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Crude glycerin inclusion in Santa Inês ewes' diet before and during the breeding season: its effects on physiological parameters, hematological variables, and reproductive performance

Inclusão de glicerina bruta na dieta de ovelhas Santa Inês antes e durante a estação de monta: Seus efeitos nos parâmetros fisiológicos, variáveis hematológicas e desempenho reprodutivo

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Highlights:
Impact of crude glycerin (CG) on physiological and reproductive performance.
All ewes exhibited estrus during the breeding season.
CG did not drastically change the hematological and biochemical variables.
CG can be used up to 10% in replacement of ground corn.

Abstract

In this study, we aimed to evaluate the effect of concentrate supplementation containing crude glycerin (CG) before and during the breeding season on the physiological and blood biochemical variables as well as on the reproductive performance of Santa Inês ewes. A total of 24 ewes (12 pluriparous and 12 nulliparous ewes, 4 and 1.5 years old, weighing an average of 40 ± 5 and 27 ± 3 kg, respectively) were randomly assigned to three treatments consisting of different dietary concentrations of CG (0%, 5%, and 10 % of the total dry matter). The experiment lasted 63 days and was divided into three 21-day phases. In the first and second phases, the animals were subjected to flushing. In the second and third phases, we evaluated the animals' reproductive performance. Weather, physiological, and blood biochemical variables were also studied. The results indicated that the inclusion of CG did not influence significantly ($P > 0.05$) either the ewes' body weight or their body condition score. All ewes exhibited estrus during the breeding season. Their respiratory rate was significantly influenced by the time of day ($P < 0.0001$). A 10% CG supplementation did not drastically change the hematological and biochemical variables, which were within the reference ranges. However, the week of supplementation influenced serum

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metabolites ($P < 0.05$). Thus, these results indicate that CG could be used in up to 10% in replacement of ground corn without significantly affecting the physiological, hematological, and biochemical parameters of the ewes or their reproductive performance before and during the breeding season.
Key words: Estrus. Flushing. Hemogram. Metabolites. Thermoregulation.

Resumo

Neste estudo, objetivamos avaliar o efeito da suplementação concentrada contendo glicerina bruta (GB) antes e durante a estação de monta sobre as variáveis bioquímicas e fisiológicas, bem como sobre o desempenho reprodutivo das ovelhas Santa Inês. Um total de 24 ovelhas (12 pluríparas e 12 nulíparas, de 4 e 1,5 anos de idade, pesando em média 40 ± 5 e 27 ± 3 kg, respectivamente) foram distribuídas aleatoriamente em três tratamentos constituídos por diferentes concentrações dietéticas de GB (0, 5 e 10% da matéria seca total). O experimento durou 63 dias e foi dividido em três fases de 21 dias. Na primeira e segunda fase, os animais foram submetidos ao flushing alimentar. Na segunda e terceira fase, avaliamos o desempenho reprodutivo dos animais. Variáveis climáticas, fisiológicas e bioquímicas sanguíneas também foram estudadas. Os resultados indicaram que a inclusão de GB não influenciou significativamente ($P > 0,05$) no peso corporal das ovelhas nem em seu escore de condição corporal. Todas as ovelhas exibiram estro durante a estação reprodutiva. A frequência respiratória foi significativamente influenciada pela hora do dia ($P < 0,0001$). Uma suplementação com 10% de GB não alterou drasticamente as variáveis hematológicas e bioquímicas, que estavam dentro do intervalo de referência. No entanto, a semana de suplementação influenciou os metabólitos séricos ($P < 0,05$). Sendo assim, esses resultados indicam que a GB pode ser utilizada em até 10% na substituição do milho moído, sem afetar significativamente os parâmetros fisiológicos, hematológicos e bioquímicos das ovelhas assim como o desempenho reprodutivo antes e durante a estação de monta.
Palavras-chave: Estro. Flushing. Hemograma. Metabolitos. Termorregulação.

Introduction

Reproductive functions are impaired by nutritional imbalance during both the pre-pubertal and the active reproductive stage in animals (Kenny & Byrne, 2018). Therefore, energy supplementation both before and during the breeding season plays a significant role in determining the reproductive efficiency of sheep. However, supplementing conventional feeds has become less frequent mainly owing to the high cost of supplements and the use of alternative feeds (by-products). A variety of alternative feeds that increase the energy supply and lead to improvements in the reproductive and production capacity of the animals have been studied (Lage et al., 2014).

The biodiesel industry is the source of these alternative feeds. This industry has grown rapidly along with the increasing demand for renewable and sustainable energy sources as well as lower power pollution (Popp et al., 2016). Among these by-products, crude glycerin (CG) stands out as an energy source used to replace part of the energy components of the diet. The nutritional value of glycerol makes it suitable for use in ruminant diets, as it acts as a precursor to gluconeogenesis, thus emerging as an alternative feed in sheep diets (Merlim et al., 2015).

Many studies have evaluated the use of CG in ruminant diets in terms of nitrogen balance (Oliveira et al., 2016), meat quality (Lage et al., 2014), fatty acid profile (Terré, Nudda, Casado, & Bach, 2011), milk composition (Paiva et al., 2016), and rumen metabolites (Barton et al., 2013); the origin and concentration of glycerol has resulted in different research outcomes. Limited information is available regarding the effects of dietary glycerol as part of flushing (the practice of increasing an animal's nutrient intake and body condition prior to and during breeding in an effort

to increase its ovulation and fertilization rates) on ewes' performance during the reproductive season. Furthermore, as it is a by-product with high energy density, it can be used to reduce heat production, acting on the adaptive capability of ewes in semi-arid regions. Therefore, the present study attempted to generate information about the influence of CG originating from residual frying oils from biodiesel production on the physiology of ewes both before and during the breeding season. Further, we also evaluated the ewes' hematological and biochemical profiles as indicators of metabolic balance as this information will allow us to map the animals' nutritional condition. Additionally, this information will assist in the assessment of the adequacy of nutritional management and the physiological responses of the ewes in terms of the local reality; consequently, this research may lead to the adoption of technologies that will increase the productivity and the reproductive efficiency of the herd.

We hypothesized that CG with high fat content (264.3 g kg^{-1} DM) and low glycerol content (306.2 g kg^{-1} DM) could be used as an energy source in the diet of Santa Inês ewes before and during the breeding season without compromising their adaptive capability, reproductive performance, and productivity. Therefore, the aim of this study was to evaluate the effect of CG supplementation before and during the breeding season on the reproductive, physiological, hematological, and biochemical parameters of Santa Inês ewes.

Materials and Methods

The experiment was carried out at the Didactic-Productive Module of small ruminants of the Technical College of Bom Jesus, Federal University of Piauí, located in Bom Jesus, PI, Brazil (09º04'28 "S 44º21'31"W).

Animals, experimental diets, and procedures

Twenty-four Santa Inês ewes (12 pluriparous and 12 nulliparous, 4 ± 2 and 1.5 ± 0.5 years old, and with an average body weight of 40 ± 5 and 27 ± 3 kg, respectively), non-pregnant, and clinically healthy, were treated with disophenol 20% (Ibasa®, Porto Alegre, RS, Brazil) to control internal and external parasites and were vaccinated against enterotoxemia.

The study lasted 63 days and was divided into three 21-day phases. In the first and second phases, the ewes were subjected to flushing and in the second and third phases, their reproductive performance was evaluated. Further, in the third phase, all ewes received the same concentrate, i.e., a diet formulation with 0% CG, as is shown in Figure 1.

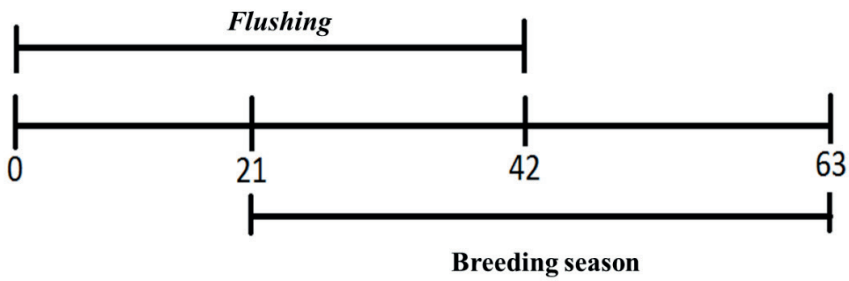

Figure 1. Scheme of feeding and reproductive management during the experimental period.

With the help of a vasectomized male, the first estrus manifestation was observed from the 22nd until the 63rd day of the experiment. Two daily observations (in the morning and in the afternoon) were performed. The sheep that allowed the vasectomized male to mount them were considered to be in estrus and after 12 h they were subjected to natural breeding. After coverage, the ewes were reintroduced to the group and were not monitored for estrus thereafter. Thirty days after mating, their pregnancy was confirmed through ultrasonography. Their prolificacy was calculated by the ratio of the number of lambs born to the number of sheep lambing.

During the experimental period, the ewes remained in a collective stall (4 × 10 m), equipped with a feeder and a water fountain. They were fed corn silage and had *ad libitum* water access. In the late afternoon, the sheep were transferred to individual stalls, where they received concentrate supplementation containing CG, the composition of which is described in Table 1.

Table 1
The ingredients and chemical composition of the concentrate diet

Item	Levels of crude glycerin (% of dry matter)		
	0	5	10
Ingredient (% DM)			
Ground corn	75	70	65
Soybean meal	20	19.8	19.6
Crude glycerin	0	5	10
Limestone	1	1	1
Mineral Supplement[1]	4	4	4
Urea	-	0.2	0.4
Nutrient (%DM)			
Dry matter (g kg $^{-1}$ MN)	89.97	87.48	85.13
Crude protein	16.15	16.23	16.3
Neutral detergent fiber	12.05	11.4	10.76
Ether extract	4.3	5.36	6.43
Mineral matter	6.48	6.43	6.38
Non-fibrous carbohydrates	61.02	60.58	60.13

[1] Guarantee levels per kg of product: 267 g of calcium; 61 g of phosphorus; 35 g of sulfur; 20 g of magnesium; 610 mg of fluorine; 6000 mg of zinc; 350 mg of copper; 23 mg of selenium; 500 mg of molybdenum; 2000 mg of manganese; 60 mg of chromium; 80 mg of iodine; 20 mg of cobalt e 3000 mg of iron.

CG replaced ground corn for 42 days in the experimental treatments in the following proportions: 0%, 5%, and 10% of the total dry matter (DM). The CG (Table 2) used originated from residual frying oils from biodiesel production and was provided by the Sewage Water Treatment Agency of the Piauí State (AGESPISA). The CG was manually homogenized and incorporated to the concentrate mixture in the aforementioned substitution levels.

Table 2
Chemical composition of crude glycerin from the biodiesel production from frying oils

Item	(g kg^{-1} Dry matter)	Analytical method
Humidity (g kg^{-1} natural matter)	427.6	Association of Official Analytical Chemistis [AOAC] (1990) - method: 930.15
Mineral matter	7.5	AOAC (1990) - method: 942.05
Crude protein	9.1	AOAC (2007) - method: 992.15
Ether extract	264.3	AOAC (2007) - method: 920.39
Glycerol	306.2	USP (2015) - method: 169
Methanol	1.11	USP (2015) - method: 467
Sodium	1.6	FDA (2010) - method: 0159
Gross energy (kcal kg^{-1} Dry matter)	3.787	bomb calorimeter
Total Fatty Acids (g 100^{-1} fat)	29.43	AOAC (2007) - method: 996.06

During the supplementation period, the body weight of the ewes was measured weekly using a mobile mechanical scale for small ruminants (Balanças Cauduro Ltda. - 499 SU, Cachoeira do Sul, RS, Brazil). Their body condition score (BCS) was determined according to the methodology described by Thompson and Meyer (2006), using a 1-5 scale, where BCS of 1 and 5 represented a thin and a very fat animal, respectively.

Measurement of climatic and physiological variables

During the experimental period, the climatic variables were monitored continuously using a thermo-hygrometer (temperature and relative humidity, RH), a dry bulb (dry bulb temperature, DBT), a wet bulb (wet bulb temperature), and a black globe (black globe temperature, BGT), which were installed along the animals' height. The black globe-humidity index was calculated using the following equation: BGHI = BGT + [0.36 × DPT (DBT - (100-RH%)/5)] + 41.5 described by Buffington, Collazzo-Arocho and Canton (1981), wherein DPT = dew point temperature. The environmental variables were measured at 07:00 and 15:00 h, three times per week.

The physiological parameters [rectal temperature (RT) and respiratory rate (RR)] were measured three times per week, in the morning (07:00 to 08:00 h) and in the afternoon (15:00 to 16:00 h). To obtain the RT a veterinary clinical thermometer (Incoterm®, Porto Alegre, Rio Grande do Sul, Brazil) was used; this was introduced into the animal's rectum where it remained for two minutes and the result was expressed in Celsius degrees (ºC). The RR (breath/minute) was measured using a flexible stethoscope at the level of the laryngotracheal region.

Blood sampling

Blood samples were collected to determine the hematological (5 mL) and biochemical (10 mL) profiles of the animals. Blood samples were collected at weekly intervals from all the animals before they set out to graze. Blood samples were collected by jugular venipuncture, using disposable needles (25 × 8 mm; Greinerbio-onne®, Americana, São Paulo, Brazil) and deposited in vacutainer tubes. The blood samples were collected and kept in two different tubes; one tube contained an anticoagulant agent (ethylenediamine tetraacetic acid) to evaluate the hematological variables, while the other tube did not contain an anticoagulant agent in order to evaluate the biochemical variables. The blood samples were kept in iceboxes until their arrival at the Laboratory of Veterinary Clinical Pathology of the Veterinary Hospital (HVU-CPCE). The biochemical profile samples were centrifuged at 3500 rpm/15 min,

packed as serum aliquots into Eppendorf tubes, and stored in a freezer at -20 °C for subsequent analysis. The laboratory analyses were carried out at the Laboratory of Veterinary Clinical Pathology of the University Veterinary Hospital (UFPI-CPCE), located in the city of Bom Jesus, Piauí, Brazil.

Measurement of hematological parameters

Red blood cell (RBC) and leukocyte (Le) counts were performed in a Neubauer improved cell counting chamber as recommended by Vallada (1999). The hematocrit (Ht) was determined by the microhematocrit technique and the result was expressed as a percentage (%). Plasma samples were used to measure the total proteins (TP) using a refractometer and analyzed by the degree of light refraction technique (Thrall, Weiser, Campbell, & Allison, 2015). The cyanmethemoglobin method was used to determine the hemoglobin content, after the samples had been diluted in Drabkin's solution. The values obtained by counting the number of red blood cells and determining the hematocrit and hemoglobin contents were used to establish the values of the absolute hematimetric indexes: mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). The differential leukocyte count of two blood smears from each sample was measured with the use of Romanowsky-type stains (Panótico Rápido; Laborclin® Ltda, Pinhais, Paraná, Brazil), according to the standard technique for animals described by Viana et al. (2002). In each blood smear, 100 leukocytes were identified, classified according to their morphological and color characteristics, and read in a 1000× magnification microscope to differentiate among neutrophils, eosinophils, basophils, lymphocytes, and monocytes.

Measurement of biochemical parameters

The metabolites evaluated were creatinine (Labtest Diagnóstica® S.A, Lagoa Santa, Brazil), urea (urea liquiform; Labtest Diagnóstica® S.A.) by the colorimetric enzymatic method, cholesterol (cholesterol liquiform; Labtest Diagnóstica® S.A.), glucose (glucose liquiform; Labtest Diagnóstica® S.A.), and triglycerides (triglycerides liquiform; Labtest Diagnóstica® S.A.). The enzyme evaluated was aspartate aminotransferase by the UV kinetic method (AST/GOT Liquiform; Labtest Diagnóstica® S.A.). The total protein level was estimated by the biuret method (Labtest Diagnóstica® S.A), while the total serum protein was estimated by the bromocresol green method. All biochemical analyses were performed in a semi-automatic biochemical analyzer (Spectrum®, São Paulo, Brazil).

Statistical analysis

The experimental design was completely randomized with measures repeated over time; these measures were three levels of CG inclusion (0%, 5%, and 10% of the total dry matter), two parturition orders (PO) (nulliparous and pluriparous), and nine weeks of evaluation. The data were analyzed using the MIXED procedure of SAS (version 9.0) (SAS Inst. Inc., Cary, NC, USA), including in the model the level of CG, PO, and their interactions as fixed effects. Week was considered as a measure repeated in time (REPEATED = week). The animal nested within the treatment was considered as a random effect.

In the analysis of the physiological and climatic parameters, the effect of the time of day was considered. Several covariance structures for the residues were compared; among them, the covariance structure of first order autoregression (AR1) was identified as better considering the Bayesian criterion (BIC).

The treatment effects on the analyzed variables were compared by Tukey's adjusted test (option PDIFF ADJUST), being considered significant at $P < 0.05$. When the interactions were significant, the F test was conducted using the option SLICE of LSMEANS for the unfolding of the interactions. The residues were plotted against predicted values and were used to verify the assumptions of the homoscedasticity model, which were independence and normality of

the errors. One data point was considered an outlier and removed from the database when the studentized residual was outside the ± 2.5 range.

Results and Discussion

The CG levels did not affect the body weight and BCS of the ewes during the experiment (P = 0.75 and P = 0.43, respectively); however, pluriparous ewes had a higher BCS (P = 0.007) compared with nulliparous ewes. Further, the effect of the supplementation week on the body weight (BW) (P = 0.001) and the BCS (P = 0.007) was verified with a gradual increase of these parameters (Table 3). Although the ewes were in early pregnancy, these values probably resulted from hormonal and uterine changes, such as the progression of pregnancy, the gradual increase of the gravid uterus, the embryonic annexes, and fetal growth (Osol & Mandala, 2009).

Table 3
Body weight and body condition score of Santa Ines ewes supplemented with crude glycerin before and during breeding season

Item	Body weight (kg)	Body condition score
Overall average	39.7	2.62
Parturition order		
Nulliparous	34.27b	2.48b
Multiparous	45.17a	2.74a
SEM	2.02	0.06
Crude glycerin		
0%	39.80	2.53
5%	41.00	2.66
10%	38.37	2.64
SEM	2.47	0.08
Week of supplementation		
1	36.87d	2.41d
2	37.20d	2.50cd
3	37.32d	2.56bc
4	37.80d	2.64ab
5	39.48c	2.62bc
6	41.04b	2.66ab
7	42.19a	2.64bc
8	42.00ab	2.68b
9	43.59a	2.79a
SEM	1.55	0.07
P-value		
parturition order (PO)	0.001	0.007
Level of crude glycerin (CG)	0.75	0.43
Week (S)	<0.0001	0.01
Interaction	ns	Ns

Means followed by different letters in the same column differ by Tukey test (P < 0.05); SEM = standard error of the mean; ns = not significant.

The use of flushing in reproductive management aims, indirectly, to increase the BW (by nutrient supplementation) of the ewes for the breeding season, thus improving their BCS and bringing it to levels suitable for reproductive activity in order to increase their ovulation and fertilization rates (Branca, Molle, Sitzia, Decandia, & Landau, 2000). Flushing also increases the coverage and prolificacy rates owing to the storage of nutrients in the muscular and adipose tissues of the animals, thus being a great reproductive management tool (Kenny & Byrne, 2018). The established superiority of pluriparous ewes in terms of BW and BCS could be attributed to the fact that these were adult, well-developed females. On the other hand, nulliparous ewes were in constant growth and their organisms required nutrients to support both their development and their pregnancy. In nulliparous sheep and especially those that are raised in pasture-based production systems, the birth of lambs with lower BW, lower milk production, and lambs with delayed growth are common occurrences. The lack of supplementation directly affects both the sheep and the lambs; it also affects the next reproductive cycle by increasing the anestrus period and the lambing interval and by negatively affecting the reproductive performance of ewes. Thus, flushing is recommended not only during but also before the breeding season and during the maternal-dependent phase, aiming for a better development of the animals and positive impacts on their reproductive performance (Torreão et al., 2014).

All ewes exhibited estrus during the breeding season. In the first phase (days 21-41), 83.3% of the ewes in heat were flushed and 16.7% were not. In the second phase (days 42-63), 12.5% of the ewes in heat were flushed and 87.5% were not (Figure 2). There was no treatment effect on either the estrus or the prolificacy percentages. This result may be associated with the fulfillment of the ewes' nutritional requirements as the animals had access to quality roughage (corn silage) as well as mineral supplementation (Table 4).

Table 4
Identification of estrus and prolificity of Santa Inês sheep supplemented with crude glycerin before and during breeding season

Item	Identification of estrus (Days)	Prolificacy (Lambs/ewe)
Overall average	32.12	1.33
Parturition order		
Nulliparous	31.75	1.25
Multiparous	32.50	1.42
SEM	2.08	0.14
Crude glycerin		
0%	33.37	1.50
5%	27.25	1.37
10%	35.75	1.12
SEM	2.55	1.17
P-value		
Parturition order (PO)	0.8	0.42
Crude glycerin (CG)	0.08	0.33
Interaction (CG*PO)	ns	ns

Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant.

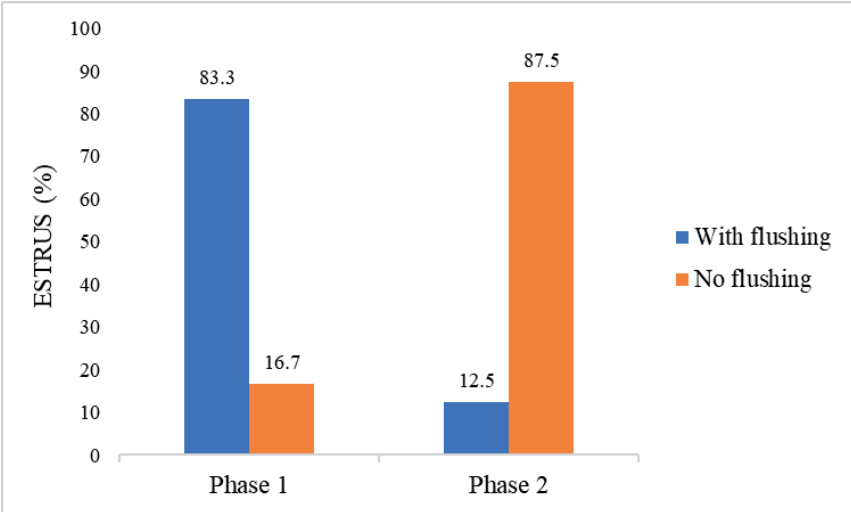

Figure 2. Occurrence of estrus (%) in Santa Ines ewes supplemented with crude glycerin before and during breeding season (phase 1 = 21 to 41 days; phase 2 = 42 to 63 days).

The thermal comfort zone of sheep is between 20 and 30 °C (Baêta & Sousa, 2010). In the current study, the climatic variables studied triggered heat stress during the afternoon thereby initiating the thermoregulatory mechanisms of the animals as reflected by the increase in their RR (Table 5). In optimum temperatures for sheep, 20% of heat loss occurs through breathing. On the other hand, in temperatures above 35 °C, heat loss via respiration constitutes 60% of the total heat loss (Sousa et al., 2008).

Table 5
Climatic variables during the experimental period

Item	Tmax (ºC)	Tmin (ºC)	RH (%)	DBT (ºC)	WBT (ºC)	BGHI
Morning (06:00 - 07:00 h)	28,07b	26,61b	47,92a	27,11b	24,16b	74,24b
Afternoon (14:00 - 15:00 h)	45,28a	40,31a	25,26b	39,76a	30,09a	89,22a
SEM	0,79	0,81	2,01	0,44	0,51	0,6
P-value						
Period of the day	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
Week	0,8	0,28	0,006	0,04	0,46	0,37
Interaction (Period of the day x week)	ns	ns	ns	ns	ns	ns

Tmax - maximum temperature; Tmin - minimum temperature; RH = relative humidity; DBT - dry bulb temperature; WBT - wet bulb temperature; BGHI = black globe-humidity index. Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant.

The CG supplementation that aimed to increase the energy density of the diet did not alter the animals' RR and RT. The higher energy density aims to compensate for the lower neutral detergent fiber intake in animals under heat stress and to reduce the impact of heat load from the intake, digestion, and metabolism of nutrients on animal physiology. This process, known as caloric increase, generates heat. In the case of arid and semi-arid regions, thermal stress is considered the most intriguing factor to negatively influence animal production (Pereira et al., 2014; Rocha et al., 2014). Heat stressed animals reduce their caloric intake, thereby reducing their basal heat production, which, consequently, results in the activation of all the physiological variables such as RR, RH, RT. The magnitude of this effect is determined by the environmental and climatic conditions as well as by the physiological, nutritional, and reproductive stages of the animals (Silva et al., 2016). In this context, the adaptive capacity of the breed under study should be considered, as in native breeds, such as Santa Inês, the response to supplementation may not be as pronounced as in exotic breeds.

The time of day affected ($P < 0.0001$) all climatic variables, with higher averages reported in the afternoon, except for the average RH value, which was higher in the morning (Table 5). The DBT ($P = 0.04$) and RH ($P = 0.006$) were influenced by the weeks of evaluation. The BGHI values in the two periods of the day (morning and afternoon) were elevated during the experimental period; however, this was not perceived as a dangerous situation for the ewes, as their RT was within the established average for the species, in a manner characteristic of homeothermic maintenance. The BGHI values were similar to those reported by Silva et al. (2006) and Silva, Souza, Brandão, Marinho and Benício (2011). The RH values were outside the comfort zone of the sheep In the semi-arid region, which has common characteristics with semi-arid tropical environments, the RH values are outside the comfort zone (50 to 70%) of sheep (T. P. D. Silva et al., 2016). In most cases, the adaptive capability of animals was compromised by affecting the most sensible physiological variables such as heart rate and RR. It is important to note that in semi-arid regions this impact on animal physiology is prolonged for a large part of the year and affects negatively the reproductive rates and, consequently, the performance of the animals (Wolfenson & Roth, 2019).

The animals' RR was influenced by the time of day ($P < 0.0001$) with oscillation along the experimental weeks ($P < 0.0001$) (Table 6), probably owing to the daily variation of climatic parameters. In the afternoon, the RR recorded were above the normal values described for sheep (between 16 and 34 breaths/min) (Souza et al., 2008). High temperature, due to the high intensity of solar radiation, was probably responsible for the RR increase in the afternoon as described by Silva et al. (2013, 2016). In these cases, the higher the ambient temperature (AT) and the RH variation, the more difficult it will be for the animals to lose heat by non-evaporative mechanisms, such as conduction, convection, and radiation, resulting in heat loss only through evaporative mechanisms culminating in increased RR. The RT was not influenced by the treatments ($P > 0.05$), being 38.9 ºC on average (Table 6), which is within the normal range for sheep (38.3 to 39.9 °C) (Ribeiro et al., 2008). It is important to mention that a 1 °C increase in RT is enough to reduce the performance of most domestic animal species (McDowell, 1972). An RT increase corresponds to heat accumulation in the body caused by the heat from the environment coupled with the internal heat generation during the day and the inability of the thermoregulatory mechanisms to dissipate the excess heat (Baêta & Sousa, 2010).

Table 6
Respiratory rate and rectal temperature of Santa Inês ewes supplemented with crude glycerin before and during breeding season

Item	Respiratory rate (breath/min)	Rectal temperature (ºC)
Overall average	46.67	38.91
Crude glycerine		
0%	45.85	38.60
5%	47.08	38.61
10%	47.08	39.46
SEM	1.61	0.45
Parturition order		
Nulliparous	45.97	38.69
Multiparous	47.37	39.09
SEM	1.31	0.37
Period of the day		
Morning	31.73b	38.89a
Afternoon	61.60a	38.88a
SEM	0.98	0.37
Week of supplementation		
1	39.36e	38.99a
2	48.66b	38.84a
3	46.59b	38.60a
4	47.38b	38.52a
5	57.38[a]	38.62a
6	46.22bc	38.56a
7	43.75c	38.55a
8	42.63d	38.52a
9	48.04b	38.39a
SEM	1.29	0.79
P-value		
Crude glycerin (CG)	0.82	0.33
Period of the day (PD)	<0.0001	0.98
Parturition order (PO)	0.46	0.46
Week (W)	<0.0001	0.39
Interaction	PD*W (<0.0001); CG*W (0.01)	ns

Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant.

The hematological parameters (erythrogram and leukogram) of Santa Inês ewes are shown in Tables 7a and 7b. The TP, Ht, and MCV were higher in pluriparous ewes compared with nulliparous ewes. There was an effect ($P < 0.05$) of CG on Ht, MCV, leukocytes, neutrophils, and lymphocytes. Despite the effect of different CG levels on the Ht and MCV counts, we cannot suggest that this alteration resulted from the use of lipid sources. In ewes supplemented with 10% CG there was no reduction

($P > 0.05$) in TP, RBC, MCHC, and eosinophil counts. Furthermore, it is important to emphasize that all hematological parameters evaluated were within the normal ranges for sheep (Kramer, 2006). Age was the most likely contributing factor in the increase of the serum parameters of pluriparous ewes (Reece, 1996).

Table 7a
Erythrogram of Santa Ines ewes supplemented with crude glycerin before and during breeding season

Item	TP (g dL^{-1})	RBC ($x10^6$ **µl**)	Hb (g dL^{-1})	Ht (%)	MCV (fL)	MCHC (g dL^{-1})	Platelets ($x10^3$ **µl**)
Reference values*	4 -8	8.0-18.0	8 - 12	22-38	28-40	31-38	300-600
Overall average	6.48	8.09	11.37	27.55	35.4	41.07	411
Parturition order							
Nulliparous	6.23b	7.92a	11.26a	26.86b	34.57b	41.65a	406a
Multiparous	6.71a	8.20a	11.47a	28.23a	36.23a	40.47a	416a
SEM	0.07	0.16	0.1	0.42	0.59	0.5	172
Crude glycerin							
0%	6.58a	8.28a	11.57a	28.31a	35.05b	40.72a	419a
5%	6.44a	8.14a	11.19a	26.24b	33.87b	42.25a	437a
10%	6.41a	7.76a	11.34a	28.07a	37.28a	40.21a	377a
SEM	0.09	0.2	0.12	0.51	0.72	0.62	211
Week of supplementation							
1	6.35a	8.14b	10.98bc	25.59d	31.91c	42.63ab	400b
2	6.33a	7.63bc	10.98bc	26.58cd	35.60b	40.49ab	344bc
3	6.52a	7.36b	10.90c	26.11d	36.33b	42.25ab	376b
4	6.63a	6.50d	11.61a	28.91a	43.68a	40.18b	412ab
5	6.60a	6.57d	11.84a	27.81bc	43.27a	42.81a	361b
6	6.46a	7.24c	11.59a	27.01bd	37.99b	41.07ab	278c
7	6.59a	9.51a	11.52ab	29.06a	31.67c	39.95b	524a
8	6.48a	9.67a	11.47ac	28.24ab	29.23c	40.47ab	509a
9	6.30a	9.93a	11.40ac	28.56ab	28.91c	39.70b	494a
SEM	0.08	0.26	0.12	0.54	1.13	0.71	318
P-value							
Parturition order (PO)	0.0001	0.24	0.16	0.03	0.05	0.1	0.67
Crude glycerin (CG)	0.37	0.18	0.12	0.02	0.006	0.07	0.11
Week (W)	0.8	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Interaction	CG*W (0.007)	ns	CG*W (<0.0001)	ns	ns	CG*S (0.03)	ns

Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant; TP= total Proteins; RBC= red blood cell; Ht= hematocrit; Hb= hemoglobin; MCV= mean corpuscular volume; MCHC= mean corpuscular hemoglobin concentration; * Reference range for sheep (Kramer, 2006).

Table 7b
Leukogram of Santa Ines ewes supplemented with crude glycerin before and during breeding season

Item	Le (/µl)	Ns (/µl)	Ly (/µl)	Eos (/µl)	Bas (/µl)	Mon (/µl)
Reference values*	4000-12000	700 - 6000	2000 - 9000	0-1000	0-300	0-750
Overall average	5600	3046	2263	216.82	11.36	64.01
Parturition order						
Nulliparous	5928a	3293a	2302a	218.03a	0	55.00a
Multiparous	5272b	2800b	2224a	181.86a	53	76.43a
SEM	163	114	82	24.42	-	9.4
Crude glycerin						
0%	5744a	3083ab	2392a	217.82a	0	65.50a
5%	6119a	3314a	2448a	290.99a	0	67.00a
10%	4936b	2742b	1948b	172.03a	53	77.50a
SEM	200	140	101	29.91	-	13.05
Week of supplementation						
1	5900ab	3738a	1810c	108.43c	0	40.50a
2	5260b	3228ab	1861c	134.40c	0	0.00a
3	6020ab	3054ab	2782a	164.92bc	0	76.00a
4	4811b	2631b	1903c	210.17b	0	38.00a
5	4932b	2646b	2093bc	185.67b	0	92.50a
6	5014b	2541b	2077bc	226.42ab	0	66.67a
7	5627ab	3096ab	2316abc	303.58ab	53	63.00a
8	5968ab	2830b	2721ab	247.93b	0	95.50a
9	6864a	3652a	2801a	373.21a	0	82.50a
SEM	307	188	157	34.97	-	15.68
P-value						
Parturition order (PO)	0.006	0.004	0.5	0.3	-	0.62
Crude glycerin (CG)	0.0004	0.02	0.002	0.51	-	0.89
Week (W)	0.0003	<0.0001	<0.0001	<0.0001	-	0.92
Interaction	PO*W (0.0002)	CG×W (0.004) CG×PO (0.04)	T×S (0.01)	CG×PO (0.04); CG×W (0.02)	-	ns

Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant; Le= Leukocytes, Ns= Segmental Neutrophils, Lin= Lymphocytes, Eos= eosinophils, Bas= basophils, Mon= monocytes. *Reference range for sheep (Kramer, 2006).

Regarding the serum biochemical profile, only the cholesterol and urea concentrations were influenced by the CG level; thus, it was evident that the effect of the dietary energy intake was reflected directly in these concentrations, demonstrating that the blood parameters are true indicators of animal health (Table 8). Thus, in the blood level (central compartment of nutrient reserves), nutrients are readily available for metabolism. This compartment is continuously supplied with nutrients by the digestive system, through the dietary supply, organs, and tissues as well as through the kinetics of the elements, i.e., by the nutritional mobilization due to the organism's needs, aiming at homeostasis. This signifies the importance of determining the essential hemato-biochemical parameters for

health and nutrition assessment and the consequent adequacy of animal management, if necessary. Urea is an indicator of protein metabolism and its concentration in the blood has been used to monitor dietary protein. Again, the age factor is most likely responsible for the differences observed in the blood concentrations of the indicators, as in nulliparous ewes the development process promotes higher protein use metabolism, consequently causing a decrease of nitrogen compounds (Henriques, Gregory, Rizzo, Hasegawa, & Meira, 2016).

Table 8
Serum biochemical profile (mg dL $^{-1}$) of Santa Ines ewes supplemented with crude glycerin before and during breeding season

Item	Glucose	Triglycerides	Cholesterol	Urea	Creatinine	AST
Reference values*	50-80	set/30	52-76	17.12-42.8	1.2-1.9	60-280
Overall average	66.9	18.9	60.2	19.12	1.2	135.78
Parturition order						
Nulliparous	69.51a	19.99a	57.50b	17.02b	1.18a	133.95a
Multiparous	64.43b	17.87b	62.89a	21.22a	1.20a	137.44a
SEM	0.88	0.44	1.7	0.7	0.02	3.43
Crude glycerine						
0%	66.75a	18.89a	57.47b	19.50ab	1.19a	140.77a
5%	68.10a	19.08a	59.30ab	17.37b	1.23a	132.37a
10%	66.05a	18.82a	63.81a	20.50a	1.16a	133.95a
SEM	1.08	0.54	2.09	0.86	0.22	4.2
Week of supplementation						
1	60.34e	20.91ab	47.56d	11.94e	1.15a	130.35ab
2	70.74bc	18.86bc	45.90d	15.79c	1.22a	136.37ab
3	68.02c	17.41ce	48.44d	20.95b	1.23a	140.27ab
4	70.14bc	15.48e	59.46c	17.80cd	1.11a	138.89ab
5	67.24cd	17.39ce	62.53c	25.67a	1.16a	143.69a
6	76.73a	19.03bc	63.19c	19.98b	1.23a	145.79a
7	52.95f	20.00bc	69.62b	24.48a	1.19a	137.73ab
8	72.75b	22.80a	76.93a	19.46bd	1.20a	124.22b
9	63.83de	18.49bc	68.09b	16.03c	1.23a	123.95b
SEM	1.35	0.67	2.02	0.93	0.03	4.13
P-value						
Parturition order (PO)	0.0002	0.002	0.03	0.0002	0.32	0.47
Crude glycerin (CG)	0.4	0.94	0.04	0.04	0.07	0.34
Week (W)	<0.0001	<0.0001	<0.0001	<0.0001	0.07	0.0001
Interaction	ns	CG*W (0.0001)	ns	CG*W (0.03)	CG*W (0.02)	CG*PO (0.04)

Means followed by different letters in the same column differ by Tukey test ($P < 0.05$); SEM = standard error of the mean; ns = not significant; *Kaneko, Harvey and Bruss (2008).

Added ingredients in supplements with high lipid content and their influence on the energy metabolism of ruminants have been evaluated in terms of the lipid profile (Fernandes et al., 2012). In this context, serum triglyceride levels in ruminants are low compared with non-ruminants, reflecting their low hepatic synthesis capacity (Fernandes et al., 2012). The cholesterol concentration reflects the energy metabolism in the liver, particularly the export of lipids in the form of Very low density lipoproteins (Ndlovu et al., 2007). The decrease in serum cholesterol levels in this study indicates an energy deficit, while increases occur in response to the ingestion of high energy foods in the form of lipids (Fernandes et al., 2012). In the present study, the serum triglyceride levels were not altered as a result of the diet; however, ewes fed 5% and 10% of CG had increased cholesterol. In nulliparous ewes, higher triglyceride concentrations were established, probably owing to the increase of glucose and its conversion (excess) to lipids. Pluriparous ewes, on the other hand, had higher serum cholesterol, owing to the higher energy intake that caused high lipid deposition; this can be justified by the pluriparous ewes' larger size and body weight and higher BCS. However, it should be emphasized that the values obtained are within the species' normal range (Kaneko et al., 2008).

The variation of serum metabolites throughout the weeks of supplementation had a direct effect on the reproductive performance of sheep. This was in agreement with the findings reported in sheep by Viñoles et al. (2005). The effect of short-term nutritional supplementation on the follicular development of ewes may have occurred due to the increase in plasma glucose concentrations acting directly on the ovaries. The stage of follicular development under maximum glucose and metabolic hormone concentrations may be the determining factor for the increase in the ovulation rate of ewes.

Conclusions

CG with high fat and low glycerol content can substitute ground corn up to 10% in Santa Inês ewes' diet before and during the breeding season without causing significant changes on their physiological and hematological variables and while preserving their reproductive performance.

Conflict of interest

The authors declare that they have no conflict of interest.

Approval of the Bioethics and Biosafety Committee

This study complied with all ethical principles in research involving animals and it was approved by the Bioethics Committee of the UFPI (PROPESQ-UFPI-047/2013) and the Ethics Committee on Animal Use (Protocol ID: 016/14).

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