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Ovarian and follicular variables used to determine ewes with different *FecG*^{E} genotypes

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ABSTRACT

Based on ovarian and follicular variables, there was determination of ewes with different *FecG*^{E} genotypes. Based on the *FecG*^{E} genotype, 65 Santa Inês ewes were assigned to three experimental groups: homozygous wild-type (n = 25; *FecG*$^{+/+}$), mutant heterozygous (n = 27; *FecG*$^{+/E}$) and mutant homozygous (n = 13; *FecG*$^{E/E}$). The ewe's ovaries were weighed and measured, then the follicles (oocyte, nucleus and nucleolus) were histologically evaluated for morphometry and morphology. Morphologically normal follicles, in the primordial and transitional stages, explained 70.18% of the variability morphological characteristics between mutant and wild-type ewes. Conducting the morphometric evaluation resulted in a more precise determination of the genotype groups when there was assessment of the primordial and secondary follicular developmental stages. The diameter of the oocyte and the oocyte nucleus of the primordial follicles explained 36.76% of the variability in follicular morphology between ewes with the mutation and those with the wildtype group. Similarly, the core diameter of oocytes in secondary follicles explained 10.63% of the variability in follicular morphology among *FecG*$^{E/E}$, *FecG*$^{+/E}$ and *FecG*$^{+/+}$ ewes. Thus, morphologically normal follicles in the primordial and transitional stages of development are the variables that allow for a more precise differentiation of Santa Inês ewes with the *FecG*^{E} mutation. These variables may be evaluated to make more efficient the adoption of biotechniques that when conducted there is utilisation of follicles in the initial developmental stages as a physiological basis for classifying whether specific follicles are useful when conducting the techniques.

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1. Introduction

The identification of animals with superior genotypes based on molecular markers has allowed for an increase the frequency of genes associated with greater productive efficiency in genetic breeding programs (Coutinho et al., 2010). Thus, identifying and selecting for fecundity genes (Fec) that affect ovulation rate are an alternative to increase the performance in sheep flocks (Abdoli et al., 2016).

The Fec genes have an important function in regulating folliculogenesis through growth factors. Mutations in these genes, however, can affect the gene expression and reproductive performance of animals (Dong et al., 1996; Monniaux, 2016). Among the Fec genes, ovine growth differentiation factor (GDF-9), contained in Chromosome 5, codes for a protein (GDF-9) that is essential for folliculogenesis (Juengel et al., 2002).

Homozygous Santa Inês ewes with the mutation in Exon 2 of GDF-9, denominated $FecG^{E}$ (variant F345C), have greater ovulation rates and prolificacy (Silva et al., 2011). If the homozygous $FecG^{E}$ mutation is present, there is a greater reproductive performance of the animals with the mutation when compared to those homozygous ewes with other mutations in the same or other genes (Hanrahan et al., 2004; Nicol et al., 2009).

Although the presence of $FecG^{E}$ mutation results in improvement in ovulation rate and prolificacy, the resulting change in regulation of folliculogenesis and ovarian variables are still not completely understood. Ovarian and follicular variables from Santa Inês $FecG^{E}$ ewes were evaluated, therefore, to determine the genotype groups. This information should contribute to elucidation of the physiological functions for which there is alterations as a result of expression of this gene and possibly the use of the gene mutation as a molecular marker associated with increased reproductive performance.

2. Material and methods

This research was conducted after evaluation and approval of the Committee of Ethics in Animal Use, Brazilian Agricultural Research Company - Embrapa, Tabuleiros Costeiros Unit, Aracaju-SE, Brazil (License: 13072016.006).

2.1. Experimental location and animals

There was evaluation of 65 Santa Inês ewes produced in the *in situ* Conservation Ovine Center in Embrapa Tabuleiros Costeiros, located in Pedro Arle Experimental Field (Frei Paulo-SE, Brazil). The latitude and longitude where this experiment was conducted are 10°36′15″ South and 37°38′29″ West, respectively.

The ewe's age and body weight were 52.8 ± 30.0 months and 50.6 ± 10.0 kg, respectively. The ewes were genotyped for the $FecG^{E}$ mutation using the polymerase chain reaction (PCR/RFLP) technique, utilising to the methodology proposed by Silva et al. (2011). After genotyping, which was conducted in Embrapa Recursos Genéticos e Biotecnologia, Brasília-DF/Brazil, the ewes were assigned to three experimental groups as follows: homozygous wild-type ($n = 25$; $FecG^{+/+}$), mutant heterozygous ($n = 27$; $FecG^{+/E}$) and mutant homozygous ($n = 13$; $FecG^{E/E}$).

The ewes were slaughtered, and the aseptically collected ovaries ($n = 130$) were transported in an antibiotic-containing buffer medium to the Embrapa Tabuleiros Costeiros Biotechnology Laboratory, Aracaju-SE/Brazil.

2.2. Ovarian morphometry

In the laboratory, the ovaries were weighed in a precision scale (Mars AW220, Minas Gerais, Brazil) with a sensitivity of 0.0001 g. Measurements of the gonads were performed using a calliper rule, considering the width (mm) between the pedicle and the opposite end of the ovary and the length (mm) on its major axis.

2.3. Morphometry and morphology of ovarian follicles

Ovarian tissue fragments (3 × 3 × 1 mm) were removed at random locations from the cortex of the right and left sides of each animal to be processed histologically, using the procedures of Martins et al. (2010). After fixing these fragments in paraffin, serial cuts of 7 μm thickness were made with a microtome (Leica RM 2125RT, Heidelberg, Germany) to prepare microscope slides. Subsequently, the slides were stained with haematoxylin and eosin as described by Cunha et al. (2018). The slides were screened every 28 μm using an optical microscope (Olympus BX41TF, Tokyo, Japan) with a magnification of 100 or 400×.

Considering the morphometry, 9,322 follicles were classified using the methods of Silva et al. (2004) as: primordial (oocytes surrounded by granulosa cells); transitory (oocytes surrounded by granulosa cells and three or more cuboid cells); primary (oocytes surrounded by a complete layer of cuboid cells); secondary (oocytes surrounded by two or more layers of cuboid cells); and initial antral follicle (oocytes surrounded by two or more layers of cuboid cells with antrum formation). The follicles were classified as normal after morphological analysis, taking into account the organisation of granulosa cells, absence of pycnotic nuclei and retraction of cytoplasmic oocytes, as described previously by Costa et al. (2014).

Images of follicles considered morphologically normal were obtained using an optical microscope (Olympus, Tokyo, Japan) coupled to a digital camera. For each genotype (E/E, +/E and +/+), 50 follicles in the primordial, transition and primary stages were assessed. In the secondary stages, 51 follicles (13 in $FecG^{E/E}$, 24 in $FecG^{+/E}$ and 14 in $FecG^{+/+}$) were assessed, while for the initial antral stage, there were 35 follicles (eight in $FecG^{E/E}$, 11 in $FecG^{+/E}$ and 16 in $FecG^{+/+}$) that were assessed. The follicles,

Table 1
Morphometric variables of Santa Inês ewe ovaries genotyped for *FecG*^{E} (mean ± standard error).

Variable	*FecG*^{E} Genotypes		
	+/+	+/E	E/E
Weight (g)	1.57 ± 0.14	1.43 ± 0.07	1.57 ± 0.15
Length (mm)	12.28 ± 0.52	12.08 ± 0.37	12.11 ± 0.64
Width (mm)	15.68 ± 0.71	15.13 ± 0.51	16.60 ± 1.07

+/+: Homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype.

oocytes, nuclei and nucleoli were measured for length and width using a pachymeter rule and the ZEN software. Based on these two measurements, the diameter of each structure was calculated using the methods described by Lundy et al. (1999).

2.4. Statistical analysis

For data analysis, each ewe was considered as a replicate within each genotype group. The ewe's age, average ovarian measurements (weight, length and width), average of the total morphologically normal follicles and number of morphologically normal secondary follicles were considered as covariates for analyses. The multivariate evaluation was performed by applying the discriminant analysis technique using the XLStat software, version 2014.

The relationship among values for ovarian variables, follicular morphology and the effect of the genotype groups on these variables were assessed using the Pearson's correlation and univariate analyses. The data were evaluated regarding normal distribution and homoscedasticity by using the Lilliefors and Bartlett tests, respectively. When necessary, the data were transformed based on the Box-Cox methodology (1964). An ANOVA was used for parametric variables, followed by the use of the Tukey's test. For the non-parametric variables, the Kruskal-Wallis test was conducted, followed by use of the Dunn's test. The IBM SPSS Statistic software, version 20, was used to conduct the previously described analyses, considering the significance level of 5%.

3. Results

The genotype did not affect ($P > 0.05$) the average values of the ovarian variables (weight, length and width; Table 1). For these variables, there were moderate, positive and significant ($P < 0.05$) correlations between weight and length ($r = 0.52$), weight and width ($r = 0.54$) and length and width ($r = 0.61$). The values are included in Table 2 for correlation coefficients (r) among the different developmental stages of morphologically normal follicles.

During primordial and transitional stages, morphologically normal follicles had a greater correlation with the F1 discriminant functions, which explained 70.18% of the variability between the ewes with the mutation (*FecG*$^{+/E}$ and *FecG*$^{E/E}$) and those without (*FecG*$^{+/+}$) the mutation. The data for ovarian weight and width, as well as the morphologically normal secondary and initial antral follicles, indicated there was a greater correlation with the F2 discriminant function. These variables accounted for 29.82% of the variability between the *FecG*$^{+/E}$ ewes in comparison to *FecG*$^{E/E}$ and *FecG*$^{+/+}$ (Fig. 1).

The results from the morphometric analysis indicate the female genotype affects ($P < 0.05$) the primordial and secondary follicle stages (Table 3). The primordial follicles of ewes with *FecG*$^{E/E}$ mutation had a larger diameter ($P < 0.05$) than those of ewes with *FecG*$^{+/E}$ and *FecG*$^{+/+}$ mutations. When considering the secondary follicles and the oocytes, there were smaller ($P < 0.05$) diameters in ewes with the *FecG*$^{E/E}$ than in those with *FecG*$^{+/E}$ genotype but the diameters were similar ($P > 0.05$) in ewes with *FecG*$^{+/+}$ genotype as those with the other two genotypes. In the primary follicles, the oocyte nucleoli had a smaller diameter ($P < 0.05$) in the ewes with *FecG*$^{+/E}$ when compared to those with the *FecG*$^{+/+}$ genotype. The ewes with the *FecG*$^{E/E}$ genotype had similar ($P > 0.05$) values compared with those of the other genotypes.

Based on the diameter of the follicles and oocytes, as well as the internal structures (nucleus and nucleolus), there was a more precise group determination when assessing the primordial and secondary follicular stages. The diameter of the primordial follicle accounted for 63.24% of the variability among ewes with the *FecG*^{E} (*FecG*$^{E/E}$ and *FecG*$^{+/E}$) genotype. The oocyte and oocyte diameters of the primordial follicles accounted for only 36.76% of the variability among the ewes with *FecG*^{E} (*FecG*$^{E/E}$ and *FecG*$^{+/E}$)

Table 2
Correlation coefficients (r) among the viable follicles, according to the follicular development stage, in Santa Inês ewes genotyped for *FecG*^{E}.

Total Follicles	Viable	Primordial	Transitional	Primary	Secondary	Antral
Viable	1.000	0.876*	0.775*	0.497*	0.264	0.447*
Primordial		1.000	0.582*	0.442*	0.133	0.333*
Transitional			1.000	0.391*	−0.086	0.232
Primary				1.000	0.245	−0.045
Secondary					1.000	0.453*
Antral						1.000

* $P < 0.05$.

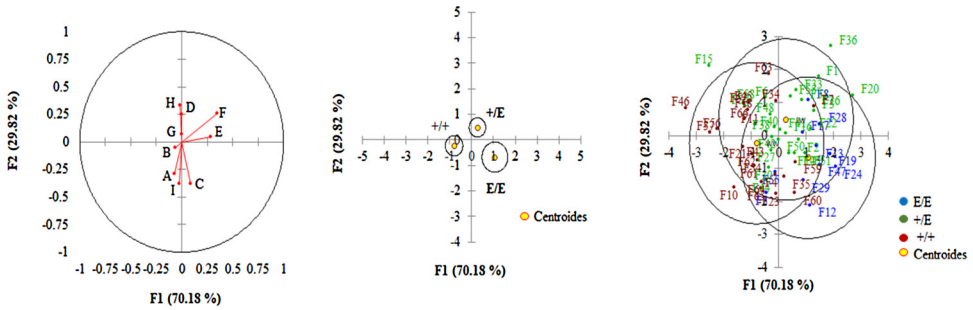

Fig. 1. Graphic representations of the discriminant analysis of the ovarian characteristics and of the morphologically normal follicles; +/+: homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; A: ovary weight; B: ovary length; C: ovary; D: total normal follicles; E: normal primordial follicles; F: normal transitional follicles; G: normal primary follicles; H: normal secondary follicles; I: normal antral follicles.

Table 3
Values for morphometric variables (mean ± standard error) of follicles, oocytes, nuclei, and nucleoli of Santa Inês ewes genotyped for *FecG*^{E}.

Follicular stage	*FecG*^{E} Genotypes		
	+/+	+/E	E/E
	Follicle diamater (μm)		
Primordial	21.30 ± 0.64^{b}	20.30 ± 0.62^{b}	24.62 ± 1.50^{a}
Transitional	28.02 ± 1.53	24.92 ± 0.81	28.77 ± 4.1
Primary	40.31 ± 3.11	41.54 ± 2.80	42.55 ± 3.40
Secondary	118.26 ± 6.4^{b}	159.35 ± 12.65^{a}	82.56 ± 9.13^{b}
Antral	324.76 ± 44.79	241.21 ± 26.51	226.84 ± 37.59
	Oocyte diameter (μm)		
Primordial	15.76 ± 0.69	16.38 ± 0.76	18.51 ± 1.04
Transitional	19.95 ± 1.23	18.14 ± 0.72	19.99 ± 1.61
Primary	27.18 ± 1.41	26.54 ± 1.31	27.45 ± 1.47
Secondary	44.73 ± 2.16^{b}	65.88 ± 5.38^{a}	41.58 ± 4.81^{b}
Antral	65.17 ± 7.57	67.43 ± 6.26	64.48 ± 2.31
	Nucleus diameter (μm)		
Primordial	9.32 ± 0.84	9.77 ± 0.45	11.02 ± 0.48
Transitional	11.54 ± 0.71	10.44 ± 0.38	11.23 ± 0.57
Primary	14.42 ± 0.87	14.44 ± 0.95	13.23 ± 0.56
Secondary$^{(*)}$	17.58 ± 2.08	28.77 ± 2.23	–
Antral$^{(*)}$	36.83 ± 8.45	–	18.33 ± 2.17
	Nucleolus diameter (μm)		
Primordial	2.74 ± 0.34	2.73 ± 0.24	2.67 ± 0.17
Transitional	3.02 ± 0.21	2.60 ± 0.13	2.49 ± 0.17
Primary	4.07 ± 0.30^{a}	3.18 ± 0.18^{b}	3.01 ± 0.21^{ab}
Secondary	–	–	–
Antral	–	–	–

+/+: Homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; $^{(*)}$ Follicular stage with insufficient number of replicates for statistical analysis; (–) Structure not made visible; Different letters in the same row indicates a difference ($P < 0.05$).

genotype in comparison to the ewes without the mutation (*FecG*$^{+/+}$; Fig. 2).

In the secondary follicular category (Fig. 3), the diameters of the follicles and oocytes were the variables for whith there was the greatest correlation with the discriminant factor F1, which accounted for 89.73% of the variability between the groups E/E and +/+ compared to +/E. Furthermore, the diameter of the oocyte nucleus accounted for only 10.63% of the variability among the ewes with the mutation (*FecG*$^{E/E}$ and *FecG*$^{+/E}$) compared with those with the wild-type genotype (*FecG*$^{+/+}$; Fig. 3). In the other stages of follicular development, it was not possible to determine whether genotyped ewes had the *FecG*^{E} mutation because the centroids of the genotype groups were overlapping (Fig. 4).

The data for Mahalanobis distances between genotypes, based on the follicular development, are included in Table 4. Based on these distances, it was possible to ascertain the matrix of values "P" for Fisher distance for which there were significant results in the morphometric analyses of primordial and secondary follicles.

4. Discussion

Genotypic differences among Santa Inês *FecG*^{E} ewes are associated with greater or lesser rates of ovulation and prolificacy (Silva

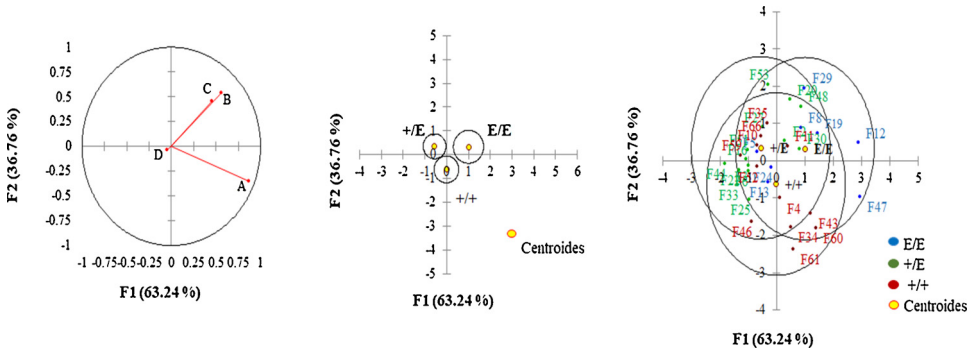

Fig. 2. Graphic representations of the discriminant analysis of the primordial follicle morphometry. +/+: homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; A: follicle diameter; B: oocyte diameter; C: oocyte nucleus diameter; D: oocyte nucleolus diameter.

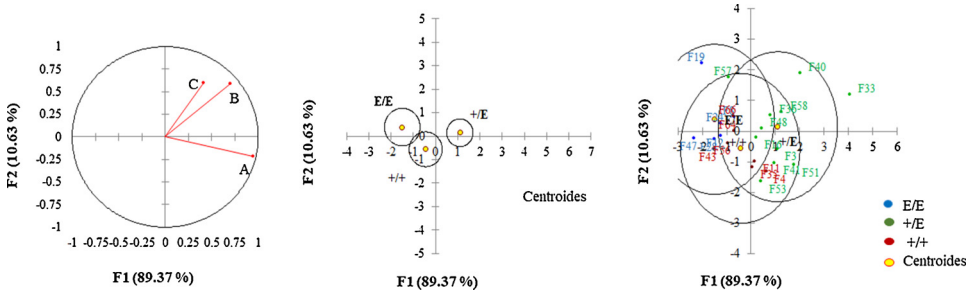

Fig. 3. Graphic representations of the discriminant analysis of the secondary follicle morphometry. +/+: homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; A: follicle diameter; B: oocyte diameter; C: oocyte nucleus diameter.

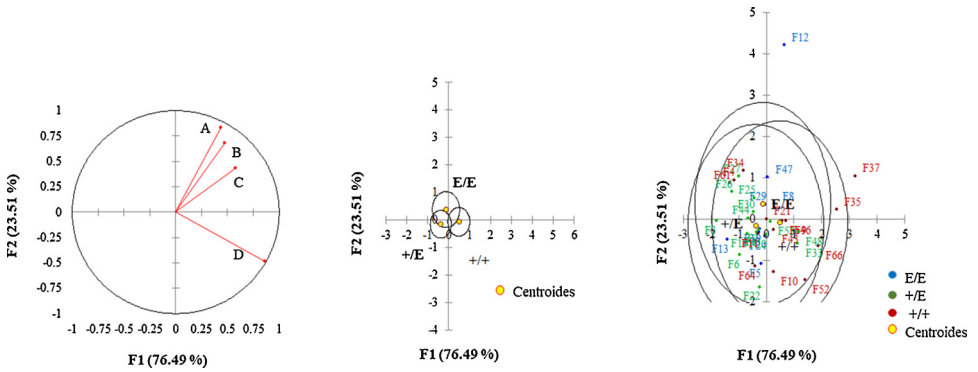

Fig. 4. Graphic representations of the discriminant analysis of the transitional follicle morphometry. +/+: homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; A: follicle diameter; B: oocyte diameter; C: oocyte nucleus diameter; D: oocyte nucleolus diameter.

Table 4
Mahalanobis distance between the genotypes based on the morphometric results of the follicular development stages.

Follicular stage	Genotype $FecG^E$		
	+/+ *vs* E/E	+/+ *vs* +/E	+/E *vs* E/E
Primordial	1.9	1.2	2.4*
Transitional	0.5	0.7	0.3
Primary	1.9	0.9	0.8
Secondary	2.0	2.8*	6.7*
Antral	0.9	0.7	0.06

+/+: Homozygous wild-type genotype; +/E: mutant heterozygous genotype; E/E: mutant homozygous genotype; (*) Significant difference ($P < 0.05$).

et al., 2011). The physiological mechanisms that result in these phenotypic differences resulting from the *FecG*^{E} mutation are not yet fully understood. In view of these aspects, it was hypothesised in the present study that the histological evaluations of the ovaries and follicles would allow for a precise determination of the genotypic groups of ewes with the mutation (*FecG*$^{E/E}$ and *FecG*$^{+/E}$) from those without the mutation (*FecG*$^{+/+}$).

The greater correlation between the total morphologically normal follicles and the morphologically normal primordial and transitional follicles is consistent with the fact that the greater quantity/quality of follicles present in the ovary occurs in the early stages of follicular development (Slot et al., 2006). Furthermore, the correlation between morphologically normal primordial and antral follicles that was ascertained in the present study is consistent with the findings of Gougeon et al. (1994) and Silva et al. (2014). In these previous studies, the primordial follicles were associated with the total number of antral follicles recruited during the wave in follicular development. Additionally, it has been reported that the relatively greater ovulation rate in prolific ewes is likely due to the greater number of recruited follicles (Gonzalez-Bulnes et al., 2004). Thus, in the present study it was hypothesised that the greater number of follicles in *FecG*$^{E/E}$ ewes from which ovulations occurred, as reported by Silva et al. (2011), is related to the increased recruitment of follicles.

Morphologically normal primordial and transitional follicles were the main variables that allowed for a more precise determination of ewes with the *FecG*^{E} mutation. This finding supports the hypothesis for the present study and may be, among other factors, a consequence of the GDF-9 functions on follicle viability by regulating the uptake of energetic substrates for oocytes and decreasing the granulosa cell apoptosis (Eppig, 2001; Juengel et al., 2002; Sugiura et al., 2005; Orisaka et al., 2006).

Follicular growth in the early stages (primordial, transitional and primary) of folliculogenesis is regulated by factors produced by oocytes (McNatty et al., 2001), which stimulate oocyte growth (Leitão et al., 2009). Thus, the larger diameter of the primordial follicles that was detected in the present study may be an outcome of an increased GDF-9 gene expression by ewes with the *FecG*$^{E/E}$ mutation in follicle oocytes during this developmental stage, thus, enhancing the initial growth of the oocytes. The smaller size of the secondary follicle of the ewes with *FecG*$^{E/E}$ and *FecG*$^{+/+}$ compared to those with the *FecG*$^{+/E}$ genotype may be related to the smaller diameter of the oocytes in the follicles of the secondary follicles. Furthermore, the difference observed in the nucleolus diameter of the primary follicle oocytes of ewes with the *FecG*$^{E/E}$ and *FecG*$^{+/+}$ compared with those of *FecG*$^{+/E}$ genotype may be related to the functional status of oocyte protein activities (Schwarzacher and Wachtler, 1983) independent of the mutation.

According to the Mahalanobis distance, it was possible to verify that the ewes have different reproductive characteristics when considering the stage of follicular development analysed independent of the genotype of ewes. Thus, physiological functions that are modified as a result of expression of the *FecG*^{E} gene does not negate the effects of growth factors and hormones on follicular development.

Although some follicular variables (normal morphology of primordial and transitional follicles, diameter of primordial and secondary follicle structures) allowed for more precise determination of the genotype groups, it was anticipated that other ovarian variables, such as initial antral follicle morphometry, could contribute to the variations in reproductive rate of these ewes. This hypothesis, however, was not supported by the findings in the present research. Likewise, the centroids of the genotype groups were very close, regardless of the variable evaluated. This overlap among the centroids increases the possibility of follicular characterisation errors of the groups analysed.

McLeod et al. (1997) were able to determine which Inverdale ewes were homozygous from those that were heterozygous based on gonadotropin concentrations. Similarly, Guo et al. (2018) determined which ewes had the Booroola gene by the metabolites present in the follicular fluid. Folliculogenesis occurs as a result of both intra- and extra-ovarian factors. Because the metabolites reflect the enzymatic activity of cumulus oocyte complexes, quantifying concentrations of hormones and/or metabolites in the follicular fluid will contribute to a greater capacity to determine which ewes have the various *FecG*^{E} genotypes.

5. Conclusion

Morphologically normal follicles in the primordial and transitional stages of development are the variables that allow for determining the genotypes of Santa Inês ewes with the *FecG*^{E} mutation. In addition, these variables may be utilised to make more efficient the adoption of biotechniques for uses of the follicles in the initial developmental stages with decisions about follicle use being determined with a physiological basis.

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