

Blood and follicular fluid metabolic profile and oocyte quality in primiparous F1 Holstein x Gyr cows in lactation

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Abstract

The blood and follicular fluid metabolic profiles and their correlation with the quality of aspirated oocytes were studied at different periods after parturition in primiparous F1 Holstein x Gir lactating cows. Follicular aspirations were performed on 29 F1 Holstein x Gir crossbred heifers on days 30 ± 2 , 60 ± 2 , and 100 ± 2 after parturition. On the same days as oocyte collection, blood and follicular fluid from the dominant follicle were collected for glucose, NEFA, BHBA, and urea assays. Oocyte recovery rates, in vitro maturation rates, and apoptosis rates were evaluated. The average glucose concentration on day 30 postpartum was higher in follicular fluid compared to blood plasma. Urea concentrations in plasma and follicular fluid did not differ between days 30, 60, and 100. For BHBA and NEFA, no difference was observed between the concentrations in follicular fluid and blood serum between days 30, 60, and 100 postpartum. There was no difference in the oocyte recovery rate between collection times; however, it was observed that collections performed on days 30 and 60 showed a lower percentage of viable oocytes. No difference was observed in TUNEL negativity between the analyzed times; however, it was evidenced that oocytes collected on day 30 postpartum showed a higher maturation rate compared to those collected on day 100. It is concluded that primiparous F1 Holstein x Gir cows, from 30 days postpartum onwards, have the capacity to enter reproductive routine, as they present oocytes of quality even in an earlier postpartum period.

Introduction

The reduction in fertility of high-production dairy cows has been reported (Kim, 2014; Opsomer, 2015), and the higher milk production capacity has been considered one of the main causes of reproductive underperformance in females (Butler, 2003). However, other studies have demonstrated that not only is milk production related to fertility, but also that other factors such as nutrition, body condition, and endocrine and metabolic changes directly and indirectly affect animal health and, consequently, reproductive performance (Butler, 2000; Carvalho et al., 2014; Bakshi et al., 2017; Barletta et al., 2017). The metabolic and hormonal changes in high-production Holstein cows, especially during negative energy balance, which leads to greater mobilization of body tissues, are directly related to the composition of follicular fluid, particularly glucose, non-esterified fatty acids, beta-hydroxybutyrate, and urea (Leroy et al., 2004). The intensity of body condition score loss after calving is associated with higher milk production, but also with postpartum anestrus, fertility, days open, risk of uterine infections, and metabolic disorders that will interfere with the cow's reproductive health (Roche et al., 2009). Possibly, the reduction in fertility of high-production cows that lose body condition more intensely after calving is mainly due to the poor quality of oocytes and, consequently, of embryos (Leroy, 2005). The objective of the present study was to describe the metabolic profile in blood and follicular fluid and correlate it with the quality of aspirated oocytes at different periods after calving in primiparous lactating F1 Holstein x Gir cows.

Materials and Methods

Twenty-nine pregnant crossbred F1 Holstein x Gir (HG) heifers, with an average age and live weight of 20.36 ± 0.96 months and 505.5 ± 37.5 kg, respectively, were used. At 30 days before the expected calving date, the heifers were housed in maternity paddocks formed by *Cynodon dactylon* x *nlemfuensis*, which had 12 electronic feeders (AF-1000, Intergado® Ltda, Contagem, Minas Gerais, Brazil) and two automatic waterers with a body weight scale platform (VW-1000, Intergado® Ltda). Ear tags with electronic transponders (TAG) were affixed to the animals' ears for identification during access to feeding troughs and waterers. The diet was offered ad libitum twice daily throughout the experimental period.

After calving, the animals were transferred to a free-stall barn divided into five modules with a capacity for 12 animals each, equipped with 12 feeders (AF-1000, Intergado® Ltda, Contagem, Minas Gerais, Brazil) and two automatic waterers with a body weight scale platform (VW-1000, Intergado® Ltda). Thus, individual diet intake and live weight were recorded daily in an automated manner. The animal's daily live weight was considered the average of all weighings performed on that day. Body condition score was evaluated by inspection and palpation of the rump, ileum, ischium, and lumbar and costal regions (Scale 1 = very thin, 5 = very fat; Roche et al., 2009) at 30, 60, and 100 days postpartum. The animals received a complete diet (Table 1) ad libitum, twice daily, formulated according to NRC (2001), with the first feeding provided at 07:00 AM and the second at 3:00 PM.

Table 1
Ingredients and chemical composition of experimental diets in the
pre- and postpartum period.

Ingredients (g/kg of DM)	Prepartum	Postpartum
Corn silage		593,0
Cynodon silage	834,6	-
Tifton hay		23,0
Soybean meal	89,8	198,8
Ground corn	44,0	164,0
Prepartum mineral mix ¹		-
Lactation mineral mix ²	21,8	18,0
Urea		2,4
Dicalcium phosphate	7,3	-
Magnesium oxide	1,0	-
Salt	1,0	-
Ammonium sulfate	0,7	-
Chemical composition (g/kg of DM)		
Dry matter	332,38	536,4
Organic matter	887,05	928,0
Crude protein	110,23	169,7
Neutral detergent fiber	663,76	323,7
Ether extract	21,78	33,5
Ash	112,97	71,9
Non-fibrous carbohydrates	91,26	400,7
Gross energy (Mcal/kg DM)	4,6	4,4

¹Prepartum mineral mix: calcium (136.9 g/kg), phosphorus (20.8 g/kg), magnesium (43.4 g/kg), sulfur (53.2 g/kg), sodium (25 g/kg), iron (500 mg/kg), copper (500 mg/kg), zinc (2,000 mg/kg), manganese (1,333.40 mg/kg), iodine (50.6 mg/kg), cobalt (10 mg/kg), selenium (13.30 mg/kg), maximum fluoride (208 mg/kg), vitamin A (400,000 IU/kg), vitamin D3 (400,000 IU/kg), vitamin E (10,000 IU/kg).

²Mineral mix: calcium (150 g/kg), phosphorus (60 g/kg), magnesium (15 g/kg), sulfur (17 g/kg), sodium (57 g/kg), iron (1,500 mg/kg), copper (1,300 mg/kg), zinc (3,000 mg/kg), manganese (1,300 mg/kg), iodine (100 mg/kg), cobalt (100 mg/kg), selenium (30 mg/kg), fluoride (600 mg/kg), vitamin A (220,000 IU/kg), vitamin D3 (60,000 IU/kg), vitamin E (1,000 IU/kg).

After parturition, cows received oral electrolyte solution, via an oro-esophageal tube, composed of 160g of sodium chloride, 20g of potassium chloride, 10g of calcium chloride, and 300mL of propylene glycol, in 30 liters of water. Subsequently, the animal was taken to the free stall barn, and the milking routine was established. The average total milk production during lactation (305 days) and daily production of primiparous F1 Holstein x Gir cows were $4,695.4 \pm 1,257.2$ kg and 15.4 ± 4.1 kg, respectively.

Oocyte and follicular fluid (FF) collections were performed by ultrasound-guided follicular aspiration (7.5MHz microconvex transducer; DP 2200 Vet®, Mindray, Shenzhen, China), transvaginally, on days 30 ± 2 , 60 ± 2 , and 100 ± 2 after parturition. A 20G needle (WTA®, São Paulo, Brazil) connected to a WTA® aspiration system was used, consisting of an 80cm long, 1.7mm diameter Teflon circuit. The circuit was connected to a vacuum system adjusted to a pressure between 50 and 70 mmHg, coupled to a 50 mL Falcon tube maintained at a controlled temperature of 37 °C throughout the aspiration procedure.

To synchronize follicular wave emergence and obtain a larger number of aspirated oocytes, cows underwent aspiration of follicles with diameters greater than 7 mm, considered dominant, approximately three days before (day 0) the scheduled follicular aspiration for oocyte and follicular fluid collection. For ultrasound-guided ovarian follicular aspiration, animals received sacrococcygeal epidural anesthesia using 5mL of 2% lidocaine (Lidovet®, Bravet, RJ, Brazil). Recovered oocytes from aspiration were kept in a tube containing 50 mL of phosphate-buffered saline (PBS) and 1% fetal bovine serum, combined with 125 IU of sodium heparin/mL (Liquemine®, Roche, São Paulo, SP, Brazil). Five days after follicular aspiration (day 8) for oocyte collection, cows underwent a new follicular aspiration to collect follicular fluid (FF) from the dominant follicle. The recovered follicular fluid was centrifuged at 800G for 10 minutes, and the resulting supernatant was stored at -20 °C until the analysis of glucose, non-esterified fatty acids, urea, and beta-hydroxybutyrate.

In the laboratory, cumulus-oocyte complexes (COCs) were classified (Viana et al., 2004) according to cytoplasmic appearance and the number of cumulus oophorus cell layers, following these criteria: grade 1 - compact COC with more than three cell layers and an oocyte with homogeneous cytoplasm; grade 2 - compact COC with one to three cell layers and/or an oocyte with slightly heterogeneous cytoplasm; grade 3 - compact COC with less than three cell layers and an oocyte with slightly heterogeneous cytoplasm; denuded - oocyte without any cumulus oophorus cell layers; degenerated - oocyte with many cytoplasmic granules and lighter coloration; expanded - COC with expanded cells. Only grade 1 and 2 oocytes were used.

Oocytes were transported in 1.5 mL tubes containing TCM199 with Earle's salts supplemented with 10% fetal bovine serum, in an oocyte transporter (WTA®, São Paulo, Brazil) at 37°C. In vitro maturation was performed for 22–24 hours in an incubator (Thermo Scientific®, USA) with a humidified atmosphere of

5% CO₂ and 95% humidity, at 38.8°C. Maturation was carried out in maturation medium composed of TCM199, supplemented with 10% FBS + 22µg/mL sodium pyruvate + 5µg/mL LH + 1µg/mL FSH and 50µg/mL amikacin. Nuclear maturation of oocytes was evaluated by the meiotic progression stage after IVM. For this, Hoechst (33342) stain was used, added simultaneously during slide mounting for apoptosis analysis by the TUNEL technique. Oocytes in metaphase II (MII), i.e., those presenting the first polar body, were considered mature. Structures that did not progress through meiotic stages (metaphase, anaphase, and telophase I) were considered immature, according to Roth and Hansen (2005). Slides were evaluated under a fluorescence microscope (Olympus Provis®, Tokyo, Japan) at 400X magnification, using a blue filter (460nm), observing the cells stained by Hoechst.

After in vitro maturation (IVM), oocytes were fixed in 4% paraformaldehyde solution for 25 minutes and subsequently stored under refrigeration in Eppendorf tubes containing PBS or 0.9% NaCl physiological solution until analysis. Apoptosis was evaluated using the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) technique. Procedures were based on the methodology described by Sollecito et al. (2019), using a commercial kit Dead End Fluorimetric TUNEL System (Promega®, Madison, WI, USA). For slide preparation, oocytes were initially permeabilized by immersion in phosphate-buffered saline with 0.2% TritonX-100 and incubated in equilibration buffer (180µL) with nucleotide mix (20µL), rTdT enzyme (4µL), and fluorescein-12-dUTP, which is responsible for labeling fragmented DNA. Oocytes remained for 60 minutes in a humid chamber at 37°C. Negative control oocytes were incubated in equilibration buffer without the rTdT enzyme, and for the positive control, oocytes were incubated in equilibration buffer containing fluorescein-12-dUTP enzyme and 2.7 U/µL of DNase to promote cellular DNA degradation. TUNEL-positive cells, with fragmented DNA (marked in green), and TUNEL-negative cells, without DNA fragmentation, were considered.

Blood collections were performed in tubes containing EDTA, fluoride (glucose assay), and tubes without anticoagulant. Collections were made by coccygeal venipuncture on days 30 ± 2, 60 ± 2, and 100 ± 2 after parturition. After collection, blood and follicular fluid were centrifuged at 300G for 15 minutes, and plasma, serum, or follicular fluid were stored at -20°C until analysis. Glucose, urea, beta-hydroxybutyrate, and non-esterified fatty acid levels were measured in blood and follicular fluid. Glucose was analyzed by the enzymatic glucose oxidase method (Vet Glicose Liquiform, Labtest®, Brazil), and urea was analyzed by enzymatic photometry in ultraviolet using a two-point kinetic assay (Vet Ureia UV Liquiform, Labtest®, Brazil), using a semi-automatic biochemical analyzer TP Analyzer Plus (Thermo Plate®). Serum non-esterified fatty acids and beta-hydroxybutyrate were measured in samples collected on day - 7 pre-partum, and on days 7, 14, 21, 30, 60, and 100 post-partum. In follicular fluid, NEFA, BHBA, urea, and glucose were measured on days 30, 60, and 100 post-partum. Non-esterified fatty acids and beta-hydroxybutyrate were measured using an enzymatic kit (Randox NEFA FA115 and Randox BHBA RB1007, Randox® Laboratories Limited, Antrim, Ulster, United Kingdom), using an automatic biochemical analyzer Cobas Mira Plus (La Roche® Ltda, Basel, Switzerland), following the manufacturer's recommendations.

All statistical analyses were performed using R software version 3.6.1 (R Core Team, 2019). To test differences between groups as a function of time, or between times for each group, generalized estimating equations models were fitted for each response variable (Guimarães and Hirakata, 2012). Time was treated as a discrete predictor variable, with means estimated for each time point and for each group. The binomial distribution was used to model dichotomous qualitative variables. For all variables, a compound symmetry temporal autocorrelation structure was used. After model fitting, the global effect of time (overall) was tested, and the mean values and their respective 95% confidence intervals were calculated. Pairwise differences were evaluated using Tukey's test.

Results

No differences were found for milk production and dry matter intake at 30, 60, and 100 days postpartum ($P > 0.05$). Live weight and BCS ($P < 0.05$) increased with days in lactation, being higher at 100 days when compared to 30 and 60 days postpartum (Table 2).

Table 2
Average live weight (kg), body condition score, dry matter intake (kg), water intake (L/day), in primiparous crossbred F1 Holstein x Gir cows at 30, 60, and 100 days postpartum.

Variable	Days in lactation			P Value
	30	60	100	
Live weight (kg)	577,89 ± 52,14 a	596,83 ± 53,09 a	627,49 ± 55,94 b	0,004
Body condition score (1–5)	3,80 ± 0,22 a	3,88 ± 0,28 a	4,03 ± 0,28 b	0,005
Dry matter intake (kg/day)	15,15 ± 2,67	16,19 ± 2,18	14,51 ± 2,78	0,057
Water intake (L/day)	62,59 ± 16,97	61,58 ± 10,38	57,36 ± 12,09	0,322
Milk yield (kg/day)	18,81 ± 5,25	18,31 ± 5,30	16,34 ± 4,29	n.s.

a, b, c Different lowercase letters in the row differ from each other ($P < 0.05$) according to Tukey's test.

Plasma glucose concentrations on days 30, 60, and 100 postpartum did not differ ($P > 0.05$). The comparison of glucose concentrations in blood plasma and follicular fluid differed ($P < 0.05$) at 30 days postpartum (Table 3).

Table 3

Mean concentrations of glucose (mg/dL), urea (mg/dL), beta-hydroxybutyrate (BHBA), and non-esterified fatty acids (NEFA) in blood and follicular fluid at 30, 60, and 100 days postpartum of primiparous crossbred F1 Holstein x Gir cows.

Metabolites	Days postpartum		
	30	60	100
Plasma glucose (mg/dL)	67,1 ± 1,78 Ba	70,6 ± 3,51 Aa	71,6 ± 2,04 Aa
Follicular fluid glucose (mg/dL)	75,5 ± 1,95 Ab	69,2 ± 1,60 Aa	73,0 ± 2,18 Aab
Plasma urea (mg/dL)	28,7 ± 1,22 Ba	27,3 ± 1,44 Ba	29,1 ± 1,05 Ba
Follicular fluid urea (mg/dL)	19,8 ± 0,85 Aa	21,4 ± 1,29 Aa	20,5 ± 0,82 Aa
Serum BHBA (mmol/L)	0,66 ± 0,05 Aa	0,77 ± 0,09 Aa	0,66 ± 0,06 Aa
Follicular fluid BHBA (mmol/L)	0,64 ± 0,04 Aab	0,76 ± 0,07 Ab	0,61 ± 0,04 Aa
Serum NEFA (mmol/L)	0,21 ± 0,04 Aa	0,20 ± 0,03 Aa	0,15 ± 0,02 Aa
Follicular fluid NEFA (mmol/L)	0,16 ± 0,03 Aa	0,15 ± 0,02 Aa	0,12 ± 0,05 Aa

^{a,b,c} Different lowercase letters in the rows differ from each other ($P < 0.05$) according to Tukey's test.

^{A, B} Different uppercase letters in the columns differ from each other ($P < 0.05$) according to Tukey's test.

For follicular fluid, there was a difference ($P < 0.05$) in concentrations between days 30 and 60 postpartum, with higher concentrations on day 30. The average urea concentrations in follicular fluid were significantly lower ($P < 0.05$) than in plasma, but no variations were observed between collection days postpartum. The primiparous crossbred F1 Holstein x Gir cows in the present study maintained significantly ($P < 0.05$) lower urea concentrations in follicular fluid compared to blood plasma at 30, 60, and 100 days after calving.

The concentrations of beta-hydroxybutyrate in follicular fluid were similar to serum concentrations at 30, 60, and 100 days postpartum ($P > 0.05$). However, the highest concentration was observed at 60 days, and although it differed ($P < 0.05$) from that found on day 100 postpartum, the values are considered within the normal range. The average NEFA concentrations in follicular fluid and blood serum were similar and did not differ from each other ($P > 0.05$) for the collection days at 30, 60, and 100 postpartum.

In the present study, no differences ($P > 0.05$) were observed in the number of aspirated oocytes between days 30, 60, and 90 postpartum (Table 4).

Table 4
Production, viability, and apoptosis and maturation rates of oocytes collected on days 30, 60, and 100 postpartum from primiparous F1 Holstein x Gir cows.

	Days postpartum			<i>P</i> value
	30	60	100	
Number of oocytes collected	14,3 ± 1,3	11,8 ± 1,1	12,0 ± 1,2	0,277
Oocyte viability (%)	29,5 ± 1,8 a	37,3 ± 4,7 a	50,9 ± 2,8 b	0,001
Oocyte apoptosis rate (%)	40,0 ± 9,4	63,5 ± 11,0	64,6 ± 7,6	0,085
Oocyte maturation rate (%)	55,2 ± 4,9 a	35,0 ± 7,2 ab	23,8 ± 4,5 b	0,001

^{abc} Different letters in the rows indicate statistically significant differences ($P < 0.05$) according to Tukey's test.

Significant differences ($P < 0.05$) were observed in the percentage of viable oocytes on days 30 and 60 compared to day 100 postpartum, when the mean percentage of viable oocytes increased by 21% on day 100, relative to day 30 postpartum. No differences ($P > 0.05$) were found in the percentage of TUNEL-negative (viable) oocytes among the different collection days. The in vitro maturation rate differed significantly ($P < 0.05$) between oocytes collected on days 30 and 100, with a mean 31% lower on day 100.

Discussion

In the present study, glucose concentrations in follicular fluid were lower on days 60 and 100 postpartum, similar to those observed in plasma. Both plasma glucose concentrations (Carvalho et al., 2009) and those in follicular fluid are within the parameters reported by authors for F1 Holstein x Zebu crossbred cows (Santos et al., 2019). Primiparous $\frac{3}{4}$ Holstein x Gir cows showed higher glucose concentrations in follicular fluid compared to $\frac{7}{8}$ Holstein x Gir cows (Santos et al., 2019). The differences observed may be due to milk production and the genetic composition of the animals (Santos et al., 2019). High urea values in plasma and follicular fluid were observed in high-producing multiparous Holstein cows in the first weeks of lactation, which could compromise oocyte quality and reproductive health in dairy cows (Leroy et al., 2004).

Data from lactating primiparous $\frac{3}{4}$ and $\frac{7}{8}$ Holstein x Gir cows, kept under confinement, showed slightly higher urea concentrations in follicular fluid, 26.49 mg/dL and 23.65 mg/dL, respectively (Santos et al., 2019), when compared to the values found in the present study. Similarly, Leroy et al. (2004) found no differences in beta-hydroxybutyrate concentrations between serum and follicular fluid across postpartum days but observed that in follicular fluid, between days 14 and 46 postpartum, beta-hydroxybutyrate concentrations were above normal values.

Because it is a blood ultrafiltrate (Butler, 2003), NEFA (non-esterified fatty acid) concentrations in follicular fluid were always lower than those observed in serum. Similar data were found by Leroy et al. (2004) in high-producing multiparous lactating Holstein cows; however, the concentrations were higher than those found in the present study. Santos et al. (2019) found lower blood NEFA concentrations in confined, lactating, primiparous 3/4 and 7/8 Holstein x Gir cows compared to the present study: 0.11 mmol/L and 0.10 mmol/L, respectively, and values of 0.04 mmol/L in follicular fluid for both 3/4 and 7/8 Holstein x Gir animals.

Santos et al. (2019), evaluating lactating primiparous 3/4 and 7/8 Holstein x Gir cows, found significant differences in the total number of oocytes collected between the two genetic groups on days 14, 25, 42, and 56 postpartum, with higher numbers for 3/4 females (8.54 ± 1.24) compared to 7/8 (3.56 ± 1.3), and the overall mean of oocytes recovered in both genetic groups was 6.15 ± 0.58 . These values are lower than those found in the present study with primiparous F1 Holstein x Gir cows. Usually, the total number of oocytes aspirated per female differs between genetic backgrounds, being higher for zebu cows (Viana et al., 2004) compared to taurine cows, which could be exemplified by the differences found among Holstein x Gir 1/2, 3/4, and 5/8 genetic groups (Pontes et al., 2010).

Santos et al. (2019) found no significant differences in oocyte viability according to collection days; however, oocyte viability varied according to the blood degree of primiparous 3/4 and 7/8 Holstein x Gir cows, with higher viability found in 3/4 cows compared to 7/8 females (76.87% vs 69.94%). These results are superior to those found in the present study with primiparous F1 Holstein x Gir cows.

Dairy Holstein cows show reduced reproductive performance after calving, and as the days in lactation progress, fertility rates improve (Butler, 1998). Sales et al. (2015) found no significant differences between oocyte quality and apoptosis rates in multiparous *Bos taurus taurus* and *Bos taurus indicus* lactating cows. However, *Bos taurus indicus* cows produced higher quality oocytes compared to *Bos taurus taurus* cows, which had higher apoptosis rates. The percentage of TUNEL-negative oocytes showed the same behavior as the percentage of viable oocytes classified based on morphology, immediately after their recovery by follicular aspiration, throughout the days postpartum.

The body condition score of the female affects oocyte production. Adamiak et al. (2005) reported, in a study with dairy Holstein heifers, that animals with higher body condition (3.6 vs 4.1) have higher insulin concentrations (18.0 vs 48.3 $\mu\text{UI/mL}$) compared to those found in heifers with lower body condition score, indicating that fat heifers are under a hyperinsulinemic effect. In the primiparous cows of the present study, an increase in body condition score and live weight was observed after calving, which could explain the differences in maturation percentage due to lactation. It is observed that lactating primiparous cows had, on average, body condition scores of 3.75 ± 0.21 , 3.88 ± 0.28 , and 4.03 ± 0.28 at 30, 60, and 100 days postpartum, indicating that, probably, as the body condition score increased during the weeks of lactation, there was a reduction in the in vitro maturation rate of the oocytes.

A study with cows of different breeds showed that of the 64,826 oocytes sent to the laboratory, after being selected according to morphological structure (Viana et al., 2004), 16,924 viable embryos were

obtained for transfer. However, the embryo production per number of aspirated oocytes was 18.8% for F1 Holstein x Gir crossbred cows; that is, of the 7,035 viable aspirated oocytes, only 1,033 developed into embryos (Pontes et al., 2010). In the present study, only the in vitro maturation rate was evaluated, and the cleavage rate and the percentage of embryos produced were not measured. Santos et al. (2014) found that cleavage and blastocyst rates were higher for oocytes aspirated on day 14 postpartum compared to those obtained on days 28, 42, and 56 in lactating primiparous 3/4 and 7/8 Holstein x Gir cows kept under confinement. Under the current conditions of this study, we conclude that primiparous F1 Holstein x Gir cows, from 30 days postpartum, can enter a reproductive routine since they present good-quality oocytes, even during the early postpartum period. Higher body condition score in primiparous F1 Holstein x Gir cows may compromise oocyte quality and viability, which could be one of the causes of decreased reproductive success in dairy production systems. Well-fed primiparous F1 Holstein x Gir cows do not show loss of body condition and live weight in the first 100 days after calving; however, the diet should be adjusted to avoid excessive body condition, which could negatively affect reproduction. The feeding regimen used in the present study allowed maintenance of body condition and live weight, which likely justifies the normal reproductive function, with no negative energy balance observed during the peripartum period.

Declarations

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Compliance with ethical standards

The Animal Care Committee of Embrapa Gado de Leite approved the study design (protocol number # 9264220317/17), and it was conducted according to the principles of the Sociedade Brasileira de Ciência em Animais de Laboratório, which regulates conditions for experiments involving animals.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability declaration: Not applicable

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