Ovarian characteristics in sheep with multiple fecundity genes

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Abstract

Ewes heterozygous for combinations of the Inverdale (FecX^I; I+), Booroola (FecB; B+) and Woodlands (FecX^W; W+) mutations have ovulation rates higher than each mutation separately. The aims of the experiments described herein were to examine the ovarian phenotypes in I+B+ and I+B+W+ ewes and to compare these with the appropriate ++ (controls), I+ and BB animals available for this study. The mean $\pm$ S.E.M. ovulation rates in the ++ ($n=23$), I+ (10), I+B+ (7), I+B+W+ (10) and BB (3) animals were 1.8 ± 0.1 , 2.5 ± 0.2 , 6.6 ± 1.0 , 9.6 ± 0.9 and 9.7 ± 0.9 respectively. The maximum number of granulosa cells per follicle in the ++ and I+ genotypes was accumulated after exceeding 5 mm diameter, whereas in I+B+, I+B+W+ and BB animals, this was achieved when follicles reached $>2-3$ mm. The number of putative preovulatory follicles, as assessed from those with LH-responsive granulosa cells, 24 h after the induction of luteolysis, was higher ($P < 0.01$) in the I+B+ and I+B+W+ compared to the ++ and I+ genotypes. The median follicular diameters of these follicles in the ++, I+, I+B+, I+B+W+ and BB genotypes were 6, 5, 3, 3 and 3 mm respectively. The total number of granulosa cells in the putative preovulatory follicles when added together, and total mass of luteal tissue, did not differ between the genotypes. Thus, despite large ovulation rate differences between animals with one or more fecundity genes, the total cell compositions over all preovulatory follicles and corpora lutea, when added together, are similar to that from the one or two such follicles in the wild types.

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Introduction

Over the past 15 years, an extensive range of genetic mutations have been identified in sheep that affect both ovulation rate and litter size (Juengel *et al.* 2013, Souza *et al.* 2014). Most of these mutations have been located in genes of the transforming growth factor beta (TGFB) superfamily or a related receptor. The most prevalent of these are inactivating mutations present within the bone morphogenetic protein 15 (BMP15) or growth differentiation factor 9 (GDF9) gene. The ovulation rate phenotypes for the heterozygous and homozygous BMP15 and GDF9 mutations that lead to increased ovulation rate and sterility respectively, can be replicated by partial or full immuno-neutralisation of the BMP15 or GDF9 proteins *in vivo* (Juengel *et al.* 2002, 2004). In addition to the aforementioned growth factors, a point mutation (Q249R) in the type I BMP receptor, (BMPR1B) also known as activin-receptor-like kinase 6 (ALK6), increases ovulation rate in both the heterozygotes and homozygotes of animals referred to as Booroola (B) sheep. This mutation alters the signalling response to BMP ligands *in vitro* and reduces the level of BMP15 but not GDF9 mRNA (Fabre *et al.* 2003, Campbell *et al.* 2006, Crawford *et al.* 2011). Within the

ovary, BMP15 and GDF9 are expressed in oocytes but not cumulus cells in sheep, cattle, rats, mice, pig and deer (Crawford & McNatty 2012). In other reports, BMP15 and GDF9 mRNA was detected in cumulus and/or granulosa cells of cattle, goats and pigs using qPCR (Silva *et al.* 2004, Paradis *et al.* 2009, Hosoe *et al.* 2011). In these studies, validation of intact, undamaged oocytes from individual follicles from which cumulus or granulosa cells were recovered was not described. Therefore, without this key control, these findings will need further validation to eliminate the possibility of false positives (Mester *et al.* 2015). In contrast, BMPR1B is known to be expressed more widely in the ovary including oocytes, granulosa cells and theca cells (Wilson *et al.* 2001). In addition to the above mutations, there is another, yet to be identified, X-linked mutation (Davis *et al.* 2001), that has been found in Woodlands (W) ewes (FecX^W): both the heterozygous and homozygous carrier animals have higher ovulation rates and litter sizes (Davis *et al.* 2001, 2005). Interestingly, in these animals, the expression levels of BMP15 and BMPR1B mRNA were both lower, those of ALK5 mRNA was higher and GDF9 and BMPR2 mRNA were not different from the wild type (Feary *et al.* 2007).

Sheep carrying various heterozygous combinations of some of these mutations appear to have either additive or multiplicative effects on ovulation rate compared to the wild types (++). For example, in animals heterozygous for both the Inverdale variant of the *BMP15* mutation (I+) and the *BMPR1B* (Booroola, B+) mutation (i.e. I+B+ animals), the mean ovulation rate was found to be 267% above that for the ++, whereas for I+ and B+ ewes, the increases were 67% and 132% respectively (Davis *et al.* 1999). Animals with the three mutations, I+B+W+ also appear to be at least additive with ovulation rates as high as 13 compared to 1–2 for the wild type (Davis *et al.* 2008). The additive or multiplicative effect of these genes may be related to a reduction in BMP15 concentrations as lower *BMP15* expression levels in oocytes have been shown in the BB and W+ genotypes.

Investigations of the ovarian phenotypes in the heterozygous and homozygous Booroola mutant animals (B+/BB) indicate that follicles reach preovulatory diameters at consistently smaller sizes than in the wild type. This maturation at smaller follicular diameters is also accompanied with fewer granulosa cells per follicle and an earlier acquisition of LH responsiveness by the granulosa cells as measured by cAMP output *in vitro* (McNatty *et al.* 1986, Henderson *et al.* 1989). The acquisition of LH receptors by granulosa cells is known to be a key marker for identifying the presumptive preovulatory follicle (Webb & England 1982). Moreover, this maturation of the preovulatory follicle at a smaller diameter occurs in BB animals with no change in FSH receptor-binding characteristics, or FSH responsiveness, with respect to cAMP synthesis (McNatty *et al.* 1989, Crawford *et al.* 2011). For the heterozygous Inverdale phenotype (I+), the acquisition of LH responsiveness by granulosa cells was also obtained in some follicles at smaller diameters, whereas other follicles were at a similar size to those in ++ animals (McNatty *et al.* 2009). In general, the numbers of granulosa cells for a given follicular diameter in the I+ genotype were lower than that in the ++ animals (Shackell *et al.* 1993). Additionally, the acquisition of LH receptivity in granulosa cells also occurred without any change in FSH receptor-binding characteristics or FSH responsiveness with respect to cAMP synthesis (McNatty *et al.* 2009). In both Booroola and Inverdale ewes, the changes in ovarian phenotype associated with increases in ovulation rate were not associated with any overall change in ovarian secretions of oestradiol during the follicular phase and/or progesterone during the luteal phase (McNatty *et al.* 1986, Shackell *et al.* 1993). With regards to the heterozygous or homozygous Woodland-mutant ewes (W+ or WW), little is known about the cell numbers or functional properties of the developing antral follicles with respect to LH and FSH responsiveness by granulosa cells. However, it is known that the proportion of non-atretic antral follicles,

but not total number of follicles, is higher in W+ than in ++ animals. Moreover, when the oocytes of the antral follicles in the W+ ewes were expressed as a fraction of the follicular diameter, they were significantly larger (Feary *et al.* 2007).

The aims of the experiments described herein were to examine, in more detail, the ovarian phenotypes in I+B+ and I+B+W+ ewes and to compare these with the appropriate ++ controls and with I+ and BB animals that were available for this study. Given the possibility that the BMP15 protein levels might be lower in all the mutant genotypes, the hypothesis being tested was that each of the heterozygous genetic mutations (W+, B+ and I+) when present in individual animals, would increase ovulation rate by increasing the proportion of follicles maturing at smaller diameters and with fewer numbers of granulosa cells that develop an earlier responsiveness to LH with respect to cAMP synthesis. It is also hypothesised that the total mass of corpora lutea formed for each genotype would not differ from that in the wild type.

Materials and methods

Animals

All experiments were performed with the approval of the AgResearch Invermay Animal Ethics Committee in accordance with the Animal Welfare Act Regulations of New Zealand. The animals in this study were mixed-aged ewes, primarily with a Romney background, consisting of ++ (controls, $n=23$), I+ ($n=10$), I+B+ ($n=7$), I+B+W+ ($n=10$) and BB ($n=3$) ewes. The presence or absence of the Booroola mutation and the Inverdale mutation were determined through genotyping (GenomNZ, Mosgiel, New Zealand; www.genomznz.co.nz). The background ovulation rate of the homozygous Booroola flock ranged from 4 to 13. Whether this flock contains another, as yet unidentified, genetic mutation has not been determined. Ewes carrying the Woodlands mutation, which is present on the X chromosome, were generated using phenotyped Woodlands carrier rams as described (Davis *et al.* 2001). Some of the I+B+ ewes ($n=3$) and all of the I+B+W+ ewes were progeny of Romney ewes crossed with sires that were half Coopworth-Texel and 13 of the aforementioned ++ control ewes contained the same background. Animals with the W+, B+ genotypes were not available and only two W+I+ animals were available for this study: the data for the W+I+ animals are only briefly referred to in the results. The animals in this study had access to pasture and water *ad libitum*. All animals, during the breeding season, were synchronised with two PGF2 α (cloprostenol; Bayer Animal Health, Auckland, New Zealand) injections 10 days apart. Oestrus checks were undertaken after the second PGF2 α injection to ensure the animals had synchronised. The ovaries were recovered 24 h after a third PGF2 α given 9–12 days after the second injection. The variation in timing of the third PGF2 α injection was to ensure that only ovaries from three sheep per day were available for follicular dissection. Irrespective of the timing of

the third injection, the progesterone concentrations at ovarian removal, had declined by >75% relative to the levels at the time of the third injection. The time between recovery of the ovaries and initiation of follicular dissection was <30 min.

FSH and LH reagents

An in-house highly purified ovine FSH preparation (oFSH Wal) was used to test FSH responsiveness of granulosa cells (Moore *et al.* 1997, Fidler *et al.* 2003). This preparation was >90% pure with a bioactivity of 1.4× USDA-oFSH-10-SIAFP RP2 or 33,000 IU/mg when the second human FSH reference preparation 78/549 was used as the standard in a radio-receptor assay. The level of LH contamination was <0.002% as determined by bioassay. The LH preparation used to test LH responsiveness of granulosa cells was an hCG preparation (CR121; 13,450 IU/mg; NICHD, Bethesda, MD, USA).

Ovarian studies

Ovaries were transported to the lab in warmed saline (37°C), rinsed briefly in 70% ethanol to remove any bacterial contamination, and then in saline before being immersed in dissection media (DM2; Dulbecco's MEM with 0.1% bovine serum albumin (BSA; ICPbio Ltd, Auckland, New Zealand), 20 mM Hepes buffer and 0.2 nM 3-isobutyl-1-methylxanthine). Apart from BSA, all other media reagents were obtained from Sigma-Aldrich. All ovaries were weighed and then all corpora lutea were individually dissected and weighed. Thereafter, all follicles ≥1.0 mm diameter, in all animals, were individually dissected and their diameters were measured. Subsequently, each follicle was placed in a separate petri dish (35 mm diameter) and punctured to release the follicular fluid, most of which was removed without extracting the oocyte-cumulus cell complex (COC) or granulosa cells. Thereafter, 1 mL of the above-mentioned DM2 media was added and the COC was located and classified based on their morphological appearance. Thereafter, each COC was transferred into a well of a four-well culture dish, (Nunc, Thermo Scientific) containing 0.5 mL of HEPES-buffered M199 media with 0.1 M kanamycin and 0.04% BSA and maintained at 38°C. The COCs were used for a separate study and not included herein. Once the COC was removed, the granulosa cells were carefully isolated by dispersal from the internal follicular wall using a platinum loop and then an aliquot was counted by haemocytometer. Removal of the granulosa cells under a dissecting microscope made it possible to ensure that damage to the internal follicular wall

Table 1 Effect of genotype on ovulation rate, mean CL weight per ewe and total CL weight per ewe.

Genotype (n)	Ovulation rate	Mean individual weight of CL per ewe (g)	Mean total weight of CL per ewe (g)
++ (23)	1.8 ± 0.1 ^a	0.490 ± 0.027 ^a	0.896 ± 0.048 ^a
I+ (10)	2.5 ± 0.2 ^b	0.377 ± 0.035 ^c	0.801 ± 0.054 ^a
I+B+ (7)	6.6 ± 1.0 ^c	0.105 ± 0.012 ^d	0.816 ± 0.086 ^a
I+B+W+ (10)	9.6 ± 0.9 ^d	0.108 ± 0.022 ^d	1.107 ± 0.136 ^a
BB (3)	9.7 ± 0.9 ^d	0.117 ± 0.032 ^d	1.123 ± 0.384 ^a

Values are means ± S.E.M. Numbers in columns not sharing common alphabetical superscripts are significantly different from one another: avb, $P < 0.05$; all other comparisons were $P < 0.01$.

n, number of ewes; g, grams.

was negligible. When isolating granulosa cells from individual follicles, the following variables were recorded namely: the extent of vascularity of the follicle wall; presence or absence of debris in follicular fluid; the total number of granulosa cells recovered and the morphological appearance of the COC (i.e. intact COC, few cumulus cells, misshapen oocyte etc.; McNatty *et al.* 1986).

Granulosa cell incubation and cAMP assay

The isolated granulosa cells from each follicle in 1 mL of DM2 media were centrifuged at 450 g at 4–6°C for 10 min. Thereafter, the media were discarded and the cells were resuspended in 1 mL of fresh DM2 media for recounting by haemocytometer. Recoveries of cells at this stage averaged around 60%. Subsequently, the individual cell concentrations were adjusted so that a final concentration of approximately 60,000 cells per culture was achieved. Any cell preparations with fewer numbers of cells than those allowed for one treatment, with the appropriate control, to be tested were discarded. The cells were then incubated in 48-well plates with or without FSH (1000 ng/mL) or hCG (100 ng/mL) in a final volume of 600 µL in a 38°C water bath for 45 min. The doses of FSH and hCG were selected to elicit maximal cAMP responses based on previous dose response studies in wild type, I+ and BB ewes (Henderson *et al.* 1989, McNatty *et al.* 1990, 2009, Shackell *et al.* 1993): from these dose response studies, no genotype differences were noted (Henderson *et al.* 1987, 1989). Subsequently, the cultures were heated at 80°C for 15 min. Samples were stored at –20°C until assayed for cAMP by radioimmunoassay (RIA). The assay employed was that described previously by Jolly and coworkers (Jolly *et al.* 1997) using an in-house rabbit anti-0,2-monosuccinyl-3',

Table 2 Effect of genotype on mean ± S.E.M. number of granulosa cells (in millions) with respect to follicular diameter in sheep.

Genotype (n)	Follicular diameter (mm)				
	1–2	>2–3	>3–4	>4–5	>5
++ (23)	0.71 ± 0.03 ^a [120]	1.29 ± 0.05 ^b [103]	1.88 ± 0.16 ^{b,c} [23]	3.12 ± 0.51 ^{c,d} [8]	4.29 ± 0.24 ^d [32]
I+ (10)	0.61 ± 0.02 ^a [86]	0.87 ± 0.04 ^b [43]	1.47 ± 0.19 ^{b,c} [11]	2.09 ± 0.21 ^{c,d} [18]	3.70 ± 0.30 ^d [10]
I+B+ (7)	0.41 ± 0.03 ^a [81]	0.82 ± 0.06 ^b [51]	1.03 ± 0.09 ^b [20]	0.89 ± 0.12 ^b [5]	– [0]
I+B+W+ (10)	0.35 ± 0.01 ^a [132]	0.85 ± 0.07 ^b [37]	0.98 ± 0.05 ^b [42]	1.16 ± 0.13 ^b [9]	– [0]
BB (3)	0.36 ± 0.03 ^a [37]	0.97 ± 0.11 ^b [14]	0.77 ± 0.11 ^b [7]	1.20 ^b [2]	– [0]

n, number of ewes, [n] refers to number of follicles. For each genotype separately, the granulosa cell numbers in each row not sharing an alphabetical superscript are significantly different from one another, $P < 0.01$.

5'-cyclic monophosphate antibody with the separation of bound and free cAMP involving the use of an in-house sheep anti-rabbit second antibody. The antibody cross-reacted 9% with dibutyl cAMP and <0.0014% with cGMP and <0.0001% with AMP, ADP or ATP. The detection limit was 0.2 pmol/million cells and the intra- and inter-assay coefficients of variation were both <10%. Follicles with granulosa cells producing cAMP ≥ 5 pmol/ 10^6 cells in response to hCG were considered to be LH responsive (McNatty *et al.* 2009).

Statistical procedures

The data concerning the effect of genotype on ovulation rate and CL weight were analysed by ANOVA and when appropriate, the Newman–Keuls *post hoc* test was applied. For determining genotype differences in numbers of granulosa cells for a given follicular diameter, each follicle was considered a discrete variable irrespective of whether it was isolated from the same or a different animal. This was considered to be a valid assumption as few, if any, follicles were identical either in function or in composition within each animal (McNatty *et al.* 2010). Only follicles devoid of visible debris in follicular fluid and intact COC were used for the analyses of granulosa cell number and cAMP responsiveness. These data together with those for LH and FSH responsiveness were analysed by Kruskal–Wallis and the Dunn's multiple range test.

Results

Effect of genotype on ovulation rate and the mean weight of corpora lutea (CL)

These data are summarised in Table 1. The ovulation rate in Table 1 is based on the number of regressing CL at 24 h after inducing luteolysis with a PGF 2α derivative. The ovulation rate in I+B+W+ ewes was equivalent to that in BB ewes and greater than that of I+B+ animals which in turn was greater than that in I+ and ++ genotypes. In contrast, the mean weight of each CL was lighter in the I+B+, I+B+W+ and BB animals compared to that in I+ and ++ animals. For each genotype, the total weight of CL tissue per ewe was not different.

Effect of genotype on the number (mean $\pm$ S.E.M.) of follicles ≥ 1 mm diameter

The numbers of follicles in ++, I+, I+B+, I+B+W+ and BB genotypes ≥ 1 mm diameter were 31 ± 3 , 36 ± 4 , 38 ± 4 , 34 ± 6 and 24 ± 8 respectively. These values were not significantly different from one another.

The effect of genotype on the mean $\pm$ S.E.M. number of granulosa cells with respect to follicular diameter are summarised in Table 2. In the ++ and I+ genotypes, the mean numbers of granulosa cells increased as follicular diameter increased with the highest mean numbers per follicle found in those >4–5 mm. Contrary to this pattern, for the I+B+, I+B+W+ and BB genotypes,

Figure 1 Number of follicles with LH-responsive granulosa cells represented by Box and Whisker plots. Box and Whisker plots with different alphabetical superscripts are significantly different from one another: avc, $P < 0.001$; bvc, $P < 0.01$; avc $P < 0.05$. The plots indicate that in the I+B+ and I+B+W+ genotypes, there were between 5 and 12 follicles that could be considered to be preovulatory, whereas in BB, there were between 4 and 7, in I+ between 1 and 6 and in the ++ between 1 and 4.

the total number of granulosa cells increased when comparing 1–2 mm follicles with those >2–3 mm but the total cell numbers plateaued thereafter. In these three genotypes, the highest mean numbers of granulosa cells per follicle were noted after follicles exceeded 2 mm diameter. Over all ranges of follicular diameters, the mean numbers of granulosa in I+ ewes

Figure 2 Effect of genotype on diameters of follicles with LH-responsive granulosa cells represented by Box and Whisker plots. Box and Whisker plots with different alphabetical superscripts are significantly different from one another, $P < 0.01$. The plots indicate that >75% of all LH-responsive follicles in the ++ and I+ genotypes are >4 mm diameter, whereas in the I+B+, I+B+W+ and BB ewes >75% of all such follicles were <4 mm diameter.

Table 3 Effect of genotype on hCG- or FSH-induced cAMP synthesis by granulosa cells from the putative preovulatory follicles.

Hormone	Genotype				
	++ [41]	I+ [24]	I+B+ [42]	I+B+W+ [38]	BB [14]
hCG	14.1 (10.6, 18.1)	12.1 (9.3, 18.0)	10.1 (8.4, 12.2)	10.8 (9.0, 13.1)	10.6 (7.8, 14.5)
FSH	7.7 (5.7, 10.3)	6.4 (4.4, 9.4)	4.4 (3.7, 5.3)	6.1 (4.4, 8.3)	4.5 (3.2, 6.3)

The cAMP values are expressed as pmol per 10^6 cells. Values are geometric means (and 95% confidence limits). Numbers in square brackets refer to the number of follicles.

were consistently lower than those in ++ animals. In the I+B+, I+B+W+ and BB genotypes, the mean number of granulosa cells for each range of follicular diameter (1–2, >2–3 and >3–4 mm) were not different from one another: follicles >5 mm were not observed. Moreover, for the I+B+, I+B+W+ and BB genotypes, the mean numbers of granulosa cells in the largest follicles were about one-third of those accumulated in the largest follicles of the ++ and I+ ewes. Animals with the W+ genotype were not available for inclusion in this study. Only two animals with the W+I+ genotype were investigated, and these two animals had ovulation rates of 8 and 4 and with the largest follicles reaching 5.0 and 5.3 mm in diameter and with granulosa cell numbers of 1.25 and 2.98 million respectively: these animals were not investigated further.

Effect of genotype on the number of follicles with LH-responsive granulosa cells

These data are summarised in Fig. 1. The median number of follicles with LH-responsive granulosa cell populations was highest in the I+B+ and I+B+W+ genotypes compared to either the ++ or I+ genotypes at 24 h after induction of a new follicular phase with a PGF 2α derivative. The number in the BB genotype was not different from I+, I+B+ and I+B+W+ but was significantly higher than that in ++ ($P < 0.05$).

Effect of genotype on follicular diameter and total numbers of granulosa cells in follicles with LH-responsive granulosa cells

The data with respect to the effect of genotype on follicular diameter are summarised in Fig. 2. The diameter of follicles in the I+B+, I+B+W+ and BB genotypes were not different from one another but all were significantly lower than that in the ++ and I+ animals at 24 h after induction of a follicular phase with PGF 2α . In the ++, I+, I+B+, I+B+W+ and BB genotypes, the median diameters of LH-responsive follicles were around 6, 5, 3, 3 and 3 mm respectively. The geometric mean (and 95% confident limits) total number of LH-responsive granulosa cells over all follicles in each of the ++, I+, I+B+, I+B+W+ and BB genotypes was 6.5 (5.4, 7.9), 6.7 (5.8, 7.8), 5.7 (3.9, 8.4), 6.9 (5.8, 8.3) and 5.3 (3.6, 7.9) million cells respectively: these numbers were not significantly different from one another.

There were no significant differences between the genotypes with respect to the geometric mean (and 95% confidence limits) levels of cAMP produced from the LH-responsive granulosa cells in the presumptive preovulatory follicles (Table 3).

Effect of genotype on FSH responsiveness of granulosa cells

Overall, there was no effect of follicular diameter (i.e. 1–2, >2–3, 3–>4, >4–5 and >5 mm) on the maximal levels of cAMP synthesised in response to FSH *in vitro*. In I+B+ and I+B+W+ genotypes, there were no follicles >5 mm diameter and for BB, no follicles >4 mm diameter were present. The only exception to this was noted for the ++ genotype where the mean FSH response from granulosa cells recovered from the >5 mm diameter follicles was significantly greater than that from cells recovered from 1–2 and >2–3 but not the >3–4 or >4–5 mm diameter follicles ($P < 0.05$; data not shown). In the LH-responsive granulosa cell populations (i.e. the putative preovulatory follicles), there was no effect of genotype on the FSH-induced cAMP responses (Table 3).

Discussion

The ovulation rate in I+B+ animals was significantly higher than that in I+ and ++ animals and intermediate between those for I+B+W+ and BB genotypes. The incorporation of both the I+ and B+ mutations within an animal appeared to be at least additive for ovulation rate and similar to that noted previously by Davis and coworkers (Davis *et al.* 1999). Animals with the W+ genotype have a mean ovulation rate that is ~25% above that of the wild type (Davis *et al.* 2001). In the present study, animals that contained the W+ together with the I+ and B+ polymorphisms were found to have an ovulation rate that was 45% above that of the I+B+ animals indicating that the inclusion of W+ was at least additive. As mentioned earlier, W+ genotype animals were not available for inclusion in this study and only two W+I+ animals were available. However, from examining the ovaries from these animals (i.e. ovulation rate, the size of the largest follicles and numbers of granulosa cells), the evidence suggests that these were intermediary in ovarian phenotype between I+ and I+B+ animals.

The key findings from this study were that the ovarian characteristics in I+B+ and I+B+W+ compared to the ++ genotype, as assessed by the responsiveness of granulosa cells to LH, were indicative of follicles maturing at significantly smaller diameters. This finding is consistent with previous studies examining ovarian characteristics of the B+, I+ and W+ genotypes separately (McNatty *et al.* 1986, Shackell *et al.* 1993, Feary *et al.* 2007), with the responses of individual mutations being less apparent than those observed in ewes with multiple mutations. The more dramatic effects observed in ewes with multiple mutations is likely caused by the additive, or potentially synergistic, influences of the *BMP15*, *BMPR1B* and *W+* mutations (Juengel *et al.* 2013).

The number of putative ovulatory follicles in the different genotypes at 24 h after an induced luteolysis, as assessed by the numbers of follicles with LH-responsive granulosa cells, was consistent with the ovulation rate determined from the previous cycle. An exception to this might have occurred for the BB animals in that the number of putative preovulatory follicles varied from 4 to 7, whereas the number of corpora albicans from the previous cycle varied from 8 to 11. Nevertheless, the presence of 4–7 putative preovulatory follicles lies within the range of ovulation rates observed in this flock of Booroola ewes. Importantly, all animals in this study contained follicles with LH-responsive granulosa cells. In the I+B+ and I+B+W+ animals, the maximum number of granulosa cells accumulated in individual follicles was achieved when follicles exceeded 2 mm in diameter. Moreover, in these animals, the largest follicles at 24 h after PGF α treatment never exceeded 5 mm diameter: this was similar to that observed for the BB animals. Moreover, the diameters of follicles in the I+B+, I+B+W+ and BB genotypes when LH responsiveness was acquired in the granulosa cells, occurred between 1.2 and 5.0 mm in diameter. In I+ and ++ animals, LH responsiveness was identified in follicles between 2.3 and 6.4 mm and 3.1 and 8.2 mm respectively. Despite the greater number of putative preovulatory follicles in I+B+, I+B+W+ and BB relative to the I+ and ++ genotypes, the total numbers of LH-responsive granulosa cells between these genotypes when added together were not different from one another. The geometric mean cAMP responses/ 10^6 granulosa cells in response to LH also did not differ between the genotypes. And, no differences in FSH-induced cAMP responses were noted in these putative preovulatory follicles. Collectively, these observations indicate that despite the high ovulation rates in I+B+ and I+B+W+ animals, the overall level of ovarian endocrine signalling with respect to hormones such as oestradiol, inhibin and progesterone secretion is likely to be similar to that in the BB genotype and not different from that noted for ++ animals with ovulation rates of 1 or 2 (McNatty *et al.* 1986, 1992, Niswender *et al.* 1990).

When using a highly purified FSH preparation, devoid of LH contamination, there was no evidence

to indicate that the higher proportion of follicles with LH-responsive granulosa cells in I+ compared to ++ ewes was associated with any genotype difference in FSH responsiveness (McNatty *et al.* 2009): this was in contrast to an earlier report when a less pure preparation of FSH was used (Shackell *et al.* 1993). Using the same highly purified FSH (and LH-free) preparation in the present study as reported by McNatty and coworkers (McNatty *et al.* 2009), no differences in FSH-induced cAMP responsiveness with respect to follicular diameter was noted for the I+B+, I+B+W+ or BB ewes. The only exception to this was an effect noted in the ++ animals whereby the cAMP response to FSH was greater in >5 compared to 1–3 mm diameter follicles. This raises the possibility of an effect of FSH with respect to follicular diameter in the I+, I+B+, I+B+W+ and BB genotypes but that this might occur in follicles <1 mm diameter. However, it is worth noting that the effect of follicular diameter on FSH-induced cAMP production has not been consistently observed. Previous studies that used highly purified FSH, devoid of LH, were unable to observe any effect on follicular diameter in ≥ 1 mm diameter follicles in ++ ewes (McNatty *et al.* 2009, Crawford *et al.* 2011, Juengel *et al.* 2011).

In a physiological context, the ovulation rates in I+B+ and I+B+W+ ewes are superior to those achieved using superovulation regimes. For example, after a superovulation treatment in wild-type Romney ewes, the range of follicular diameters in follicles with LH-responsive granulosa cells with respect to cAMP synthesis was between 2 and 9.5 mm (McNatty *et al.* 2010). Moreover, the number of LH-responsive follicles varied between 1 and 10 and the total number of granulosa cells when added together from these putative preovulatory follicles varied between 3.2 and 13.6×10^6 . Sheep, during a superovulation regimen, using exogenous FSH-rich preparations, acquire LH-responsive follicular granulosa cells in follicles >1 mm diameter (McNatty *et al.* 1993). Given that each of these follicles will accumulate between 1×10^6 and 6×10^6 granulosa cells, it becomes evident that as the ovulation rate increases, the total weight of the ovaries and the total accumulated masses of follicular cells increases with concomitant increases in ovarian hormone secretion. Given the high variability in subsequent pregnancy or embryo transfer success, it is reasonable to assume that the increase in preovulatory follicular development may not always be accompanied by a concomitant and synchronous maturation of oocytes. This is in contrast to what happens in I+B+, I+B+W+ and BB ewes where the high ovulation rates are achieved without altering the total number of granulosa cells from all ovulatory follicles or luteal cell masses and with a high level of repeatability in individual animals. Moreover, as indicated previously, this is most likely achieved without increasing the net ovarian secretions of oestradiol, inhibin or progesterone as these are highly correlated with the

number of granulosa or luteal cells (McNatty *et al.* 1986, 1993, Niswender *et al.* 1990, Shackell *et al.* 1993). Importantly, the evidence from embryo transfer studies in the I+B+ and I+B+W+ ewes indicates that the yields of good quality embryos are high. In I+B+ donors ($n=9$) with a mean $\pm$ S.E.M. ovulation rate of 7.8 ± 0.4 , the number of embryos recovered was 5.9 ± 0.6 . In I+B+W+ donors ($n=9$), the mean $\pm$ S.E.M. ovulation rate was 9.0 ± 0.7 and the mean $\pm$ S.E.M. embryo recovery was 5.8 ± 0.9 , with one ewe producing no viable embryos (JL Juengel, AR O'Connell & LD Quirk, Unpublished observations, Juengel *et al.*). It is also reasonable to propose that the superior 'superovulation-like' outcome in these genotypes is due to a lower concentration of oocyte-derived BMP15 as this effect can be replicated, at least in part, by partial immuno-neutralisation of BMP15 (Juengel *et al.* 2002). It is also of interest to note that applying a superovulation treatment to ewes immunised against BMP15, to partially neutralise this secreted growth factor, does not further advance ovulation rate (Juengel *et al.* 2011).

In summary, these unique sheep models provide evidence in support of the hypothesis that lowering endogenous BMP15 concentrations, perhaps by the application of long-acting BMP15 antagonists, might be an attractive alternative to exogenous gonadotrophins for generating consistently high embryo yields (McNatty *et al.* 2014).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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