**ANDRÉIA FERREIRA MACHADO**

# EFFECTS OF SUPPLEMENT CRUDE PROTEIN LEVEL ON PERFORMANCE AND REPRODUCTION OF REPLACEMENT HEIFERS MANEGED IN INTENSIVE GRAZING SYSTEMS

Dissertation submitted to the Universidade Federal de Viçosa as partial fulfillment of the requirements of the Graduate Program in Animal Science to obtain the degree of *Magister Scientiae*.

Adviser: Marcos Inácio Marcondes

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## BIOGRAPHY

ANDRÉIA FERREIRA MACHADO, daughter of Sérgio Machado and Maria José Dutra Ferreira Machado was born in Bicas, MG-Brazil on July 20, 1995.

She started the undergrad in Veterinary Medicine at Universidade Federal de Viçosa in 2013, and obtained a Bachelor of Science degree in Veterinary Medicine in January of 2018. In March of 2018, she started the Master Science program in Animal Science at the same university, with major in Ruminant Production and Nutrition.

## ABSTRACT

MACHADO, Andréia Ferreira, M.Sc., Universidade Federal de Viçosa, February, 2020. **Effect of protein supplement level on performance and reproduction of replacement heifers managed in intensive grazing systems.** Adviser: Marcos Inácio Marcondes.

Evaluations of the interaction between nutrition and reproduction of grazing dairy heifers are scarce. Thus, we aimed to evaluate performance, muscle and mammary gland development, oocyte quality, and in vitro production of embryos of crossbred heifers grazing an intensively managed pasture and supplemented with high or low protein concentrates. Eighteen pubertal crossbred heifers (Holstein x Gyr) with an initial weight of 350 ± 8.0 kg were used in a 90-day trial. Two supplement types, 12% crude protein (S12CP) or 24% CP (S24CP), and a control treatment (mineral mixture, CON) were randomly distributed to the heifers. Throughout the experiment, four digestibility trials were performed over four consecutive days. Four ovarium pick-ups were performed to evaluate oocyte quality and in vitro embryo production. Lastly, ultrasounds of carcasses and mammary glands were performed. The intakes of dry matter, digestible energy, and CP were greater for supplemented (SUP) compared with CON heifers. The SUP heifers had a greater average daily gain (645 versus 390 g/d) and rib eye area (58.78 versus 53.32 $cm^2$) than the CON heifers. Oocyte recovery, quality, and follicle features were not affected by supplementation strategy. However, the cleavage rate (47.17% versus 30.31%) and blastocyst rate (27.91% versus 10.12%) were negatively affected by supplementation. The S12CP presented a blastocyst rate much lower than the S24CP (3.02% versus 17.23 %). Carcass ultrasonography indicated a trend for greater rib eye area for S24CP and mammary ultrasonography indicated no effects of supplementation on mammary gland development. In summary, supplementation with concentrate containing 24% CP seems to be an appropriate strategy for satisfactory performance, with greater muscle deposition and no negative impacts on mammary gland development. However, in vitro embryo production was impaired.



Keywords: Supplementation. Oocyte quality. Embryo production.

## RESUMO

MACHADO, Andréia Ferreira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Efeitos da suplementação com diferentes níveis de proteína bruta na performance e reprodução de novilhas de reposição manejadas em sistema de pastejo.** Orientador: Marcos Inácio Marcondes.

Avaliações sobre a interação entre nutrição e reprodução de novilhas leiteiras em pastejo são escassas. Assim, objetivou-se avaliar o desempenho, o desenvolvimento muscular e da glândula mamária, a qualidade oocitária e a produção in vitro de embriões de novilhas cruzadas em sistema de pastejo intensamente manejado e suplementadas com concentrado de alto e baixo teor proteico. Dezoito novilhas cruzadas (Holândes × Gir) púberes com peso inicial de 350 ± 8,0 kg foram utilizadas no período experimental, com duração de 90 dias. Dois tipos de suplemento (12 e 24% de proteína bruta, S12CP e S24CP) e o tratamento controle (mistura mineral, CON) foram aleatoriamente distribuídos às novilhas. Ao longo do experimento quatro ensaios de digestibilidade foram realizados, durante quatro dias consecutivos. Quatro seções de aspiração folicular foram realizadas para avaliação da qualidade oocitária e da produção in vitro de embriões. Por último, avaliações ultrassonográficas da carcaça e da glândula mamária foram realizadas. O consumo de matéria seca, energia digestível e proteína bruta foram maiores para as novilhas suplementadas (SUP) comparado com as novilhas CON. As novilhas SUP obtiveram melhor ganho médio diário (645 versus 390 g/d) e maior área de olho de lombo (58,78 versus 53,32 cm²) do que as novilhas CON. A taxa de recuperação e qualidade oocitária e as características foliculares não foram afetadas pela estratégia de suplementação. Entretanto, a taxa de clivagem (47,17 versus 30,31%) e taxa de blastocistos (27,91 versus 10,12%) foram negativamente afetadas pela suplementação. O tratamento S12CP apresentou a taxa de blastocistos inferior ao tratamento S24CP (3,02 versus 17,23%). A ultrassonografia da carcaça indicou uma tendência de maior área de olho de lombo para o tratamento S24CP e a ultrassonografia da glândula mamária não indicou efeitos da suplementação no desenvolvimento da glândula mamária. Em resumo, a suplementação com concentrado contendo 24% PB demostra ser uma estratégia apropriada para uma performance satisfatória durante a estação chuvosa em clima tropical, com maior deposição muscular e sem impactos negativos ao desenvolvimento da glândula mamária. Por outro lado, a produção in vitro de embriões foi prejudicada.



Palavras-chave: Suplementação. Qualidade oocitária. Produção de embriões.

## LISTA DE ABREVIATURAS

18S 18S ribosomal RNA

ADG Average Daily Gain

BMP15 Bone Morphogenetic Protein 15

BR Blastocyst rate

BR/CO Blastocysts per Cleaved Oocytes

CO Cleaved Oocytes

COC Cumulus Oocyte Complexes

CON Control Treatment

CP Crude Protein

CPI/DMI Crude Protein Intake per Dry Matter Intake

CPmic Microbial Crude Protein

CR Cleavage Rate

DE Digestible Energy

DM Dry Matter

DMI Dry Matter Intake

DMI/BW Dry Matter Intake per Body Weight

EMS Efficiency of Microbial Crude Protein Synthesis

FV Follicles Visualized

GDF9 Growth and Differentiation Factor 9

IVP In Vitro Production

IVPE In Vitro Produced Embryos

NDF Neutral Detergent Fiber

NDFap Neutral Detergent Fiber corrected for ash and protein contents

NDFI/BW Neutral Detergent Fiber Intake per Body Weight

OPU Ovum Pick-Up

OR Oocytes Recovered

PI Pasture Intake

PI/BW Pasture Intake per Body Weight

RDP Rumen Degradable Protein

RR Recovered Rate

S12CP Supplement with 12% Crude Protein

S24CP Supplement with 24% Crude Protein

SR Substitution Rate

SUP Supplemented

THI Temperature Humidity Index

VO Viable Oocytes

VO/OR Viable Oocytes per Oocytes Recovered

**SUMÁRIO**

*Interpretive summary:* **Effect of protein supplement level on performance and reproduction of replacement heifers managed in intensive grazing systems.** By Machado et al**.** Evaluations of the interaction between nutrition and reproduction of grazing dairy heifers are scarce. Therefore, we aimed to examine the effect of protein supplementation (12% and 24% crude protein in the supplement) on the performance and reproduction of dairy heifers in an intensive grazing system. The supplement with 24% crude protein improved the heifers' performance. However, embryo production was impaired with that level of protein supplementation.

PROTEIN SUPPLEMENTATION FOR GRAZING HEIFERS

**Effect of protein supplement level on performance and reproduction of replacement heifers managed in intensive grazing systems**[1]

[1]Manuscript written to be submitted to the *Journal of Dairy Science*

## ABSTRACT

Evaluations of the interaction between nutrition and reproduction of grazing dairy heifers are scarce. Thus, we aimed to evaluate performance, muscle and mammary gland development, oocyte quality, and in vitro production of embryos of crossbred heifers grazing an intensively managed pasture and supplemented with high or low protein concentrates. Eighteen pubertal crossbred heifers (Holstein x Gyr) with an initial weight of 350 ± 8.0 kg were used in a 90-day trial. Two supplement types, 12% crude protein (**CP**) (**S12CP**) or 24% CP (**S24CP**), and a control treatment (mineral mixture, **CON**) were randomly distributed to the heifers. Throughout the experiment, four digestibility trials were performed over four consecutive days. Four ovarium pick-ups were performed to evaluate oocyte quality and in vitro embryo production. Lastly, ultrasounds of carcasses and mammary glands were performed. The intakes of dry matter (**DM**), digestible energy (**DE**), and CP were greater for supplemented (**SUP**) compared with CON heifers. The SUP heifers had a greater average daily gain (**ADG**) (645 versus 390 g/d) and rib eye area (58.78 versus 53.32 $cm^2$) than the CON heifers. Oocyte recovery, quality, and follicle features were not affected by supplementation strategy. However, the cleavage rate

(47.17% versus 30.31%) and blastocyst rate (27.91% versus 10.12%) were negatively affected by supplementation. The S12CP presented a blastocyst rate much lower than the S24CP (3.02% versus 17.23 %). Carcass ultrasonography indicated a trend for greater rib eye area for S24CP and mammary ultrasonography indicated no effects of supplementation on mammary gland development. In summary, supplementation with concentrate containing 24% CP seems to be an appropriate strategy for satisfactory performance, with greater muscle deposition and no negative impacts on mammary gland development. However, in vitro embryo production was impaired.

**Key words:** supplementation, oocyte quality, embryo production

## INTRODUCTION

Replacement heifers represent the future lactating cows of a dairy herd. However, as this phase is long and expensive, an earlier age at first calving has been sought for better economic returns (Ettema and Santos, 2004; Hutchison et al., 2017). One way to reduce the costs of replacement heifers is to use grazing systems (Lowe et al., 2016). In the tropics, the feeding of dairy heifers is usually based on pasture systems (Paciullo et al., 2011), as increasing the proportion of forage in the diet reduces feeding costs without affecting growth rates when adequate supplementation is provided (Emmanuel et al., 2015).

An adequate supplementation program should be established for grazing heifers, as tropical grasses can rarely be considered a balanced diet (Detmann et al., 2014a) and can often lead to low performance (Boval et al., 2015). In grazing systems, there is an excess of energy in the grasses during the rainy season, and protein supplementation is frequently needed (Detmann et al., 2010, 2014a;b). Previous studies with beef heifers showed that supplementary nitrogen improved their performance (Batista et al., 2016; Sotelo et al., 2018). Additionally, Machado et al. (2019) observed that protein supplementation improved the digestibility and performance of Holstein heifers grazing intensively managed tropical grass (*Panicum maximum* cv. Mombaça).

However, diets with a high concentration of nitrogen compounds have been associated with impaired reproductive performance of cows (Jordan and Swanson, 1979; Butler et al., 1996; Lean et al., 2012). Grazing dairy cows usually ingest large amounts of rapidly rumen degradable protein RDP (Diskin et al., 2006), allowing an increase in the levels of ammonia and circulating urea, which causes reproductive damage as there is a strong correlation between urea nitrogen concentration in follicular fluid and in blood (Leroy et al., 2008). Thus, oocyte quality may be

influenced by the excess of urea nitrogen circulating in the bloodstream (Leroy et al., 2004; Hammon et al., 2005). Diets that generate high concentrations of plasma urea nitrogen may impair oocyte competence (Sinclair et al., 2000; De Wit et al., 2001; Ocon and Hansen, 2003) and impair embryonic development (Fahey et al., 2001; Papadopoulos et al., 2001; Ferreira et al., 2011).

The hypothesis of this study is that crossbred heifers (Holstein x Gyr) in intensively managed grazing systems supplemented with a high protein concentrate will have lower oocyte quality and lower embryo production than animals supplemented with a low protein concentrate, despite having similar performance. Thus, the aim of this study was to evaluate performance, nutrient intake and digestibility, muscle and mammary gland development, blood metabolic profile, oocyte quality, and in vitro production of embryos of crossbred heifers in an intensively managed grazing system supplemented with high or low protein concentrates.

## MATERIALS AND METHODS

All animal handling and procedures of the present study were approved by the Ethics Commission on the Use of Farm Animals of Universidade Federal de Viçosa (Viçosa, MG, Brazil), under protocol no. 022/2018.

### *Animals, Experimental Design, and Feeding*

Eighteen pubertal crossbred heifers (¾ Holstein x Gyr), with an initial weight of 350 ± 8.0 kg and age of 19 ± 1.0 months, were kept in an intensively managed rotating grazing system on *Panicum Maximum* cv Mombaça. Eighteen paddocks of 1000 m² each were used, where all animals always grazed the same paddock. The puberty of females was confirmed by the presence of the corpus luteum on gynecological examination. The experimental period occurred during the rainy season, from January to April.

The animals were randomly distributed among three treatments, which were: mineral mixture (**CON**; control treatment), concentrate supplement with 12% CP (**S12CP**), and concentrate supplement with 24% CP (**S24CP**). Concentrate was offered at 0.5% of body weight (**BW**), while water and mineral mixture were supplied ad libitum. The concentrate was formulated based on corn meal and soy meal and was fed individually according to the pre-established treatment; the composition of the diet is presented in Table 1. Every day at 1100 h

animals were removed from the paddocks, separated, and the supplement was individually fed. No orts were allowed. The amount of supplement offered to each animal was adjusted for each period according to their weight. Animals from the CON treatment were also led to individual stalls but received no supplementation.

The calculations to determine the size and management of the paddocks followed the same methodology presented by Machado et al. (2019). We expected a production of approximately 73 kg DM/ha/d during the experimental period, based on the previous pasture yield of the same area. Therefore, for a total of 90 d (30 d for adaptation and 60 d for the experimental period), using 18 heifers/paddock/d, 18 paddocks of 1000 m² were necessary (considering a grazing efficiency of approximately 70%). The fertilization procedure was distributing 20 kg of N/ha and 15 kg of $K_2O$/ha in the paddock after each grazing cycle. The pre-grazing sward height target was 70 cm, and the post-grazing height target was 35 cm. Thus, every day before the entrance to a new paddock and after removing the animals from the previous one, the mean height of the pasture was obtained at ten different points using a graduated ruler. The actual herbage allowance is presented in Table 2.

The experimental period lasted 90 d, 30 d of adaptation to the experimental diet, so that the dietary effects could be observed and 60 d subdivided into four periods of 15 d each. Animals were kept in the same treatment group for the entire study. Initially, the animals were kept in the area for a period of 30-d for adaptation, during this adaptation period all heifers were fed the same concentrate containing 18% CP at 0.5% BW.

***Performance Estimates***

To estimate the ADG, animals were weighed for three consecutive days at the beginning and end of the experiment. On the first day of each period, intermediate weightings were performed to adjust the concentrate supply. All weightings were performed after supplementation, before the animals returned to the paddock.

***Total Apparent Digestibility Trial, Analysis, and Calculations***

Titanium dioxide was used as a marker to determine fecal excretion, which was provided orally for eight days at 15 g/d per animal and started on d 1 of each period. After the five-day period of marker provision, fecal collection started (d 6). Four spot collections of feces were

performed at 1800 h of d 6, 1400 h of d 7, 1000 h of d 8, and 0600 h of d 9. On the same days, spot urine samples (approximately 50 mL) were obtained by stimulated urination. To pool the urine samples, 10 mL of pure urine was diluted into 40 mL of sulfuric acid (0.036 *N*) and stored (−20 ºC) to prevent purine derivative degradation (Valadares et al., 1999).

The composition and availability of the grazing stratum were estimated between d 6 and d 9 through two isolation cages (1.0 × 1.5 m), which were placed in the paddock before the animals' entrance, in a place representative of the pasture height and density, in the same way used by Machado et al. (2019). On the following day, after the animals left the paddock, the average height of the pasture was used so that the height of the forage inside the cage was cut/sampled at the same height of the pasture consumed by the animals (Brandao et al., 2018; Machado et al., 2019). At the end of each digestibility trial, samples of pasture and feces were pooled and stored at −20 ºC.

Samples of forage and feces were partially dried in a forced-air drying oven at 55 °C for 72 h (Detmann et al., 2012). Concentrate, forage, and feces samples were grounded in a Willey mill (model TE-680, brand TECNAL, Piracicaba, São Paulo, Brazil) in 2 mm and 1 mm screens (Detmann et al., 2012). The 1-mm ground samples were analyzed for DM (AOAC International, 2005; method 934.01), CP (AOAC International, 2005; method 990.13), ash (AOAC International, 2005; method 942.05), and NDF corrected for ash and protein contents (**NDFap**) (Detmann et al., 2012; INCT-CA methods F-002/1, N-004/1, and M-002/1). While the 2-mm ground samples were used to determine the indigestible NDF, which was used as an internal marker to estimate pasture intake. Briefly, samples of feeds and feces were incubated in the rumen of a cow, using non-woven textile bags (100 g/m$^2$) for 240 h, and NDF was estimated in the post-incubation material (Valente et al., 2011).

The urine samples were analyzed for the content of creatinine, measured using the colorimetric endpoint method, with the use of picrate and acidifier in commercial kits (Labtest Diagnóstica S.A. Lagoa Santa, Minas Gerais, Brazil). In addition, the concentration of uric acid and allantoin in urine were determined according to Fujihara et al. (1987) and Chen and Gomes (1992), respectively. The total daily urinary excretion was estimated using the daily creatinine excretion as proposed by Chizzotti et al. (2008) for Holstein heifers ($\mathrm{CE} = 32.2 - 0.0109 \times \mathrm{BW}$). The ruminal microbial CP (**CPmic**) synthesis was estimated as a function of absorbed purines, which was calculated from the excretion of the purine derivatives, uric acid, and allantoin, according to the equations proposed by Prates et al. (2012) for Holstein heifers.

***Real-time Carcass Trait Evaluation***

On d 1 and d 90 of the experimental period, an ultrasound device was used to measure the *gluteus medius* and the *biceps femoris* muscle intercessions, located between the ischial and the ileal tuberosities in the P8 region and to scan the *longissimus dorsi* between the 12th and 13th ribs. We used an 18-cm linear array ultrasound instrument (Aloka SSD-500V, Aloka Co., Ltd., Tokyo, Japan) operated at a frequency of 3.5 MHz. A standoff (Aloka long standoff guide-beef, Aloka Co., Ltd. Tokyo, Japan) and vegetable oil were used for adequate acoustic contact between the transducer, the standoff, and the animals' skin. Ultrasound images were recorded and later analyzed for back fat thickness and rib eye area (**REA**) using the BioSoft Toolbox® II for 200 Beef (Biotronics Inc., Ames, Iowa, USA) software.

***Blood Sampling and Analysis***

Blood samples were collected on d 15 of each period from coccygeal venipuncture. We used vacutainer tubes of 10 mL containing separator gel and clot activator (silica) for serum collection, and tubes of 5 mL containing sodium fluoride for plasma collection. The tubes were kept on ice until centrifugation (3,000 × *g* at 4 ºC for 20 min). The serum and plasma were pipetted into Eppendorf tubes and stored (−20 ºC) until analysis.

The serum sample was used for analysis of blood urea, total protein, albumin, total cholesterol, triglycerides, insulin, and IGF-I, and the plasma sample was used for glucose analysis. Concentrations of urea, glucose, total protein, albumin, total cholesterol, and triglycerides were measured by biochemical multi-analyzer (HumanStar 300; Human GmbH, Wesbaden, DEU) with Bioclin Kits. Analyses of insulin and IGF-I were performed using chemiluminescence immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, USA).

***Ultrasound-Guided Transvaginal Follicular Aspiration***

On d 9 of each period, the heifers were submitted to a synchronization protocol with 0.5 g intravaginal progesterone device (Primer – Tecnopec) and 2 mg estradiol benzoate (Sincrodiol, OuroFino). On d 13, the animals received 0.5 mg of cloprostenol sodium (Sincrocio, OuroFino) and ovum pick-up (**OPU**) occurred on d 15, corresponding to four OPUs every 15 days. An

ultrasound device (B-mode) equipped with a micro-convex transducer working at a frequency of 6.5 MHz (DP2200, Mindray, China) coupled to a guide (WTA) was used. A 20 G needle and a 1.2 m follicular aspiration system (WTA, Cravinhos, SP, Brazil) were added to this system (Galli et al., 2001). Before starting the aspiration process, the follicles present in each ovary were counted and measured. Only follicles of 8 mm or less were aspirated. The aspiration system was subjected to negative pressure of 14 mL per minute (96 mmHg) through a vacuum pump (WTA). All follicles (≤ 8 mm) were punctured and their contents were collected in a 50 mL vial containing 10 mL of 0.9% saline plus 10 IU sodium heparin/mL, and preserved at 35–36 °C. The aspirated fluid was poured into an 80 µm mesh filter (WTA, Cravinhos, SP, Brazil). After filtration, the contents were transferred to a petri dish (dimensions 90 × 15 mm) with 0.9% saline solution and were screened in a stereomicroscope. The cumulus oocyte complexes (**COC**) were morphologically evaluated and classified as viable or inviable based on oocyte cytoplasm characteristics and the number of cumulus cell layers (adapted from Leibfried and First, 1979). Oocytes that presented layers of cumulus or were partially denuded with homogeneous cytoplasm were considered viable and oocytes nude or in degeneration that presented heterogeneous cytoplasm were considered inviable.

***In Vitro Oocyte Maturation, Fertilization, and Culture***

The oocytes referring to the last 3 OPUs were destined for in vitro embryo production (**IVP**). The oocytes were classified and those classified as viable were transferred to the maturation medium in a 95% humidity and 5% $CO_2$ atmosphere at 38.5 °C for 22–24 hours in an oocyte carrier (Gonçalves et al., 2007), used to transport the oocytes to the commercial laboratory (BH Embriões, Belo Horizonte, Minas Gerais), where in vitro production was performed. After maturation, the mature COCs were fertilized with frozen sexed semen from a single batch of a single proven in vitro fertility Holstein bull. The sperm concentration was adjusted to $2 \times 10^6$ sperm/mL and kept in the medium with the oocytes for a period of 18–20 hours. Possible zygotes were transferred to the culture medium, prior to this step the possible zygotes were stripped (removal of cumulus cells by successive pipetting). The cleavage rate (**CR**) was determined 72 hours after fertilization and the blastocyst rate (**BR**) evaluated 192 hours after fertilization (Gonçalves et al., 2007). All media used in the procedure were purchased from Origem Embriões (Uberaba, Minas Gerais).

***Oocyte Transcript Quantification***

The oocytes obtained at the first OPU were used for gene expression analysis. A pool of four oocytes (viables) from each female was quickly frozen in liquid nitrogen after going through the denudation process (pipetting). For total RNA extraction and cDNA synthesis, the Cells-to-cDNA kit (Ambion – Austin, USA) was used according to the manufacturer's recommendations. Quantitation of cDNA concentration was performed using 1 µL of sample in a NanoVue Plus spectrophotometer (GE Healthcare). Finally, the samples were diluted to 10 ng/µL concentration and the material was stored at −20 °C for real-time PCR analysis.

***Relative Quantification by Real-Time PCR***

Relative quantification was performed in duplicate on an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using GoTaq qPCR Master Mix (Promega Corporation, Madison, USA) according to the manufacturer's recommendations. The amplification efficiency of each gene was calculated by constructing a cDNA serial dilution curve at concentrations of 25, 75, and 225 ng cDNA and concentrations of 100, 200, and 400 ng primer per reaction. The reactions were considered efficient when the amplification efficiency of the target gene and the reference gene were approximately equal, with a tolerance of 10% variation in relation to the reference gene, as described by Livak and Schmittgen (2001). Amplification conditions for all systems were 95 °C for two minutes, 40 denaturation cycles at 95 °C for 15 seconds, and extension at 60 °C for 60 seconds.

The expression for each gene was calculated using the ΔCt method (target gene Ct − Ct endogenous reference) for all individual samples, where Ct reflects the PCR cycle number at which the fluorescence generated crosses an arbitrary threshold. The gene expression differences were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). Target genes evaluated in the current study were Bone Morphogenetic Protein 15 (*BMP15*) and Growth and Differentiation Factor 9 (*GDF9*) which are important regulators of ovarian follicular development and ovulation rate (McNatty et al., 2004), and the reference gene was 18S ribosomal RNA (*18S*). Primer pairs for all genes are listed in Supplementary Table 1.

***Mammary Gland Ultrasound***

On d 1 and d 90 of the experimental period we collected ultrasound images of the mammary glands. Mammary gland ultrasound images were taken using a micro-convex transducer (Mindray DP2200, Shenzhen, China), operating at a frequency of 6 MHz (Albino et al., 2017a). Images were taken of each mammary quarter in a standardized position, with an inclination of 45º in relation to teat insertion, and recorded in bitmap (BMP) format, a technique described by Albino et al. (2017a). Mammary gland ultrasound images were evaluated for pixel value in an 8-bit format using ImageJ® (NIH, Bethesda, MD, USA) software.

The pixel value of each mammary quarter was obtained as the mean from three squares (16 mm² each) randomly collected near the ductal structures and mammary fat pad from each image. Then, the pixel of the mammary gland was obtained as an average value of the mammary quarters.

***Statistical Analysis***

All variables were analyzed using the GLIMMIXED procedure of SAS (Statistical Analysis System, version 9.4). Intake and digestibility data, blood parameters, and oocyte and embryonic parameters were analyzed as a completely randomized design, and the period was included as a repeated measure in the model:

$$Y_{ijkl} = \mu + T_i + \delta_{ij} + P_k + (T \times P)_{ik} + \varepsilon_{ijkl}$$

Where $\mu$ = general mean; $T_i$ = fixed effect of the treatment i; $\delta_{ij}$ = random error with a mean of zero and variance of $\sigma^2$, the variance among animals within treatment, equal to the covariance among repeated measures within animals; $P_k$ = fixed effect of period; $(T \times P)_{ik}$ = fixed effect of the interaction between treatment i and period e; and $\varepsilon_{ijkl}$ = random error with a mean of zero and variance of $\sigma^2$, the variance among measures between animals.

Seven variance–covariance structures (AR1, CS, UN, TOEP, VC, ARH1, TOEPH) were tested, and the one that provided the best fit based on the Akaike information criterion was used.

Reproductive characteristics and gene expression data did not follow a normal distribution and data were analyzed using Poisson (for reproduction variables), exponential (*GDF9*), or beta (*BMP15*) distributions.

Performance data, mammary gland, carcass, and gene expression were analyzed as a completely randomized design, model:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where $\mu$ = general mean; $T_i$ = fixed effect of the treatment i; $\varepsilon_{ij}$ = random error.

Means were compared by orthogonal contrasts as follows:

Contrast 1: Effect of supplementation (non-supplemented animals – CON vs supplemented animals – SUP);

Contrast 2: levels of CP in the concentrate (S12CP vs S24CP).

Contrasts were considered significant when $P \leq 0.05$ and tendency was used when $0.05 < P < 0.10$.

## RESULTS

The SUP had a greater dry matter intake (**DMI**) ($P = 0.014$; Table 3) and digestible energy (**DE**) intake when compared with CON ($P < 0.01$; Table 3). Additionally, the CON had a tendency to greater pasture intake (**PI**) and PI per BW (**PI/BW**) when compared with SUP ($P = 0.05$; Table 3). There was no interference of supplementation on NDF intake (4.68 kg/d), relative DMI (20.66 g/kg of BW), and relative NDF intake (12.15 g/kg of BW). The SUP had a greater CP intake when compared with CON (P = 0.008; Table 3), and there was no difference in CP digestibility between SUP and CON. Both CP intake and digestibility were greater in S24CP when compared with S12CP ($P > 0.01$; Table 3).

The SUP animals had greater DM digestibility when compared with CON ($P = 0.001$; Table 3). The NDF digestibility was not different between SUP and CON ($P = 0.864$; Table 3). The CPmic and efficiency of microbial crude protein synthesis (**EMS**) were also not affected by treatment and were, on average, 524.08 g/d and 117.17 g/kg of total digestible nutrient intake, respectively.

We observed a period effect for NDF intake, relative DMI, PI, and NDF intake, DM, CP, and NDF digestibility and EMS ($P < 0.05$; Table 3). The NDF intake, relative DMI, PI, and NDF intake were greater in periods 1 and 3 when compared with periods 2 and 4. The DM, CP, and NDF digestibility were greater in period 1when compared with other periods. However, the EMS was lower in period 1 when compared with other periods. CP intake per DMI (**CPI/DMI**) presented a treatment-by-period interaction, and S24CP had the greater CPI/DMI and S12CP the lowest CPI/DMI in all periods ($P = 0.005$; Table 3).

The ADG ($P = 0.003$; Figure 1) and REA ($P = 0.040$; Table 4) were affected by supplementation, and SUP had greater ADG and REA when compared with CON. We also observed a tendency for greater REA in S24CP animals when compared with S12CP ($P = 0.07$; Table 4). The back fat thickness did not vary across treatments ($P = 0.952$; Table 4) and was,

on average, 2.73 mm. The mammary glands were also not affected by treatments, either in the parenchymal tissue or in the fat pad mammary tissue ($P > 0.05$; Table 4).

The SUP animals had higher albumin ($P = 0.008$; Table 5) when compared with CON. Total protein ($P = 0.873$; Table 5), total cholesterol ($P = 0.697$; Table 5), triglycerides ($P = 0.427$; Table 5), insulin ($P = 0.534$; Table 5), and IGF-I ($P = 0.959$; Table 5) were not affected by treatments. Nevertheless, triglycerides, insulin, and IGF-I ($P < 0.05$; Table 5) were affected by period. Total protein had a decreased concentration in periods 3 and 4 when compared with other periods, insulin was higher during period 4 and IGF-I was lower during period 1. In addition, urea and glucose had a treatment-by-period interaction. The urea was higher in S24CP during period 4 when compared with S12CP and CON (Figure 2). The glucose was higher in S12CP during the first period when compared with CON, and, in period 4, glucose was higher in S12CP when compared with S24CP ($P = 0.031$; Figure 2).

The number follicles visualized (**FV**) did not differ between SUP and CON (15.01) ($P = 0.273$; Table 6). However, there was a difference in the FV between S24CP and S12CP, and we observed, on average, five more follicles in S24CP when compared with S12CP ($P = 0.016$; Table 6). The provision of supplement (when compared with CON) did not change the number of oocytes recovered (**OR**) (12.54) ($P = 0.132$; Table 6) and the recovery rate (**RR**) (90.39%) ($P = 0.306$; Table 6). Similarly, the number of viable oocytes (**VO**) was not affected by the CP content in the supplement ($P = 0.926$; Table 6). Additionally, the VO per oocytes recovered (**VO/OR**) tended to be greater in CON animals (50.64%) when compared with SUP (42.26%) ($P = 0.078$; Table 6). The period affected the VO ($P = 0.007$; Table 6), since period 1 had the lowest VO.

The cleaved oocytes (**CO**) tended to be 28.11% greater in CON when compared with SUP ($P = 0.099$; Table 6). Moreover, S24CP tended to have 39.04% greater CO when compared with S12CP ($P = 0.082$; Table 6). The CR was 35% lower in SUP when compared with CON ($P = 0.017$; Table 6). Additionally, the CR was not different between S24CP and S12CP (30.31%) ($P = 0.102$; Table 6).

The supplementation negatively interfered with the BR, which was 63.72% lower in SUP animals ($P = 0.001$; Figure 3). The S24CP (17.23%) had a greater BR when compared with S12CP (3.02%) ($P = 0.012$; Figure 3). The number of in vitro produced embryos (**IVPE**) was also negatively affected by supplementation, and CON had 51.8% more IVPE compared to SUP ($P = 0.016$; Table 6). In addition, S24CP presented a greater IVPE (1.33 embryos) when compared with S12CP (0.27 embryos) ($P = 0.011$; Table 6). The CON had a high BR per

number of CO (**BR/CO**) when compared with SUP ($P = 0.019$; Table 6). In addition, S24CP presented a tendency to greater BR/CO when compared with S12CP ($P = 0.055$; Table 6). The BR/CO averages were 58.30%, 15.26%, and 44.57% for CON, S12CP, and S24CP, respectively. Neither of these characteristics had a treatment-by-period interaction ($P > 0.05$; Table 6).

Finally, the gene expression of *BMP15* and *GDF9* was similar across treatments ($P > 0.05$; Figure 4).

## DISCUSSION

The CON presented a tendency to greater PI, followed by S24CP. Similar results were observed by Machado et al. (2019), who evaluated protein and energy supplementation for grazing dairy heifers and observed that protein supplementation promoted greater PI, since greater levels of rumen ammonia promoted greater forage digestibility (Moraes et al., 2006). Other authors also suggested that supplements with greater energy content, as occurred in S12CP, tend to cause a reduction in PI (Faverdin et al., 1991; Stockdale, 2000; Machado et al., 2019). This information confirms the effect observed in this study, where supplementation with a greater protein level (S24CP) increased the PI. Furthermore, the greater energy intake from supplementation in S12CP triggered a greater substitution rate (Bargo et al., 2003) when compared with S24CP. We observed a substitution rate of 0.59 in S12CP, and 0.29 in S24CP heifers, which represents a two-fold greater substitution rate. In addition, the CON presented a tendency to greater relative PI compared with SUP. Therefore, CON animals required a greater relative PI to meet their nutritional needs. Since CON did not receive supplementation, it has been shown that these animals will try to compensate for the lower dietary energy by increasing PI (Silveira et al., 2008; Machado et al., 2019).

According to Dixon and Stockdale (1999) when you have medium and high quality forages, containing adequate microbial substrates, changes in voluntary forage intake are likely to be a consequence of changes in the rate of rumen digestion of forage components and the rate of removal of feed residues from the rumen. This indicates that S12CP might have a reduced digestion rate of forage fiber due to the drop in pH caused by the consumption of the high energy supplements, which leads to a longer time to remove rumen forage, leading to less PI observed in these animals.

Supplementation was associated with a greater DMI and DEI, which was reflected in greater DM digestibility and ensured better ADG. The greater ADG associated with supplementation confirms the fact that supplementation improves performance even during periods of greater forage quality (Poppi and McLennan, 1995; Paulino et al., 2008; Miorin et al., 2016) and that the greater energy intake is the reason for the higher performance, since performance is directly affected by energy intake (Brown et al., 2005). The greater CP intake and CP digestibility of S24CP was provided by the greater CP intake, consequently diluting the endogenous fraction of N, impacting CP digestibility. According to Moraes et al. (2006), protein supplementation creates a synchronism between the fermentable organic matter in the rumen and the use of nitrogen by the ruminal microorganisms. However, CPmic and EMS were not affected by treatments, what lead us to think that the excess protein of the S24PB was not used in rumen N metabolism and was exported to the bloodstream as ammonia, since the blood urea was on average 12% higher in the S24CP when compared with S12CP and CON.

The greater DMI and DM digestibility likely promoted the greater ADG and REA observed in SUP when compared with CON animals. SUP animals had greater energy intake, which is closely linked with ADG. It has previously been described that greater energy intake stimulates muscle protein tissue synthesis, since protein turnover is stimulated by a higher content of energy consumed (Reeds, 1989). On the other hand, S24CP animals had a greater CP intake and CP digestibility, which is closely linked to REA gain, thus gain composition, indicating that our protein supplementation (S24CP) favored a greater protein synthesis in animal muscle (Rosenvold et al., 2001). The increase in protein synthesis is usually related to greater ADG, since the energy cost to deposit protein is lower than lipid deposition (Owens et al., 1995).

We also observed a higher albumin concentration in SUP, which may be linked to their greater DMI and DM digestibility. Usually blood albumin is related to a higher concentration of nutrients available for absorption, which requires a higher concentration of blood albumin for the transport of substances such as free fatty acids and amino acids (González et al., 2000). Consequently, we observed a high blood urea, especially during periods 2 and 4 when the pasture had a greater CP content. The high concentration of glucose, especially during the first period, was the same period in which we observed a greater digestibility of DM and NDF, and greater relative PI and DM, providing higher levels of circulating glucose. The difference found between CON and S12CP is possibly due to the greater intake of gluconeogenic precursors by S12CP animals. There was no difference in glucose concentration between CON and S24CP, despite the supplementation received by the animals of S24CP; this was possibly due to the

higher energy cost of these animals for the synthesis of urea (Butler, 1998) and due to the greater proportion of muscle tissue, which demands greater glucose uptake for muscle turnover. During period 3, there was a drop in circulating glucose, which coincided with the period of lower DM and NDF digestibility, which may explain this drop despite the greater availability of pasture (Table 2). The same reason given above explains the difference in glucose concentration between S24CP and S12CP during period 4.

The FV number depends on follicular recruitment during the growth wave and improvements in nutritional condition lead to increased recruitment of small follicles (Gutiérrez et al., 1997; Gong, 2002). Metabolic hormones such as IGF-I and insulin can act to control follicular development stages independently or in synergy with gonadotropins by modulating follicular recruitment (Gong et al., 1996; Armstrong et al., 2001). For this reason, despite the difference found in FV between S24CP and S12CP, we could not associate this difference with the responses to treatments, since both groups received supplementation and we did not find variations in blood IGF-I and insulin between them. The OR of our Holstein × Gyr crossbred heifers was close to the number of OR from Holstein heifers (Gimenes et al., 2014) and Holstein cows (Pontes et al., 2010; Sales et al., 2015). Pontes et al. (2010) observed that crossbred ½ Holstein × Gyr animals had an OR pattern close to that found for Zebu animals, which was greater than that observed in this study (31.40 OR on average). Thus, we speculate that ¾ Holstein × Gyr heifers may have an ovarian physiology close to what we observe in Holstein heifers, but future studies should look more closely into this issue. Differences in ovarian physiology between *Bos taurus* and *Bos indicus* were associated with the lower number of COCs recovered by OPU in *Bos taurus* animals (Viana et al., 2012). *Bos taurus* animals had fewer follicles recruited per follicular wave, fewer follicular waves per cycle, and greater persistence and diameter of the dominant follicle (Sartori et al., 2016), characteristics that interfere with the smaller number of follicles along the estrous cycle compared to Zebu breeds (Viana et al., 2012).

The VO was also close to that observed in Holstein heifers by Gimenes et al. (2014). However, when compared with other authors, our VO was lower than observed in Holstein cows (Pontes et al., 2010; Sales et al., 2015). Considering that the heifers were kept in a grazing system, we speculate that this lower oocyte quality might be a result of an increased heat stress (Supplementary Table 2). Nevertheless, our results were similar to those found by Souza-Cácares et al. (2019), who worked with grazing ¾ Holstein × Gyr cows. Additionally, the VO/OR we observed is in agreement with that obtained by Fialho et al. (2018), in environmental

conditions of THI between 69 and 72 for crossbred Holstein × Gyr animals in grazing conditions (Supplementary Table 2). Hyperthermia can directly affect follicle function, leading to changes in follicular development, dominance, steroidogenesis, and gonadotropin secretion (Roth et al., 2000), indicating that the climatic conditions of the experiment may have influenced the low oocyte quality. This may also indicate that, in conditions of heat stress, expenditures to maintain body temperature may mask any benefit from nutrition (greater protein and energy intake) (Broucek et al., 2009). Once again, we could not link VO and VO/OR results with our nutrition responses, since differences in energy and protein intake across our treatments could not be associated with the differences we observed.

The IVPE and BR obtained were in agreement with information obtained for Holstein animals (Gimenes et al., 2014; Sales et al., 2015). The greater IVPE in Zebu breeds was previously associated with a better intrinsic quality of oocytes in these animals or with a positive cooperative effect from a higher number of COCs in the culture (Viana et al., 2012). Additionally, the greater CPI/DMI presented by treatments CON and S24CP did not affect the BR. However, only the CON presented a BR close to the average IVP found at the commercial level for *Bos taurus* breeds of 25.60% (Viana et al., 2012). Lean et al. (2012) observed, in a meta-analysis, that increased dietary CP or increased CP degradability reduced the chances of conception in lactating cows. Studies suggest that the deleterious effects of urea on embryo quality are probably due to deleterious changes in the follicle or oviduct (Sinclair et al., 2000; Fahey et al., 2001; Rhoads et al., 2006). The values of VO and gene expression had no difference across treatments for oocytes, only a trend of greater VO/OR was observed for CON. However, considering that the smallest BR was found in S24CP compared with CON, we suspect that the higher blood urea due to the greater CP intake and digestibility led to losses in the IVPE of the S24CP, since the concentration of blood urea interferes in the composition of the follicular microenvironment (Leroy et al., 2004, 2008; Hammon et al., 2005). Although S12CP had the lowest IVPE, the blood urea concentration in this treatment was the lowest. On the other hand, considering the value presented by Leroy et al. (2008), the serum urea concentration above 20 mg/dL could result in reproductive losses, so all treatments in this experiment could present some level of reproductive loss.

Mikkola et al. (2005) obtained an improvement in embryo quality with a moderate long-term increase in protein content (18% CP) of Ayrshire heifers' diets. Other authors also found no effect of excess CP on long-term embryonic quality, suggesting that cows can adapt to a high urea content over a 10-day period, which prevents reproductive damage (Dawuda et al., 2002;

Laven et al., 2004). In our study, the supplement was provided for a period of 30 days before the start of collections, what might explain the BR of the S24CP, indicating that although the BR was lower in the S24CP compared to the CON, we suspect there was an adaptive effect to the high blood urea, thus without a negative impact on BR.

We could not identify the reason for the low BR observed in S12CP animals. Three animals in this treatment did not present any oocytes that reached the blastocyst phase during the in vitro embryo production, greatly reducing the average rate in S12CP. During the experiment or in vitro embryo production, we could not observe anything that could elucidate these low results. Therefore, we do not have a physiological explanation for such a low rate, and we have never faced such a low rate in our farm conditions.

The absence of differences in *GDF9* and *BMP15* gene expression among treatments confirms that there were no differences in these genes that could interfere with embryo production. *BMP15* and *GDF9* are members of the *TGFβ* family and are expressed at all stages of bovine follicular development (Juengel and McNatty, 2005). They are fundamental for activation of primordial follicles and for cell development and differentiation (Hanrahan et al., 2004; Yoshino et al., 2006; Gendelman et al., 2010). Authors have demonstrated that mutation in these genes triggers reproductive defects (Shimasaki et al., 2004; Su et al., 2004; McNatty et al., 2005), confirming their importance for follicular development. Therefore, other genes must be studied in an attempt to seek the answer to the reduction of IVPE in SUP animals.

Lastly, parenchymal and fat pad growth were also not affected by the treatments. Although effects on mammary gland development are more pronounced during the allometric growth phase during pre-puberty (Sinha and Tucker, 1969), studies have shown that diet may have similar effects on mammary gland development among heifers of different growth stages (Meyer et al., 2006; Albino et al., 2015). Silva et al. (2018) showed no difference in the composition of the mammary glands of pubertal Holstein heifers when receiving diets with different levels of metabolizable protein. Likewise, supplemental CP levels do not influence tissue deposition in the mammary glands. Considering the ADG among 600 to 700 grams of the supplemented groups, the absence of damage to the mammary glands of crossbred animals with this ADG is not in accordance with the results found by Albino et al. (2017b). They observed that ½ Holstein × Gyr animals with an ADG of 0.9 kg/d showed impaired mammary gland development. This difference may have occurred due to the genetic merit of their animals, since Albino et al. (2015) and Silva et al. (2018), using Holstein heifers, did not find any

impairment in mammary gland development with an ADG close to 1 kg. We suspect that ¾ Holstein × Gyr heifers might behave closer to purebred Holstein heifers regarding mammary gland development and, as long as an adequate metabolizable protein is supplied, might not have any detrimental effect on mammary gland development in high ADG.

In summary, grazing Holstein × Gyr heifers on intensively managed pasture supplemented with a concentrate containing 24% CP seems to be the appropriate strategy for satisfactory performance, as it was responsible for optimizing performance without negative impacts on mammary gland development. On the other hand, although it does not influence oocyte quality, the amount of 24% of CP in the heifers' supplement was associated with a low CR and BR, which highlights the importance of further research to better understand the nutrition–reproduction relationship in conditions of intensively managed pastures in tropical areas.

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## TABLE AND FIGURE

Table 1: Pasture (*Panicum maximum*, cv Mombaça) characteristics, pre and post-grazing sward height of 18 days (average days of cycle) of grazing activities.

| Item[1] | Period[2] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Accumulated herbage (kg DM/ha/cycle) | 1998.16 | 1387.28 | 2135.00 | 1746.02 |
| Accumulated herbage (kg DM/paddock/cycle) | 160.48 | 131.47 | 208.65 | 165.79 |
| Herbage DM allowance (kg DM/animal/day) | 8.91 | 7.30 | 11.59 | 9.21 |
| Grazing efficiency (%) | 80.69 | 88.32 | 63.27 | 68.30 |
| PreGH (cm) | 69.11 | 73.35 | 76.29 | 66.00 |
| PostGH (cm) | 36.84 | 36.95 | 37.16 | 32.18 |

[1] Grazing efficiency = DMI (sum of all animals)/accumulated herbage (kg DM/paddock/cycle) × 100; PreGH = pre-grazing sward height; PostGH = post-grazing stubble height.
[2] 1 = February 13 to February 27, 2019; 2 = February 28 to March 14, 2019; 3 = March 15 to March 29, 2019; 4 = March 30 to April 13, 2019.

Table 2: Pasture (*Panicum maximum*, cv Mombaça) and concentrate chemical composition (DM basis).

| Item, %DM otherwise stated[1] | Pasture – Period[2] | | | | Concentrates | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | S12CP | S24CP |
| DM (%) | 15.15 | 16.47 | 15.66 | 15.13 | 89.61 | 89.55 |
| NDF | 66.43 | 62.71 | 66.16 | 68.92 | 11.62 | 13.98 |
| iNDF | 13.34 | 13.77 | 13.56 | 13.21 | 0.89 | 0.92 |
| CP | 17.39 | 17.52 | 17.09 | 18.27 | 11.47 | 24.96 |
| Ash | 12.43 | 12.44 | 12.20 | 12.34 | 1.90 | 3.82 |

[1]DM = dry matter; NDF = neutral detergent fiber; iNDF = indigestable neutral detergent fiber; CP = crude protein.
[2]1 = February 13 to February 27, 2019; 2 = February 28 to March 14, 2019; 3 = March 15 to March 29, 2019; 4 = March 30 to April 13, 2019.

Table 3: Intake and diet digestibility of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

| Item[1] | Supplement | | | SEM[2] | *P*-Value[3] | | | |
|---|---|---|---|---|---|---|---|---|
| | CON | S12CP | S24CP | | CON × SUP | S12CP × S24CP | PER | SUP × PER |
| Intake | | | | | | | | |
| DM, kg/d | 7.36 | 8.04 | 8.58 | 0.307 | 0.014 | 0.220 | 0.091 | 0.360 |
| Pasture, kg/d | 7.36 | 6.36 | 6.84 | 0.307 | 0.051 | 0.270 | 0.064 | 0.339 |
| NDF, kg/d | 4.86 | 4.41 | 4.78 | 0.201 | 0.285 | 0.188 | 0.027 | 0.306 |
| CP, kg/d | 1.28 | 1.30 | 1.63 | 0.053 | 0.008 | 0.001 | 0.327 | 0.408 |
| CP/DM, kg/kg | 0.17 | 0.16 | 0.19 | 0.0004 | 0.043 | 0.001 | 0.001 | 0.005 |
| DE, Mcal/d | 18.12 | 21.23 | 22.33 | 0.839 | 0.001 | 0.355 | 0.050 | 0.512 |
| DM/BW, g/kg of BW | 19.65 | 20.81 | 21.54 | 0.001 | 0.278 | 0.646 | 0.006 | 0.297 |
| P/BW, g/kg of BW | 19.65 | 16.49 | 17.23 | 0.001 | 0.056 | 0.641 | 0.004 | 0.301 |
| NDF/BW, g/kg of BW | 12.99 | 11.42 | 12.04 | 0.0007 | 0.176 | 0.553 | 0.002 | 0.259 |
| Digestibility | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| DM, g/kg | 566.10 | 611.20 | 614.40 | 0.009 | 0.001 | 0.814 | 0.001 | 0.875 |
| NDF, g/kg | 696.10 | 698.20 | 696.60 | 0.005 | 0.864 | 0.844 | 0.001 | 0.893 |
| CP, g/kg | 677.30 | 636.70 | 698.60 | 0.015 | 0.608 | 0.010 | 0.001 | 0.472 |
| Microbial Synthesis | | | | | | | | |
| CPmic, g/d | 532.10 | 512.40 | 527.74 | 36.954 | 0.794 | 0.772 | 0.924 | 0.284 |
| EMS, g/kg | 131.34 | 109.28 | 110.89 | 8.985 | 0.072 | 0.900 | 0.001 | 0.5632 |

[1]DM = dry matter; NDF = neutral detergent fiber; CP = crude protein; DE = Digestible energy; CP/DM = Crude protein per dry matter; DM/BW = dry matter per body weight; P/BW = pasture per body weight; NDF/BW = neutral detergent fiber per body weight; CPmic = microbial crude protein; EMS = efficiency of microbial crude protein synthesis (g of CPmic/kg of total digestible nutrients intake).
[2]Standard error of the mean.
[3]CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement; PER = effect of period; SUP×PER = interaction effect between supplementation and period.

Table 4: Carcass trait and mammary gland pixels' pattern of Holstein × Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

| Item | Supplement | | | SEM[1] | *P*-Value[2] | |
|---|---|---|---|---|---|---|
| | CON | S12CP | S24CP | | CON × SUP | S12CP × S24CP |
| Rib eye area, $cm^2$ | 53.32 | 56.00 | 61.56 | 1.983 | 0.040 | 0.070 |
| Back fat thickness, mm | 2.72 | 2.81 | 2.66 | 0.216 | 0.952 | 0.663 |
| Parenchymal, pixels/$mm^2$ | 4.65 | 4.67 | 4.66 | 0.076 | 0.920 | 0.920 |
| Fat pad, pixels/$mm^2$ | 5.09 | 5.11 | 5.09 | 0.018 | 0.540 | 0.490 |

[1]Standard error of the mean.
[2]CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement.

Table 5: Blood parameters of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

| Item | Supplement | | | SEM[1] | *P*-Value[2] | | | |
|---|---|---|---|---|---|---|---|---|
| | CON | S12CP | S24CP | | CON × SUP | S12CP × S24CP | PER | SUP × PER |
| Urea, mg/dL | 33.45 | 31.75 | 37.08 | 1.286 | 0.551 | 0.010 | 0.001 | 0.002 |
| Glucose, mg/dL | 72.87 | 76.75 | 74.66 | 1.647 | 0.180 | 0.385 | 0.001 | 0.031 |
| Albumin, mg/dL | 2.86 | 3.05 | 3.17 | 0.066 | 0.008 | 0.233 | 0.358 | 0.621 |
| Total protein, mg/dL | 7.37 | 7.45 | 7.37 | 0.177 | 0.873 | 0.782 | 0.063 | 0.486 |
| Total Cholesterol, mg/dL | 89.62 | 93.91 | 78.87 | 6.656 | 0.697 | 0.130 | 0.941 | 0.850 |
| Triglycerides, mg/dL | 7.66 | 8.00 | 8.70 | 0.694 | 0.427 | 0.485 | 0.002 | 0.342 |
| Insulin, µUI/mL | 2.25 | 2.58 | 2.55 | 0.406 | 0.534 | 0.963 | 0.009 | 0.846 |
| IGF-I, ng/mL | 227.08 | 231.87 | 225.75 | 27.545 | 0.959 | 0.877 | 0.002 | 0.804 |

[1]Standard error of the mean.
[2]CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement; PER = effect of period; SUP×PER = interaction effect between supplementation and period.

Table 6: Reproductive parameters of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

| Item[1] | Supplement | | | SEM[2] | *P*-Value[3] | | | |
|---|---|---|---|---|---|---|---|---|
| | CON | S12CP | S24CP | | CON × SUP | S12CP × S24CP | PER | SUP × PER |
| FV, no | 13.31 | 12.83 | 17.89 | 1.100 | 0.273 | 0.016 | 0.992 | 0.998 |
| OR, no | 10.68 | 12.15 | 14.79 | 1.129 | 0.132 | 0.257 | 0.956 | 0.967 |
| RR, % | 83.92 | 99.31 | 87.95 | 7.395 | 0.306 | 0.298 | 0.919 | 0.066 |
| VO, no | 6.07 | 5.39 | 7.25 | 1.572 | 0.926 | 0.420 | 0.007 | 0.547 |
| VO/OR, % | 50.64 | 44.34 | 40.17 | 3.651 | 0.078 | 0.398 | 0.081 | 0.090 |
| CO, no | 2.81 | 1.53 | 2.51 | 0.406 | 0.099 | 0.082 | 0.666 | 0.528 |
| CR, % | 47.17 | 23.92 | 36.71 | 5.716 | 0.017 | 0.102 | 0.676 | 0.359 |
| IVPE, no | 1.66 | 0.27 | 1.33 | 0.281 | 0.016 | 0.011 | 0.845 | 0.721 |
| BL/CO, % | 58.30 | 15.26 | 44.57 | 9.436 | 0.019 | 0.055 | 0.214 | 0.666 |

[1]FV = follicles visualized; OR = oocytes recovered; RR = recovery rate; VO = viable oocytes; VO/OR = viable oocytes per oocytes recovered; CO = cleaved oocytes; CR = cleavage rate; IVPE = in vitro produced embryos; BL/CO = blastocysts per cleaved oocytes

[2]Standard error of the mean.

[3]CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement; PER = effect of period; SUP×PER = interaction effect between supplementation and period

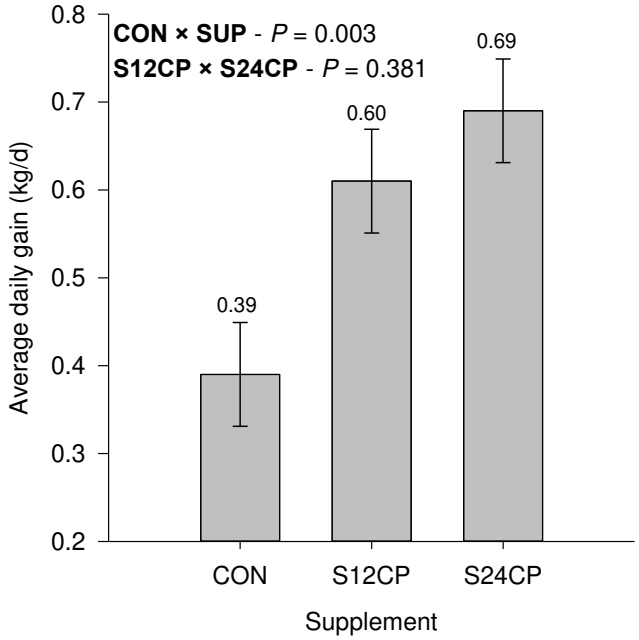


Figure 1: Average daily gain of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

CON × SUP = effect of supplementation; S12CPB × S24CP = effect between protein levels in the supplement.

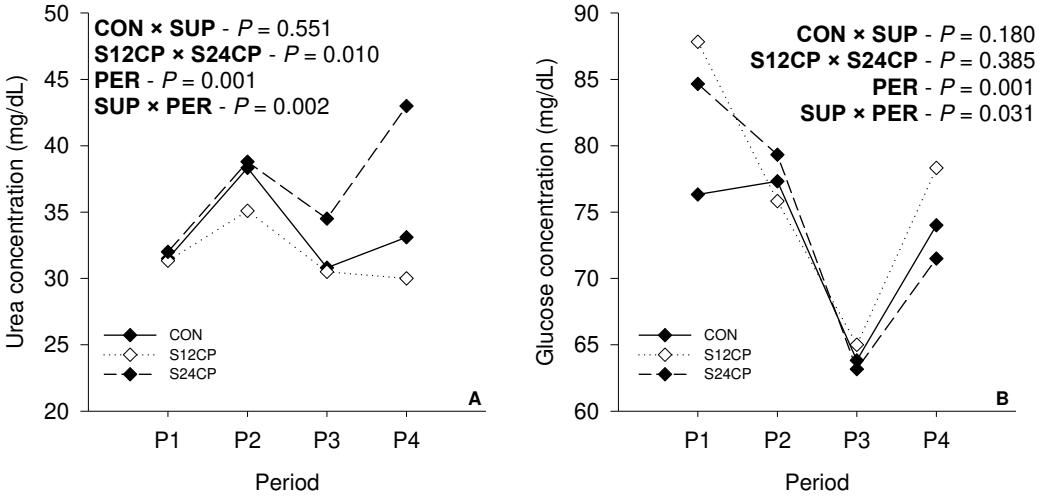


Figure 2: Blood urea (A) and glucose (B) concentration of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

CON × SUP = effect of supplementation; S12CPB × S24CP = effect between protein levels in the supplement; PER = effect of period; SUP×PER = interaction effect between supplementation and period.

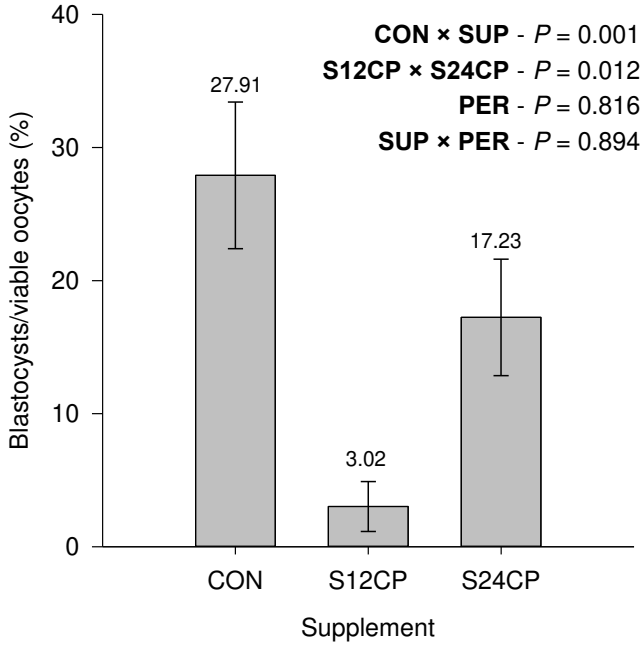


Figure 3: Blastocyst rate of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement; PER = effect of period; SUP×PER = interaction effect between supplementation and period.

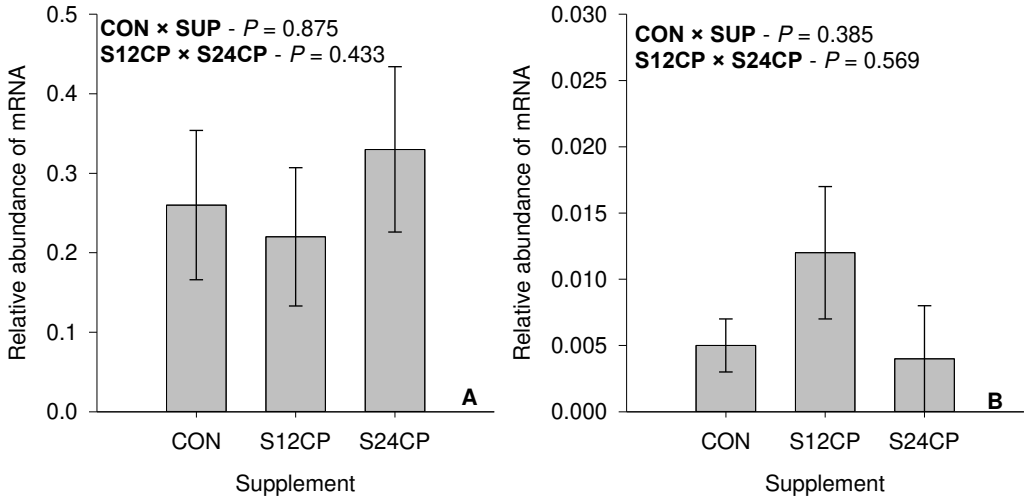


Figure 4: Relative abundance of A) BMP15 and B) GDF9 obtained by real-time PCR of oocytes of Holstein x Gyr crossbred heifers not supplemented (CON) or supplemented with concentrate containing 12% CP (S12CP) or 24% CP (S24CP) on a rotational grazing system *Panicum maximum* cv. Mombaça pasture.

CON × SUP = effect of supplementation; S12CP × S24CP = effect between protein levels in the supplement.

# APPENDIX 1

Supplementary Table 1: Gene names, accession numbers and primers sequences.

| Gene[1] | Accession no.[2] | Primer sequence[3] |
|---|---|---|
| BMP15 | NM_001031752.1 | F: CACATACAGACCCTGGACTTTC<br>R: GGTGGGAATGAGTTAGGTGAAG |
| GDF9 | NM_174681.2 | F: CCAGATGACAGAGCTTTGAG<br>R: GCCGAACAGTGTTGTAGAG |
| 18S | NM_001033614 | F: CCTGCGGCTTAATTTGACTC<br>R: AACTAAGAACGGCCATGCAC |

[1]BMP15 = bone morphogenetic protein 15; GDF9 = growth and differentiation factor 9; 18S = ribosomal RNA.

[2]Accession number in GenBank (http://www.ncbi.nlm.nih.gov).

[3]F = forward; R = reverse.

Supplementary Table 2: Environmental conditions throughout periods

| Item[1] | Period[2] | | | | |
|---|---|---|---|---|---|
| | Adaptation | 1 | 2 | 3 | 4 |
| Average Temperature, °C | 24.35 | 22.94 | 23.40 | 21.37 | 22.05 |
| Minimum Temperature, °C | 18.27 | 19.53 | 19.58 | 18.15 | 18.65 |
| Maximum Temperature, °C | 32.01 | 29.73 | 31.52 | 27.94 | 28.66 |
| Rainfall, mm | 77.60 | 81.00 | 97.60 | 23.60 | 92.00 |
| Relative humidity, % | 68.64 | 82.93 | 81.86 | 83.25 | 85.30 |
| THI | 72.90 | 72.03 | 72.68 | 69.50 | 70.77 |

[1]THI = Temperature humidity index.

[2]Adaptation = January 13 to February 12, 2019; 1 = February 13 to February 27, 2019; 2 = February 28 to March 14, 2019; 3 = March 15 to March 29, 2019; 4 = march 30 to April 13, 2019.