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DOES A HIGH OR MODERATE NUTRITIONAL SUPPLEMENTATION AFFECT THE PUBERTY AND PRODUCTIVE PERFORMANCE OF NELLORE HEIFERS?

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Abstract
The effects of higher supplementation levels for young Nellore heifers fed tropical forages including their influence on puberty, need to be understood. This study investigated the influence of high and moderate supplementation levels on puberty onset and the productive performance of Nellore heifers. Thirty-six Nellore heifers (225 ± 3.52 kg) were used in a completely randomized design. The heifers were kept in nine 1.0 ha paddocks with *Brachiaria brizantha* cv. Marandu grass, with three different supplementations: (I) mineral salt (control group), (II) concentrate supplement at 4 g/kg body weight (BW); (III) concentrate supplement at 8 g/kg BW. It was evaluated intake, digestibility, performance, onset of *corpus luteum* and carcass characteristic by ultrasound methodology. Increased supplement level led to a linear increase in average daily gain ($P < 0.05$). The high supplementation level (8 g/kg BW) had the highest proportion of corpus luteum presence (82% of heifers), compared to the 4 g/kg BW treatment group (67%) and the control group (33%; $P < 0.05$). Our findings indicate that providing a high level of energy supplementation to Nellore heifers for approximately 100 d improves performance and increases the proportion of heifers that reach puberty.

Keywords: Beef heifers. Nutrition. Pasture.

1. Introduction

Feeding supplementation is an effective livestock management tool that can have a high impact on heifer development. For *Bos taurus taurus* females, the positive impact of early nutrition in puberty age using the level of feeding to change average daily gain in heifers that are weaned early has been documented (Gasser et al. 2006; Cardoso et al. 2014). However, for Zebu cattle, such as the Nellore breed, pre-weaning supplementation strategies have been evaluated as having little or no effect on animal performance and no impact on puberty onset (Nepomuceno et al. 2017). Therefore, it would not be advantageous to implement management strategies that increase pre-weaning growth to increase pubertal heifer proportions (Silva et al. 2017).

Some evidence suggests that nutritional plans during the post-weaning period efficiently improve heifers' average daily gain and increase the number of heifers that reach puberty (Barcellos et al. 2014).

Accordingly, researchers have been directing their efforts toward elucidating questions regarding the amount of supplement required for heifers to reduce their age at puberty. Studies have investigated different supplementation levels, with some indicating better performance and reproductive variables when heifers were offered 3, 4, or 6 g supplement per kg of body weight (BW) (Couto et al. 2010; Cabral et al. 2014; Silva et al. 2017). However, the effects of higher supplementation levels on puberty onset and performance in heifers fed with forages remains poorly understood.

Thus, we hypothesized that a higher level of supplementation before the breeding season increases growth rate and expedites the onset of puberty in Nellore heifers. This study, therefore, investigated the influence of high and moderate supplementation levels on puberty onset, and the productive performance of Nellore heifers.

2. Material and Methods

Animals, diets, and experimental design

All experimental procedure was authorized by the Ethical Committee of Animal Use in Experiments, CEUA/UFRA (protocol number: 011/2015). The experiment was conducted in the facilities of the Universidade Federal Rural da Amazônia – UFRA, Campus Parauapebas (6°4′25.53′′S, 49°48′54.57′′W) during April to July months. Data on the rainfall and average temperatures were collected throughout the experiment at the Meteorological Station of the UFRA (Table 2). Animals were adapted for 15 days to the diet and pasture areas, and then spent 122 days in the experimental trial. Thirty-six Nellore heifers that were 16 months old and had a BW of 225 $\pm$ 3.52 kg were used (12 experimental units per treatment). The heifers were selected for the experiment had the absence of the corpus luteum determined by an ultrasonographic assessment of their ovaries and follicle diameters in two evaluations separated by a 10-day interval. Only heifers that did not have a corpus luteum in both evaluations were considered pre-pubertal and were used in the trial.

The study was performed as a completely randomized design, with the initial BW as a covariate. Experimentation comprised three treatments and twelve replications, namely (I) mineral salt (control), (II) 4 g/kg BW of concentrate supplement, and (III) 8 g/kg BW of concentrate supplement (Table 1). Supply of crude protein was fixed to meet 70% of the protein requirement for a daily mean gain of 0.800 kg (Valadares Filho et al. 2016). The energy level was 2.5 times greater in the 0.8% BW treatment. The control group received the mineral mixture only *ad libitum*. The heifers were kept in nine 1.0 ha paddocks with *Brachiaria brizantha* cv. Marandu grass and with a drinker and feeders. Animals (and their respective treatments) were rotated among the paddocks every seven days to prevent possible paddock effects on the treatments. Supplements were offered to heifers at 10:00 each day in a collective trough located in each paddock during the experiment.

Heifers were weighed after fasting at the beginning and end of the experiment to determine their average daily gain and were weighed every 15 days during the experimental period for performance monitoring and adjustment of the amount of supplement offered.

Forage characterization

Pasture chemical composition was assessed using hand-plucked samples taken every two weeks (de Vries 1995). For quantitative evaluation, the forage was sampled by cutting it at 10 cm above ground level in areas delimited by a 0.5 × 0.5 m (McMeniman 1997) metal square placed at four random points in each experimental paddock. The samples were weighed and homogenized, and two aliquots were taken: one for evaluating the availability of total dry matter and another for analyzing the composition of available herbage mass, after being separated into green leaves, stems, and senescence material (Table 2). Thereafter, the samples were weighed and dried in forced air incubator (55ºC), and processed grinding mil (1 and 2 mm). From the aliquot destined to estimation of forage total availability, the percentage of potentially digestible dry matter (pdDM) was calculated, which was obtained using the indigestible residual in neutral detergent fiber (iNDF) evaluated after in situ incubation of the samples for 288 hours (Valente et al. 2011). The potentially digestible dry matter (pdDM) was estimated following Paulino et al. (2008): pdDM = [0.98x(100-NDF)] + (NDF - iNDF).

Table 1. Ingredients and chemical composition of supplements and forage.

Item	Supplements			Forage[1]
	Mineral salt (control)	4 g/kg BW	8 g/kg BW	
Ingredients (g/kg as fed basis)				
Corn	-	126.5	690.4	-
Soybean meal	-	756.8	252.1	-
Mineral salt[2]	100	70.8	34.9	-
Urea	-	45.9	22.6	-
Chemical composition (g/kg dry matter basis)				
Dry matter	999.0	856.6	865.8	331.0
Organic matter	-	905.9	867.5	907.5
Ether extract	-	1.6	2.8	2.0
Crude protein	-	507.7	232.9	112.4
Neutral detergent fiber$_{ap}$	-	161.1	160.4	641.2

[1] Forage sample from manual grazing simulation; [2] Mineral salt: 8.7% calcium, 9.0% phosphorus, 18.7% sodium, 9.0% sulfur, 2400 mg/kg of zinc, 800 mg/kg of copper, 1600 mg/kg of manganese, 40.0 mg/kg of iodine, 8.00 mg/ kg of cobalt, and 8.16 mg/kg of selenium.

Table 2. Values of rainfall levels and monthly mean of maximum, medium, and minimum temperatures, and morphological characteristics of *B. brizantha* cv. Marandu during the experimental period.

	April	May	June	July
Climatic characteristics				
Rainfall level (mm)	166.4	120.2	22.8	60
Days of rain	17	18	5	7
Medium temperature	26.1	26.5	26.8	26.9
Morphological characteristics				
pdDM (kg/ha)	309	320	195	208
Green leaves (g/kg)	226	203	148	124
Stem (g/kg)	178	157	074	073
Senescence material (g/kg)	060	074	044	076
Herbage mass (kg/ha)	4640	4351	2662	2763

Intake and digestibility

To evaluate the nutritional characteristics of the diet, two 10-day digestibility trials were performed, the first beginning on the 51st day of the experimental period and the second beginning on the 110th day. The three-marker method was employed as follows: chromic oxide (Cr_2O_3) was used to estimate the animals' fecal excretion. The Cr_2O_3 was wrapped in paper cartridges at a dose of 15 g/animal/day and administered using a metal probe via the esophagus at 08:00 (Sampaio et al. 2011). Titanium dioxide (TiO_2) was used to estimate the individual supplement intake, provided via supplement at a proportion of 10 g/kg of supplement (Titgemeyer et al. 2001). To estimate the pasture dry matter intake, indigestible neutral detergent fiber (iNDF) was used as an internal marker.

The first six days of each trial were used for animal adaptation to TiO_2 and Cr_2O_3. Fecal samples were collected immediately after defecation or directly from the rectum of the animals in the last four days of the digestibility period (one sample per day) at 16:30, 13:30, 10:30, and 07:30 on the respective day (Sampaio et al. 2011). After collection, feces were dried at 55°C for 72 h and ground using a 1- and 2 mm knife mill (Willye type). A composite sample was prepared for each animal and stored for subsequent analysis.

Assessment of the presence of the corpus luteum

To determine the approximate time of first ovulation, each animal was examined every 15 days during the experimental period to determine those that had attained puberty, and to measure the diameter of the largest follicle by means of transrectal ultrasonography using a portable ultrasound machine (Aquila model, Esaote-Pie Medical), coupled with a linear transducer with a frequency of 7.5 MHz. The largest follicle was measured by taking the mean width and length from a frozen image. Puberty was defined by the presence of a corpus luteum detected by ultrasonography. Heifers that attained puberty (i.e. presence of the corpus luteum) were removed from the experiment at this time (Nepomuceno et al. 2017).

Chemical analyses and calculations

The forage, fecal, and supplement samples were analyzed following procedures for dry matter (DM, index INCT-CA G-003/1), mineral mater (MM, index INCT-CA M-001/1) crude protein (CP, index INCT- CA N-001/1), ether extract (EE, index INCT-CA G-005/1) and neutral detergent fiber was determined and corrected for residual ash and protein (NDFap; indexes INCT-CA F-002/1, INCT-CA M-002/1, and INCT-CA N-004/1), and the non-fiber carbohydrate level (NFCap) was calculated using the following equation:

NFC = 100- [MM + EE + NDFap + (CP – CPu + U)], in which: NFC: non-fiber carbohydrates; MM: mineral matter; EE: ether extract; NDFap: neutral detergent fiber corrected for ashes and protein; CP: crude protein; CPu: crude protein from urea, and U: urea content.

Indigestible NDF (iNDF) contents were evaluated as the residual NDF remaining after 288 h of ruminal in situ incubation using the samples processed to pass the 2-mm screen sieve and F57 filter bags (Ankom®, Macedon, NY, USA), according to Valente et al. (2011).

Total digestible nutrients were calculated by the equation: TDN (%) = DP+DNDF+ DNFC+2.25DEE, in which: DP: digestible protein; DNDF: digestible neutral detergent fiber; DNFC: digestible non-fiber carbohydrates; DEE: digestible ether extract.

The fecal samples were analyzed for levels of chromic oxide using atomic absorption spectrophotometry (index INCT-CA M-005/1), and for titanium dioxide using colorimetry (index INCT-CA M-007/1) (Detmann et al. 2012).

The excretion of fecal dry matter was estimated based on the quantity of the indicator (Cr_2O_3) provided (15g/day) and their concentration in the feces samples.

The estimation of individual supplement intake was obtained by the equation: SupIC = (($FE \times TiO_{2\,feces}/ TiO_{2\,sup}$)) x Sup, in which: SupIC = individual supplement intake (g/day); FE = fecal excretion (g/day); $TiO_{2\,feces}$ = concentration of TiO_2 in the feces (g/g); $TiO_{2\,sup}$ = TiO_2 present in the supplement for the animal group (g/day); Sup = supplement amount provided to the animal group (g/day).

For estimation of the forage intake, iNDF was used, quantified by *in situ* incubation procedures with Ankon (F57) bags by 288 hours in the samples processed to 2 mm. The forage intake was estimated, according to the equation: FI = [($FE \times iNDF_{feces}$) – $iNDF_{sup}/iNDF_{forage}$ in which: FI = forage intake (kg/day); FE = fecal excretion (kg/day); $iNDF_{feces}$ = concentration of iNDF in the feces (kg/kg); $iNDF_{sup}$ = consumption of iNDF from supplement (kg/day), and $iNDF_{forage}$ = concentration of iNDF in the forage (kg/kg).

The total intake was determined as the sum of forage and supplement intake.

Evaluation of carcass traits by ultrasonography

On the last day of the experimental period, carcass traits of the heifers were evaluated by ultrasound (model: SSD 500v, linear probe =18 cm; Aloka). Carcass images were obtained from the right side of the animal, specifically the ribeye area, and subcutaneous fat thickness measurements were taken from a transverse section of the longissimus dorsi muscle (between the 12^{th} and 13^{th} thoracic vertebrae) (Peña et al. 2014). Images were analyzed using ImageJ software.

Statistical analyses

Statistical analyses were performed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The treatments were compared using polynomial orthogonal contrasts, which were partitioned in linear (-1 +0 +1) and quadratic (+1 -2 +1) contrasts. The least squares (LS) means of fixed effects (i.e., treatments) were estimated using the LSMEANS statement of the MIXED procedure. Initial BW was used as a covariate. Differences were considered significant at $P < 0.05$.

Follicle diameter was analyzed in a split-plot arrangement, with subdivisions based on time (i.e., day of the experiment). Heifers exhibiting the corpus luteum were removed from this analysis. The Kenward-Roger method (Kenward and Roger 1997) was used to calculate the residual degrees of freedom. The covariance structures of compound symmetry, heterogeneous compound symmetry, and components of variance were tested, and the best fit was selected using the Akaike information criterion (Akaike 1973)

The individual effects ($P < 0.05$) were analyzed using the CONTRAST statement, with polynomial contrast of the linear and quadratic effects for the temporal subdivisions, and the PDIFF statement was used to compare the treatments.

Fisher's exact test was used to evaluate the number of heifers with a corpus luteum on each day of data collection. Differences were considered significant at $P < 0.05$.

3. Results and Discussion

From April to May a greater production was noticed (98.2g/kg of BW) of potentially digestible dry matter (pdDM), associating quantity and quality of forage. However, from June to July its availability was reduced to 59.2 g/kg of BW (Table 1). Although the supply of pdDM was reduced, the offered forage was greater than recommended for zebu (40-50 g/kg of body weight) to express the potential weight gain (Paulino et al. 2008). Also, in this study, the forage indigestible neutral detergent fiber was lower than 270 g/kg DM (Almeida et al. 2022), which indicate good forage conditions to maximize the average daily gain in the tropics.

There was no supplementation effect ($P > 0.05$) on total dry mater and neutral detergent fiber intake (Table 3). However, when supplement level increased, forage intake decreased linearly, and the crude protein and total digestible nutrients intake increased linearly ($P < 0.05$). These results indicate the occurrence of substitutive effect due to the increase of consumption of total digestible nutrients by the linear increase of supplement consumption associated with reduction of forage consumption (Table 3). A possible cause for this effect could be the high ratio of mean stocking rate of the paddock (2.26 animal/hectare) associated with the least performance of the heifers that received mineral supplement (0.355kg/animal/day), which probably enabled the animals to have less possibility of forage selection, altering the proportion of morphological components, like less relation leaf/stem, indicating that there was the necessity by part of these animals to elevate the forage intake with decreasing of the canopy forage height (Barbero et al. 2015).

The indigestible neutral detergent fiber intake decreasing linearly ($P < 0.05$, Table 3). The animals supplemented with 0.8% of body weight could have had ingested less proportion of stem, without differences on the consumption of neutral detergent fiber (NDF) (Table 3), which confirms the theory that animals receiving higher levels of concentrated consumes less proportion of forage lignified cell wall (Martins et al. 2017). Despite the observed linear effect for crude protein intake, the similarity observed between treatments 4 g/kg and 8 g/kg BW (1.01 versus 1.03 kg/d) was expected. The supplements were formulated to provide the same amount of this nutrient, regardless of the supplement intake. On the other hand, as already mentioned, higher energy intake in treatments with higher supplementation was expected.

The digestibility coefficient of dry matter, non-fibrous carbohydrate, total digestible nutrients increase linearly ($P < 0.05$; Table 3) according to the levels of supplementation. The alterations are related to the increase in supplement intake. The chemical composition of the ingredients increases microbial enzyme efficiency by exposing alpha bonds in the polysaccharide composition, enabling greater effectiveness of degradation, thereby leading to increased digestibility (Detmann et al. 2014; Reis et al. 2016). However, the digestibility coefficient of crude protein showed quadratic effect ($P < 0.05$) in function to the supplementation levels, being the highest value found for level 0.4% of body weight, showing 68.07% of digestibility.

The proteins from the ingredients of the concentrated supplement have, as a rule, qualitative characteristics above that from forages produced during the dry season. This way, since most of the dietetic protein of treatments 0.4% and 0.8% of BW were from concentrated ingredients, it seems to exist a direct effect of high digestibility from the supplement itself for the animals of these treatments.

Table 3. Intake and apparent digestibility according to amount of supplement fed to Nellore heifers.

Item	Supplements			SEM	P-value	
	Mineral salt	4 g/kg BW	8 g/kg BW		Linear	Quadratic
		Intake (kg/day)				
Total intake	5.82	5.37	6.35	0.25	0.354	0.245
Forage intake[1]	5.78	4.36	4.14	0.19	<0.001	0.177
Supplement intake	0.04	1.01	2.21	0.10	-	-
Crude protein[2]	0.66	1.01	1.03	0.04	<0.001	0.050
Non-fibrous carbohydrate$_{ap}$	0.96	0.97	1.91	0.07	<0.001	0.010
Neutral detergent fiber$_{ap}$	3.47	2.88	2.87	0.13	0.072	0.279
Indigestible NDF	1.87	1.38	1.14	0.06	<0.001	0.330
Total digestible nutrients[3]	2.62	2.82	3.71	0.15	0.005	0.427
		Digestibility (g/kg)				
Dry matter[4]	467.8	498.3	544.6	0.89	<0.001	0.824
Crude protein[5]	557.0	680.7	624.1	0.83	<0.002	<0.001
Non-fibrous carbohydrate$_{ap}$	52.05	63.55	74.22	1.28	<0.001	0.964
Neutral detergent fiber$_{ap}$	48.43	48.52	49.63	0.85	0.493	0.962
Total digestible nutrients[6]	441.9	513.2	574.4	0.81	<0.001	0.567

4g/kg BW = supplement concentrate at 4 g/kg of body weight (BW); 8 g/kg BW = supplement concentrate at 8 g/kg of body weight. SEM = standard error of mean; ap = corrected for contents of ashes and insoluble protein. [1]$y = 5.14-1.99x$ ($R^2 = 0.30$); [2]$y = 0.71+0.47x$ ($R^2 = 0.34$); [3]$y = 2.50+1.37x$ ($R^2 = 0.24$); [4]$y = 46.52+9.60x$ ($R^2 = 0.28$); [5]$y = 55.69+53.46x-56.33x^2$ ($R^2 = 0.55$); [6]$y = 44.36+16.56x$ ($R^2 = 0.58$).

Increased supplement level led to a linear increase in daily weight gain (P < 0.05; Table 4). The high supplementation level (8 g/kg BW) had the highest average daily gain (ADG; 0.579 kg/day) but was still below our expectations (less than 0.800 g/day). Even though, the animal's growth was linear increasing, probably because the supplementation provided the protein and energy that was lacking from the forage only. It is worth mentioning that the additional gain of supplemented animals compared to the ones that received the mineral supplement makes the systems efficient in reducing the age of first ovulation (Figure 1) because it maximizes the weight gain rate during the dry season (Silva et al. 2017).

Table 4. Productive performance and body composition *in vivo* of Nellore heifers fed with different levels of supplement.

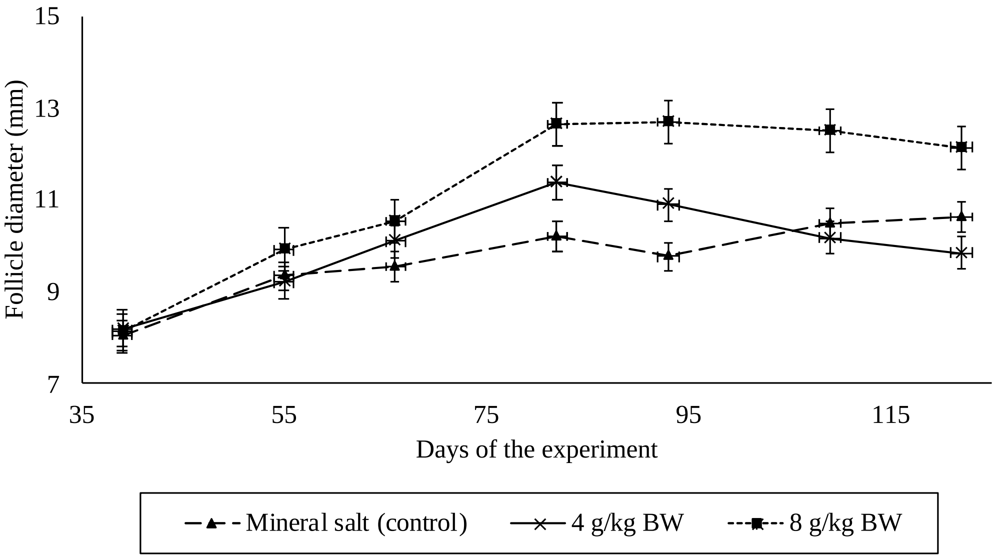
Item	Supplements			SEM	P-value	
	Mineral salt	4 g/kg BW	8 g/kg BW		Linear	Quadratic
Initial body weight	231	218	225	3.52	-	-
Final body weight[1]	276	280	298	2.43	<.001	0.359
ADG[2] (g/day)	0.355	0.493	0.579	0.019	<.001	0.360
REA[3]	42.16	47.97	46.56	2.499	0.026	0.133
SFT	0.31	0.30	0.34	0.27	0.330	0.262

4g/kg BW = supplement concentrate at 4 g/kg of body weight (BW); 8 g/kg BW = supplement concentrate at 8 g/kg BW; ADG = average daily gain; REA = ribeye area (cm^2); SFT = subcutaneous fat thickness (mm); SEM = standard error of mean; [1]$y = 273.70+27.18x$ ($R^2 = 0.09$); [2]$y = 0.36+0.27x$ ($R^2 = 0.39$); [3]$y = 43.16+6.55x$ ($R^2 = 0.20$).

The ribeye area measured in the longissimus dorsi increased linearly as a function of supplementation level (P < 0.05) but had no significant effect on subcutaneous fat thickness. This alteration in the deposition of muscle tissue can be related to the enhancement of body weight gain, which may have influenced the number of heifers that developed the corpus luteum (Figure 2). Age at puberty determined in this study are similar to those obtained by Barcellos et al. (2014), who verified earlier puberty when fed heifers with higher nutritional levels. However, although some results suggest a positive relationship between the increase in adipose tissue and ovulation rates (Bailey et al. 2014). This relationship did not occur in the present study, possibly due to the age of the heifers once Zebu breed has fat deposition belatedly (Cooke et al. 2020).

There was no interaction between time and treatment for follicle size (P > 0.05; Figure 1b). The supplementation level affected (P < 0.05) the follicle size of the heifers; those receiving high energy supplementation (8 g/kg BW) showed the largest follicles. There was no difference in follicle size between heifers that received moderate energy supplementation (4 g/kg BW of supplement) and the control group (no concentrate). It is known that measurements of follicle size should ideally be performed during the entire follicular wave. Although there were differences in follicle size measured in this experiment, measurement every 15 d gave a general impression of the size variation of the follicle, because the days of measurement would have coincided with different phases of the follicular wave (Nepomuceno et al. 2017).

A



B

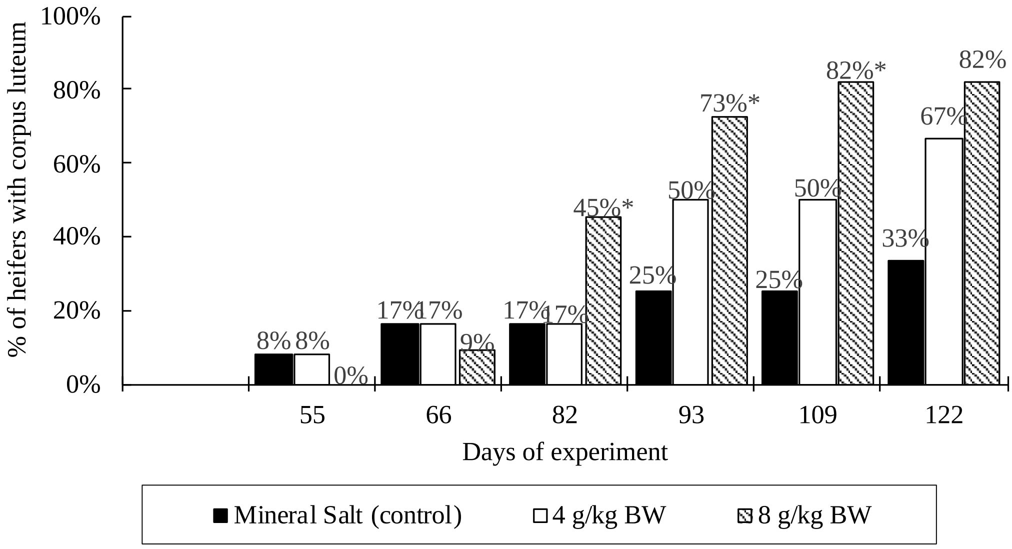


Figure 1. A - Effect of the different supplementation levels on follicle diameter as a function of experimental days. The largest follicle was verified in heifers that received 8 g/kg BW of supplement, regardless of time. The follicle size had a quadratic effect over time (linear P value < 0.001; quadratic P value = 0.021); B - Proportion of Nellore heifers with a corpus luteum as a function of experimental days. Bars with asterisk (*) represent differences (P < 0.05) between treatments, as measured by Fisher's exact test.

Differences in the number of heifers that reached puberty were verified after 82 d of supplementation (Figure 1a). The supply of 8 g/kg BW of supplement (high energy supplementation) resulted in a greater number of heifers with CL at 82, 93, 109, and 122 d of the experiment (P < 0.05). These data suggest that supplementation for approximately 100 d should be adopted as ideal to increase the percentage of heifers with CL. Therefore, associated with biological responses the producer needs to evaluate the prices of ingredients and cost of supplements for their make the better choice. According Ciccioli et al. (2005) diets with greater amounts of starch may induce puberty in heifers that have inadequate BW at the start of breeding season. These authors used 16.5% more starch in isocaloric diets and observed that increased amounts of starch in the diet may induce puberty. In current study, the high energy supplement had corn starch in the composition which could increase propionate concentration and release of splanchinic glucose increasing energy retention in beef heifers.

It is evident that the increase in energy supplementation triggered a greater ADG for heifers, and that both (energy supply and greater ADG were associated with an increase in the number of heifers with CL present. These results agree with Pereira et al. (2017), who found that a greater correlation between body weight gain during weaning and puberty in heifers.

High energy supplementation in the period preceding the breeding season might have influenced follicle diameter and the presence of corpus luteum. The energy level changes the heifer's body size, which signs to organism to increase IGF-1 and insulin concentration. These hormones are essential to activate and differentiate primordial follicles for one to become dominant. And stimulate steroidogenesis by increasing estradiol secretion from the dominant follicle (Barcellos et al. 2014, Silva et al. 2017)

4. Conclusions

The findings of this study indicate that providing high energy supplementation levels (8 g/kg BW) with ground corn as starch source for Nellore heifers for approximately 100 d is a nutritional strategy that can be adopted to improve average daily gain and increase the proportion of heifers that reach puberty.

Authors' Contributions: DA MATA, V.J.V.: acquisition of data and drafting the article; DE OLIVEIRA, L.R.S.: drafting the article and critical review of important intellectual content; DETMANN, E.: drafting the article and critical review of important intellectual content; MEZZOMO, R.: drafting the article and critical review of important intellectual content; ALVES, K.S.: acquisition of data and analysis and interpretation of data; LACERDA, N.G.: acquisition of data and drafting the article; SANTANA, K.R.S.: acquisition of data and drafting the article; GOMES, D.I.: conception and design, drafting the article and critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: All experimental procedures followed the ethical principles of animal experimentation, approved by the Ethics Committee of Animal Use, CEUA/UFRA (protocol number: 011/2015).

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