Effects of equine chorionic gonadotropin and GDF9 gene on the reproductive performance of Katahdin sheep

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ABSTRACT

Objective. To determine the influence of equine chorionic gonadotropin (eCG) application and the presence of exon 2 of the GDF9 gene on reproductive variables in Katahdin sheep in the transition period. Materials and methods. 63 sheep with average age and weight of 2.83±0.89 years and 45.32±5.44 kg, respectively, were used. A 2x2 factorial arrangement under a completely randomized design was used, where the factors and levels were: the absence or presence of exon 2 of the GDF9 gene and the application or not of the eCG hormone in the synchronization protocol. Results. The application of eCG showed a significant favorable effect on the reproductive behavior of sheep, but not for the presence of exon 2 of the GDF9 gene. Treatments T2 and T4 were superior in the variables % of gestation, % of lambing, fertility and prolificacy with respect to T1 and T3. The best reproductive response was obtained with the application of eCG regardless of the presence or absence of the gene. Conclusions. This study shows evidence of the null interaction of exon 2 of the GDF9 gene and eCG to improve the reproductive performance of Kathadin sheep. The application of eCG is recommended in estrus synchronization protocols for Kathadin sheep in the transition period. Faced with the few genomic reports on Katahdin ewes, evidence is shown of the need to carry out other research on genetic variants and their relationship with reproduction in sheep.

Keywords: Estrus; exon; fertility; gene; gonadotropin; sheep; prolificacy (Source: USDA, ICYT animal biology).

RESUMEN

Objetivo. Determinar la influencia de la aplicación de gonadotropina coriónica equina (eCG) y la presencia del exón 2 del gen GDF9 en variables reproductivas en ovejas de la raza Katahdín en época de transición. Materiales y métodos. Se utilizaron 63 ovejas con edad y peso promedio de
INTRODUCTION

Sheep production is critical for the meat industry in central and southeastern Mexico. Although the quantity of sheep meat produced has increased in recent years, it has not covered domestic demand; therefore, it is necessary to import meat from other countries such as New Zealand and the United States (1,2). Sheep farming is a vital source of resources and food for low-income producers with small plots, low-quality fodder (3,4), fruit orchards, and forestry activities (2). Nutritional, genetic, and reproductive factors are critical to efficient animal production. In particular, proper reproductive management with timing estrus protocols is indispensable for obtaining outstanding calving and weaning rates (5). In sheep, exogenous hormone use in synchronization protocols allows control of estrus presence and the pregnancy rate in the reproductive and non-reproductive periods.

Progesterone (P4) impregnated in an intravaginal controlled internal drug release (CIDR) device is used for the synchronization of estrus, supported by the application of prostaglandins to cause lysis of the corpus luteum in cyclic sheep (5). In addition, to obtain greater prolificacy (6), the protocols are complemented by the application of eCG, which promotes follicular growth and development and steroidogenic activity (7). In addition, treatment with gonadotropin-releasing hormone (GnRH) at the end of the protocols improves ovulation and pregnancy rates (5,8).

In addition to synchronization protocols in sheep, new biotechnologies have emerged to improve reproductive behavior. The development of molecular biology and genomics tools has been key to the genetic improvement of reproductive characteristics in sheep (9). In particular, researchers have been interested in reproductive advancement in Katahdin sheep, standing out for their reproductive qualities similar to those of the Pelibuey breed, such as maternal ability and good prolificacy (10). The fertility rate of 1.8 births per year and the adequate production of milk to nurse their young, at least up to three lambs, are characteristics of the Katahdin breed (3).

Genes and mutations associated with sheep prolificacy and fecundity have also been identified (11). These are called fertility genes: growth and differentiation factor 9 (GDF9, composed of exons 1 and 2 and an intron), bone morphogenetic protein-15 (BMP15), and bone morphogenetic protein-1B receptor (BMPR1B), which belong to the gene family of β-type transforming growth factor (12,13). In particular, the GDF9 gene (14) has been reported to have a direct effect on hypothalamic-adenohypophyseal-ovarian axis functions, proliferation, and organization of teak cells (15), folliculogenesis, and the proliferation and differentiation of granulosa cells (16). Five genetic variants or mutations, including dominant heterozygous, recessive, or wild-type alleles, have been identified in exon 2 of the GDF9 gene in wool sheep, such as high fertility (FecG\(^\text{H}\)) (12), Icelandic Thoka (FecG\(^\text{I}\)) (17), Vecaria (FecG\(^\text{V}\)) (18), and Finnsheep (FecG\(^\text{WS}\) or FecG\(^\text{W}\)) (19,20), and hair breed, Santa Inés (Embrapa or FecG\(^\text{E}\)) (21), affecting reproductive variables. In particular, the genotype GG of variant Embrapa or FecG\(^\text{E}\) in exon 2 has been reported to considerably affect the ovulation rate and the prolificacy of hair sheep (21,22).
Knowledge of the influence and evaluation of fertility genes promotes the application of genetic improvement strategies in sheep and the eventual improvement of ovulation rate, lambing, and prolificacy rates. Therefore, the objective of this study was to determine the influence of the application of eCG and the presence of exon 2 of the GDF9 gene on synchronization and return to estrus, pregnancy and lambing percentage, prolificacy, and fecundity rate in Katahdin sheep during the transition from the reproductive season to anestrus period.

**MATERIALS AND METHODS**

**Location.** The study was conducted from February to August 2020 in the town of Zempoala, Hidalgo, Mexico, with coordinates 19°54′56″ N, 98°40′12″ W, and an altitude of 2500 m (23). A place with a temperate-cold climate, an annual average temperature and precipitation of 14.3°C and 494 mm, respectively, and a rainy period from June–September (24). The laboratory phase was performed in the facilities of the Genetics Laboratory, located in the Colegio de Postgraduados, Campus Montecillo, Texcoco, Mexico.

**Animals.** We used 63 sheep of the Katahdin breed with an average age of 2.83±0.89 years, with records of 1.97±0.88 consecutive average births, and an average weight of 45.32±5.44 kg. We selected 32 sheep carrying exon 2 of the GDF9 gene and 31 non-carrier sheep. The selected sheep were healthy, with a body condition ranging from 3–3.5, on a scale of 1–5 (25), and were dewormed intramuscularly with vitamin levamisole (L-Vermizol®, Laboratorio Aranda, Mexico) at 1 mL 24 h, along with vitamin levamisole (L-Vermizol®, Laboratorio Aranda, Mexico) at 72 h, in concentrate (Borrega Plus®, Laboratorio Aranda, Mexico) at 1 mL 24 h, as well as saline water. They were kept in different pens based on the treatment, fed isonitrogenous diets containing 9.5% raw protein per day and 419 KJ kg⁻¹ dosage. They were kept in different pens based on the treatment, fed isonitrogenous diets containing 9.5% raw protein per day and 419 KJ kg⁻¹ PC⁺ (26), plus flushing with 0.32 kg concentrate (Borrega Plus®, 15% raw protein and 2.4 Mcal per kg) per sheep for 24 d, 15 d before and 9 d after synchronization, and ad libitum access to clean water.

**Blood sample collection.** For the identification of exon 2 of the GDF9 gene, 3 mL of blood was collected from each sheep by jugular vein puncture with sterile syringes, according to the criteria of the Official Mexican Standard NOM-062-ZOO-1999 (27) on technical specifications for the product care and use of laboratory animals, following regulations for the use and care of research animals, approved by the General Academic Council of the College of Postgraduates, Mexico (28). The collected blood (0.5 mL) was placed on Whatman FTA® FTA® (Flinders Technology Associates; WB 120055, GE Healthcare®, United Kingdom). The cards were dried in the shade and kept at room temperature following the manufacturer’s specifications until processing in the laboratory.

**DNA purification and amplification of the GDF9 gene.** For DNA purification, cuts of approximately 1 mm² of the blood-impregnated Whatman FTA® MiniCard were made and deposited in a 0.2 mL Eppendorf tube, followed by three 200 µL washes with FTA (GE Healthcare®) purification reagent at room temperature for 5 min each. Then, two washes were performed with 200 µL of Tris (hydroxymethyl aminomethane)-EDTA (ethylenedinitrotetraacetate acid) or TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer at room temperature, for 5 min. The reaction mixture for polymerase chain reaction (PCR) was prepared with a final volume of 12.5 µL, with 6.25 µL of GoTaq® Colorless Master Mix 2X (Promega® Madison, WI, USA), 0.625 µL of each primer [GDF9; exon 2: For-5'-GGAGAAAAAGGAGCAGAACG-3’ and Rev-5‘-ACGACAGGTACATTAGT-3’ (21); 10 µM; IDT®, Illinois, EUA] and 5 µL of trehalose dihydrate 10% (nuclease-free water and proteases, Merck® KGaA 64271, Darmstadt, Germany).

Amplification of exon 2 of the GDF9 gene was performed using PCR technique. Tubes with processed MiniCard fragments and reaction mixtures were placed in a thermostyler (GeneAmp® PCR System 9700 Applied Biosystems, California, USA). The amplification conditions were as follows: GDF9, exon 2 initial denaturation, 93°C, 3 min, plus 35 cycles (denaturation, 93°C, 40 s; alignment, 56°C, 40 s; extension, 72°C, 40 s), and final extension at 72°C for 5 min. Of the PCR products or amplicons, 3 µL was mixed with a 3 µL Loading Dye 6x load damper. For the size assessment of the amplicons, a molecular marker ΦX174 DNA-Hae III (New England BioLabs, MA, USA) was used. Subsequently, this mixture of amplicons and dye was transferred to a 1% agarose gel at 90 V for 40 min to visualize the size of the fragment (1034 bp) of exon 2 in the GDF9 gene. The fragments were visualized under an ultraviolet light transilluminator (MiniBis Pro, DNR Bio-Imaging Systems, Neve Yamin, Israel) using GelCapture software.
**Treatments.** Sheep were distributed randomly to each of the four treatment groups: T1 (n=15), sheep not carrying the exon 2 of the GDF9 gene and not treated with eCG (Control, 0/0); T2 (n=16), sheep without the presence of the exon 2 of the GDF9 gene and treated with eCG (0/1); T3 (n=16), sheep carrying the exon 2 of the GDF9 gene and not treated with eCG (1/0); and T4 (n=16) sheep carrying the gene and treated with eCG (1/1; Figure 1).

**Estrus synchronization.** The estrus synchronization was initiated in sheep with a progesterone-based intravaginal CIDR device (0.35 g CIDR®, Pfizer) application on day −9 before device removal. On day −2, 1 mL of analog prostaglandin F₂α (250 mcg of Celosil, Schering-Plough Veterinary) and 1.75 mL of equine chorionic gonadotropin (eCG) and gonadotropin-releasing hormone (GnRH) were administered intramuscularly, according to the treatment assigned (Figure 1). Withdrawal of the CIDR was considered as day 0. All sheep received 1 mL of gonadotropin (50 μg, GnRH, Sanfer) 24 h after withdrawal of the CIDR.

**Estrus detection followed by artificial insemination.** Estrus was detected based on the presence of four male markers using rams with aprons as teasers, introduced every 6 h (with sessions of 30 min) after the removal of the CIDR device, and continued for 72 h, with the recording of hours passed after the withdrawal of the CIDR device to the beginning of estrus. Artificial insemination was performed intrauterine in sheep that presented estrus with fresh semen and doses of 100 million sperm using laparoscopy. All inseminations were performed 12 h after the female presented with estrus by accepting the male mount without coitus.

**Pregnancy diagnosis.** Pregnancy diagnosis was made at 45 d post-insemination, using 6–8 MHz real-time ultrasound with a rectal transducer (Universal Medical Systems, Inc. UMS 900). The number of pregnant ewes was recorded for the number of inseminated sheep and monitored up to the delivery time to record the number of sheep that reached the calving stage.

**Prolificacy and fertility.** Deliveries were attended by recording the number of lambs born per lamb and the total number of sheep per treatment to determine prolificacy and fecundity variables, respectively. The proportions were obtained as a measure of the variables.

**Statistical analyses.** The sheep were assigned to the pens using a 2 × 2 factorial array under a completely randomized experimental design where the factors and levels were the absence or presence of the exon 2 of the GDF9 gene and the application or lack of the application of eCG hormone in the estrus synchronization protocol, respectively. Statistical analyses were performed using software R, version 4.1.3 (29). The variable hours at the beginning of estrus were analyzed using the `aov` function of the agricolae package (30) in the following statistical model:

\[
Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}
\]

for \(i=2; j=2; k=16\), where \(Y_{ijk}\) represents the value of the variable hours at the beginning of estrus, \(\mu\) is the general mean, \(A_i\) is the effect of the level of (presence or absence of the gene), \(B_j\) is the effect of the level of (application or not of eCG), \(AB_{ij}\) corresponds to the effect of the interaction of the two factors and \(e_{ijk}\) is the random error that obeys

Using the model, we determined the significance of each factor and its interaction at the beginning of estrus. Subsequently, Tukey’s means were compared using Tukey’s HSD function of the `stats` package (29) (\(p<0.05\)). For hours at the beginning of estrus, a graph of survival curves was constructed using the `survfit` (survival) (31) and `ggsurvplot` (survminer) (32) functions, which showed the probability of time in hours for the onset of estrus once the CIDR was removed.
Using logistic regression and a \( X^2 \) test, the variable response, presence of estrus, return to estrus, pregnancy percentage (%), and lambing percentage (%) variables were analyzed with a binomial model. Fertility and prolificacy were analyzed with the Gaussian model, using the functions \( glm \) and \( anova \) of stats (29), to determine the statistical significance of the explanatory variables of the presence or absence of the GDF9 gene and application of eCG in the response variables.

The presence and return to estrus, pregnancy percentage, and lambing percentage variables were analyzed using the \( prop.test \) function of the \texttt{stats} package (29) to determine the significance (\( p<0.05 \)) of the difference between treatments. For fecundity and prolificacy, means were compared using the \textit{bootstrapping} method (29).

**RESULTS**

**Effects of the GDF9 gene and eCG hormone application on the reproductive behavior of Katahdin sheep.** Table 1 shows statistical evidence of the significant impact of eCG application on the reproductive behavior of Katahdin sheep (\( p<0.05 \)) but not for the presence of exon 2 of the GDF9 gene (\( p>0.05 \)). However, there was no interaction between \( GDF9 \times eCG \) factors (\( p>0.05 \)). For the return to estrus, there was no significant effect on any of the studied factors.

**Table 1.** The \( p \)-values for fully randomized 2 x 2 factorial ANOVA\(^1\) statistical tests and logistic regression (binomial models\(^2\) and gaussiano\(^3\)) of the effects of the GDF9 gene, the eCG hormone, and the \( GDF9 \times eCG \) interaction in the reproductive variables of Katahdin sheep.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( GDF9 )</th>
<th>eCG</th>
<th>( GDF9 \times eCG )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours at the start of estrus(^1)</td>
<td>0.62 ( 2.63 \times 10^{-9} )</td>
<td>0.45</td>
<td>0.97</td>
</tr>
<tr>
<td>Presence of estrus(^2)</td>
<td>0.90 ( 3.78 \times 10^{-12} )</td>
<td>0.31</td>
<td>0.14</td>
</tr>
<tr>
<td>Return to estrus(^2)</td>
<td>0.36</td>
<td>0.19</td>
<td>0.02</td>
</tr>
<tr>
<td>Fecundity</td>
<td>0.19</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>Prolificacy</td>
<td>0.51</td>
<td>SC</td>
<td>SC</td>
</tr>
<tr>
<td>% of pregnancy(^2)</td>
<td>0.72</td>
<td>0.72</td>
<td>0.00</td>
</tr>
<tr>
<td>% of lambing(^2)</td>
<td>0.37</td>
<td>0.37</td>
<td>0.99</td>
</tr>
</tbody>
</table>

The significance of \( p \)-values\(^*\) was ascertained at \( p<0.05 \). SC=\( p \)-values not calculated because of a lack of observations at one of the factor levels.

**Variables associated with estrus.** Treatments with eCG resulted in better reproductive behavior associated with estrus. Table 2 shows that for the presence of estrus, T2 and T4 (both with eCG application) were the best (\( p<0.05 \)), more than 12 times higher than T1 and T3, which shows evidence of their superiority. For hours at the beginning of estrus, T2 and T4 showed superiority over T1 and T3. Although T1 showed evidence of a shorter time for this variable, the standard error suggests that this value was measured from a single observation; therefore, the result is not considered definitive. A similar situation occurs with return to estrus, where T1 showed ideal values; however, the average is the result of a single observation, so it is also not a conclusive result. Finally, for return to estrus, T2 (with eCG) presented the lowest percentage (without considering T1), indicating the superiority of this treatment compared to the other treatments for this variable.

**Table 2.** Percentages and averages (\( \pm \) standard error) by treatment of reproductive variables associated with Katahdin sheep estrus.

<table>
<thead>
<tr>
<th>( GDF9 )/eCG treatments</th>
<th>Estrus presence at 72h (%)</th>
<th>Hours at start of estrus (h)</th>
<th>Return to estrus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 0/0</td>
<td>6.67±6.67(^a)</td>
<td>18.00±0.00(^a)</td>
<td>0.00±0.00(^a)</td>
</tr>
<tr>
<td>T2 0/1</td>
<td>87.50±8.54(^a)</td>
<td>24.86±3.13(^b)</td>
<td>14.29±9.71(^b)</td>
</tr>
<tr>
<td>T3 1/0</td>
<td>6.25±6.2(^b)</td>
<td>36.00±0.00(^c)</td>
<td>100.00±0.00(^c)</td>
</tr>
<tr>
<td>T4 1/1</td>
<td>87.50±8.54(^a)</td>
<td>21.43±2.65(^b)</td>
<td>21.43±11.38(^b)</td>
</tr>
</tbody>
</table>

\( T1 = \) no gene and no eCG (Control, 0/0); \( T2 = \) without gene and with eCG (0/1); \( T3 = \) with gene and without eCG (1/0); and \( T4 = \) with gene and with eCG (1/1). Values with different superscripts (\(^a\), \(^b\), \(^c\)) per column indicate significant differences (\( p<0.05 \)).

As shown in Figure 2, there were no differences between the probabilities of the hours at the beginning of estrus between the four treatments (\( p>0.05 \)) due to the great variation in risk values between treatments. However, eCG treatments showed fewer hours at the onset of estrus; that is, for eCG treatments (T2; 0/1 and T4; 1/1), estrus is more likely to occur before 18 h after CIDR has been withdrawn, whereas, for treatments without eCG (T1; 0/0 and T3; 1/0), estrus is more likely to occur after 15 h (Figure 2).
Probability curve with risk value for hours at the onset of estrus by treatment (Trat) in Katahdin sheep, constructed from Kaplan–Meier survival estimators.

**Figure 2.**

**Pregnancy and lambing percentages.** There was evidence of the superiority of T2 over the other treatments for fertility variables (Table 3). At T1 and T3, there were no pregnant or lambing ewes. Meanwhile, T2 was significantly higher than T4 for pregnancy and lambing percentage variables (p<0.05).

**Table 3.** Percentages (± standard error) of pregnancy and lambing by treatment in Katahdin sheep.

<table>
<thead>
<tr>
<th>GDF9/eCG treatment</th>
<th>Pregnancy (%)</th>
<th>Lambing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 0/0</td>
<td>0.00±0.00c</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>T2 0/1</td>
<td>62.50±12.50a</td>
<td>75.00±1.18a</td>
</tr>
<tr>
<td>T3 1/0</td>
<td>0.00±0.00c</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>T4 1/1</td>
<td>56.25±12.81b</td>
<td>56.25±12.81b</td>
</tr>
</tbody>
</table>

T1= no gene and no eCG (Control, 0/0); T2= without gene and with eCG (0/1); T3= with gene and without eCG (1/0); and T4= with gene and with eCG (1/1). Values within columns with different superscripts (a, b, c) indicate significant differences (p<0.05).

**Prolificacy and fecundity rates.** As in the previous sections, T2 and T4 (with eCG) stood out for their high values (Table 4). There was evidence of the superiority of T2 and T4 over T1 and T3 in fecundity and prolificacy (p<0.05).

Although T2 is greater than T4 by 0.38 and 0.14 for fecundity and prolificacy, respectively; there are no significant differences between treatments (p>0.05).

**Table 4.** Rates (± standard error) of fecundity and prolificacy by treatment in Katahdin sheep.

<table>
<thead>
<tr>
<th>GDF9/eCG treatment</th>
<th>Fecundity</th>
<th>Prolificacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 0/0</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
</tr>
<tr>
<td>T2 0/1</td>
<td>1.19±0.21a</td>
<td>1.58±0.13a</td>
</tr>
<tr>
<td>T3 1/0</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
</tr>
<tr>
<td>T4 1/1</td>
<td>0.81±0.23a</td>
<td>1.44±0.18a</td>
</tr>
</tbody>
</table>

T1= no gene and no eCG (Control, 0/0); T2= without gene and with eCG (0/1); T3= with gene and without eCG (1/0); and T4= with gene and with eCG (1/1). Values within columns with different superscripts (a, b) indicate significant differences (p<0.05).

**DISCUSSION**

Although recent studies have shown an effect on the reproductive behavior of some variants of exon 2 of the GDF9 gene in Luzhong, Rambouillet (FecG B), and African Rahmani (FecG H) sheep (33) and a significant effect on ovulation rate and prolificacy in Pelibuey sheep carrying the mutated variant Embrapa (GG) compared to the wild group (AA) and non-gene group (sG) (22) and in ovulation rate with the mutated group or Embrapa (GG) in Santa Inés sheep (21), for the present study the effects of exon 2 of the gene GDF9 in Katahdin sheep were not significant. These results are similar to those reported in Santa Inés sheep (34), where they used eCG in sheep with the presence of the FecG E allele in exon 2 of the GDF9 gene, and the authors reported no improvement in embryo production in superovulation protocols.

The null significance of the GDF9 gene factor may be due to the existence of intragenic regions independent of exon 2, which negatively affects fertility and was not characterized in this study. Studying the complete GDF9 gene in Cambridge and Belclare sheep, sterile sheep were reported to have homozygous genotypes associated with sterility in the polymorphic region G8 (12). Mutations present in exon 2 of the GDF9 gene positively affect reproductive behavior in some breeds of sheep, such as high fertility (12), Icelandic Thoka (17), Vecaria (18), Finnsheep (19,20), and Embrapa or Santa Inés (21), have not been reported in Katahdin sheep. The exclusive analysis for exon 2, without the determination of the genetic variants indicated...
above, could cause a bias in the estimates since the sequence and polymorphisms negatively associated with reproductive variables for the breed, such as the G8 polymorphism, are not known (12). In addition, this study did not characterize the sheep population for exon 1, which has also been reported to have favorable effects on prolificacy (11).

There is a possibility of intergenic interaction or epistasis as one of the causes of the absence of significant effects of exon 2 of GDF9 in the reproductive variables of Katahdin sheep. In vitro studies using cluster cells showed evidence of the association of the GDF9 gene with BMP15 and BMPR1B genes in the regulation of prolificacy in ruminants (35,36). Studies have reported high levels of GDF9 and BMPR1B in the ovarian tissue of Small Tail Han sheep (with deliveries of two or more lambs); whereas BMP15 levels in these conditions were lower (p<0.01). Conversely, the expression of BMP15 was higher (p<0.01) than that of GDF9 and BMPR1B in Sunite sheep with single lamb deliveries (14). Genetic improvement of reproductive behavior in sheep is complex; in this particular study, it may depend on polymorphisms or genetic variants present in the intragenic region of the entire GDF9 gene and not only exon 2, in addition to intergenic interaction and differentiated mutations between breeds.

Treatments that included eCG application had greater results in reproductive variables than treatments that only included the GDF9 gene and the control treatment. When applying eCG in short and long treatments of up to 14 d, it has been observed that it shortens the manifestation of estrus with good ovulation quality compared to treatments that only use CIDR (progesterone) for more than 5 d (37). Better results were also obtained for other reproductive variables, mainly those related to the stimulation of follicular growth in superovulation and embryo transfer programs (38), and the development of oocytes and follicles in vitro (7). In general, a higher dose of eCG corresponds to a better response in the following variables: hours of presence and response to estrus, increase in the rate of pregnancy, and prolificacy (6).

The eCG hormone is a viable alternative to the use of follicle-stimulating hormone (FSH), as it has a long half-life and increases multiple ovulations with a higher yield of follicular growth, participates in the formation of antilogs, improves steroidogenic activity, and may cause the better incidence of competent meiotic oocytes (7). In contrast, in Katahdin sheep in Mexico, possible disadvantages have been reported in the combination of FSH with decreasing doses of eCG, which generates a greater number of oocytes that become degenerated embryos in the non-reproductive period (39). In the present study, a dose of 350 IU of eCG improved the efficacy of Katahdin sheep. Prolificacy in T2 (1.58±0.13) and T4 (1.44±0.18) was similar to the reported prolificacy range in hair sheep from 1.2–2.3, to which eCG 250 and 400 UI were applied, respectively (6). Therefore, to increase the number of lambs in the flock, a minimum application of 350 IU of eCG in estrus synchronization protocols in Katahdin sheep during the transition period is recommended.

In contrast, treatments that were not administered eCG (T1 and T3) resulted in zero reproductive yields. Such a low response was not expected for groups without eCG application, as in other studies without hormonal treatment, reproductive behavior was higher (40). This negative response may have been due to the quality of the supplement or flushing, and probably to the transitional period. In a study with Katahdin sheep (41), palm almond flour was offered as a supplement during the anestrus period; the authors obtained better results than in the present study with a prolificacy of 1.0±0.2 against 0.0±0.0 of the present study. This may have been due to the caloric difference between supplements offered, since in this study a commercial product was offered with 2.4 Mcal kg⁻¹, in contrast to palm almond flour with 3.3 Mcal kg⁻¹.

The caloric deficiency of the commercial product and forage may have resulted in low levels of nutrients during the follicular phase. It is reported that increased blood nutrients through short-term supplementation stimulate folliculogenesis and ovulation by increasing FSH levels and decreasing estradiol levels; this effect is associated with the intramolecular metabolic system of insulin, glucose, insulin-like growth factor (IGF), and leptin at the ovarian level (42).

In dairy Sardinian sheep in the anestrus terminal phase, supplemented for four days with a mixture of glycerol and propylene glycol (substances that generate increased blood glucose), there were no differences in prolificacy and fecundity compared to a group of sheep of the same breed, to which 200 IU of eCG was
applied (43). Therefore, in the present study, blood nutrient thresholds may not have been reached, limiting reproductive performance in Katahdin sheep in the T1 and T3 treatments. An adequate caloric level during flushing in the non-reproductive season or transitional period can trigger the rupture of the shallow anestrus in Katahdin sheep to overcome the hormonal effects of long days, such as low concentrations and hormonal frequencies of LH and FSH at the systemic level as follicular, thus manifesting estrus derived from follicular maturation, starting the estrus cycle (44,45). Katahdin sheep have reproductive parameters similar to those of Pelibuey sheep (10). Both breeds in Mexico have little marked anestrus from January to June (41,46), with better estrus activity in summer and autumn (47). Despite the little marked anestrus in the Katahdin breed, the sheep (T1 and T3) of the present study in the transition period presented deficient reproductive parameters, possibly attributable to the low energy level of the supplement, compared to other studies with similar conditions (41). It has been reported that nutrition is a non-genetic factor that significantly affects the prolificacy of sheep from birth to the reproductive season (48,49). Therefore, it is important to identify the genetic factors of the flock to design strategies that improve reproductive performance through their interaction with environmental factors to achieve genetic improvement in sheep (50,51).

The present study provides evidence of the null interaction of exon 2 of the GDF9 gene and eCG to improve the reproductive performance of Katahdin sheep for the following variables: synchronization and return to estrus, % of pregnancy and lambing, prolificacy, and fecundity. These results are not definitive, as the additive, dominant, and epistatic effects of the genes associated with GDF9 in the breed have been little studied. For the GDF9 gene in the Katahdin breed, extensive research on gene characteristics (genotypes and polymorphisms) and molecular characterization of its interactions with other genes are needed. However, regardless of the null effect of the GDF9 gene on the response of reproductive variables, the results with the application of eCG make it advisable to apply it to estrus synchronization protocols for Katahdin sheep during the transition period. The results of this study are considered preliminary and inconclusive for some reproductive parameters, given the sample size in some of the measurements. Despite the above, this study serves as a background for future research, in particular, genome-wide association studies to identify new candidate genes associated with sheep reproduction, as exon 2 of the GDF9 gene in this study had no influence on reproductive variables in Katahdin sheep.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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