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# Identification of an ionotropic glutamate receptor AMPA1/GRIA1 polymorphism in crossbred beef cows differing in fertility<sup>1,2</sup>

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**ABSTRACT:** A proposed functional polymorphism in the ionotropic glutamate receptor AMPA1 (GRIA1) has been reported to influence antral follicle numbers and fertility in cows. Repeat breeder cows that fail to produce a calf in multiple seasons have been reported to have reduced numbers of small (1 to 3 mm) antral follicles in their ovaries. Therefore, we tested the hypothesis that this GRIA1 polymorphism was affecting antral follicle numbers in repeat breeder cows. Repeat breeder cows ( $n = 64$ ) and control cows ( $n = 72$ ) that had always produced a calf were housed in a dry lot and observed twice daily for behavioral estrus. Blood samples were collected, and cows were genotyped for this GRIA1 polymorphism and for a polymorphism in the GnRH receptor (GnRHR) that was proposed to influence age at puberty. On d 3 to 8 after estrus cows were slaughtered, and reproductive organs were collected to determine antral follicle count, ovary size, and uterine horn diameter. Repeat breeder cows were older at first calving than control cows ( $P = 0.006$ ). The length ( $P = 0.03$ ) and height ( $P = 0.02$ ) of the ovary contralateral to the corpus luteum (CL) were greater in control cows than repeat breeder cows. The endometrial diameter in

the horn ipsilateral to the CL was greater in the control cows than the repeat breeder cows. Repeat breeder cows had fewer small (1 to 5 mm) antral follicles than control cows ( $P = 0.003$ ); however, there was no association between GRIA1 genotype and antral follicle number. The GnRHR polymorphism was associated with age at first calving because cows that were homozygous for the C allele had a greater age at first calving than heterozygous cows or cows that were homozygous for the T allele ( $P = 0.01$ ). In the granulosa cells from small (1 to 5 mm) antral follicles, mRNA abundances of 2 markers of oocyte quality, anti-Müllerian hormone and pentraxin 3, did not differ between fertility groups ( $P \geq 0.12$ ). We conclude that this GRIA1 polymorphism exists in beef cows but that it does not influence antral follicle numbers. The association between GnRHR genotype and age at first calving is likely not causal as this polymorphism is not functional. The utility of this polymorphism as a genetic marker for early conception in heifers will require further validation. Screening postpartum cows by ultrasonography to determine antral follicle numbers may aid in making culling decisions.

**Key words:** antral follicle count, cattle, reproductive longevity

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## INTRODUCTION

Failure to become pregnant is the primary reason a beef cow is removed from the production herd (Mathews and Short, 2001), and risk of pregnancy failure increases with age (Erickson et al., 1976; Renquist et al., 2006). In cows, reproductive aging is associated with decreased numbers of follicles in the ovary (Erickson, 1966; Malhi et al., 2005; Cushman et al., 2009) and decreased luteal function (Malhi et al., 2005; Echterkamp et al., 2009). Similar observations were reported for repeat breeder beef cows that failed to produce a calf in 2 consecutive years (Echterkamp and Maurer, 1983; Maurer and Echterkamp, 1985). These cows had fewer small antral follicles (1 to 3 mm) and decreased serum progesterone concentrations compared with contemporary herd mates at a similar age, suggesting an advanced state of reproductive aging.

A polymorphism in the ionotropic glutamate receptor AMPA1 (**GRIA1**) has been associated with antral follicle numbers and decreased conception to AI in cows (Sugimoto et al., 2010). This polymorphism was reported to be functional because transfection of this form of the receptor into immortalized hypothalamic cells resulted in decreased GnRH secretion in response to glutamate in vitro compared with cells transfected with the wild-type receptor. Therefore, we hypothesized that this GRIA1 polymorphism would associate with decreased follicle numbers in repeat breeder cows. Secondly, we examined the influence of a polymorphism in the GnRH receptor (**GnRHR**) on reproductive traits in repeat breeder cows. This GnRHR polymorphism was investigated because it was proposed as a genetic marker of fertility and age at puberty because of the greater frequency of the C allele in *Bos indicus* cattle compared with *Bos taurus* cattle (Lirón et al., 2011).

## MATERIALS AND METHODS

All procedures were approved by the U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committee.

### Cows

Mature (3 to 13 yr of age) nonlactating crossbred beef cows (primarily Angus and Hereford with some Simmental and Charolais) that had exhibited standing estrus twice in a 18 to 23 d period were identified as repeat breeder cows ( $n = 66$ ) or contemporary herd mates that had always produced a calf (control,  $n = 73$ ) on the basis of calving records. Repeat breeder cows were defined according to the criteria of Echterkamp

and Maurer (1983) as cows that had failed to produce a calf in the previous 2 breeding seasons. Breeding seasons were approximately 60 d in length, which would represent 5 to 6 opportunities to become pregnant over a 2 yr period.

Cows were moved from the pasture to a dry lot approximately 1 wk before the start of the study and were provided ad libitum access to silage. In the dry lot, cows were observed twice daily for behavioral estrus with no synchronization of estrus. On d 3 to 8 after observed estrus, cows were transported to a local abattoir for slaughter and the reproductive organs were collected. Reproductive organs were transported (1 h) to the laboratory at ambient temperature for further evaluation and tissue collection. Reproductive organs with gross abnormalities or adhesions were excluded from further analysis ( $n = 2$  repeat breeder cows and 1 control cow). All cows used in this study tested negative for Y chromosome (McDaneld et al., 2012). The corpus luteum (CL) was dissected from the ovary, weighed, and frozen in liquid nitrogen. Ovaries were measured, and surface antral follicles ( $>1$  mm) were counted. Small antral follicles (1 to 5 mm) were aspirated, the follicular fluid was centrifuged at  $164 \times g$  for 5 min at room temperature, the follicular fluid was removed from the granulosa cell pellets, and the granulosa cells pellets were frozen for real-time reverse transcription PCR (RT-PCR). To measure the endometrial diameter, we measured the distance from the edge of the myometrium interface to the edge of the opposite myometrium interface. The endometrial diameter was measured at the widest point of the uterine horn ipsilateral to the CL with digital calipers, 1 to 2 cm from the uterine body at an anatomical location similar to that described previously when measuring endometrial volume or endometrial thickness using ultrasound (Jimenez-Krassel et al., 2009; Souza et al., 2011). We chose to report endometrial diameter because this is closest to the measurement used in evaluating reproductive tract scores (Holm et al., 2009; Mee et al., 2009). Reproductive tracts were all in the luteal phase with no fluid in the uterine bodies or uterine horns to affect these measurements.

### Cow Body Weights

All cows were weighed when they were born and when they were weaned as part of standard management practices at USMARC. These BW were stored in the USMARC database and retrieved for analysis. In addition, cows were weighed on the day that they were moved to the dry lot to determine their BW at the start of the study.

### RNA Extraction and Real-Time RT-PCR

Total cellular RNA was extracted from the granulosa cell pools derived from the small (1 to 5 mm) follicles from a subset of the cows (control = 25 and repeat breeders = 32) with Qiagen RNeasy miniprep kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. The subset was chosen randomly from the total set to decrease the costs of the experiment and to save some samples as backups in case of RNA extraction failures or for other biochemical analysis if desired. The difference in numbers between control and repeat breeders represents the difference in the numbers of control and repeat breeder cows in the entire data set. Final concentration was determined by spectrophotometry at 260 nm on a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). After determination of concentration, 500 ng of mRNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. After reverse transcription, the cDNA was diluted to a final concentration of 10 ng/ $\mu$ L and stored at  $-20^{\circ}\text{C}$  until used for real-time RT-PCR.

Real-time RT-PCR was performed on cDNA from granulosa cells for each cow with the Chromo4 real-time PCR detection system (Bio-Rad) using previously published primers for anti-Müllerian hormone (AMH; Ireland et al., 2009), pentraxin 3 (PTX3; Chitko-McKown et al., 2004), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; Klipper et al., 2004; Cushman et al., 2007). We chose AMH and PTX3 for characterization in the granulosa cells of small (1 to 5 mm) antral follicles of repeat breeder cows because they have been reported to be biomarkers of oocyte quality (Zhang et al., 2005; Ireland et al., 2009).

Briefly, all real-time RT-PCR was run in 25- $\mu$ L reactions containing 12.5  $\mu$ L PerfeCTa SYBR Green Supermix with ROX (Quanta BioSciences, Gaithersburg, MD), 0.2  $\mu$ M of the appropriate forward and reverse primer (Table 1), and 2  $\mu$ L of cDNA (20 ng total per reaction). For each gene product, samples were run in duplicate, and the samples were run on two 96-well plates with common pools run on each plate. The PCR conditions were denaturation at  $95^{\circ}\text{C}$  for 5 min followed by amplification ( $95^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 15 s, and  $70^{\circ}\text{C}$  for 45 s) for 40 cycles. The average intra-assay CV was 8.8%, 4.9%, and 9.8% for AMH, PTX3, and GAPDH, respectively, and the average interassay CV was 11.7%, 2.2%, and 13.4% for AMH, PTX3, and GAPDH, respectively. Relative abundances of gene products were calculated using the  $2^{-\Delta\Delta\text{CT}}$  method as described by Livak and Schmittgen (2001).

**Table 1.** Primer sequences for real-time reverse transcription PCR and source references

Gene <sup>1</sup>	Primer <sup>2</sup>	Sequence	Source
AMH	F	5'-AAGGTGGTCAAGTCACTCAG-3'	Ireland et al. (2009)
	R	5'-CAGGGAAGAAGTCTTCAGCA-3'	
GAPDH	F	5'-GGCGTGAACCACGAGAAGTAT-3'	Klipper et al. (2004)
	R	5'-CGTGGACAGTGGTCATAAGT-3'	
PTX3	F	5'-TCTTTATTTATCTTGGCAAAT- ACTGAGTAA-3'	Chitko-McKown et al. (2004)
	R	5'-AAGCACCATGGCATAAAATCT- AGTAA-3'	

<sup>1</sup>AMH = anti-Müllerian hormone; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; PTX3 = pentraxin 3.

<sup>2</sup>F = forward primer; R = reverse primer.

### Genotyping

Blood samples (10 mL) were collected by jugular venipuncture from the cow into 10-cm<sup>3</sup> syringes with EDTA and equally divided into five 2.0-mL screw cap microcentrifuge tubes (Sigma-Aldrich, St. Louis, MO). The DNA was extracted from these samples with the Biosprint 96 DNA purification kit and procedure (number 940057, Qiagen). An assay was designed to genotype these GRIA1 and GnRHR polymorphisms using primer extension assays on a Sequenom MassArray analyzer (Sequenom, San Diego, CA). The GnRHR polymorphism was included in the assay because it was proposed as a marker of fertility and age at puberty because of the greater frequency of the C allele in *Bos indicus* cattle compared with *Bos taurus* cattle (Lirón et al., 2011).

### Statistical Analysis

Weight and growth traits were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC) with fertility group (repeat breeder or control) as a fixed effect. Antral follicle count and reproductive organ parameters were analyzed using the MIXED procedure of SAS with fertility group as a fixed effect and age and day of the estrous cycle as covariates. Relative abundance of AMH and PTX3 were analyzed using the MIXED procedure of SAS with fertility group as a fixed effect and age and day of the estrous cycle as covariates. Relationship between antral follicle count and GRIA1 genotype was analyzed using the MIXED procedure of SAS with the genotype, fertility group, and the interaction as fixed effects and age and day of the estrous cycle as covariates. Relationship between age at first calving and GnRHR genotype was analyzed using the MIXED procedure of SAS with the genotype, fertility group, and the interaction as fixed effects.

**Table 2.** Comparisons of BW between repeat breeder cows and control cows

Weights	Repeat breeder	Control	<i>P</i> -value
Cows, <i>n</i>	64	72	—
Birth weight, kg	38.4 ± 0.6	37.3 ± 0.6	0.23
Weaning weight, kg	217.1 ± 3.6	222.4 ± 3.4	0.29
Weight on study, kg	671.4 ± 9.0	642.8 ± 8.5	0.02

## RESULTS

There was no difference in birth or weaning weights between repeat breeder cows and control cows (Table 2;  $P \geq 0.23$ ); however, at the start of the study the repeat breeder cows were heavier ( $P = 0.02$ ). There was a greater interval from when they had last weaned a calf ( $P < 0.0001$ ; Table 3). There also was no difference between fertility groups in the age that the cows first went to breeding (Table 3;  $P = 0.74$ ); however, there was a difference in age at first calving ( $P = 0.006$ ), with the repeat breeder cows calving later in their first season. As would be expected, control cows were older when they produced and weaned their last calf ( $P < 0.0001$ ).

The length and height of the ovary contralateral to that containing the CL were smaller for repeat breeder cows as well (Table 4;  $P \leq 0.03$ ). Weight of the CL was influenced by day of the estrous cycle ( $P < 0.0001$ ) but not by age of cow ( $P = 0.94$ ) or fertility group ( $P = 0.47$ ;  $3.22 \pm 0.36$  vs.  $3.66 \pm 0.39$  g for control and repeat breeder cows, respectively). Finally, the endometrial diameter, defined as the diameter between one edge of the myometrium and the opposite edge of the myometrium measured 1 to 2 cm anterior to the uterine body on the horn ipsilateral to the CL, was smaller for repeat breeder cows compared with control cows ( $P < 0.0001$ ) when corrected for age and day of the estrous cycle. Repeat breeder cows had fewer small antral follicles than control cows (Fig. 1;  $P = 0.003$ ); however, follicle number was not influenced by GRIA1 genotype ( $P = 0.43$ ). Cows that were homozygous for the C allele of the GnRHR had a greater average age at first calving

**Table 4.** Comparisons of reproductive organ measurements between repeat breeder cows and control cows

Organ measurements	Repeat breeder	Control	<i>P</i> -value
Cows, <i>n</i>	64	72	—
Day of the estrous cycle	5.9 ± 0.2	5.7 ± 0.2	0.44
Ovary length, <sup>1</sup> mm	28.7 ± 1.8	34.7 ± 1.5	0.03
Ovary height, <sup>2</sup> mm	18.2 ± 1.2	22.5 ± 1.0	0.02
Endometrial diameter, <sup>3</sup> mm	11.5 ± 1.1	18.9 ± 1.0	<0.0001

<sup>1</sup>Length of the ovary contralateral to the corpus luteum.

<sup>2</sup>Height of the ovary contralateral to the corpus luteum.

<sup>3</sup>The distance from the myometrium interface on 1 side to the opposite myometrium interface measured 1 to 2 cm anterior to the uterine bifurcation.

**Table 3.** Comparisons of production traits between repeat breeder cows and control cows

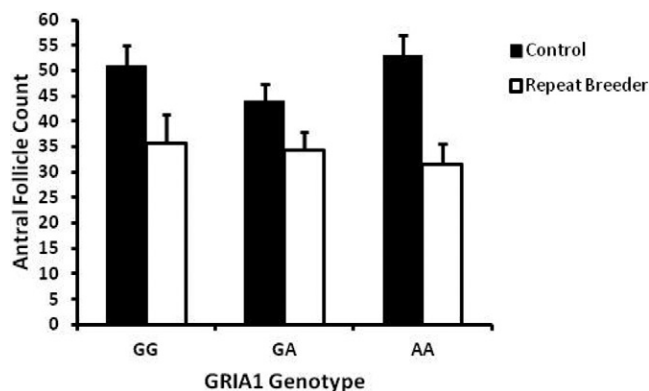
Performance trait	Repeat breeder	Control	<i>P</i> -value
Cows, <i>n</i>	64	72	—
Age at first breeding, d	426.5 ± 2.1	425.5 ± 2.3	0.74
Age at first calving, d	758.8 ± 11.3	715.9 ± 10.6	0.006
Age at last calving, d	1710.9 ± 103.4	3467.2 ± 97.5	<0.0001
Days postpartum	475.6 ± 18.9	249.1 ± 17.8	<0.0001
Days postweaning	325.24 ± 20.1	115.2 ± 18.9	<0.0001

than cows that were heterozygous or were homozygous for the T allele (Fig. 2;  $P = 0.01$ ).

There were no differences in the relative abundances of mRNA for AMH or PTX3 in the granulosa cells of small antral follicles between repeat breeder and control cows (Fig. 3;  $P \geq 0.12$ ). Furthermore, relative abundances of these mRNA did not differ between GRIA1 or GnRHR genotypes ( $P \geq 0.43$ ; data not shown).

## DISCUSSION

In the present study, we were unable to identify a difference in the number of small (1 to 5 mm) antral follicles in the ovaries of cows due to GRIA1 genotype. However, repeat breeder cows had lower numbers of surface follicles (current study and Maurer and Echterkamp, 1985), ovaries that were smaller, and endometrial diameters that were decreased when compared with contemporary herd mates that always produced a calf. The lower number of antral follicles in repeat breeder cows continues to support the decreased fertility observed in low antral follicle count heifers and cows both in vivo (Cushman et al.,



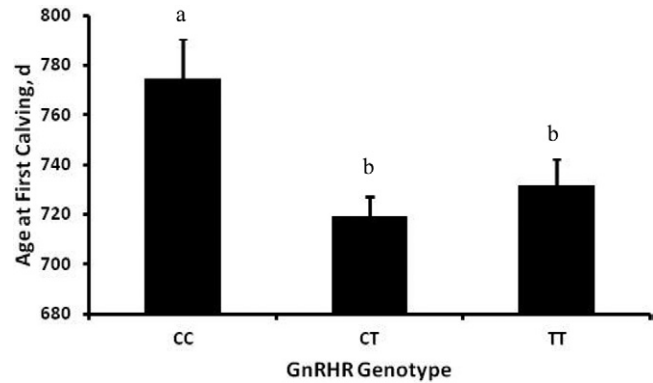
**Figure 1.** The total number of small (1 to 5 mm) antral follicles counted on d  $5.8 \pm 0.2$  of the estrous cycle in the paired ovaries of a cow at slaughter. Genotype of ionotropic glutamate receptor AMPA1 (GRIA1) did not influence follicle number ( $P = 0.43$ ); however, there was a significant effect of fertility group ( $P = 0.003$ ) because repeat breeder cows had fewer small follicles in their ovaries than control cows when corrected for day of the estrous cycle. Number of cows in each genotype is as follows: GG: repeat breeder = 9, control = 19; GA: repeat breeder = 27, control = 26; AA: repeat breeder = 20, control = 18.

2009; Mossa et al., 2012) and in vitro (Tessaro et al., 2011) that could be due to decreased oocyte quality or differences in serum progesterone concentrations (Jimenez-Krassel et al., 2009).

Although the age that these cows entered their first breeding season did not differ between fertility groups, the age at first calving was greater in repeat breeder cows when compared with contemporary herd mates that always produced a calf. The later age at first calving is assumed to be due to a later age at puberty; however, in the present study age at puberty was not determined. The C allele of the GnRHR that had a greater frequency in *Bos indicus* than *Bos taurus* cattle in a previous study (Lirón et al., 2011) was associated with an increased age at first calving in homozygous cows in the present study. Because calving early in the first calving season is associated with improved lifetime productivity (Burriss and Priode, 1958; Lesmeister et al., 1973), this polymorphism may be useful for selecting heifers that will conceive early and remain in the production herd longer. However, it must be noted that no functional mechanism is associated with this polymorphism and further validation of its utility as genetic marker of early conception in heifers is required.

Behavioral estrus was observed in all cows used in this study, and at slaughter, ovulation was confirmed by the presence of a CL. This is in agreement with a report that low-fertility cows that had failed to produce a calf in either 1 or 2 previous seasons did not demonstrate a failure in ovulation (Warnick and Hansen, 2010). Because conception rates are relatively high in beef cows (Maurer and Chenault, 1983), this would suggest a greater rate of embryonic loss in repeat breeder cows, as has been reported previously (Maurer and Echterkamp, 1985; Warnick and Hansen, 2010). Thus, oocyte quality may be the connection between decreased follicle numbers and subfertility in heifers and cows. Indeed, the demonstration of a number of oocyte-derived growth factors that positively influence granulosa cell function supports this hypothesis (Eppig, 2001).

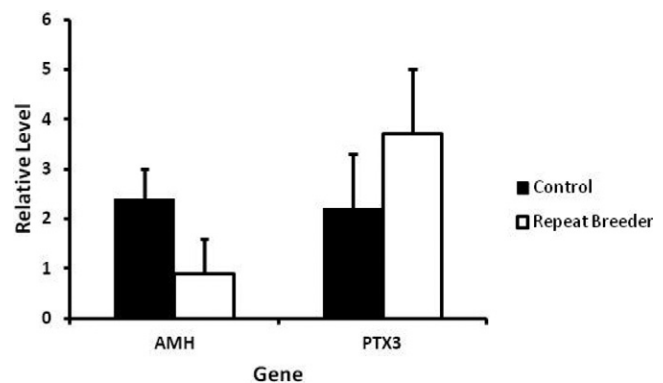
Therefore, we examined the expression of 2 granulosa cell markers of oocyte competence, AMH and PTX3. Granulosa cells of small antral follicles (<3 mm) from women with premature ovarian failure produced less AMH as determined by immunohistochemistry when compared with women without premature ovarian failure (Meduri et al., 2007). Similarly, the expression of AMH mRNA in granulosa cells of medium (5 to 7 mm) bovine antral follicles was decreased in heifers with low antral follicle numbers compared with heifers with high antral follicle numbers (Ireland et al., 2009). However, we observed no differences in relative abundances of AMH mRNA from the granulosa cells of small (1 to 5 mm) antral follicles of repeat breeder cows



**Figure 2.** Influence of GnRH receptor (GnRHR) genotype on age at first calving in days. Cows that were homozygous for the C allele had a greater average age at first calving than heterozygotes or cows that were homozygous for the T allele ( $P = 0.01$ ). <sup>a,b</sup>Bars with different superscripts are different. Number of cows in each genotype is as follows: CC = 16, CT = 67, TT = 39.

compared with control cows. Rico et al. (2011) observed no difference in follicular fluid concentrations of AMH from 3 to 5 mm follicles from cows with a high (>15 CL) superovulatory response compared with cows with a low (<10 CL) superovulatory response. This suggests that fertility status influences the production of AMH by bovine granulosa cells more in the later stages of follicular development than the early stages of follicular development. It appears that in this way cows may differ from women with low follicle counts. Alternatively, the decrease in granulosa cell function that results in lower AMH mRNA production by the granulosa cells of heifers with low numbers of antral follicles may not be occurring in repeat breeder cows or cows with low superovulatory response. Although follicle numbers are decreased in repeat breeder cows, they are not as greatly decreased as in the ovaries of heifers with low numbers of follicles.

Pentraxin 3 was also an ideal candidate gene to examine for differences related to oocyte quality and fertility. In humans, PTX3 mRNA abundance in the cumulus cells was reported to be a marker of oocyte



**Figure 3.** Relative abundances of anti-Müllerian hormone (AMH) and pentraxin 3 (PTX3) gene expression in the pooled granulosa cells from repeat breeder ( $n = 32$ ) and control ( $n = 25$ ) cows. Fertility group did not influence the relative abundances of AMH ( $P = 0.12$ ) or PTX3 ( $P = 0.44$ ) mRNA produced by the granulosa cells.

competence (Zhang et al., 2005), and women carrying a polymorphism in the PTX3 gene had greater fertility (May et al., 2010). There was no change in PTX3 mRNA abundances in bovine cumulus cells as follicle diameter increased, suggesting that PTX3 could be a marker of inherent oocyte competence at all stages of follicular development (Caixeta et al., 2009). The oocyte-derived growth factor growth and differentiation factor-9 (**GDF-9**) stimulated production of PTX3 in cultured mouse mural granulosa cells, and PTX3 mRNA abundances were decreased in the granulosa cells of transgenic mice that did not produce GDF-9 compared with wild-type controls (Varani et al., 2002). Therefore, we hypothesized that inherent difference in oocyte quality in repeat breeder cows might be reflected by decreased PTX3 mRNA production by the mural granulosa cells from the small follicles. However, that was not the case. Thus, differences in fertility in repeat breeder cows are not due to differences in PTX3 production by the mural granulosa cells, and PTX3 does not appear to be a biomarker of oocyte quality in these cows.

In summary, the results from the present study confirm previous reports that the numbers of small antral follicles are decreased in the ovaries of repeat breeder cows (Maurer and Echternkamp, 1985). However, we were unable to identify an association of a polymorphism in the GRIA1 gene with follicle numbers in cows, in contrast to a previous report (Sugimoto et al., 2010). Repeat breeder cows were older at first calving than control cows and had a polymorphism in the GnRHR associated with age at first calving. Relative abundances of AMH and PTX3 mRNA in the granulosa cells of small antral follicles did not differ between repeat breeder and control cows, suggesting that any relationship between the production of these proteins by bovine granulosa cells and oocyte quality exists only at the later stages of follicular development. From this, we conclude that heifers that calve late in their first calving season are at risk of failing to produce enough calves to reimburse their development costs and that the polymorphism in GnRHR should be further investigated to confirm its association with age at first calving and its use as a genetic marker to select heifers that will calve early in their first calving season. Reproductive tract scoring using ultrasonography has been proposed in postpartum cows to improve reproductive management (Mee et al., 2009). We further propose that decreased numbers of antral follicles should be considered as a screening method for making culling decisions when ultrasonographic examinations of the reproductive tract are being performed in postpartum cows.

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