

Follicular dynamics and gene expression in granulosa cells, corpora lutea and oocytes from gilts of breeds with low and high ovulation rates

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Abstract. Follicular dynamics and the expression of candidate genes using real-time polymerase chain reaction (PCR) were compared during the oestrous cycle of pig breeds with high (commercial line; $n = 24$) and low (local Brazilian Piau; $n = 21$) ovulation rates and prolificacy. Gilts were killed on Days 0, 4, 10 and 18 of the oestrous cycle and visible ovarian follicles were classified by follicular diameter. Recovered cumulus–oocyte complexes were classified as normal or atretic and frozen in liquid nitrogen until RNA extraction. Low ovulation rates and/or prolificacy in Piau gilts was associated with a different pattern of follicle development, with lower numbers of small follicles on Day 18, fewer large follicles on Days 0 and 18 ($P \leq 0.05$) and a higher proportion of atretic follicles on Days 0 and 18 ($P \leq 0.05$). Compared with commercial line gilts, less-prolific Piau gilts exhibited higher expression of apoptotic genes during luteolysis (*CASP3* and *FASL*; $P \leq 0.05$), decreased expression of *TGFBR2* and *BAX* mRNA in the corpus luteum ($P \leq 0.05$), higher expression of apoptotic genes (*FAS*, *BCL2* and *CASP8*; $P \leq 0.05$) in granulosa cells and a greater abundance ($P \leq 0.05$) of genes controlling oocyte-secreted factors (*GDF9*, *BMP15* and *BMP6*), suggesting underlying mechanisms controlling differences in follicular development, ovulation rate and inherent prolificacy in this pig breed.

Additional keywords: growth factors, litter size, ovulation.

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Introduction

As in sheep, differences in ovulation rate in different breeds of pigs are presumably linked to differences in intraovarian regulatory mechanisms and overall prolificacy. For example, according to Manabe *et al.* (2004), the higher ovulation rate in the Meishan sow is related to differences in both follicular recruitment and atresia. The same group reported that a lower ovulation rate and a smaller number of pigs born in local Hungarian Mangalica sows compared with other European breeds was also related to differences in follicle recruitment and atresia (Rátky *et al.* 2005). It has been postulated that follicles of Meishan sows provide a 'better' environment for oocyte maturation than follicles of Large-White hybrid sows, which may also contribute to the prolificacy of Meishan sows (Bazer *et al.* 1988a; Haley and Lee 1993; Hunter and Picton 1995). Similarly, different patterns of follicle and oocyte development may exist between prolific commercial breeds and the less prolific

Brazilian Piau breed, and comparative studies using these breeds may contribute to the identification of genes related to ovulation rate, oocyte quality and consequently embryonic development and survival in the pig. Preliminary studies comparing different traits in contemporary commercial sows with sows from the less-prolific Brazilian Piau breed (Silva *et al.* 2011) have revealed differences in ovulation rate (15.5 ± 1.9 vs 11.1 ± 2.4 , respectively; $P \leq 0.05$), the number of total pigs born (TB; 14.3 ± 3.7 vs 9.3 ± 2.7 , respectively; $P \leq 0.05$) and pigs born alive (BA; 12.7 ± 3.1 , 7.9 ± 2.6 , respectively; $P \leq 0.05$). Because the Piau breed has never been subjected to intensive genetic selection, it may carry allelic variants that are no longer found in highly selected and more prolific commercial lines.

The growth and development of ovarian follicles requires a series of coordinated events that lead to follicular somatic cell differentiation, oocyte maturation and ovulation (Bonnet *et al.* 2008). In farm species, the number of ovulatory follicles is

regulated mainly by the pituitary gonadotrophins FSH and LH (Hunter *et al.* 2004). However, it is well established that the ovulatory process is also influenced by endocrine and paracrine pathways that involve local growth factors secreted from granulosa cells (GC) and the growing oocytes (Manabe *et al.* 2004). Most recent studies have focused on the role of bone morphogenetic proteins (BMPs), which belong to the transforming growth factor (TGF)- β family, in the control of follicle development in mammals (Glistner *et al.* 2004; Feary *et al.* 2007; Paradis *et al.* 2009). The BMPs modulate a wide range of cellular functions, such as proliferation and differentiation, steroidogenesis, metabolism and apoptosis (Shimasaki *et al.* 2004; Juengel and McNatty 2005; Gilchrist *et al.* 2008; Gilchrist and Ritter 2011), and can also affect the quality of the oocyte and consequently embryonic development and survival (Hunter *et al.* 2004; Hunter and Paradis 2009). Bidirectional communication between oocyte and somatic cells is also considered to be essential for follicular and theca cell development, as well as for oocyte maturation (Singh *et al.* 1993; Gilchrist *et al.* 2004). However, once having initiated development, most follicles become atretic before ovulating due to apoptosis of GC, irrespective of the stage of follicular development (Tilly 1996; Guthrie 2005). Therefore, the fate of follicles during follicular development is determined by the balance between pro-apoptotic and survival molecules. The molecules and the processes in which they are involved may be summarised as follows: (1) atresia: Bcl2 family members (Bcl2), tumour necrosis factor (TNF) and caspases; (2) follicle selection: Bcl2, Bax, FSH, inhibin, Fas ligand (*FASL*) and caspases; and (3) luteolysis: Fas/FasL, caspase 3 (*CASP3*), Bax and BMP ligands and receptors (Hussein 2005). However, the precise role of these proteins and genes in regulating follicle selection and apoptosis in the pig has not been completely elucidated (Inoue *et al.* 2011). Because ovulation rate and oocyte quality are important determinants of reproductive efficiency, understanding the regulation of follicular growth leading to ovulation is crucial (Webb *et al.* 2007).

Because there are no gene expression data available to explain the lower prolificacy of the Brazilian Piau breed, the aims of the present study were to: (1) investigate and compare the length of the oestrous cycle and the dynamics of follicle growth between the less prolific Piau and commercial line sows; and (2) elucidate the expression pattern of candidate genes during the oestrous cycle using quantitative real-time polymerase chain reaction (PCR) in both genetic lines as a means of better understanding the mechanisms controlling ovarian follicular development in the pig.

Materials and methods

Animals

This experiment was conducted with approval from the Universidade Federal de Viçosa Animal Care Committee (Protocol #34/2010). The 24 commercial line (Landrace \times Large-White \times Pietrain) and 21 Brazilian Piau breed gilts used in the study were obtained from the pig farm at the Universidade Federal de Viçosa (Viçosa, Brazil). Oestrous behaviour was evaluated during the first to seventh consecutive oestrous periods. The

onset of oestrus was checked once a day using a mature boar and was designated as Day 0. Groups of gilts were then killed by electrical stunning on Days 0, 4, 10 and 18 of the oestrous cycle ($n = 6$ per day of the oestrous cycle for commercial gilts; $n = 5$ gilts per day of the oestrous cycle for the Piau breed, except for Day 18 when $n = 6$). The selected time frame covers the initial phase of follicle development (Days 0–4), which is characterised as being gonadotrophin independent and during which initial recruitment and growth of primordial follicles and pre-antral follicle relies mostly on local ovarian factors (Foxcroft *et al.* 1994). Days 10–18 of the cycle correspond to the final selection phase when the preovulatory follicle population has been established (Grant *et al.* 1989; Hunter and Wiesak 1990). The gilts were fed to appetite with a standard diet twice daily and had free access to water.

Tissue collection

Ovaries were collected immediately after the gilts had been killed and were transported on ice to the laboratory within 20 min. Before processing the ovaries, the number of CL was recorded in order to estimate ovulation rate. All visible ovarian follicles were classified on the basis of follicular diameter as small (≤ 3 mm), medium (3–6 mm) or large (≥ 6 mm), and individual follicles were aspirated using a 21-gauge needle attached to a 1-mL disposable syringe. Recovered cumulus–oocyte complexes (COCs) were classified on the basis of morphological criteria under a stereomicroscope as: (1) surrounded by intact layers of cumulus cells; (2) having a partial cumulus cell layer; (3) naked oocytes; (4) having expanded cumulus cells; or (5) degenerated oocytes. Because of the limited amount of material available, oocytes of the same size and classified morphologically as Group 1, 2 or 3 were pooled within sow and classified as relatively normal or healthy; similarly, oocytes classified morphologically as Group 4 or 5 were pooled within sow and classified as relatively degenerated or atretic oocytes. Oocytes classified as normal exhibited homogeneous cytoplasmic pigmentation and unexpanded zona pellucida, regardless of whether they were surrounded by cumulus cells; in contrast, atretic oocytes had an abnormal shape, lacked cytoplasmic pigmentation and did not have an intact zona pellucida. After oocyte isolation, the follicular fluid was centrifuged at 6000g for 5 min at 4°C to collect GC that were classified as either normal or atretic depending on the COCs from which they were harvested. The aspirated oocytes were denuded from the attached cumulus cells by repeated pipetting in 1 \times phosphate-buffered saline (PBS) with 0.1% (w/v) polyvinyl alcohol (PVA) and frozen immediately in liquid nitrogen until RNA extraction. The CL from each sow were dissected and treated with RNA-later (Ambion, Austin, TX, USA) and stored at -20°C for 48 h. After freezing, the CL of each sow were thawed and ground in liquid nitrogen, and the ground tissue was stored at -70°C until RNA extraction.

Isolation of RNA and cDNA synthesis

Immediately after classification of COCs, GC from follicles with the same classification from each animal were washed twice in 1 \times PBS by centrifugation at 5000g for 6 min at 4°C,

stored in RLT Buffer provided in the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and frozen at -20°C until RNA extraction. The GC samples were thawed at 36°C for 5 min and extracted according to the manufacturer's instructions. RNA was isolated from luteal tissue using 30 mg ground tissue using the RNeasy Mini Kit (Qiagen). Similarly, oocytes were thawed on ice and total RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. All samples were DNase treated using on-column DNase digestion with the RNase free DNase Set (Qiagen) and RNA was eluted in 30 μL for GC and CL, and in 10 μL for oocytes. Samples were quantified using a NanoVue Plus spectrophotometer (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and RNA integrity was verified using an Agilent 2100 Bioanalyser Nano Kit (Agilent Technologies, Palo Alto, CA, USA). Oocyte, GC and CL total RNA was reverse transcribed using a ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions with 5 μM of oligo(dT). The cDNA was synthesised using 1 μg GC and CL total RNA and 6 μL oocyte total RNA. The cDNA was then stored at -20°C until analysis by quantitative real-time PCR.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SyBr Green GoTaq qPCR Master Mix (Promega, Madison, WI, USA)

according to the manufacturer's instructions. The primer oligonucleotides used for the reactions were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA, USA) from swine sequences available in GenBank (<http://www.ncbi.nlm.nih.gov>, accessed 3 November 2010). In the present study, *GAPDH* was used as a reference gene against which all gene expression was normalised. The list of primer sequences and expected PCR product lengths are given in Table 1. Reactions were performed in duplicate in 96-well optical reaction plates sealed with optical adhesive film using 12.5 μL of $2 \times$ SyBr Green GoTaq qPCR Master Mix (Promega). Prior to quantification by quantitative real-time PCR, the amplification efficiency and optimal primer concentration was determined for each gene using serial dilution of cDNA from each cell type. The PCR efficiencies for all primers pairs were obtained using the formula $E = 10^{-1/\text{slope}}$, where E is efficiency and slope is the gradient of the dilution series in the linear phase. Samples were amplified separately using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the following amplification program: 40 cycles of 30 s of melting at 95°C followed by 30 s of annealing and extension at 60°C . After the 40 amplification cycles, all samples were subjected to a melting curve analysis, in which they were heated in increments of 1°C per 30 s from 60°C to 94°C to validate the absence of non-specific products.

Table 1. List of primer sequences and quantitative real-time polymerase chain reaction quality control data for pig candidate gene expression analysis

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; *BMP6*, bone morphogenetic protein 6; *FASL*, Fas ligand; *TGFBR2*, transforming growth factor- β receptor 2; *CASP8*, caspase 8; *CASP3*, caspase 3; GC, granulosa cell; CL, corpus luteum

Gene	GenBank Accession no.	Cell type	Primer (5'–3')	Product size (bp)	R^2	Slope of calibration curve	Efficiency (%)
<i>GAPDH</i>	AF017079	GC	Forward: GCAAAGTGGACATTGTGCGCCATCA	124	0.99	–3.33	2.00
		CL	Reverse: GCTTCCCATTCTCAGCCTTGACT		0.99	–3.32	1.99
		Oocyte			0.99	–3.21	2.05
<i>GDF9</i>	NM001001909	Oocyte	Forward: TGGTGCAGAACATCATCCACGAGA	100	0.99	–3.32	2.00
			Reverse: GGCTCAATGGCCAACACACTCAAA				
<i>BMP15</i>	NM001005155	Oocyte	Forward: AAGCTTGGACGGAGATGGATGTCA	162	0.99	–3.53	1.92
			Reverse: GAAGGCAGTGTCCAGGGATGAAA				
<i>BMP6</i>	EU693015	Oocyte	Forward: GGCGGTGACGGCTGCAGAAT	150	0.98	–3.42	1.96
			Reverse: CACACGACGCGGGTGTCCAA				
<i>FASL</i>	NM213806	CL	Forward: AGGCCTGTGTCTCCTTGTGATGTT	125	0.99	–3.30	2.00
			Reverse: TTTGGCTGGCAGACTCTCTGAGTT				
<i>FAS</i>	NM213839	GC	Forward: AGGTGATGATGCCAAGTGACTGA	149	0.99	–3.53	1.92
		CL	Reverse: AGTCAGCATGTTTCCGTTTGCCAG		0.99	–3.55	1.91
<i>BCL2</i>	NM214285	GC	Forward: TACGGAAACAATGCAGCAGCTGAG	123	0.99	–3.41	1.96
		CL	Reverse: TGGTCATTTCCGACTGAAGAGCGA		0.99	–3.55	1.91
<i>BAX</i>	NM138761	GC	Forward: TTTCTGACGGCAACTTCAACTGGG	122	0.99	–3.73	1.85
		CL	Reverse: TGTCAGCCCCATGATGGTTCTGAT		0.99	–3.28	2.01
<i>TGFBR2</i>	EF396957	GC	Forward: TGAGTCCTTCAAGCAGACGGATGT	134	0.99	–3.33	2.00
		CL	Reverse: TGGAACCAAAGGGTGGCTCATAGT		0.99	–3.76	1.85
<i>CASP8</i>	NM001031779	GC	Forward: TGCCTCCGTTACAACACTACATCCT	112	0.99	–3.31	2.01
		CL	Reverse: AACTTGAGGGAAGCCAGGTCATCA		0.99	–3.76	1.85
<i>CASP3</i>	NM214131	GC	Forward: ATGCTGCAAATCTCAGGGAGACCT	159	0.99	–3.59	1.90
		CL	Reverse: CACCATGGCTTAGAAGCACGCAAA		0.99	–3.26	2.02

Statistical analysis

The Chi-squared test of independence was used to evaluate the distribution of follicles within different follicle size classes and the sample size deviation was corrected using the Fisher's exact test. Statistical tests were performed using PROC FREQ in the SAS statistical package (SAS Institute, Cary, NC, USA), with a significance level of 5%. Confidence intervals for the independent proportions were then constructed by the PROPTEST in the R program (R Development Core Team 2008). The number of CL and data on the interoestrous interval were analysed using SAS PROC UNIVARIATE to verify differences in ovulation rate and oestrous cycle length between genetic groups.

The expression for each gene was calculated using the ΔC_t method (target gene C_t – *GAPDH* gene C_t) for all individual samples, where C_t is the PCR cycle number at which the fluorescence generated within a reaction crosses an arbitrary threshold. Differences in gene expression were estimated using the $2^{-\Delta C_t}$ method of Livak and Schmittgen (2001). For statistical analysis, six follicle classifications were used: (1) large normal; (2) large atretic; (3) normal medium; (4) medium atretic; (5) small normal; and (6) small atretic. These were compared between genetic group \times day of oestrous cycle. However, because follicle classifications varied by genetic line and day of cycle, not all comparisons were possible. The comparisons were made using the SAS GLM PROCEDURE and defined as (commercial – Piau) for GC and oocytes. For the CL, comparisons were made using the same model with balanced samples, regardless of follicle classification. Unless indicated otherwise, data are given as the mean \pm s.e.m.

Results

Dynamics of follicle development

Compared with commercial line gilts, the less-prolific Piau gilts tended to have a shorter oestrous cycle (19.4 ± 1.7 vs 20.0 ± 1.0 days, respectively; $P = 0.068$) and a different pattern of follicle development, as well as a lower ovulation rate (15.5 ± 1.9 vs 11.1 ± 2.4 , respectively; $P \leq 0.05$).

The preovulatory population of follicles for both Piau and commercial gilts reached 6–10 mm in diameter. The distribution of both small ($P \leq 0.05$) and large ($P \leq 0.01$) ovarian follicles during the oestrous cycle differed between commercial and Piau gilts throughout the oestrous cycle (see Table S1 available as Supplementary Material to this paper). The confidence interval for the difference between proportions allowed the identification of days of the oestrous cycle on which the follicular number from Piau and commercial line gilts differed significantly, as illustrated in Fig. 1. The pattern of follicle development in the Piau breed was characterised by a lower number of small follicles on Day 18 and large follicles on Day 0, reflecting a lower ovulation rate compared with the commercial line gilts.

The proportion of normal and atretic follicles during the oestrous cycle for all size follicles in commercial and Piau gilts is shown in Fig. 2. The pattern of atresia seems to differ between breeds, although the growth of large follicles on Days 18 and 0 in both breeds is marked by a significant decrease in the number of small and medium-sized follicles. In addition to the decline in follicle numbers, the incidence of estimated atresia in the total follicle population increased from 17.3% before selection

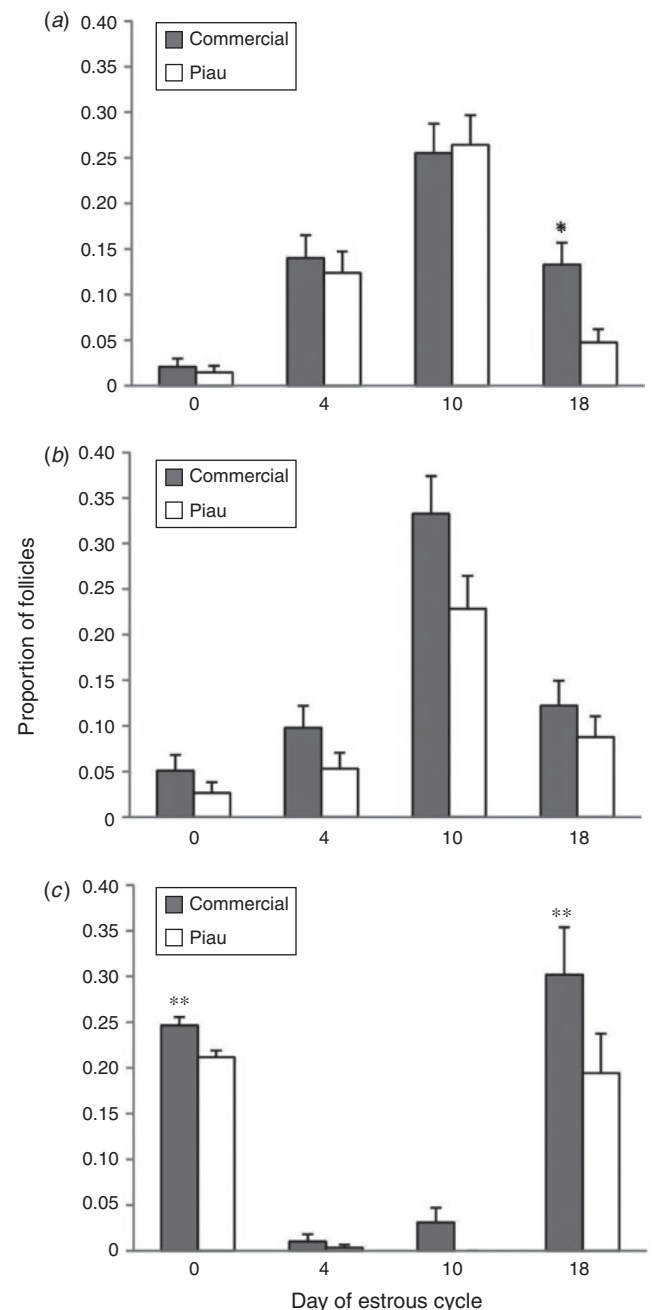


Fig. 1. Confidence intervals for the proportion of (a) small, (b) medium and (c) large follicles during the oestrous cycle in commercial and Piau gilts. Data are the mean \pm s.e.m. * $P \leq 0.05$, ** $P \leq 0.01$ compared with the Piau breed.

(Day 10) to 50% before oestrous (Day 18) in the Piau breed, compared with a decrease in atresia from 59.3% to 25.7% in the commercial line gilts.

Expression of mRNA in the CL

Few differences between the genetic lines were observed for mRNA expression of apoptosis-related genes in the CL during the oestrous cycle (Fig. 3). The abundance of *BCL2* mRNA, a major

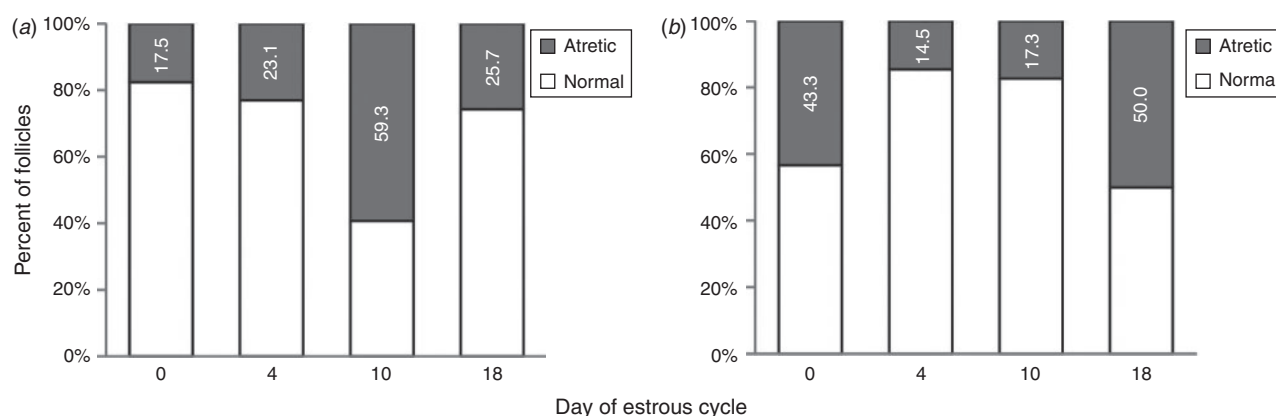


Fig. 2. Percentage of assumed normal and atretic follicles during the oestrous cycle for all follicle sizes in (a) commercial and (b) Piau sows.

anti-apoptotic molecule, was similar in commercial and Piau gilts on Day 0, whereas the mRNA abundance of the pro-apoptotic factor *BAX* was higher in commercial line gilts on Days 4 and 18 ($P \leq 0.05$ and $P \leq 0.01$, respectively). Expression of one of the components of the Fas system, namely *FASL*, was higher in the Piau breed on Day 18 ($P \leq 0.01$). However, no differences in expression were observed for *FAS* and the cascade initiator caspase-8 (*CASP8*). In contrast, the abundance of *CASP3* mRNA, one of the downstream components of *CASP8*, was higher in the Piau breed on Days 0 and 4 ($P \leq 0.05$ and $P \leq 0.01$, respectively). Similar results were observed for *TGFBR2*, with higher expression in commercial line gilts on Days 0 and 4 ($P \leq 0.0001$ and $P \leq 0.05$, respectively) than in the Piau breed.

Expression of mRNA in GC

Figure 4 shows mRNA expression in the commercial and Piau breeds of components of the death receptor apoptotic pathway, with the exception of *FASL*, which could not be detected in the GC with the current primer set, and *TGFBR2*. In large normal follicles, the abundance of *TGFBR2* mRNA was higher in commercial line gilts on Day 0; however, no significant difference was observed in large atretic follicles. Expression of *FAS* mRNA expression in large atretic follicles was higher in Piau than commercial gilts ($P \leq 0.05$) on Day 0, whereas no significant difference was observed in large normal follicles. On Day 0, medium normal follicles exhibited higher *BCL2* mRNA expression in GC from the Piau breed ($P \leq 0.01$), whereas *CASP8* mRNA expression was higher in the Piau breed for the medium normal follicle category on Day 18 of the oestrous cycle. Expression of *CASP3* mRNA was higher in commercial line gilts for medium atretic follicles on Day 18. In small normal follicles on Day 4, *CASP3* mRNA expression was higher in commercial line gilts, whereas *CASP8* mRNA expression was higher in the Piau breed. Additional comparisons between follicle size and health status were limited by the number of experimental units on specific days of the oestrous cycle.

Oocyte mRNA expression profiles

Figure 5 shows the contrast of oocyte gene expression for growth differentiation factor 9 (*GDF9*), *BMP15* and *BMP6*

between breeds according to follicle size on a particular day of the oestrous cycle. Interestingly, for all genes analysed, negative values were obtained, indicating that the expression of these genes was higher in the Piau oocytes than in oocytes recovered from commercial line gilts. The relative expression of *GDF9* in oocytes from large normal and large atretic follicles on Day 18, and in oocytes from medium normal follicles on Day 10, was higher ($P \leq 0.05$) in the Piau breed, whereas no differences in *GDF9* expression were observed for medium atretic follicles. For small follicles, no differences in *GDF9* mRNA abundance were observed in either normal or atretic follicles on the days studied. Oocyte *BMP15* mRNA expression was higher on Day 18 in the Piau breed in medium normal and small atretic follicles ($P \leq 0.05$). Only one contrast was significant for oocyte *BMP6* mRNA abundance, which was higher in the Piau breed for small normal follicles on Day 10 ($P \leq 0.05$).

Discussion

The key ovarian phenotypic traits of the less-prolific Piau breed were a lower ovulation rate and a tendency for a shorter oestrous cycle than the more prolific commercial line gilts. A shorter oestrous cycle has also been observed in the hyperprolific Meishan breed compared with less-prolific breeds (Bazer *et al.* 1988b). It has been postulated that the longer behavioural oestrus in the Meishan breed would result in the recovery of follicles at the onset of oestrus at a relatively earlier time relative to ovulation than in other breeds, and Meishan sows appear to maintain a larger number of follicles in the proliferating pool during the follicular phase, thus contributing to their high prolificacy (Bazer *et al.* 1988b). Although also showing a tendency for a shorter oestrous cycle, as observed in Meishan, the Piau breed represents the other extreme of prolificacy. Despite the lack of information about the precise time course of events during the oestrous cycle in the Piau breed, the findings of the present study indicate a decrease in the number of small follicles (≤ 3 mm diameter) in the early follicular phase (Day 18), as illustrated in Fig. 1a. This reduction in the number of small follicles, together with a higher rate of atresia on Day 18 (see Fig. 2), suggests that a block to follicle replacement in the proliferating pool, the opposite situation to that in Meishan sows, may be a primary determinant of low prolificacy in the Piau breed.

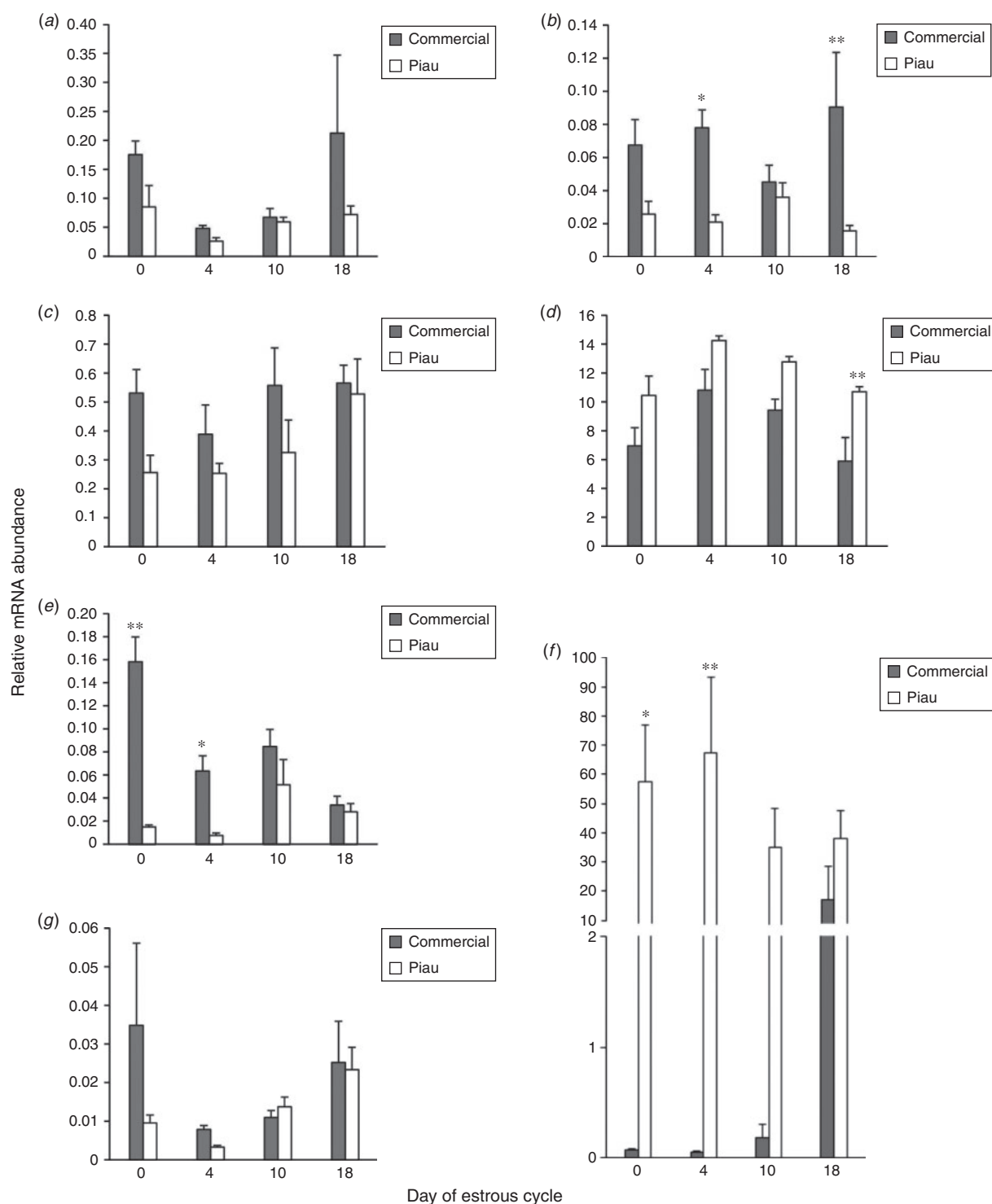


Fig. 3. Comparison of the relative mRNA abundance of (a) *BCL2*, (b) *BAX*, (c) *FAS*, (d) *FASL*, (e) *TGFBR2*, (f) *CASP3* and (g) *CASP8* in corpora lutea from commercial and Piau breeds throughout the oestrous cycle. Relative mRNA abundance is expressed as the least squares means \pm s.e.m. of $2^{-\Delta C_t}$. * $P \leq 0.05$, ** $P \leq 0.01$ compared with the Piau breed.

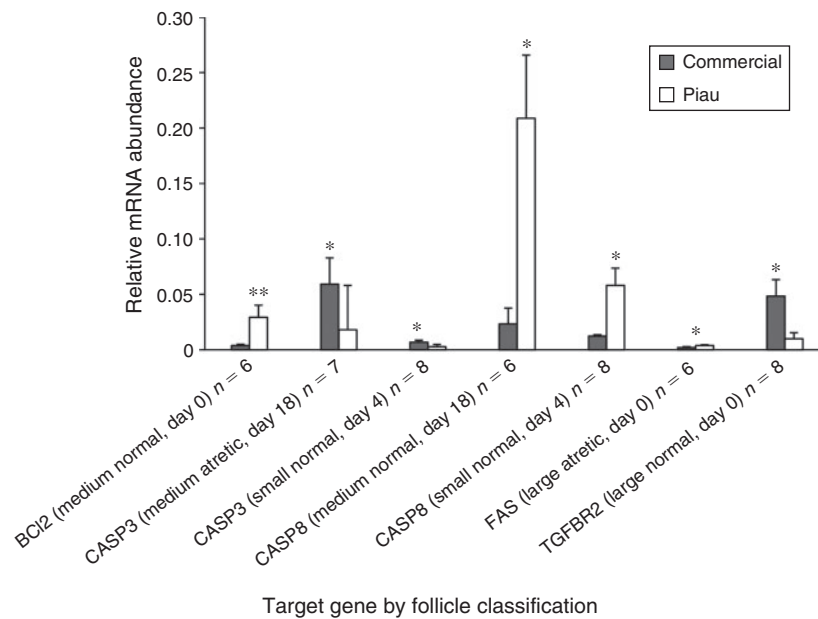


Fig. 4. Comparison of the relative mRNA abundance in granulosa cells from commercial and Piau breeds for target genes according to follicle classification on particular days of the oestrous cycle (n = number of animals represented). Relative mRNA abundance is expressed as the least squares means \pm s.e.m. of $2^{-\Delta C_t}$. * $P \leq 0.05$, ** $P \leq 0.01$ compared with the Piau breed.

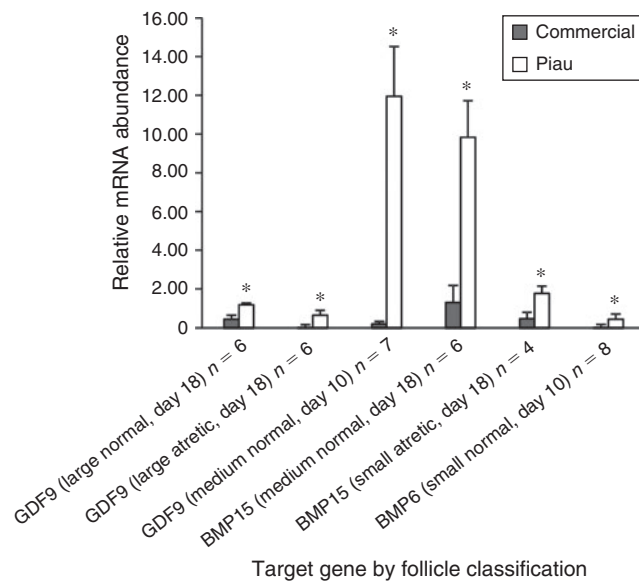


Fig. 5. Comparison of the relative mRNA abundance in denuded oocytes recovered from commercial and Piau breeds for target genes according to follicle classification on particular days of the oestrous cycle (n = number of animals represented). Relative mRNA abundance is expressed as the least squares means \pm s.e.m. of $2^{-\Delta C_t}$. * $P \leq 0.05$, ** $P \leq 0.01$ compared with the Piau breed.

The observation of fewer small follicles and the higher rate of atresia in large follicles limit the number of follicles available for selection and subsequent ovulation in the less-prolific Piau compared with commercial line gilts on Day 18 of the oestrous

cycle and is consistent with reports that sows with a higher ovulation rate have a larger number of follicles in the proliferating pool (Clark *et al.* 1973) and maintain a higher number of follicles during the follicular phase than do non-prolific breeds (Miller *et al.* 1998). However, Piau gilts also had a lower incidence of atresia in medium- and small-sized follicles on Days 4 and 10. This pool of follicles begins to grow after ovulation and will constitute the pool of follicles in the recruitment phase, which occurs on Days 14 and 16 of the oestrous cycle in pigs (Foxcroft and Hunter 1985). Collectively, these observations corroborate the suggestion that ovulation rate is a complex trait related to differences in both follicular recruitment and atresia (Manabe *et al.* 2004).

The follicular dynamics between breeds were further corroborated by gene expression analysis using quantitative real-time PCR. Genes of the TGF- β superfamily, such as those for BMPs, GDF9 and their receptors, are known modulators of mammalian folliculogenesis and were selected for investigation. The expression of *BMP6*, *BMP15* and *GDF9* transcripts, as well as that of their cell receptors, has recently been reported in porcine oocytes from preovulatory follicles (Paradis *et al.* 2009; Sun *et al.* 2010). However, less information is available about the mechanisms underlying their physiological role in follicular development between species, or differences in their expression in divergent breeds of pigs. Therefore, a comparative study using the less-prolific Piau breed and more prolific contemporary commercial line gilts provided the opportunity to identify genes related to ovulation rate and oocyte quality, and consequently embryonic development and survival, in these two breeds.

Interestingly, in the present study, the significant contrasts between two breeds identified higher expression of

oocyte-secreted factors in the Piau breed than in the commercial line. Because oocyte-secreted factors act as key regulators of oocyte maturation and folliculogenesis (McNatty *et al.* 2004; Juengel and McNatty 2005; Gilchrist *et al.* 2008), they may be related to the differences in ovulation rate between breeds. The local Piau breed exhibited higher *GDF9* mRNA abundance in large normal and large atretic follicles on Day 18, as well as in medium normal follicles on Day 10. Expression of *BMP15* mRNA was detected in the oocyte, consistent with previous studies in the pig (Quinn *et al.* 2004; Zhu *et al.* 2008; Paradis *et al.* 2009), with higher expression in medium-sized healthy follicles and small-sized atretic follicles in the Piau breed on Day 18. Working with *in vitro* oocytes, Li *et al.* (2008) reported that these two genes and their encoded proteins were differentially expressed during the maturation process, especially during cumulus cell expansion. Therefore, these results suggest that the lower prolificacy of the Piau breed may be related to differences in the expression of both oocyte-secreted factors, resulting in changes in the timing of cumulus cell expansion and potentially also affecting GC steroidogenesis. Furthermore, it is possible that the differential expression of *GDF9* and *BMP15* verified in the present study is due to relative differences in the stage of follicular maturation, because previous studies reported that the Meishan preovulatory follicle is in a more advanced state of maturation than that of the Large-White (Hunter *et al.* 1993; Faillace and Hunter 1994; Xu *et al.* 1998).

Expression of *BMP6* mRNA was detected in porcine oocytes in the present study, corroborating previous findings (Zhu *et al.* 2008; Paradis *et al.* 2009). The differential expression of *BMP6* mRNA from Piau oocytes may play an important role in FSH-dependent follicle development and in the regulation of luteinisation, again affecting the difference in ovulation rate between breeds. It has been suggested in rats that *BMP6* mRNA derived from GC is lost during selection of the dominant follicle and that *BMP6* mRNA is strongly expressed in GC during atresia (Erickson and Shimasaki 2003). Moreover, investigations into the mechanism of action found that *BMP6*, unlike *BMP15* and *GDF9*, does not have proliferative properties on rat GC and is able to suppress FSH-induced progesterone production (Otsuka *et al.* 2001). Therefore, the results of the present study suggest breed differences in *BMP6*, *BMP15* and *GDF9* mRNA expression, and indicate that a larger number of follicles can escape from atresia during early folliculogenesis in the Piau breed, resulting in the lower incidence of atresia on Days 4 and 10 compared with commercial line gilts. These results initially seem contradictory, because the Piau breed is the less prolific. However, oocyte-secreted factors may be involved in the recruitment process, leading to differences in oocyte quality (Gilchrist *et al.* 2008) and the number of tertiary and atretic follicles between lines, as suggested by Manabe *et al.* (2004). Also associated with their lower prolificacy, differences in oocyte-expressed factors may be contributing to the ability of the Piau to use smaller follicles for selection and ovulation, as described for the Meishan breed (Miller *et al.* 1998).

Once the pool of follicles initiates its growth, atresia and ovulation are the only possible fates (Knox 2005). Atresia limits the number of oocytes capable of fertilisation and embryonic development, and is physiologically important for the

elimination of degenerated oocytes (Guthrie *et al.* 1995; Guthrie 2005). It is not clear whether this process differs between pig breeds. In the present study, mRNA expression of candidate genes involved in apoptotic pathways in GC and luteal cells differed between breeds during the oestrous cycle. The Fas–FasL system has been reported in many species as the major mechanisms regulating GC (Matsuda-Minehata *et al.* 2008) and luteal cell (Juengel *et al.* 1993; Rueda *et al.* 1997; Sakamaki *et al.* 1997) apoptosis. In the present study, *FAS* mRNA was more abundant in GC from healthy than atretic follicles during the oestrous cycle, consistent with previous findings in several species (Porter *et al.* 2001; Dharma *et al.* 2003; Inoue *et al.* 2006). However, in contrast, Inoue *et al.* (2006) reported that *FAS* mRNA expression increased in GC from atretic compared with healthy follicles.

We also detected *FAS* and *FASL* mRNA expression in pig luteal tissue, as reported in murine and human studies (Kondo *et al.* 1996; Sakamaki *et al.* 1997). Interestingly, in the present study no differences were observed for the *FAS* transcript in CL between breeds, whereas *FAS* mRNA expression in GC was significantly higher in large atretic follicles from Piau gilts than from commercial line sows on Day 0. According to Manabe *et al.* (2004), differences in the initiation of GC apoptosis between species indicate local mechanisms of regulation, mainly the apoptotic stimuli induction mechanism. In this context, the present study indicates that apoptosis signalling may be differently activated in atretic follicles between distinct breeds. Therefore, it can be suggested that the follicle in the Piau breed provides a different environment for follicle apoptosis than that in commercial line follicles, which may contribute to the lower prolificacy of the Piau breed. Many reports have shown that apoptosis occurs during luteolysis and it has also been established that apoptosis plays a role in CL regression at the end of the oestrous cycle (Rueda *et al.* 1997). In the present study the amount of transcript that encodes the membrane protein Fas in the CL was similar between breeds, whereas expression of *FASL* mRNA was higher in Piau CL, which may result in a more effective apoptosis signalling towards luteolysis in this breed.

Several genes belonging to the *bcl2* family, including both anti- and pro-apoptotic family members, are known to be expressed in luteal cells of various species (Rodger *et al.* 1995; Rueda *et al.* 1997; Goodman *et al.* 1998). The *Bcl2*:*Bax* expression ratio within a cell is related to its potential to become apoptotic, and it has been proposed that Bax homodimers promote cell death, whereas Bcl2 homodimers function as repressors of cell death (Oltval *et al.* 1993). The findings of the present study indicate that *BCL2* mRNA abundance in the CL did not differ between breeds during the oestrous cycle. However, the mRNA abundance of the pro-apoptotic factor *BAX* was higher in commercial line gilts on Days 4 and 18, resulting in a higher ratio of *BAX*:*BCL2* mRNA compared with that in the Piau breed, a change consistent with bax-mediated apoptosis (Rueda *et al.* 1997). This indicates a possible occurrence of an increased ratio of apoptosis in the CL of commercial line compared with Piau sows. Similar higher expression of *BAX* mRNA in the regressing CL was reported during luteolysis in cattle and humans (Rueda *et al.* 1997; Sugino *et al.* 2000; Pretheeban *et al.* 2010). Although in the present study the

expression of *BCL2* mRNA was similar between breeds on Day 10, previous studies in humans demonstrated higher expression of *BCL2* mRNA in the mid-luteal phase CL (Sugino *et al.* 2000). Together, these findings suggest that the *BAX* gene may be important in the regulation of the CL lifespan by controlling the rate of apoptosis and that it potentially underlies differences in oestrous cycle length and cyclicity between breeds. However, it is important to emphasise that these events may also be regulated at the post-translational level and modulated by interactions with other molecules, such as p53 tumour suppressor protein (Miyashita *et al.* 1994). In addition, the abundance of transcripts is not always associated with differences in protein secretion (Griffin *et al.* 2002) and studies at the proteomic level are required.

Many findings indicate that Bcl2 family proteins modulate the apoptosis of GC in mammals, in which *BCL2* overexpression is related to reduced follicular atresia and increased litter size (Hsu *et al.* 1996; Choi *et al.* 2004). In the present study *BAX* mRNA abundance in GC was similar between breeds during the oestrous cycle, whereas *BCL2* mRNA abundance in medium-sized healthy follicles was higher in the Piau breed compared with commercial line gilts on Day 0, again suggesting decreased apoptosis in Piau GC during early follicular development. *CASP3* has been reported as an essential molecule for the apoptosis of GC (Manabe *et al.* 2004; Matsuda-Minehata *et al.* 2008). Many studies in GC have described changes in caspase-3 protein expression and activity associated with the progression of atresia in ovarian follicles (Boone and Tsang 1998; Berardinelli *et al.* 2004). In the present study, *CASP3* mRNA abundance was significantly higher in GC from medium-sized atretic and small-sized healthy follicles on Day 4 in commercial line gilts than in the Piau breed. However, little other information is available about *CASP3* mRNA expression patterns in different breeds of pigs during the oestrous cycle. During the early luteal phase in the pig, a new group of medium-sized follicles starts to grow between Days 3 and 8 (Guthrie *et al.* 1995). Although the functional lifespan of these follicles is unknown, it is likely that within a day or two after ovulation the medium-sized growing follicles will be eventually deleted by atresia, as described in cattle (Sirois and Fortune 1988). Therefore, the data on *CASP3* mRNA expression in medium- and small-sized follicles in commercial line gilts suggest that this breed may be more susceptible to atresia than the Piau breed on Day 4, driving the different pattern of follicular development observed between breeds. These findings also agree well with the higher incidence of atresia (23.1% vs 14.5%) on Day 4 in commercial line gilts compared with the Piau gilts in the total follicle population (Fig. 2).

It is also known that apoptosis occurs during luteolysis in the ovary, and it is recognised that controlled cell death is important to physical removal of the CL from the ovary at the end of the oestrous cycle (Rueda *et al.* 1997). Previous studies reported *CASP3* expression in the CL during luteolysis induced by prostaglandin in the rat (Boone and Tsang 1998) and in sheep (Rueda *et al.* 1999) CL. Moreover, abundant expression of *CASP3* in the human CL was reported and considered to be important for luteal regression (Krajewska *et al.* 1997). However, little is known regarding the expression and role of caspases in the pig CL. In the present study, *CASP3* mRNA

was expressed in luteal cells throughout the oestrous cycle and its abundance was higher in the Piau breed on Days 0 and 4. Apoptotic cell death during luteolysis is important to maintain oestrous cyclicity (Manabe *et al.* 2004). However, premature disruption of normal CL function could result in a reduction of reproductive efficiency due to irregular oestrous cycles and loss of pregnancy (Rueda *et al.* 1997). In this context, the results of the present study indicate that apoptosis mediated by *CASP3* may be differently regulated between the breeds studied and may reflect differences in the length of the oestrous cycle and the time of luteal regression. Recent findings have addressed roles for TGF- β in the follicle maturation and luteinisation processes in the pig (Paradis *et al.* 2009; Sriperumbudur *et al.* 2010). In the present study, *TGFBR2* mRNA expression was decreased in Piau CL on Days 0 and 4 compared with the commercial line, indicating that the regression process may differ between breeds. In addition, *TGFBR2* mRNA expression in the present study was higher in GC from large follicles of commercial line gilts on Day 0 and may mediate the differences in follicular dynamics leading to differences in ovulation rate and oocyte quality in gilts from the different breeds. This finding is further supported by Sriperumbudur *et al.* (2010), who suggested that *TGFBR2* may have roles in mediating the luteinisation process in postovulatory porcine follicles.

Conclusions

In conclusion, the findings of the present study indicate differences in the characteristics of the oestrous cycle and follicular development in the less-prolific Piau gilt than in commercial line gilts. The pattern of follicular growth in Piau gilts is characterised by a small population of preovulatory follicles, combined with a higher incidence of atresia in the preovulatory stage of the cycle compared with commercial line gilts. The gene expression profiles observed were consistent with the different follicular dynamics between the breeds, with the relative expression of *BCL2* and *BAX* genes influencing important decisions between proliferation and atresia in CL and GC of gilts of the different breeds. Because transcriptional profiling identifies only one level of cellular regulation, the differences in mRNA abundance observed in the present study may not necessarily correspond to differences in secreted proteins and do not provide information about possible protein modifications, activity or location (Zeng *et al.* 2004). However, the expression data suggest that further proteomic studies could be of value. In addition, from a functional perspective, techniques like RNA interference (RNAi) could be used in future experiments to downregulate the expression of specific genes of interest in *in vitro* culture systems and thus investigate the potential role of those genes in mediating cumulus cell expansion and to confirm their pro-apoptotic activity in granulosa cells during atresia. Apoptosis in the CL, mediated by differences in *FASL* and *CASP3* and by decreased expression of *TGFBR2* mRNA in Piau compared with commercial line CL on Days 0 and 4 of oestrous cycle may also reflect the tendency for a shorter oestrous cycle and faster luteal regression in the Piau breed. Finally, the higher expression of oocyte-secreted factors (*GDF9*, *BMP15* and

BMP6) in Piau oocytes may play a role in inhibiting the luteinisation process, as well as affect follicle development and induce a lower ovulation rate that is a key component of the reduced prolificacy of this breed.

Together, our results support the hypothesis that differential expression of genes and/or gene pathways controlling follicle growth mediates the different pattern of follicle development observed between the breeds studied. This may affect not only ovulation rate, but also oocyte and embryo quality.

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