

The *Fec^B* (Booroola) gene acts at the ovary: *in vivo* evidence

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The aim of this study was to differentiate between pituitary and ovarian actions of the *Fec^B* gene by measuring the ovarian response to a standardized treatment with gonadotrophins designed to mimic the changes in FSH and LH that occur in the follicular phase of the ovarian cycle in ewes, with (*Fec^{B/-}*, *n* = 6) and without (*Fec^{B/+}*, *n* = 9) the gene, that were rendered hypogonadotrophic by pretreatment with a potent antagonist of GnRH. Ewes with ovarian autotransplants were used to facilitate the assessment of follicular function by the collection of ovarian venous blood and ultrasonography. The gonadotrophin regimen resulted in concentrations of FSH and LH that were similar to concentrations found in a normal cycle and did not differ between genotypes. Follicular development and ovulation occurred in all animals, and patterns of secretion of oestradiol, androstenedione and inhibin A were normal. Despite these endocrine similarities, the antral follicle

population stimulated by FSH infusion retained the characteristic genotypic difference with the ovaries of *Fec^{B/+}* animals containing a range of follicle sizes with decreasing proportions of small (< 3.5 mm in diameter) and medium (3.5–4.5 mm in diameter) follicles as well as large follicles (≥ 4.5 mm in diameter), whereas the ovaries of *Fec^{B/-}* ewes contained no follicles of > 4.5 mm in diameter. This genotypic difference was retained after ovulation with gene carriers having more preovulatory follicles/corpora lutea (3.8 ± 0.3) of a smaller diameter (5.3 ± 0.3 mm) than did non-gene carriers (1.7 ± 0.3; 11.4 ± 0.9 mm; *P* < 0.05). As ewes carrying the *Fec^B* gene mutation were able to ovulate more follicles than non-gene carriers, despite identical concentrations and patterns of FSH and LH stimulation, the results of this study support the hypothesis that the *Fec^B* gene acts at the ovary to enhance ovarian sensitivity to gonadotrophic stimulation.

Introduction

The *Fec^B* gene is a single mutation that results in a marked increase in ovulation rate and prolificacy in sheep (Bindon, 1984). This mutation, initially termed Booroola after the name of the farm in South Eastern Australia where sheep carrying the gene were first isolated, has recently been identified as a mutation in the signalling domain of the bone morphogenic protein 1b receptor (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001). However, despite this advance the mechanisms whereby this mutation alters ovarian function in sheep are still unclear.

Early work on Booroola sheep (Bindon, 1984) was hampered by the fact that the only phenotype expressed was ovulation rate and number of offspring in the female, but it soon became clear that the gene responsible segregates as a single gene, or a closely linked series of genes, and that the heterozygote has an ovulation rate intermediate (designated F+ with ovulation rate 3–4) between the homozygote (designated FF with ovulation rate > 4) and wild type (designated ++ with ovulation

rate 1–2). Early studies on the ovarian physiology of *Fec^B* gene carriers in Merino sheep indicated that they have smaller preovulatory follicles with fewer granulosa cells and correspondingly smaller corpora lutea than do control Merino sheep (Scaramuzzi *et al.*, 1981; Baird *et al.*, 1982). These initial observations were confirmed and extended by an elegant and comprehensive series of experiments using Romney Marsh ewes carrying the gene by McNatty and colleagues who showed that small antral follicles matured precociously in gene carriers, becoming oestrogenic and developing LH receptors on the membrana granulosa at diameters of 2.5–3.5 mm compared with 4–6 mm in non-gene carriers (Henderson *et al.*, 1985; McNatty *et al.*, 1985, 1986a–c). More recently, the present authors showed that the expression of mRNA for both cytochrome P450 aromatase and the β_A -subunit of inhibin/activin can be detected in much smaller follicles in *Fec^B* gene carriers compared with controls (Campbell *et al.*, 1997).

Examination of the kinetics of folliculogenesis in *Fec^B* gene carriers during the preovulatory period has been attempted using follicular dissection (McNatty *et al.*, 1985, 1986b), ink labelling (Driancourt *et al.*, 1985) and, more recently, ultrasound imaging in ewes

with ovarian autotransplants (Souza *et al.*, 1997a). These studies collectively indicate that the differences in the ovulation rate of the *Fec^B* gene carriers are not due to differences in the total number of antral follicles, but to an extended recruitment period together with a low incidence of atresia, resulting in the ovulation of a large number of small ovulatory follicles. The smaller size of these ovulatory follicles explains the observation that ovarian secretion of oestradiol (Baird *et al.*, 1982; McNatty *et al.*, 1986b; Souza *et al.*, 1997a), androgen (Souza *et al.*, 1997a) and inhibin A (Souza *et al.*, 1997a) during the preovulatory period and luteal progesterone concentrations (Bindon 1984; McNatty *et al.*, 1985) do not differ among animals with two, one or no copies of the *Fec^B* gene. In fact, McNatty *et al.* (1985) calculated that the total population of granulosa cells in oestrogenic follicles is identical in the different genotypes.

Although precocious development of preovulatory follicles appears to explain the increase in prolificacy in *Fec^B* gene carriers, the mechanism behind this early maturation is unclear. As FSH is the primary hormone controlling follicular growth and development, it has been suggested that the *Fec^B* mutation may act by either increasing the release of FSH from the pituitary or increasing the sensitivity of follicular cells to FSH within the ovary. Studies designed to test these hypotheses have provided evidence to support both proposals. Initially it was reported that the concentrations of FSH were similar in both carriers and non-carriers of the *Fec^B* gene (Bindon *et al.*, 1985) but the development of improved FSH assays saw the publication of a number of papers supporting the hypothesis that high jugular venous FSH concentrations are associated with the *Fec^B* gene (Bindon, 1984; McNatty *et al.*, 1987). However, the fact that a number of authors have reported no consistent association between *Fec^B* and FSH (B. K. Campbell, unpublished; Boulton *et al.*, 1995; Souza *et al.*, 1997a) casts some doubt on the causality of this relationship. Equally equivocal results have been obtained from studies that have examined this question using hypophysectomized (Fry *et al.*, 1988) or hypothalamic–pituitary disconnected (HPD) and GnRH-agonist suppressed ewes (Hudson *et al.*, 1999) stimulated with exogenous gonadotrophins. Fry *et al.* (1988) showed a continued difference in ovulation rate in *Fec^B* gene carriers stimulated with equine chorionic gonadotrophin (eCG), but the study of Hudson *et al.* (1999) concluded that the *Fec^B* gene acts at both the pituitary and ovary to stimulate ovulation rate.

Testing the hypothesis that ovarian follicular cells are more sensitive to gonadotrophic stimuli in *Fec^B* gene carriers has been complicated by the precocious maturation of ovulatory follicles in gene carriers, as it is difficult to find a satisfactory comparative basis between genotypes. The existing evidence shows that smaller (2.0–4.5 mm in diameter) follicles from *Fec^B* gene carriers are more sensitive (in terms of cAMP production) to LH and FSH and have higher aromatase activity than

follicles of similar size from control ewes. In contrast, theca and granulosa cell LH binding characteristics and thecal LH-stimulated androstenedione production have not been found to differ according to genotype (McNatty *et al.*, 1985, 1986b,c). Finally, physiological serum-free culture systems that allow gonadotrophin-dependent induction of cellular differentiation *in vitro* were used to show that granulosa cells from non-differentiated small follicles of < 1 mm in diameter from *Fec^B* gene carriers are more sensitive in terms of FSH-induced oestradiol production than are follicles of similar size or medium size (1–3 mm in diameter) from non-carriers of the *Fec^B* gene (Webb *et al.*, 1995). Thus, the available evidence indicates that the *Fec^B* mutation may be acting at both an ovary and pituitary gland.

Campbell *et al.* (1999) reported the development of a model system to examine gonadotrophic requirements for ovulatory follicle development using ewes with an ovarian autotransplant treated with a potent GnRH antagonist to suppress endogenous gonadotrophins. With this model it has been possible to show that stimulatory regimens of exogenous gonadotrophins designed to mimic the gonadotrophic changes that occur during the luteal–follicular phase transition can result in normal patterns of follicular selection. Specifically, by gradually lowering FSH and, at the same time, increasing LH pulse frequency before the application of an ovulatory stimulus, it is possible to reduce the ovulatory quotient to the normal number (Campbell *et al.*, 1999). In the present study this model was used to examine further the question of the site of action of the *Fec^B* gene by suppressing endogenous gonadotrophins in carriers and non-carriers of the gene and stimulating ovulatory follicle development with an identical physiological gonadotrophic regimen designed to mimic the normal luteal–follicular phase transition.

Materials and Methods

Experimental animals

The animals used were Scottish Blackface Merino cross ewes ($n=17$) obtained from an experimental flock that had been derived in such a way that the animals differed at the Booroola locus (being either *Fec⁺Fec⁺* or *Fec^BFec^B*), but were as similar as possible for the rest of the genome to provide experimental material with little risk of biased comparisons (Haley, 1990). On the basis of pedigree, confirmed by the ovulation rate (OR) of individual animals, the ewes were divided into non-gene carriers ($OR\ 1.7 \pm 0.2$; designated *Fec^{+/+}*, $n=9$) and gene carriers ($OR\ 5.6 \pm 1.1$; $n=6$ of which four homozygote *Fec^BFec^B* and two heterozygote *Fec^BFec⁺*; by pedigree but all classified as *Fec^BFec^B* on the basis of OR; designated *Fec^{B/-}*). Collection of ovarian venous blood and monitoring of ovarian follicular development by transdermal ultrasonography was

facilitated by autotransplanting the left ovary and its vascular pedicle to a site in the neck (Goding *et al.*, 1967) and removing the right ovary. The ewes were 3–5 years of age at the time of surgery and 4–6 years of age at the time of this experiment. A detailed comparison of the endocrinology and patterns of ovarian follicular development in these animals during a normal reproductive cycle has been reported by Souza *et al.* (1997a).

The experiment was conducted during the mid-breeding season and before the start of intensive blood sampling, the animals were penned indoors under natural lighting and fed a maintenance diet consisting of concentrates and hay *ad libitum*. The anoestrous condition of the animals during the GnRH antagonist suppression period was verified by including a ram fitted with a marking harness in the flock.

On the day before the start of frequent blood sampling all animals had a Silastic cannula (1.6 mm inside diameter \times 3.2 mm outside diameter; Dow Corning, Midland, MI) inserted into the jugular vein cranial to the ovarian jugular venous anastomosis for sampling ovarian venous blood. Two smaller Silastic cannulae (0.8 mm inside diameter \times 1.7 mm outside diameter; Dow Corning) were inserted into the contralateral jugular vein for infusion of FSH and LH as described by Campbell *et al.* (1999). After cannulation, the animals were placed in metabolism crates in ventilated rooms under natural lighting and treated prophylactically with antibiotics (3 ml i.m. per 3 days; Clamoxil, SmithKline Beecham, Surrey) and heparin (5000 iu i.v. per 12 h; Leo Laboratories, Aylesbury). The animals had been habituated to the housing conditions and frequent handling before the start of the experiment.

Hormone preparations

The FSH used was NIADDK-oFSH-17, which has a biological potency of 20 U mg⁻¹ with one unit having an activity of 1 mg NIH-FSH-S1 and LH contamination of 0.04 times NIH-LH-S1 per mg. The LH used was NIADDK-oLH-26, which has a biological potency of 2.3 U mg⁻¹ with one unit having an activity of 1 mg NIH-LH-S1 and FSH contamination of <0.5% by weight. The hCG used for induction of ovulation was obtained from Intervet (Chorulon, Cambridge). All gonadotrophin preparations were dissolved in 0.9% (w/v) sterile saline with 1% (v/v) normal sheep plasma. FSH was infused continuously i.v. via one of the small jugular cannulae using Harvard infusion pumps. LH was administered i.v. via the jugular cannula on the ovarian side in a pulsatile manner using Harvard infusion pumps connected to a timer (2 min infusion at a rate of 1 ml min⁻¹). The GnRH antagonist (GnRHa; [AcDNaI¹, DCpa², DTrp³, DArg⁶, DAla¹⁰] GnRH; donated by the Salk Institute and Centre for Population Research, NICHD) was dissolved in 0.9% (w/v) sterile saline to

a concentration of 1 mg ml⁻¹ and administered s.c. (Campbell *et al.*, 1990b, 1999). Progestagen impregnated sponges (medroxyprogesterone acetate; Dunlop, Dumfries) were inserted intravaginally and changed every 10–12 days.

Experimental treatment

A hypogonadotrophic hypogonadal state was induced in all animals by combined 3-week treatment with GnRHa (50 µg kg⁻¹ s.c.) at intervals of 4–6 days and progestagen as described by Campbell *et al.* (1998). GnRHa treatment was continued throughout the experimental period. The experimental design used a gonadotrophin regimen developed by Campbell *et al.* (1999), which was designed to mimic the luteal–follicular phase transition (Fig. 1). All animals received FSH as a constant infusion at a rate of 8 µg h⁻¹ for 3 days to stimulate antral follicle development before sponge withdrawal. At the time of sponge withdrawal, the rate of FSH infusion was progressively decreased so that the animals received 6, 4 and 2 µg h⁻¹ for 0–8, 8–16 and 16–24 h after sponge withdrawal, respectively. LH was administered initially as i.v. injections of 2.5 µg at 4 h intervals for the first 3 days of FSH infusion. For the first 12 h after sponge withdrawal the frequency of LH injections was increased to 2 h without changing amplitude but thereafter all animals were treated with more frequent (hourly) injections of 1.25 µg oLH. LH injections were administered by syringe-driven pumps connected to a timer calibrated to deliver the desired dose of LH over 2 min in a volume of 2 ml. Sixty hours after sponge withdrawal, the normal duration of the follicular phase in these animals (Souza *et al.*, 1997a), all ewes received an ovulatory dose of 525 µg oLH combined with 100 iu hCG administered as an infusion over 6 h.

Collection of blood samples

Jugular venous blood samples were collected by venepuncture every 3–4 days during the 3-week period of downregulation. Over the intensive experimental period, ovarian (5 ml) and jugular (3 ml) venous blood samples were collected at 4 h intervals from the start of gonadotrophin infusion until 10 h after the end of administration of the ovulatory stimulus (76 h after sponge withdrawal; Fig. 1). Thereafter, jugular venous blood samples were collected by venepuncture every 2 days for 12 days for determination of progesterone concentrations to confirm ovulation. In addition, there were three periods when more frequent blood samples were collected at 15 min intervals near LH administration designed to stimulate ovarian steroid secretion: (i) from 30 min before until 2 h after an LH injection given 68 h after the start of gonadotrophin treatment; (ii) for 3 h from 24 to 27 h after sponge withdrawal; and (iii) for 3 h from 48 to 51 h after sponge withdrawal. All

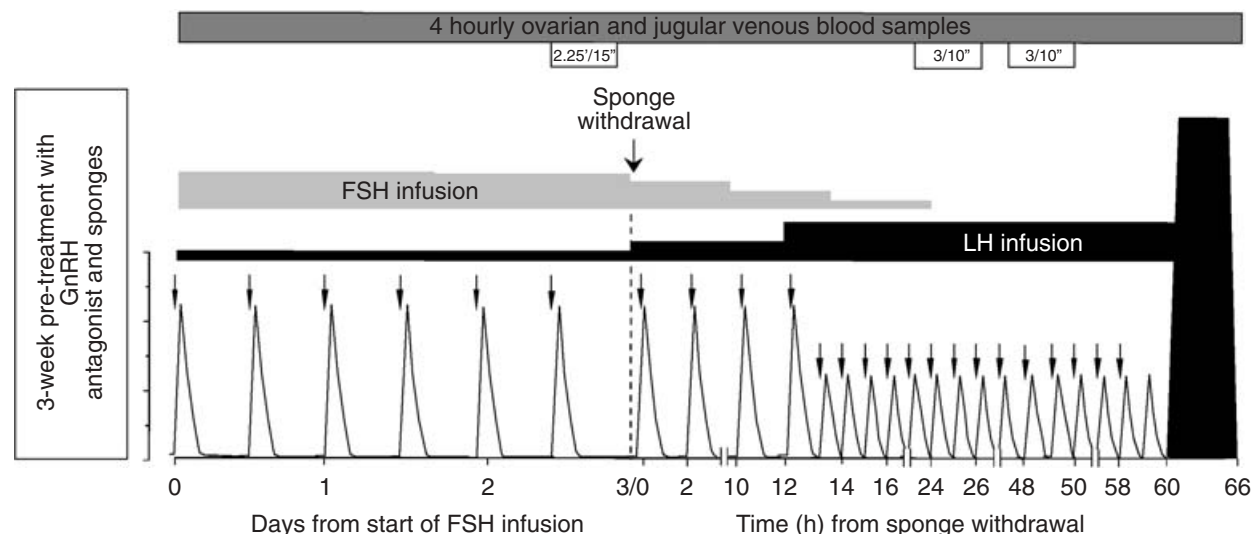


Fig. 1. Schematic representation of experimental design. Gonadotrophic regimens designed to mimic components of the luteal to follicular phase transition were applied to ewes with an autotransplanted ovary made hypogonadal by treatment for 3 weeks with GnRH antagonist ($50 \mu\text{g kg}^{-1}$ in 96 h) and progestagen sponges ($n=18$). Large antral follicle development was stimulated by FSH infusion for 3 days and, thereafter, the luteal to follicular phase transition was mimicked by staged withdrawal of FSH with a concomitant increase in LH pulse frequency. At 60 h after sponge withdrawal all animals received an ovulatory dose of oLH combined with hCG. Samples of ovarian and jugular venous blood were collected over the experimental period and there were three periods of intensive blood sampling near an LH administration. The follicle population was determined by ultrasonography performed each day.

collections of ovarian venous blood every 4 h and every hour during the intensive sampling periods were timed to allow calculation of ovarian hormone secretion rates. The samples of ovarian venous blood taken every 4 h were collected 20–30 min after LH was administered as this is the time taken for maximal steroidogenic responses to LH stimulation (Campbell *et al.*, 1990c). After correcting for the haematocrit (Collett *et al.*, 1973) and jugular contribution (inhibin only), the rate of ovarian secretion of oestradiol, androstenedione and inhibin A was calculated. The blood was centrifuged at 4°C for 30 min at 1800 *g* and the plasma stored at -20°C before radioimmunoassay.

Ovarian scanning procedure

The diameter of the antral cavity and position of all follicles of >2 mm in diameter in the medial–lateral, dorsal–ventral and cranial–caudal planes were determined every 12 h, as described by Souza *et al.* (1997a,b) and Campbell *et al.* (1998) using a combined real-time Aloka 500 ultrasound scanner with a linear 7.5 MHz transducer probe (Dynamic Imaging, Livingston). Animals were scanned at day 6 after the artificial LH surge for the presence of corpora lutea to estimate ovulation rate (Souza *et al.*, 1997b).

Hormone assays

Plasma concentrations of LH, FSH, oestradiol, androstenedione (Campbell *et al.*, 1990a,b) and progesterone

(Souza *et al.*, 1997b) were determined using previously described radioimmunoassays. The sensitivity of the assays for LH, FSH, androstenedione, oestradiol and progesterone were $0.2 \mu\text{g l}^{-1}$ (NIDDK, oLH, S23), $0.3 \mu\text{g l}^{-1}$ (USDA, oFSH, SIAFP-RP2), 175 pmol l^{-1} , 50 pmol l^{-1} and 380 pmol l^{-1} , respectively. The concentration of inhibin A in ovarian venous plasma was measured by two site ELISA modified for use in sheep plasma (Souza *et al.*, 1998) with a sensitivity of 30 ng l^{-1} . The intra- and interassay coefficients of variation for all the immunoassays were $<12\%$ in the 20–80% effective dose range.

Statistical analysis

Statistical analysis of hormonal profile data was performed by repeated sample analysis of variance and log-transformed data were partitioned on the basis of treatment and time (ANOVA). The characteristics of pulsatile LH secretion were determined using the Munro pulse analysis program (Zaristow Software, West Morham, Haddington, East Lothian). The steroidogenic pulsatile response to an LH pulse was determined by grouping the pulse data from individual profiles around the time of each LH pulse and calculating basal secretion and pulse amplitude. In addition the overall mean secretion of LH and the steroids over the periods of intensive sampling were calculated. Comparisons between treatments on these determinants of pulsatile hormone secretion were made by repeated samples

Table 1. Mean \pm SEM values for characteristics of pulsatile LH, oestradiol and androstenedione secretion determined from periods of intensive blood sampling at the end of 3 days of FSH infusion (FSH + 3 days) and during the early (sponge withdrawal (SW) + 24–27 h) and late (SW + 48–51 h) stages of the artificial ‘follicular’ phase in ewes with ovarian autotransplants that were either wild type (Fec⁺Fec⁺) or carried the Fec^B mutation (Fec⁺Fec^B)

Time window bleed Hormone/genotype	FSH + 3 days		SW + 24–27 h		SW + 48–51 h	
	Fec ^{+/+}	Fec ^{B/-}	Fec ^{+/+}	Fec ^{B/-}	Fec ^{+/+}	Fec ^{B/-}
LH (ng ml ⁻¹)						
Basal	1.07 \pm 0.21	0.94 \pm 0.07	1.65 \pm 0.29	1.30 \pm 0.24	1.41 \pm 0.25	1.21 \pm 0.18
Amplitude	2.40 \pm 0.33	2.21 \pm 0.20	1.99 \pm 0.17	1.50 \pm 0.11	1.71 \pm 0.26	1.43 \pm 0.13
Overall mean	1.40 \pm 0.20	1.21 \pm 0.13	1.65 \pm 0.18	1.41 \pm 0.20	1.49 \pm 0.21	1.20 \pm 0.09
Log oestradiol (pg min ⁻¹)						
Basal	2.79 \pm 0.15	2.40 \pm 0.17	3.30 \pm 0.13	3.13 \pm 0.15	3.54 \pm 0.13	3.24 \pm 0.15
Amplitude	3.43 \pm 0.13	3.15 \pm 0.15	3.58 \pm 0.13	3.21 \pm 0.15	3.50 \pm 0.13	3.26 \pm 0.15
Overall mean	3.23 \pm 0.12	2.87 \pm 0.14*	3.50 \pm 0.11	3.20 \pm 0.14	3.40 \pm 0.13	3.25 \pm 0.15
Log androstenedione (pg min ⁻¹)						
Basal	2.79 \pm 0.11	2.80 \pm 0.13	3.30 \pm 0.18	3.35 \pm 0.20	3.55 \pm 0.12	3.44 \pm 0.14
Amplitude	3.59 \pm 0.15	3.35 \pm 0.18	3.70 \pm 0.14	2.54 \pm 0.16	3.62 \pm 0.13	3.41 \pm 0.15
Overall mean	3.30 \pm 0.12	3.05 \pm 0.15	3.58 \pm 0.12	2.51 \pm 0.15	3.48 \pm 0.12	3.43 \pm 0.15

* $P = 0.07$ for comparison between genotypes.

ANOVA. The effect of treatment on follicle number (ANOVA) and ovulation rate (t test) was determined following $\sqrt{(x+0.5)}$ transformation.

Results

GnRH antagonist suppression

Before antagonist treatment, FSH concentrations did not differ between genotypes (Fec^{+/+} 2.7 \pm 0.6 versus Fec^{B/-} 1.7 \pm 0.1 ng ml⁻¹; not significant). GnRH antagonist treatment resulted in a marked depression ($P < 0.01$) in jugular venous FSH concentrations in both groups within 7 days of treatment and after 3 weeks of treatment FSH concentrations were similar (Fec^{+/+} 1.1 \pm 0.1 versus Fec^{B/-} 1.1 \pm 0.1 ng ml⁻¹; not significant). Similarly, LH concentrations after antagonist treatment were suppressed and no difference was evident between genotypes (Fec^{+/+} 0.6 \pm 0.1 versus Fec^{B/-} 0.6 \pm 0.1 ng ml⁻¹; not significant). Ultrasonography after 21 days of antagonist treatment revealed small ovaries containing 9.2 \pm 1.4 and 9.8 \pm 1.5 small antral follicles per ovary in Fec^{+/+} and Fec^{B/-} ewes, respectively.

The first 3 days of FSH infusion

Infusion of FSH resulted in a small but highly significant ($P < 0.001$) increase in peripheral FSH concentrations of approximately 0.5 ng ml⁻¹ in both experimental groups over the initial 8 h of treatment to concentrations similar to those observed in these animals before GnRH antagonist treatment (Fig. 2a). Thereafter, FSH concentrations remained stable until the time of sponge withdrawal and no difference between genotypes was evident. As expected, infusion of FSH stimulated antral

follicle growth in both groups ($P < 0.001$; Fig. 3) and this stimulation was reflected by an increase in ovarian inhibin A ($P < 0.05$) and LH-stimulated androstenedione ($P < 0.001$) and oestradiol ($P < 0.001$) secretion over this period (Fig. 2b–d). The comparison between genotypes over the initial period of FSH infusion showed no significant differences or genotype \times time interactions, although inhibin A concentrations in ovarian venous blood tended to be lower in gene carriers over this period.

The injection of 2.5 μ g LH every 4 h during the first 3 days of treatment resulted in normal basal LH concentrations with no difference between genotypes (Fec^{+/+} 1.6 \pm 0.1 versus Fec^{B/-} 1.4 \pm 0.1 ng ml⁻¹; not significant). Frequent sampling before sponge withdrawal revealed that the injection resulted in an LH pulse with an amplitude of 2.4 \pm 1.0 and 2.2 \pm 0.5 ng ml⁻¹ (not significant) superimposed on a baseline of 1.1 \pm 0.6 and 0.9 \pm 0.2 ng ml⁻¹ (not significant) in Fec^{+/+} and Fec^{B/-}, respectively. The amplitude and mean secretion of the oestradiol and androstenedione secretory pulses stimulated by this injection were not significantly different between genotypes before sponge withdrawal although mean oestradiol secretion tended ($P = 0.07$) to be lower in Fec^{B/-} ewes (Table 1).

After 3 days of FSH infusion, the follicular population in different genotypes differed markedly ($P < 0.01$, Fig. 3). The ovaries of Fec^{+/+} animals contained a range of antral follicles that closely resembled the follicular population observed in normal ewes during the luteal phase (Souza *et al.*, 1997), with decreasing proportions of small (< 3.5 mm in diameter; 3.2 \pm 0.7), medium (3.5–4.5 mm in diameter, 2.6 \pm 0.6) and large follicles (≥ 4.5 mm in diameter; 0.4 \pm 0.2). In contrast, the ovaries of Fec^{B/-} ewes contained mainly small follicles

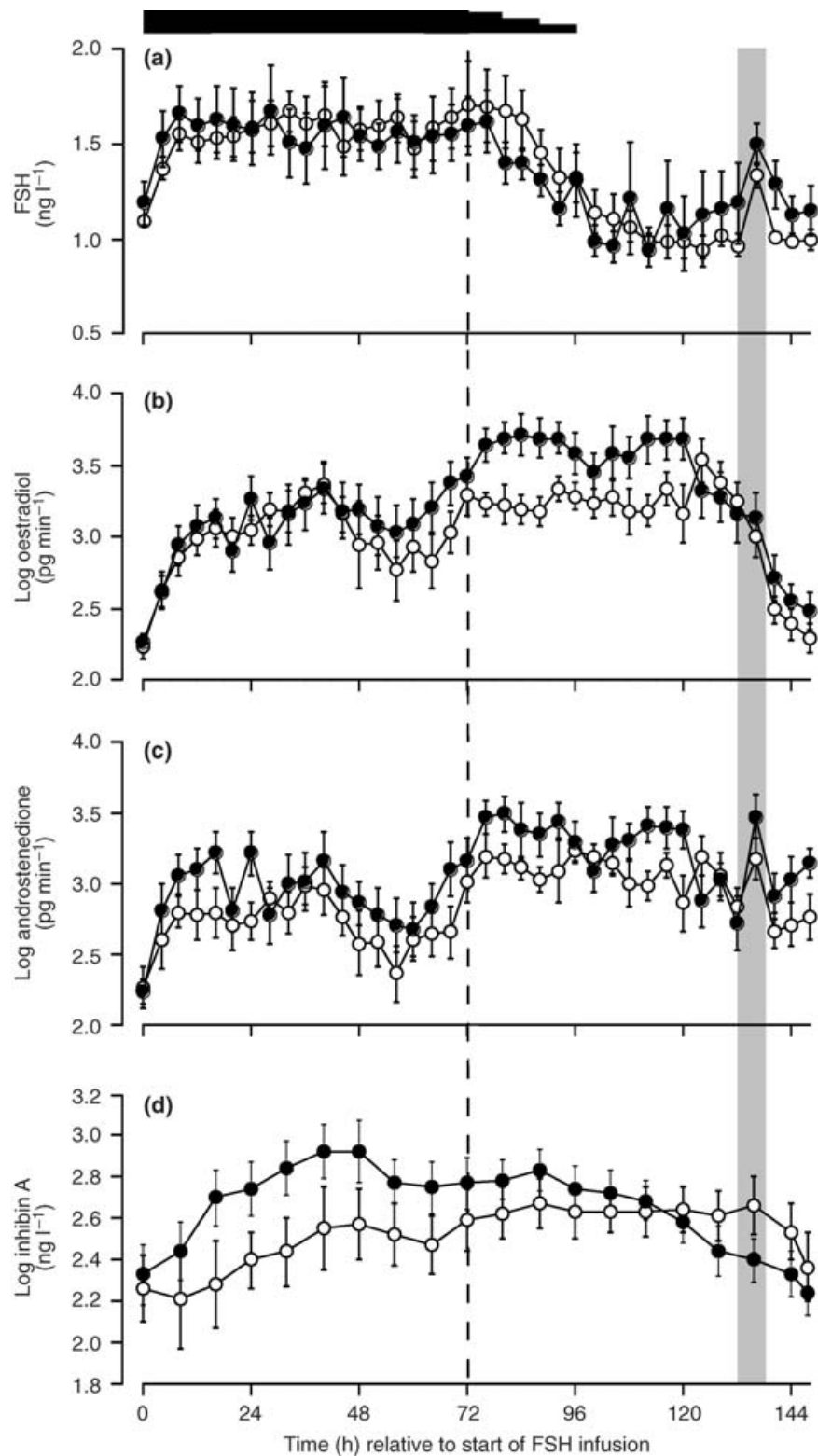


Fig. 2. (a) Jugular venous FSH and ovarian secretion of (b) oestradiol, (c) androstenedione and (d) inhibin A in wild-type ewes (●) and ewes bearing the *Fec^B* mutation (○) after FSH infusion and across the artificial follicular phase. The solid horizontal bar indicates the period and amount of FSH infusion, whereas the vertical bar indicates the period of the artificial 'LH surge'. The dashed line indicates the time of sponge withdrawal. Values are means \pm SEM.

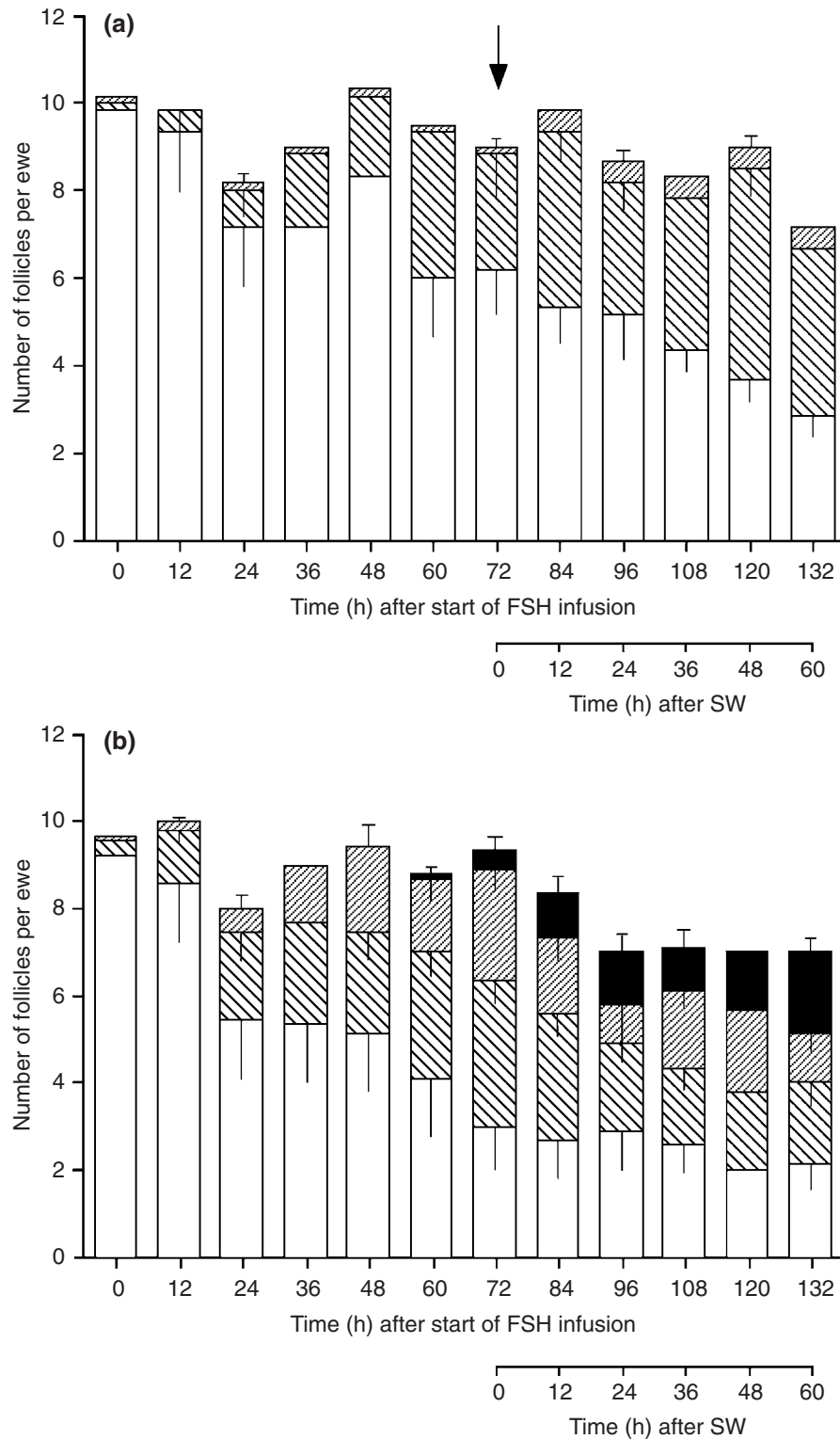


Fig. 3. Follicle populations in ewes bearing the (a) Fec^B mutation or (b) wild-type ewes after FSH infusion and across the artificial follicular phase. The arrow indicates the time of sponge withdrawal (SW). Values are means \pm SEM. □: follicles of 1.0–2.5 mm in diameter; ▨: follicles of 2.5–3.5 mm in diameter; ▩: follicles of 3.5–4.5 mm in diameter; ■: follicles of > 4.5 mm in diameter.

(4.4 ± 0.8) with a small (0.5 ± 0.3 per ewe) number of follicles of medium size and no large follicles (Fig. 3).

'Follicular phase' (sponge withdrawal to preovulatory LH surge)

The step-down in the rate of FSH infusion during the initial 24 h after sponge withdrawal resulted in a marked decline ($P < 0.001$) in peripheral FSH concentrations to pre-infusion concentrations that remained stable thereafter until the administration of an ovulatory stimulus (60 h after sponge withdrawal). There were no differences between genotypes in the timing or magnitude of this decline. In response to the concomitant increase in LH pulse frequency, oestradiol secretion increased in $Fec^{+/+}$ but not in $Fec^{B/-}$ ewes after sponge withdrawal resulting in significantly higher oestradiol ($P = 0.02$) secretion in non-gene carriers during the initial 44 h of the follicular phase (Fig. 2b). A similar trend was evident for androstenedione secretion but the difference between genotypes was not significant. Between 44 and 52 h after sponge withdrawal (116–124 h after the start of FSH infusion) oestradiol and androstenedione secretion in non-gene carriers declined ($P < 0.05$) to the same amount observed in gene carriers and steroid secretion in the two genotypes remained very similar until the start of the LH surge. Ovarian inhibin A secretion did not differ between genotypes over this period (Fig. 2d).

Frequent blood sampling during the early 'follicular' phase at about the time of LH injections confirmed the results of the samples taken at 4 h intervals. Administration of $1.25 \mu\text{g}$ LH at this stage resulted in LH pulses with amplitudes of $1.5\text{--}2.0 \text{ ng ml}^{-1}$ superimposed on a baseline of $1.3\text{--}1.7 \text{ ng ml}^{-1}$ in both genotypes (not significant; Table 1). The basal and mean secretion of both oestradiol and androstenedione estimated from periods of intensive blood sampling increased significantly ($P < 0.01$) across the luteal to follicular phase transition, whereas pulse amplitude did not change (Table 1). There were no significant genotypic differences or genotype by time interactions for these characteristics of pulsatile steroid secretion throughout the artificial follicular phase (Table 1).

Throughout the follicular phase, the endocrine changes associated with modulation of gonadotrophic stimulatory patterns in $Fec^{+/+}$ ewes resulted in a decline ($P < 0.05$) in the number of small and medium antral follicles detected during the first 24 h of the 'follicular phase' (Fig. 3). Thereafter, the total number of follicles remained unchanged in non-gene carriers, but large antral follicles represented an increasing proportion so that at the time of the LH surge, the ovaries of non-gene carriers contained 3.0 ± 0.6 ($> 3.5 \text{ mm}$) antral follicles of ovulatory size per ewe with a mean diameter of $4.2 \pm 0.3 \text{ mm}$. In contrast, the total follicle population in $Fec^{B/-}$ animals remained relatively stable until the

final 12 h before the LH surge when numbers declined, principally due to a decrease in the number of small follicles detected (Fig. 3). At the time of the LH surge, the ovaries of gene carriers contained 4.3 ± 0.6 follicles with a diameter of $> 2.5 \text{ mm}$, with a mean diameter of $3.0 \pm 0.1 \text{ mm}$.

The infusion of LH ($525 \mu\text{g}$) and hCG (100 iu) between 60 and 66 h after sponge withdrawal resulted in a marked increase in circulating LH (data not shown) and FSH (Fig. 2a). The magnitude of this 'LH surge' was 111 ± 20 and $99 \pm 8 \text{ ng ml}^{-1}$ ($P > 0.05$) in $Fec^{+/+}$ and $Fec^{B/-}$, respectively. The increase in FSH over this infusion period can be attributed to the FSH contamination in the LH preparation. The 'LH surge' resulted in a marked decline in ovarian oestradiol, androstenedione and inhibin A secretion ($P < 0.01$) similar to that observed during a normal follicular phase. There were no differences between genotypes in the magnitude and timing of this decline.

Early luteal phase

In the period after the preovulatory LH surge, ovarian oestradiol, androstenedione and inhibin A secretion remained low (Fig. 2b,c,d), whereas enlargement of ovulatory follicles was observed by serial ultrasonography. A feature of the ovarian autotransplant is that its position under the skin precludes rupture of ovulatory follicles and these will typically appear on ultrasonography during the early luteal phase as large fluid-filled bodies, the central cavity of which gradually becomes filled with echogenic tissue as the follicular cells luteinize, finally forming a characteristic corpus luteum that consists of a small central cavity surrounded by luteal tissue that is easily distinguishable from the surrounding stromal tissue. This feature of the model allowed for easy and accurate identification of ovulatory follicles/corpus luteum during the early follicular phase and the comparison between genotypes revealed that after this gonadotrophic regimen $Fec^{B/-}$ (3.8 ± 0.3) ewes had higher ($P < 0.05$) ovulation rates than $Fec^{+/+}$ (1.7 ± 0.3) ewes. Furthermore, the ovulatory structures were smaller (5.3 ± 0.3 in $Fec^{B/-}$ versus 11.4 ± 0.9 in $Fec^{+/+}$; $P < 0.05$; Fig. 4) in gene carriers, than in non-carriers as observed in these animals after normal ovulation (Souza *et al.*, 1997a).

The formation of corpora lutea, as visualized by ultrasonography, was accompanied by a normal increase in peripheral progesterone concentrations in all ewes from each group and no differences were evident between genotypes on day 12 of the luteal phase ($8.3 \pm 2.6 \text{ nmol l}^{-1}$ in $Fec^{+/+}$ versus $5.2 \pm 1.5 \text{ nmol l}^{-1}$ in $Fec^{B/-}$; $P > 0.05$). Again, this lack of difference between genotypes in progesterone concentrations despite differences in ovulation rate has been observed previously in these animals during a normal ovarian cycle (Souza *et al.*, 1997).

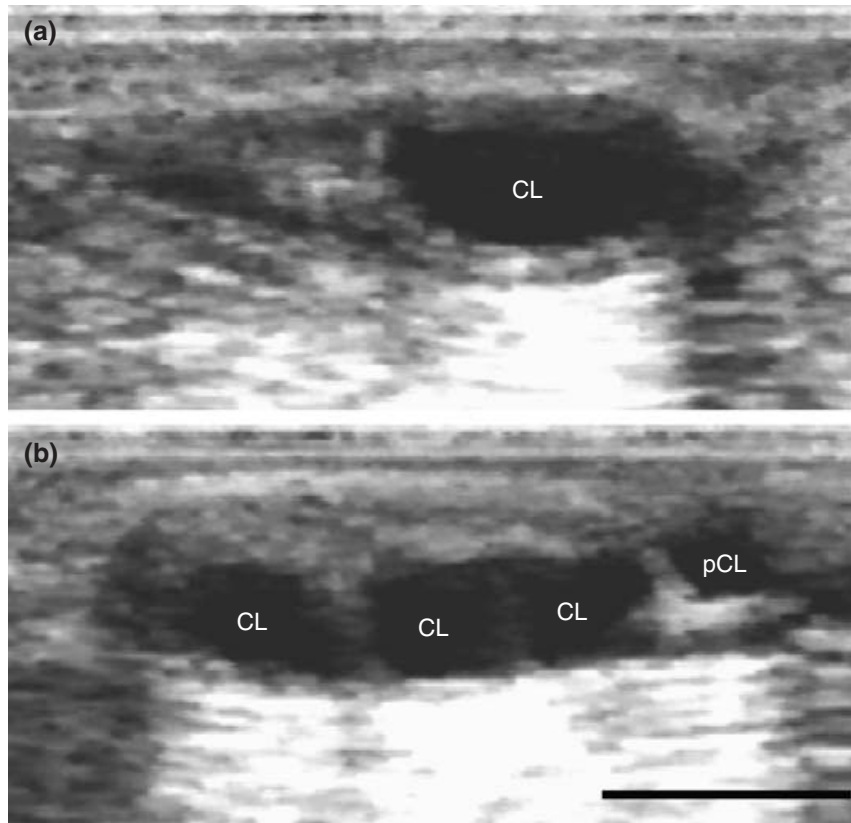


Fig. 4. Ultrasound scans through dorso–ventral plane of autotransplanted ovary captured 96 h after artificial LH surge, showing post-ovulatory enlargement of follicle during formation of corpora lutea. (a) Single large corpus luteum (CL) (8 mm × 14 mm) forming in a wild-type ewe. (b) Three small corpora lutea forming in a ewe carrying the Fec^B mutation at their maximal diameter (4–5 mm) with a partial section through a fourth at the cranial pole of the ovary (pCL). In all follicles, thickening of the wall can be observed as luteinization proceeds. Scale bar represents 10 mm.

Discussion

The results of the present study show that despite suppressing endogenous gonadotrophins and stimulating ovulatory follicle development with exactly the same pattern and level of exogenous gonadotrophic stimulation, the Fec^B gene carriers continued to display the characteristic increase in the number of ovulatory follicles that mature and ovulate at smaller diameters when compared with non-gene carriers. Therefore, these results provide compelling evidence that the Fec^B mutation acts at the ovary by increasing the sensitivity of follicular cells to gonadotrophic stimulation. Conversely, these results do not support the hypothesis that the Fec^B gene acts simply through increasing gonadotrophic stimulation.

The hypothesis that the Fec^B gene acts to increase follicular sensitivity is also supported by other lines of evidence. First, it has been possible to demonstrate using physiological culture systems designed to allow gonadotrophin-dependent induction of cellular differen-

tiation by gonadotrophins *in vitro* that both granulosa and theca cells from Fec^B gene carriers are more sensitive to gonadotrophins in terms of the dose required to induce oestradiol and androstenedione production, respectively (Webb *et al.*, 1995). Furthermore, these differences remain even when corrected for the effect of precocious differentiation on cells from different size classes of ovarian follicles. Second, Souza *et al.* (2002) have shown that the BMP1b receptor is widely expressed within the granulosa and theca cells of ovarian follicles at all stages of folliculogenesis in sheep and that bone morphogenic proteins can augment gonadotrophin-induced differentiation of cultured granulosa cells in sheep.

The results of the present study are supported by other studies that have attempted to answer the same question using a similar approach. Fry *et al.* (1988) used a small number of hypophysectomized (Hypox) ewes stimulated with large doses of eCG and showed a continued difference in ovulation rate in Fec^B carriers compared with non-carrier ewes. Hudson *et al.* (1999) used

hypothalamic–pituitary disconnected (HPD) and GnRH-agonist suppressed ewes stimulated with a mixture of gonadotrophins consisting of eCG, FSH and hCG. Although confounded by the high proportion of animals that failed to ovulate, it was concluded from this study that the *Fec^B* gene acts at both the pituitary and ovary to stimulate ovulation rate. However, a major limitation of both the Hypox and HPD models is that these animals appear to become relatively refractory to gonadotrophic stimulation and relatively large doses of gonadotrophin are needed to induce the majority of ewes to ovulate. A major strength of the GnRH-antagonist model is the acute nature of the suppression so that the level and pattern of gonadotrophic stimulation applied and the ovulatory response obtained remain within the physiological range.

Despite the physiological nature of the stimulatory regimen applied in the present study, there were differences between the patterns of follicle development and hormone secretion induced in the present study and those observed during a normal follicular cycle. The secretion of oestradiol increased rapidly in both groups within 12 h of the start of treatment with FSH and LH. This secretion is greater than would normally occur during the luteal phase and probably overestimates the daily secretion of oestradiol as samples were collected 20–30 min after the injection of LH every 4 h. The secretion of oestradiol and androstenedione is exquisitely sensitive to LH with each pulse of LH followed by a rapid increase in the secretion of steroid (Baird and Scaramuzzi, 1976). In the normal luteal phase, large follicles are stimulated to secrete oestradiol relatively infrequently due to the reduced number of LH pulses. Furthermore, in the present study oestradiol secretion was observed to be transiently higher in non-gene carriers during the early follicular phase, a difference that is not observed during the normal follicular phase (Souza *et al.*, 1997a). The most likely reason for this observation would appear to be attributable to the difference in the magnitude of the response to the initial 3 days of FSH stimulation between genotypes. Despite FSH concentrations remaining in the physiological range over this period, the non-gene carriers showed a marked response to FSH that resulted in a relatively large number (3.0 ± 0.8) of growing follicles of > 3.5 mm in diameter that would be expected to be oestrogenic (Carson *et al.*, 1979). This overstimulation is the most likely explanation for the high oestradiol secretion observed in non-gene carriers during the early ‘follicular’ phase, and the unusual depression in oestradiol secretion observed 40–44 h after sponge withdrawal in all probability reflects the point at which the ovulatory quotient is reduced to its normal level (that is, selection) under the influence of a gonadotrophic environment that consists of depressed FSH and high frequency low amplitude LH pulses. If this interpretation is correct, then the point of selection in this model system is later than during

a normal follicular phase (12–24 h: Souza *et al.*, 1997c), reflecting the exquisite sensitivity of the hypothalamic–pituitary–ovarian feedback loops that normally regulate this process (Baird, 1983; Baird and Campbell, 1998).

In contrast to the wild-type ewes, the *Fec^B* gene carriers had a normal number of follicles of ovulatory size consisting of 4.3 ± 1.1 follicles between 2.5 and 3.5 mm in diameter at the time of sponge withdrawal and a normal pattern of oestradiol secretion during the subsequent ‘follicular’ phase. This difference between the gene carriers reflects the fact that ovulatory follicles develop precociously in *Fec^B* gene carriers and indicates that the threshold concentration of FSH needed to support ovulatory follicle development is markedly lower in these animals. That is, providing extra FSH has little effect on the follicular response as the threshold concentration has already been surpassed. This concept would explain the observation that doses of FSH that induce superovulation in non-gene carriers have either no additional or negative effects in animals carrying the *Fec^B* mutation (B. K. Campbell, unpublished). Although the identity of the *Fec^B* mutation is now known, the mechanisms that allow a single point mutation to result in such a fundamental change in the mechanism of follicle selection remain to be elucidated and answering this question represents a major research objective in the near future.

In conclusion, the results of this study strongly indicate that the *Fec^B* mutation exerts its effects by markedly increasing ovulation rate by mechanisms that alter the response of the ovary to gonadotrophic stimulation rather than altering the level of the gonadotrophic stimulation applied to the ovary.

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