis and stability of the FSH molecule remain to be determined. While studies using northern blot analysis reveal no obvious differences in the amounts of pituitary mRNA for FSH β (Fleming *et al.*, 1995), it is possible that the difference is

too small to be detected reliably by this technique; the gene difference in plasma FSH, as detected by radioimmunoassay in GnRH-treated HPD-ovariectomized ewes, is only 1.6-fold (McNatty *et al.*, 1991). Perhaps a more sensitive technique, such as RNase protection assay, is needed to ensure that small differences in FSH β mRNA synthesis are not occurring. Notwithstanding this possibility, it has been shown for the HPD-ovariectomized ewe that animals homozygous for the *FecB^B* gene had higher FSH concentrations at 10 min after an exogenous GnRH injection of 250 ng compared with homozygous non-carriers (McNatty *et al.*, 1991). This finding suggests that the *FecB^B* difference is more likely to be due to a different per cell output of preformed hormone. Moreover, since the gene effect in plasma FSH concentration can arise without any measurable difference in pituitary content, it seems unlikely that there are differences in the stability of the FSH molecule (Montgomery *et al.*, 1992). Future studies may need to focus on a comparison of FSH isoforms in plasma as well as in the pituitary (Phillips *et al.*, 1995) and on *in vitro* systems to examine the intracellular regulation of GnRH-induced FSH release.

The finding that there is a significant linear relationship between pituitary volume and the number of pituitary cells or number of LH β -immunostaining cells, irrespective of genotype, suggests that the changes in pituitary volume may be a consequence of a change in the total number of cells and that the number of LH β cells is always a fixed proportion of that total number of cells. In contrast, there is no such relationship between the number of FSH β cells and either pituitary volume or total number of cells in either genotype, suggesting that the factors determining the number of FSH β cells are not the same as those for LH β . The finding of a significant linear relationship between the number of FSH β -immunostaining cells and plasma FSH concentration for each genotype separately, and the fact that the slopes of these regression lines were not different could infer that the disappearance of FSH from the circulation is likely to be the same in both genotypes. The half-life of FSH in intact sheep is estimated to be 110 min (Akbar *et al.*, 1973; Fry *et al.*, 1987), whereas that for LH is 25–30 min (Montgomery *et al.*, 1984). Presumably the longer half-life for FSH than for LH allows the aforementioned linear relationship between numbers of pituitary cell and plasma concentrations to be observed.

The finding that 10–12% of the total number of cells immunostained for FSH β is similar to that reported by Thomas *et al.* (1993) for adult sheep. For LH β , we report that about 26% of pituitary cells immunostained positively, whereas Thomas *et al.* (1993) report about 16%. Possible explanations for this discrepancy are the age, breed and reproductive status of the ewes (not defined in the Thomas *et al.* (1993) study), fixation procedure, antibodies used and method of counting. Our technique was based on the nuclear dissector method, whereas Thomas *et al.* (1993) used a random field ($\times 4$) point counting technique of individual sections. Notwithstanding these differences in ewes, the proportions of cells immunostaining for FSH β (10–12%) or LH β (16–26%) are substantially higher than noted in sheep fetuses during late gestation (120–140 days of gestation) or in the newborn (FSH β , 1–4%; LH β , 3–5%) (Messaoud-Toumi *et al.*, 1993). This is despite the fact that the plasma concentrations of FSH and LH in late gestation or soon

after birth are higher than in adult females (Smith *et al.*, 1993; Isaacs *et al.*, 1995). It would be of interest to determine the effects of plasma volume and also to establish whether the median charges and clearance rate of FSH and LH in fetal and neonatal sheep differ substantially from those in adult animals.

In conclusion, this study reports a significant linear relationship between the number of FSH β -immunostaining cells and the plasma concentrations of FSH for both homozygous carriers and non-carriers of the *FecB^B* gene, separately. This study also reports that there are no differences between the Booroola genotypes in the number or diameter of FSH β - or LH β -immunostaining cells. It is hypothesized that the differences in plasma concentration of FSH between the genotypes is due to a greater output of FSH per pituitary cell in the BB animals.

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