A new polymorphism in the Growth and Differentiation Factor 9 (GDF9) gene is associated with increased ovulation rate and prolificacy in homozygous sheep


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Summary

Brazilian Santa Inês (SI) sheep are very well-adapted to the tropical conditions of Brazil and are an important source of animal protein. A high rate of twin births was reported in some SI flocks. Growth and Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) are the first two genes expressed by the oocyte to be associated with an increased ovulation rate in sheep. All GDF9 and BMP15 variants characterized, until now, present the same phenotype: the heterozygote ewes have an increased ovulation rate and the mutated homozygotes are sterile. In this study, we have found a new allele of GDF9, named FecG\textsuperscript{E} (Embrapa), which leads to a substitution of a phenylalanine with a cysteine in a conservative position of the mature peptide. Homozygote ewes presenting the FecG\textsuperscript{E} allele have shown an increase in their ovulation rate (82%) and prolificacy (58%). This new phenotype can be very useful in better understanding the genetic control of follicular development; the mechanisms involved in the control of ovulation rate in mammals; and for the improvement of sheep production.

Keywords growth factor, Ovis aries, prolificacy.
screened for SNPs by DNA sequencing of PCR amplicons using the following primers: GDF9 (forward 5’-GGAGAAA ACGGACAAAGC; reverse 5’-ACGAGAGTACATGAGT); and BMP15 (forward 5’-GCGCTTTTCGTTGTTGTA; reverse 5’-GAGCAGCCATAGGTTAA) (see Appendix S1 for details). Seven (GI to GVII) single nucleotide polymorphisms (SNPs) were found in GDF9 (Table A1, additional data). Only the GVI polymorphism is a non-conservative change in position 345 (phenylalanine to cysteine), which was detected in 43% of the sequenced animals, and it is in the mature peptide of GDF9. This polymorphism provokes a change in a residue which is 100% conserved in the sequence of four representative mammalian species (Figure A1 (GenBank FJ429111)) according to the previous nomenclature for the FecG+ (Hanrahan et al. 2004).

To find the frequency of the FecG+ polymorphism, a total of 334 animals from a separate flock (Appendix S1) that had not been selected for prolificacy have had their genotypes identified by a PCR-RFLP strategy (Appendix S1). All data about the parturition of these ewes during the period of 2002 to 2008 were collected, and the association between the number of lamb births and genotypes was tested. The genotype distribution and allele frequency were analysed by the Chi-square test. A difference (P < 0.001) in the frequency of FecG+ and FecG++ alleles, as well as in the genotypic distribution, was observed between the randomly selected and the prolificacy-selected flocks (Table 1).

To investigate the association between the FecG+ genotypes (E/E, +/E, and +/+ ) and the ovulation rate, 39 ewes (15 FecG+/+, 15 FecG+E/ and 9 FecG+E/E) were selected from the genotyped flocks and submitted to oestrus synchronization. The animals were oestrus synchronized twice, with eCG and PGF2alpha-based protocols in a cross-over design (Appendix S1). Eleven days after the last oestrus detection, laparoscopy was performed as previously described (Killen & Caffery 1982) to infer ovulation rate by counting corpora lutea (CL). At the end of the breeding season, pregnancy status was evaluated by ultrasound. All animals submitted to laparoscopy had their GDF9 and BMP15 exons 2 sequenced to confirm the FecG+ genotyping and to verify that there was no other characterized polymorphism associated with ovulation rate. The animals were handled in accordance with pertinent Brazilian legislation and following Embrapa’s procedures for animal care.

The CL number and the number of lamb births were fitted to the GLM (Generalized Linear Model), where the Poisson distribution was attributed to the ovulation rate, pregnancy and lambing data. In this analysis, the lamb count was considered as response variable, measured for each animal in seven different breeding seasons from 2002 to 2008 (time variable). To measure the influence of genotype over the offspring number through the time, a generalized linear mixed model (GLMM, SAS software) was applied. The offspring number yij of the fth animal at the jth time was modelled as the per following model:

\[
y_{ij} | \gamma_j \sim \text{Poisson}(\mu_j)
\]

\[
\gamma_j \sim N(0, \sigma^2)
\]

\[
\mu_j = \exp(\beta_0 + \beta_1 X_1 j + \beta_2 X_2 j + \beta_3 Z_0 + \gamma_j)
\]

Var(yij | \gamma_j) = \sigma^2 \mu_j,

where yij follows the Poisson distribution conditioned to the random effect for animal \(\gamma_j\), which was assumed to be normally distributed with variance \(\sigma^2\). The expected mean \(\mu_j\) is a non-linear function of the effects of genotype (\(\beta_1 \text{ and } \beta_2\)), time when the counting was made (\(\beta_3\)) and the random effect because of each animal. The variance for \(y_{ij}\) irrespective of the random effect \(\gamma_j\) is Var(\(y_{ij}\)) = \(\sigma^2 \mu_j\), where the extra (or sub) variation is taken into account. The estimate for \(\sigma^2\) is 0.1696 (standard error = 0.0087), indicating a strong under-dispersion, but this is correctly modelled by GLMM (see Appendix S1, SAS output in additional data).

The parturition data of the 334 genotyped ewes showed a difference (P < 0.0001) in the prolificacy amongst the groups (Table 2). Regarding the ovulation rate, it was greater (P < 0.001) in the homoygote (E/E) group, which showed an 82% increase in CL average (2.22 ± 0.12, Fig. 1a), as well as the highest frequency (96.3%) of multiple-ovulating ewes (Fig. 1b), when compared with +/E and +/+ groups. The heterozygote group (+/E) presented no difference (P = 0.612) in CL average (1.34 ± 0.08) or in the frequency (31.8%) of ewes with multiple ovulations (Fig. 1a and b), when compared with the wild-type ewes (1.22 ± 0.11 and 14.6%)

### Table 1 Genotypic and allelic frequencies of FecG+ in Santa Inês flocks.

<table>
<thead>
<tr>
<th>SI Flock</th>
<th>Genotype</th>
<th>Frequency</th>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(N)</td>
<td></td>
<td>(N)</td>
</tr>
<tr>
<td>Prolific-selected</td>
<td>+/+</td>
<td>0.174 (4)</td>
<td>FecG+</td>
<td>0.478 (22)</td>
</tr>
<tr>
<td></td>
<td>+/E</td>
<td>0.609 (14)</td>
<td>FecG+</td>
<td>0.522 (24)</td>
</tr>
<tr>
<td></td>
<td>E/E</td>
<td>0.217 (5)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Randomly selected</td>
<td>+/+</td>
<td>0.656 (219)</td>
<td>FecG+</td>
<td>0.808 (540)</td>
</tr>
<tr>
<td></td>
<td>+/E</td>
<td>0.305 (102)</td>
<td>FecG+</td>
<td>0.192 (128)</td>
</tr>
<tr>
<td></td>
<td>E/E</td>
<td>0.0389 (13)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Distinct letters are different (P < 0.001) for genotype distribution.

### Table 2 The effect of of FecG+ in Santa Inês prolificacy.

<table>
<thead>
<tr>
<th>SI Flock (F1)</th>
<th>Genotype</th>
<th>Prolificacy of F1 (mean; [95% CI])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomly selected</td>
<td>+/+</td>
<td>1.13; [1.11, 1.16]*</td>
</tr>
<tr>
<td></td>
<td>+/E</td>
<td>1.44; [1.41, 1.48]b</td>
</tr>
<tr>
<td></td>
<td>E/E</td>
<td>1.78; [1.69, 1.87]c</td>
</tr>
</tbody>
</table>

Distinct letters are different (P < 0.001).

Non-selected SI; N = 334 ewes (219 +/+; 102 +/E and 13 E/E), called F1.

Prolificacy = mean of 764 offspring (F2) from the 334 genotyped ewes (F1); separated according to their genotype category.
respectively). We observed a genotype effect on the number of twins per ewe \((P=0.0136)\); E/E ewes showed 44% of twin-pregnancy, while no twin-pregnancy was observed in \(+/+\) ewes (Fig. 1c). Moreover, the E/E ewes presented no observable effect of \(\text{FecGE}\) other than the increased ovulation rate and twinning.

It has been suggested that increasing multiple births may be an efficient way to improve meat production per ewe, and an increase of 50% in total weight weaned per ewe lambing twins has been reported (Rajab et al. 1992). The increase of one extra CL and 58% more lambs born observed in E/E ewes compared with \(+/+\) was a strong evidence of the \(\text{FecGE}\) effect on ovulation rate control and prolificacy, and represents a new phenotype for \(\text{GDF9}\) in sheep. Our parturition data point to an additive effect for the \(\text{FecGE}\) allele, despite no difference being observed in the ovulation rate between \(+/+\) and E/E ewes. However, the allele interactions of \(\text{FecGE}\) are certainly distinct from the over-dominant behaviour observed in \(\text{FecGH}\) and all \(\text{FecX}\) alleles described until now. The E/E pregnancy and parturition data confirm that their oocytes were viable and fertile; which correlate with the increased prolificacy (number of lambs/ewe) observed amongst these animals. In this study, for the first time, a new SNP that increased the ovulation rate and prolificacy of homozygote sheep was documented for the \(\text{GDF9}\) gene. This new genetic variant, together with the other documented variants in \(\text{GDF9}\) and \(\text{BMP15}\), can be very useful to obtain a better understanding of the genetic control of ovulation rate in mammals. Moreover, this major gene variant can be applied in breeding programmes by gene-assisted selection (GAS), aiming towards the improvement of sheep reproductive potential and production. However, further investigation is necessary to shed light on the allelic interactions of the \(\text{FecGE}\) variant.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Appendix S1** Additional data, materials and methods.

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