

## Effect of chronic infusion of leptin and nutrition on sexual maturation of zebu heifers<sup>1</sup>

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**ABSTRACT:** The amount of fat in the carcass has been proposed as a regulator of initiation of puberty in cattle. To test if changes in energy intake and in circulating leptin concentration are each capable of altering age, BW, and body composition at puberty, 36 prepubertal Nellore heifers, 18 to 20 mo old, 275.8 ± 17.2 kg BW, and BCS of 5 ± 0.5 (1 to 9 scale), were randomly assigned to each of 3 treatments ( $n = 12$ ): High (high energy diet), Low (low energy diet), and LL [low energy diet + ovine leptin (oLeptin)]. Diets were formulated to promote BW gain of 0.4 kg/d (groups Low and LL) or 1.2 kg/d (High group). After 14 d of adjustment to diet, heifers in LL group received subcutaneous injections of oLeptin at 4.8 µg/kg BW twice a day for 56 d. Groups High and Low received similar injections of 2 mL saline solution. Age at puberty was considered to be the age on first detection of a corpus luteum, confirmed by plasma concentrations of progesterone of >1 ng/mL. Heifers were slaughtered on the second day after first corpus luteum detection. Expression of leptin gene was quantified by real-time PCR using ribosomal protein-L19 (*RP-L19*) as a control gene. Leptin administration increased

( $P = 0.04$ ) leptin serum concentration but had no effect ( $P > 0.05$ ) on age, BW, or BCS at puberty. High energy intake increased ( $P < 0.01$ ) leptin concentration, accelerated ( $P = 0.02$ ) puberty, and increased ( $P < 0.01$ ) BCS at puberty, without altering ( $P = 0.17$ ) BW at puberty. High energy intake also accelerated ( $P = 0.04$ ) follicular development. Leptin administration caused a significant ( $P < 0.05$ ) but transient increase in follicular development, which was similar to the transient increase in leptin serum concentration. Results from leptin gene expression demonstrated that high energy intake increased ( $P < 0.01$ ) and leptin administration decreased ( $P < 0.01$ ) leptin expression in 3 adipose tissues. The observed decrease in leptin gene expression after administration of leptin could explain the reduction in leptin serum concentration after 30 d of treatment and consequently the failure of leptin to accelerate puberty. Our findings did not support the hypothesis that reduced serum concentration of leptin is an important hindrance for puberty onset in malnourished zebu heifers. Although exogenous administration of leptin temporarily enhanced rate of follicular growth, it did not accelerate puberty.

**Key words:** adipose tissue, cattle, gene expression, leptin, nutrition, puberty

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### INTRODUCTION

In growing heifers, both *Bos taurus* and *Bos indicus*, puberty onset is determined by maturation of the hypothalamus, which results in reduction of the

negative feedback of estradiol, increased LH pulse frequency and, eventually, ovulation (Gasser et al., 2006). Although the physiological mechanisms involved in this maturation process are not yet fully elucidated, they are clearly influenced by BW and BCS or adiposity (Williams and Amstalden, 2010). Increase in circulating concentrations of leptin precedes the onset of puberty in several species, including cattle, and leptin has been shown to regulate the onset of puberty in rodents (Cheung et al., 2001; Perry, 2012). The

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Nellore breed is well adapted to tropical and extensive grazing conditions but presents later sexual maturation than *Bos taurus*, even when raised in the same conditions (Rodrigues et al., 2002).

Fasting decreases leptin mRNA expression in the adipose tissue of prepubertal heifers as well as the circulating concentrations of leptin and frequency of LH pulses (Amstalden et al., 2000). Administration of recombinant ovine leptin (**oLeptin**) stimulates secretion of GnRH and LH in undernourished heifers but is not capable of accelerating puberty onset in well-nourished heifers (Maciel et al., 2004b; Zieba et al., 2004). Therefore, we hypothesized that in zebu heifers older than 18 mo, raised in open range production systems and with poor body fat mass, serum concentration of leptin may be limiting GnRH secretion and delaying puberty onset.

The objective of this study was to evaluate the effects of chronic infusion of oLeptin and of dietary energy intake on age, BW, and BCS at puberty as well as on development of ovarian follicles of zebu heifers and to evaluate the effect of diet and of leptin infusion on leptin gene expression in adipose tissue.

## MATERIALS AND METHODS

All experimental procedures were in agreement with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1999), with all animal procedures approved by the University of São Paulo Animal Bioethics Committee (protocol number 1050/2007).

### Experimental Site

The experiment was conducted at the Experimental Feedlot Facility at the University of São Paulo, located at the city of Pirassununga, state of São Paulo, in south-east Brazil (21°59'46" S, 47°25'33" W, and 627 m above sea level) from June 2008 to January 2009.

### Animals and Diets

Thirty-six prepubertal Nellore heifers, 18 to 20 mo old, with an average initial BW of  $276.1 \pm 17.9$  kg and BCS of  $4.7 \pm 0.46$  (1 to 9 scale, in which 1 means emaciated and 9 means obese; Spitzer, 1986), were randomly assigned to 1 of 3 treatments: **High** (high energy diet;  $n = 12$ ), **Low** (low energy diet;  $n = 12$ ), and **LL** (low energy diet with subcutaneous administration of oLeptin;  $n = 12$ ). Heifers were housed in 2 pens according to the diet offered, under ambient light and temperature, and both diets were offered as total mixed rations once a day at 0700 h.

Diets were formulated according to NRC (1996) to fulfill maintenance requirements and promote an ADG

**Table 1.** Ingredient composition and analysis of the high- and low-energy diets

| Item                       | Diets       |            |
|----------------------------|-------------|------------|
|                            | High energy | Low energy |
| Diet composition, % of DM  |             |            |
| Corn silage                | 40.1        | 55.4       |
| Sugarcane bagasse          | –           | 31.2       |
| Ground corn                | 40.1        | –          |
| Soybean meal               | 18.2        | 11.6       |
| Urea                       | –           | 1.0        |
| Minerals and vitamins      | 1.5         | 0.8        |
| Ration analysis (DM basis) |             |            |
| aNDF, <sup>1</sup> %       | 30.7        | 57.1       |
| TDN, <sup>2</sup> %        | 75.1        | 59.5       |
| GE, <sup>3</sup> Mcal/kg   | 4.3         | 4.2        |
| ME, Mcal/kg                | 2.7         | 2.2        |
| CP, %                      | 15.6        | 12.6       |
| Ether extract, %           | 3.6         | 2.3        |
| Lignin, %                  | 1.6         | 4.7        |
| MM, <sup>4</sup> %         | 5.6         | 6.8        |

<sup>1</sup>aNDF = ash-free neutral detergent fiber (% DM).

<sup>2</sup>Estimated with NRC (2001) equations.

<sup>3</sup>Obtained in bomb calorimeter.

<sup>4</sup>MM = mineral matter.

of 0.4 kg/d (groups Low and LL) or 1.2 kg/d (group High). Both diets were offered at ad libitum intake. Composition of diets based on chemical analyses is described in Table 1.

The feed intake of the groups was controlled daily by weighing the orts and keeping it between 5 and 10% of the total offered. Heifers were weighed and had their BCS evaluated twice weekly to determine ADG and also BW and BCS at puberty. Diet ingredients and orts were sampled weekly and stored at  $-20^{\circ}\text{C}$  until further analysis. Diet composition was evaluated every week and adjusted, if necessary, to maintain specified ADG and meet NRC (1996) requirements.

Dry matter, CP, ether extract, and minerals were determined according to the Association of Official Analytical Chemists (AOAC, 1990). Lignin was analyzed according to Van Soest and Robertson (1985). Ash-free NDF was considered as percentage of NDF, determined according to Van Soest et al. (1991), omitting the sodium sulfite step, and corrected for percentage of ashes, determined after combustion of the NDF samples for at least 4 h in a muffle furnace at  $500^{\circ}\text{C}$ .

Total digestible nutrients (TDN) were estimated according to the NRC (2001) equation:  $\text{TDN} (\%) = \text{tdNFC} + \text{tdCP} + (\text{tdFA} \times 2.25) + \text{tdNDF} - 7$ , in which tdNFC represents the truly digestible nonfibrous carbohydrates, tdCP represents the truly digestible CP, tdFA represents the truly digestible fatty acids, and tdNFC represents the

truly digestible NDF. Gross energy was analyzed directly in an adiabatic bomb calorimeter.

### ***Hormonal Treatment and Reproductive Evaluation***

Highly purified ovine lyophilized recombinant leptin (Protein Laboratories Rehovot, Rehovot, Israel) was used in this study. After 14 d of acclimatizing to housing and diet, LL heifers started to receive 4.8 µg oLeptin/kg BW, subcutaneously, twice each day (at 0600 and at 1800 h) for 56 d. The volume of each injection was approximately 2 mL, varying slightly according to individual BW. Group High and Low heifers received similar injections of 2 mL saline solution.

The dose of leptin adopted in this study was based on previous studies with prepubertal heifers (Garcia et al., 2002; Maciel et al., 2004a). The aim was to raise serum leptin concentration to 5 to 10 ng/mL for a period of 8 to 12 h after a single injection. This criterion is based on the observation that serum leptin concentration of mature beef cows, cycling normally, typically is 15 to 20 ng/mL and around 6.4 ng/mL in prepubertal heifers, gaining 1.1 kg BW/d, on the week of puberty onset (Garcia et al., 2002). The oLeptin solution was prepared every 3 d by reconstitution of the lyophilized hormone in sterile saline solution and stored under refrigeration.

To determine the approximate time of first ovulation and, therefore, of puberty onset, heifers were scanned twice weekly by real-time linear ultrasonography using an Aloka SSD-550V equipment and a 7.5 MHz transrectal transducer (Aloka Corp., Tokyo, Japan). The presence of corpora lutea (CL) was observed and maximum diameter of the dominant follicle (DF) was measured in both ovaries until puberty was achieved. Ultrasonography has been shown to be reliable in measuring ovarian follicles as small as 3 mm in diameter and for observation of the following changes in growth of individual follicles (Sirois and Fortune, 1988; Knopf et al., 1989).

At the time of ultrasound evaluation, 10 mL of blood was sampled by jugular venipuncture, into dry vacuum tubes, for serum progesterone and leptin determination. Blood collections were performed without fasting the animals. Samples were centrifuged at  $1,500 \times g$  for 30 min at 4°C within 2 h of collection to minimize hormones degradation. Serum was stored in 1.5 mL tubes at -20°C until further analysis.

Age at puberty was considered as age on first detection of a CL by transrectal ultrasonography, when confirmed to be as functional by a serum progesterone concentration above 1 ng/mL (Oyedipe et al., 1986).

Serum progesterone from all heifers was determined by RIA, with Coat-A-Count direct assay kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). For determination of serum leptin, 4 heifers from each group

were randomly selected for sampling at 6 predefined time points: at the beginning of the dietary treatments, 7 d before the first leptin injection, during the leptin treatment (7, 14, and 28 d after commencing leptin injection), and 11 d after the last leptin injection. Serum leptin was determined by ELISA, specific for bovine leptin (Cusabio Biotech Co., Ltd., Hubei, PR China), according to the manufacturer's protocol (Li et al., 2012). The concentration of leptin in samples was determined using a 4 parameter logistic curve fitting algorithm option in the Master Plex QT3.0 quantification software (MiraiBio Inc., Alameda, CA). The leptin standard curve ranged from 1.56 to 100 ng/mL, and the intra- and interassay CV were 7.6 and 12.6%, respectively.

### ***RNA Extraction and cDNA Synthesis***

Eight heifers from each group were slaughtered on the second day after CL detection. The postpubertal condition of the heifers was confirmed after slaughter by confirmation of CL in the ovaries. At slaughter, samples from subcutaneous, mesenteric, and perirenal adipose tissue depots were collected, frozen in liquid N<sub>2</sub>, and stored at -80°C for subsequent analysis.

Total RNA from tissue samples was isolated using TRIzol Reagent (Life Technologies, São Paulo, Brazil), according to the manufacturer's instructions. The quality of isolated RNA was determined by measuring the absorbance at 260 and 280 nm using the GeneQuant Pro (GE Healthcare, São Paulo, Brazil) and its integrity was verified as mainly 5.8S, 18S, and 28S rRNA by electrophoresis in 1.2% (wt/vol) agarose-formaldehyde gel. To reduce genomic DNA contamination, 1.5 µg of total RNA from each tissue sample were treated with DNase I (Life Technologies). One microgram of treated RNA was submitted to cDNA synthesis using the Superscript II cDNA synthesis kit (Life Technologies), according to the manufacturer's protocol. Reactions performed without reverse transcriptase were used as negative controls for genomic contamination.

### ***Real-Time PCR Analysis***

Relative quantification of leptin (*LEP*) gene expression in the 3 adipose tissue depots was performed based on the second derivative maximum method using the StepOne Real Time PCR System (Life Technologies). Ribosomal protein-L19 (*RP-L19*) was used as control gene, and reactions without cDNA were used as negative controls.

These primers, specific for the *LEP* and *RP-L19* genes, were designed for real-time PCR based on bovine GenBank sequences using Primer Express 3.0 software (Life Technologies): *LEP* F-5'AGGTCCAGGATGACACCAAAC-3' and

R-5'TCCAAACCAGTGACCCTCTGT-3' (amplicon length 106 bp, accession number EU313203.1); RP-L19 F-5'AGGGTACTGCCAATGCTTTAATG-3' and R-5'CATGTGGCGGTCAATCTTCTT-3' (amplicon length 152 bp, accession number NM\_174243.2).

Before performing the real-time PCR reactions, a conventional PCR was performed to test the primers and verify the absence of genomic DNA contaminations. The real-time PCR reactions contained 1.0  $\mu$ L of cDNA, 10  $\mu$ L of SYBR Green master mix 2x (Life Technologies), 0.25 mM of each primer, and water up to a total volume of 20  $\mu$ L. Thermal cycling parameters were an initial denaturing step of 94°C for 10 min followed by 44 cycles of denaturing (94°C for 15 s), annealing/elongation (60°C for 1 min), a melting curve program (65 to 95°C with a heating rate of 0.3°C per cycle), and a final cooling cycle of 4°C. All reactions were performed in triplicate wells.

A dilution curve with a series of cDNA concentrations was calculated to obtain the amplification efficiency of *LEP* and *RP-L19* for each adipose tissue depots. The amplification efficiency (**E**) was constructed as  $E = 2^{(-1/\text{slope})}$ . Changes in gene expression were calculated by relative quantification using the  $\Delta\Delta\text{Ct}$  method (Livak and Schmittgen, 2001), in which Ct is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set with exponential phase of the PCR and  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{RP-L19} - \Delta\text{Ct}_{LEP}$ . If the E values were not different than 2, then *LEP* gene expression ratio (**R**) was calculated using the equation  $R = 2^{-\Delta\Delta\text{Ct}}$ . Normalized real-time PCR data were transformed into fold-change relative to the control group (low energy without leptin) and presented as such.

Before comparing gene expression, it is important to consider and, if necessary, to adjust for unequal efficiencies of cDNA amplification (Yuan et al., 2006). Regression analysis resulted in regression coefficient ( $R^2$ ) values above 0.95, indicating a linear relationship between starting cDNA concentration and Ct. Also, slopes were not significantly different than -1 (confidence intervals for slopes included the number -1). Therefore, the efficiency of amplification for *LEP* and *RP-L19* was considered to be equal to 100%. Expression of the control gene, *RP-L19*, was not affected by treatment ( $P = 0.38$ ), demonstrating its validity as a reference gene in this study.

### Statistical Analysis

All statistical analyses were conducted using SAS (SAS Inst. Inc., Cary, NC). Data were analyzed as a completely randomized design, with 3 treatments structured in classes, with 12 repetitions. Data for serum leptin had only 4 repetitions per treatment.

Analysis of variance for BW gain and age at puberty was performed using the GLM procedure of SAS according to the model  $Y = \text{Treatment} + \mu + e$ , where  $e$  is the error term for the model. Least square means (LSMEANS) were compared by contrasts (High vs. Low and LL; Low vs. LL). Body condition score data were analyzed by the nonparametric 1-way Kruskal–Wallis test.

Data for serum leptin concentration and maximum diameter of the DF were analyzed by the MIXED procedure for repeated measures of SAS, using the model  $Y = \text{Treatment} + \text{Time} + \text{Treatment} \times \text{Time} + \mu + e$ , considering treatment, time, and the interaction between treatment and time as variation sources. Day was considered as the repeated variable and animal within treatment as subject. For analysis of the treatment by time interaction, a polynomial regression was used, evaluating linear, quadratic, cubic, and cubic deviation components of the equations of serum leptin concentration and follicular diameter by time. Data for serum leptin concentration was log-transformed to meet normality assumptions. Data are presented as back-transformed.

Survival analysis was used to evaluate the ovulation rates within treatments (Prism, GraphPad Software; GraphPad Inc., San Diego, CA). Differences between treatment survival curves were tested using the Mantel-Cox test.

Data from real time PCR gene expression were analyzed as a split-plot design in a mixed model considering the fixed effect of treatment (Low, High, or LL), of the adipose tissue depots (subcutaneous, perirenal or mesenteric), and of the interaction and considering animal within treatment as a random effect. In all comparisons, significance was declared at  $P \leq 0.05$ .

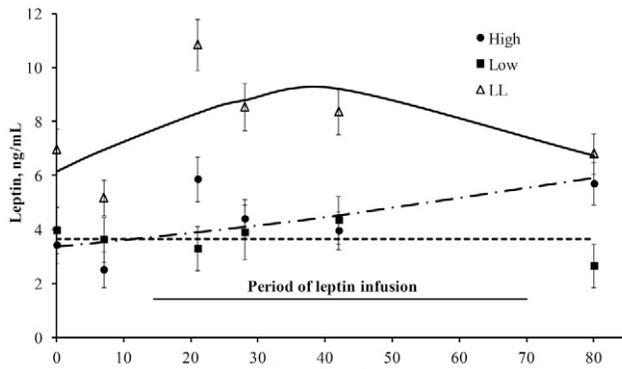
## RESULTS

### Serum Leptin Concentration

To certify that the leptin dosage applied was able to increase leptin concentration above the control group, serum samples from 4 heifers from each treatment group were randomly selected a posteriori for hormonal quantification. It was not an initial aim of this study to detect differences in serum leptin concentrations among treatments.

Leptin infusion successfully raised serum leptin concentration in prepubertal Nellore heifers in LL group (treatment  $\times$  time interaction;  $P = 0.04$ ; Fig. 1). Serum leptin concentration from heifers in LL group showed a peak ( $11.1 \pm 1.4$  ng/mL) on d 21 of the experiment, that is, 7 d after beginning leptin treatment. Thereafter, leptin concentrations decreased to  $9.4 \pm 1.5$  ng/mL on d 28 (14 d after the first leptin injection) and  $7.5 \pm 1.4$  ng/mL at 80 d (11 d after the last leptin injection).

The treatment  $\times$  time interaction demonstrated that serum leptin concentration was affected by time in High



**Figure 1.** Regression analysis of serum leptin concentration over time, from the beginning of dietary treatment (d 0) until 11 d after the end of leptin infusion (d 80). Leptin was infused twice daily from d 14 to 69. Data are presented as back transformed. High = high energy diet (●) [ $\text{Log}(\text{leptin}) = 1.75 + 0.01 \times \text{DOE}$  ( $P = 0.04$ ,  $R^2 = 0.12$ )]; Low = low energy diet (■) [ $\text{Log}(\text{leptin}) = 1.86$ ]; LL = low energy diet + ovine leptin infusion ( $4.8 \mu\text{g/kg BW}$ ;  $\Delta$ ) [ $\text{Log}(\text{leptin}) = 2.62 + 0.027 \times \text{DOE} - 0.0003 \times \text{DOE}^2$  ( $P = 0.03$ ,  $R^2 = 0.10$ )]. DOE = days on experiment.

and LL heifers ( $P < 0.01$ ) but not in treatment Low heifers ( $P = 0.50$ ), in which leptin concentrations remained constant ( $4.0 \pm 2.0 \text{ ng/mL}$ ) over the whole period of evaluation (Fig. 1). On the other hand, serum leptin of heifers receiving the high energy diet (High) increased linearly with time ( $P = 0.004$ ) whereas there was a quadratic ( $P = 0.01$ ) effect of time on serum leptin concentration for heifers in the LL treatment (Fig. 1).

### Puberty Onset

As expected, there was no difference among the 3 treatment groups (High, Low, and LL) for initial age, BW, and BCS (Table 2) as well as for initial DF maxi-

imum diameter, which was 0.74, 0.68, and 0.71 cm for treatments High, Low, and LL, respectively ( $P > 0.20$ ). These results indicate that the experimental groups were initially homogeneous. Body weight gains were similar to predicted, with heifers in the High treatment gaining significantly more BW than the those in treatment groups Low and LL ( $P = 0.001$ ; Table 2).

The energy level of the diet affected the age at first ovulation, with heifers consuming more energy (treatment High) attaining puberty earlier ( $P = 0.02$ ) than those in the lower energy diet (treatments Low and LL; Table 2). Contrary to our hypothesis, age at first ovulation did not differ between treatments Low and LL ( $P = 0.42$ ; Table 2), indicating that administration of  $4.8 \mu\text{g oLeptin/kg BW}$  twice daily for 56 d did not alter the timing of puberty. Serum progesterone concentrations were used to confirm age at puberty and averaged 1.5, 1.3, and 2.3 ng/mL at the time of slaughter for High, Low, and LL heifers, respectively.

Age at puberty was considered as age on first detection of a CL by transrectal ultrasonography, when confirmed to be as functional by a serum progesterone concentration above 1 ng/mL (Oyedipe et al., 1986).

Heifers in treatment High ovulated on average  $74.5 \pm 28.4 \text{ d}$  after the beginning of the hormonal treatment whereas the animals on treatments Low and LL ovulated on average  $142.5 \pm 63.6$  and  $134.4 \pm 62.4 \text{ d}$  after the beginning of the hormonal treatment, respectively. The survival analysis (Fig. 2) demonstrated that after 121 d in experiment all heifers on treatment High had already ovulated compared with only 55% on treatments Low and LL. All Low and LL heifers had ovulated by d 240.

**Table 2.** Age, BW, BCS and ADG of Nellore heifers according to treatments

| Variables             | Treatments <sup>1</sup> |                   |                   | Means | SEM  | P-value           |                     |
|-----------------------|-------------------------|-------------------|-------------------|-------|------|-------------------|---------------------|
|                       | High                    | Low               | LL                |       |      | Diet <sup>2</sup> | Leptin <sup>3</sup> |
| iAge, <sup>4</sup> d  | 625.7                   | 628.2             | 613.5             | 622.7 | 5.4  | 0.682             | 0.277               |
| iBW, <sup>5</sup> kg  | 273.7                   | 278.4             | 276.0             | 276.1 | 3.1  | 0.604             | 0.763               |
| iBCS <sup>6</sup>     | 4.73 <sup>a</sup>       | 4.83 <sup>a</sup> | 4.55 <sup>a</sup> | 4.71  | 0.08 | –                 | –                   |
| ADG, kg BW/d          | 1.20                    | 0.46              | 0.48              | 0.71  | 0.10 | 0.001             | 0.883               |
| ovAge, <sup>7</sup> d | 698                     | 768               | 748               | 742   | 11   | 0.017             | 0.420               |
| ovBW, <sup>8</sup> kg | 358                     | 341               | 336               | 344   | 6    | 0.174             | 0.753               |
| ovBCS <sup>9</sup>    | 5.63 <sup>a</sup>       | 4.91 <sup>b</sup> | 4.91 <sup>b</sup> | 5.10  | 0.10 | –                 | –                   |

<sup>a,b</sup>Within a row, means without a common superscript differ ( $P < 0.05$ ) using the nonparametric 1-way Kruskal–Wallis test.

<sup>1</sup>High = high energy diet, Low = low energy diet, LL = low energy diet + ovine leptin infusion ( $4.8 \mu\text{g/kg BW}$ ).

<sup>2</sup>Effect of diet (contrast High vs. Low and LL)

<sup>3</sup>Effect of leptin infusion (contrast Low vs. LL).

<sup>4</sup>iAge = initial age.

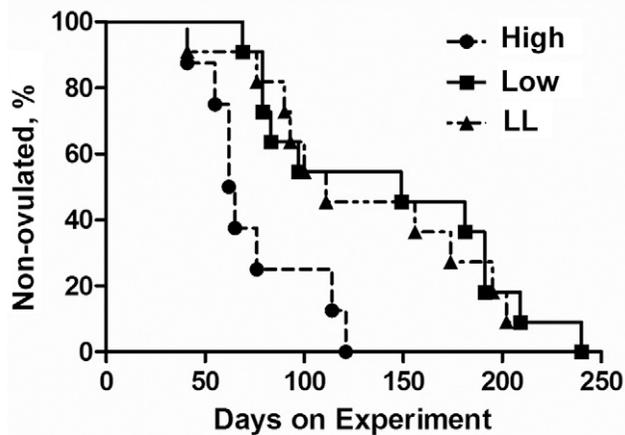
<sup>5</sup>iBW = initial BW.

<sup>6</sup>iBCS = initial BCS.

<sup>7</sup>ovAge = age at first ovulation.

<sup>8</sup>ovBW = BW at first ovulation.

<sup>9</sup>ovBCS = BCS at first ovulation.



**Figure 2.** Survival curves for ovulation rate (%) of heifers receiving 2 dietary treatments and leptin infusions. High = high energy diet (●); Low = low energy diet (■); LL = low energy diet + ovine leptin infusion (4.8 µg/kg BW; ▲).

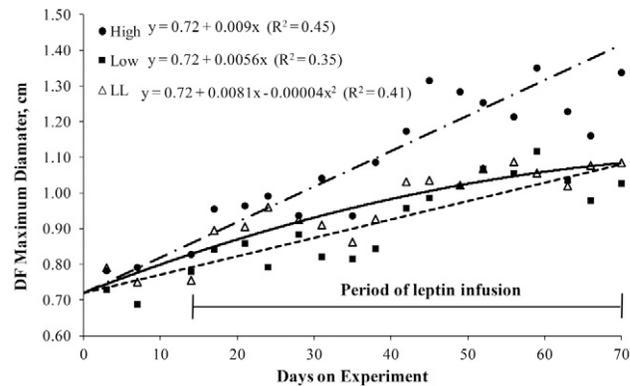
The lack of an effect of exogenous leptin on puberty in heifers on low energy intake is clearly seen in Fig. 2.

Neither the energy level of the diet ( $P = 0.17$ ) nor the exogenous administration of oLeptin ( $P = 0.75$ ) affected BW at puberty. However, heifers with greater energy intake attained puberty with greater BCS ( $P < 0.01$ ; Table 2). Leptin infusion, in turn, did not alter BCS at puberty ( $P = 0.99$ ).

### Follicular Development

An effect of treatment ( $P = 0.04$ ), of time ( $P < 0.001$ ), and an interaction between treatment and time ( $P < 0.001$ ) were all observed for DF maximum diameter. The greater energy intake accelerated follicular growth of heifers in treatment High, compared with the ones in lower energy intake (groups Low and LL). Exogenous oLeptin infusion, in turn, accelerated the follicular growth of heifers on low energy intake (LL), compared with non-leptin treated heifers (Low) on the same diet. However, the increased follicular growth rate of LL heifers was not as high as that in heifers on the greater energy intake (High). After about 30 d of treatment, the rate of follicular growth of heifers receiving oLeptin was decreased and the average DF maximum diameter approached that in the non-leptin treated animals (Fig. 3).

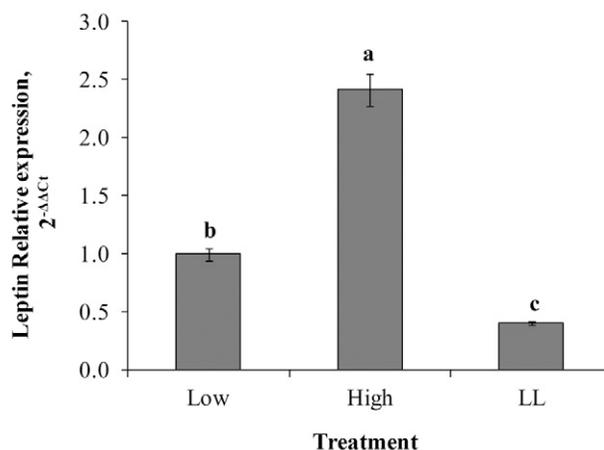
Dominant follicle maximum diameter of heifers in groups High and Low increased linearly with time ( $P < 0.01$ ) whereas for heifers in group LL, the effect was quadratic ( $P = 0.011$ ; Fig. 3). The regression curve slope of DF maximum diameter over time differed between High and Low heifers ( $P < 0.001$ ) and between Low and LL heifers ( $P < 0.05$ ), indicating that the rate of follicular growth was influenced both by energy intake and exogenous oLeptin infusion.



**Figure 3.** Regression analysis of the dominant follicle diameter over time. High = high energy diet (●); Low = low energy diet (■); LL = low energy diet + ovine leptin infusion (4.8 µg/kg BW; △).

### Leptin Gene Expression

The high energy diet increased and application of exogenous leptin for 56 d decreased *LEP* expression in adipose tissue ( $P < 0.001$ ; Fig. 4). There was no treatment  $\times$  tissue interaction ( $P = 0.39$ ) indicating that the effects of nutrition and of exogenous leptin on *LEP* expression were similar among fat depots. The high energy diet, on average, increased leptin expression 2.4-fold, on average, in the 3 adipose depots whereas exogenous leptin decreased leptin expression 2.5-fold when compared with heifers on the low energy diet without leptin administration (Fig. 4).



**Figure 4.** Effect of dietary treatment and exogenous ovine leptin (oLeptin) on *Leptin* expression. Data reported as least squares means  $\pm$  SEM for the 3 treatments (Low = low energy diet, High = high energy diet, and LL = low energy diet + oLeptin administration: 4.8 µg/kg BW). Mean mRNA abundance of leptin in 3 adipose tissue depots (subcutaneous, mesenteric, and perirenal); treatment  $\times$  tissue effect was not significant ( $P > 0.40$ ). Data were analyzed by the  $2^{-\Delta\Delta C_t}$  method, where  $C_t$  is the threshold cycle number, with low energy (Low) as the reference expression point. Significant differences ( $P < 0.05$ ) among dietary treatments are represented by different lowercase letters.

## DISCUSSION

It has been postulated that leptin acts as a permissive signal to the occurrence of puberty (Wylie, 2010). Therefore, insufficient nutrient availability or depleted body reserves followed by depressed plasma leptin would attenuate the ability of other signals to trigger puberty or even act as an absolute gating mechanism for puberty (Cunningham et al., 1999). In the present study, the effects of energy intake and of exogenous leptin infusion during the prepubertal period on the timing of puberty attainment and leptin gene expression in Nellore heifers were investigated.

Knowledge of the BW and body composition of heifers at puberty is critical for implementation of a successful heifer rearing program. Several factors, such as breed, nutrition, and environment, are known to alter age, BW, and body composition at puberty (Larson, 2007). In general, heifers receiving more energy dense diets reach puberty earlier but with greater BW (Larson, 2007), and *Bos indicus* heifers (Nellore and Brahman) are usually older, heavier, and taller at puberty than Angus or Hereford heifers (Baker et al., 1989; Hopper et al., 1993).

In the current study, high energy intake reduced age at puberty without altering BW. However, heifers on treatment High probably accumulated more adipose tissue, as evidenced by the greater BCS, indicating that energy intake altered body composition of heifers at puberty. Previous studies with different breeds also demonstrated that greater energy intake during the prepubertal period results in greater fat deposition in the carcass (McShane et al., 1989; Hall et al., 1995). In contrast to our results, most studies report an increase in BW at puberty in heifers offered greater energy diets (Baker et al., 1989; Hopper et al., 1993; Hall et al., 1995).

Contrary to our main hypothesis, exogenous leptin infusion failed to hasten puberty in heifers fed a low energy diet. The dose of leptin used in the current study (4.8 µg/kg BW twice daily or 9.6 µg/kg BW per day) was chosen based on a previous study by Maciel et al. (2004a), in which chronic administration of oLeptin at 38.4 µg/kg BW per day for 40 d generated a linear increase in leptin concentrations of treated animals, reaching plasma concentrations values 35-fold greater in treated than nontreated animals. Therefore, we chose for the current study the lowest dose of oLeptin able to significantly increase leptin concentrations above the nontreated group (9.6 µg/kg BW per day).

There was a clear increase in serum leptin concentration after the initiation of oLeptin infusion, and this was followed by a significant increase in DF diameter. However, both of these effects were transient. Diameter of the DF was greater for leptin-treated heifers up to 30 d of treatment compared with the control heifers, which

coincided with the approximate time when plasma leptin concentration started to decline in the LL group.

The follicular development pattern observed in this study, increasing linearly in time, agrees with Bergfeld et al. (1994), who demonstrated a linear increase in the DF maximum diameter of prepubertal heifers approaching puberty and also demonstrated that greater energy accelerated follicular growth. The mechanism by which energy intake influences follicle development is not yet fully elucidated. Greater energy intake increases circulating IGF-I, leptin, and LH concentrations. All 3 hormones are important factors for ovarian development (Armstrong et al., 2003). The presence of LH receptors on the surface of granulosa cells is a dominant feature of pre-ovulatory follicles and required for its continued growth in diameter (Webb et al., 2004). Leptin has been shown to regulate LH secretion in cattle (Amstalden et al., 2003; Kadokawa et al., 2006) and could enhance follicular growth indirectly by inducing LH secretion from the pituitary.

The expression of leptin receptor (**OBR**) and its isoforms in pre-ovulatory follicles as well as the presence of leptin in mature oocytes suggest that the hormone may have a local role in the ovary (Sarkar et al., 2009). Specific binding of leptin on granulosa cells has been demonstrated, and leptin has been shown to inhibit the insulin stimulation of steroidogenesis of bovine granulosa cells (Spicer and Francisco, 1997). In agreement with this concept, treatment of ewes with small doses of human leptin increased the number of large antral follicles but had no effect on the number of the small ones, suggesting that leptin probably acted on already recruited small follicles, inducing their growth (Muñoz-Gutiérrez et al., 2005). Almog et al. (2001) reported that subcutaneous infusion of leptin markedly accelerated puberty onset of immature female rats fed ad libitum. These authors also observed hypertrophy of the granulosa cell layer of antral follicles, decreased apoptosis of antral and pre-antral follicles, and increased basal concentrations of progesterone, LH, and FSH, after 14 d of leptin treatment.

The transient effect of leptin on follicle diameter observed in this study could be explained by a myriad of factors, including downregulation of LH receptors in granulosa cells (Montano et al., 2009), development of intracellular leptin resistance by increased suppressor of cytokine signaling 3 (**SOCS3**) expression (Myers et al., 2008), or saturation of the leptin transport system at the blood-brain barrier (Banks, 2004). We investigated the effect of exogenous leptin administration on endogenous leptin expression at the adipose tissue and demonstrated a suppression of leptin gene expression after administration of leptin. The observed decrease in leptin gene expression could explain the reduction in leptin serum concentration after 30 d of treatment on the LL

group and consequently the failure of the leptin treatment to continue increasing follicular development and consequently accelerate puberty.

Heifers were slaughtered on average 41 d after the end of oLeptin treatment, with just 1 heifer slaughtered during oLeptin treatment. The lasting effect of oLeptin treatment on leptin gene expression is surprising but in accordance with previously reported results with long-term effect of early-life leptin treatment (Pico et al., 2007). Leptin treatment during lactation reduced leptin gene expression in the adipose tissue of adult rats and also altered expression of hypothalamic genes (Pico et al., 2007). The mechanism explaining this early-life programming of gene expression is not known. In a subsequent study, the increase in hypothalamic expression of pro-opiomelanocortin (**POMC**) in leptin-treated animals was associated with increased methylation of POMC promoters, but there was no change in the methylation status of other promoters whose gene expression was altered in leptin-treated animals (Palou et al., 2011).

It has been shown in ruminants that hyperleptinemia is associated with decreased glucocorticoid and insulin secretion, and because both factors are stimulatory for leptin secretion, hyperleptinemia could decrease leptin secretion by adipocytes (Chilliard et al., 2001). However, it has been shown in rats that leptin administration reduces leptin gene expression in white adipose tissue without altering either insulin or corticosterone concentrations (Scarpace et al., 1998), suggesting a direct feedback through leptin receptors on adipocytes.

In conclusion, our findings did not support the hypothesis that decreased serum concentration of leptin is the most important hindrance for puberty onset in suboptimal fed zebu heifers. Although exogenous administration of leptin temporarily enhanced the rate of follicular growth, it did not accelerate puberty. Administration of leptin for 56 d decreased expression of the leptin gene in white adipose tissue, suggesting the existence of a feedback mechanism in cattle. High energy intake accelerated the rate of follicular growth, reduced age at puberty, and altered body composition of zebu heifers, increasing BCS at puberty.

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