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Research Article

Leptin and hypothalamic gene expression in early- and late-maturing *Bos indicus* Nellore heifers

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Abstract

We investigated wether early-maturing or late-maturing *Bos indicus* Nellore heifers produced more leptin mRNA in adipose tissues and altered expression of hypothalamic genes related to leptin signaling. Six prepubertal and six pubertal heifers aged about 34 months and weighing 280 kg to 300 kg each were selected from a population of 100 Nellore heifers. Real-time PCR was used to quantify the expression of the leptin gene (*LEP*) in adipose tissues and the long isoform of the leptin receptor gene (*Ob-Rb*), the NK2 homeobox 1 hypothalamic marker gene *NKX2-1*, the suppressor of cytokine signaling 3 gene (*SOCS-3*), the neuropeptide Y genes (*NPY*) and the NPY G-protein coupled receptor genes *NPY-Y1* and *NPY-Y4* in the hypothalamus. Heifers attaining puberty earlier showed significantly greater *LEP* expression in adipose tissues ($p < 0.05$) and there was tissue interaction ($p < 0.05$). Hypothalamic expression of *Ob-Rb*, *NKX2-1*, *NPY* and *SOCS-3* did not differ between groups, but in early-maturing heifers there was a tendency for lower expression of *NPY-Y1* (8.3-fold less) and *NPY-Y4* (14.3-fold less) compared to late-maturing heifers ($p = 0.1$). These results suggest that a combination of higher *LEP* expression, lower *NPY-Y1* and *NPY-Y4* expression could be a factor in regulating puberty in early-maturing *B. indicus* heifers.

Key words: Bos indicus, gene expression, hypothalamus, leptin, neuropeptide Y.

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Introduction

Older age at puberty is responsible for lower slaughter rates in cattle production systems based on *Bos indicus* (Artiodactyla, Bovidae) breeds raised on pastures (Martin *et al.*, 1992) because although *B. indicus* cattle are better adapted to harsh tropical conditions than *Bos taurus* breeds, they generally reach puberty when they are older and heavier (Thallman *et al.*, 1999; Rodrigues *et al.*, 2002). Thus, selection of early-maturing *B. indicus* heifers as well as nutritional protocols to advance puberty have received a great deal of interest from the scientific community (Vargas *et al.*, 1998; Martinez-Velazquez *et al.*, 2003).

The hypothalamic maturation process and the metabolic signal involved in regulation of puberty are not well understood (Kinder *et al.*, 1995). Leptin, a 16 kDa protein hormone coded for by the *LEP* gene (also called the obese (*ob*) gene), has a key role in regulating energy intake and expenditure and has been proposed as an indicator of body adiposity and, in rodents, has a permissive role on puberty (Smith *et al.*, 2002). Leptin acts in the hypothalamus through the long isoform of the leptin receptor (Ob-Rb). However, the molecular mechanism by which leptin signaling in the hypothalamus might be involved in initiation of puberty has not been elucidated. One possible mechanism is through neuropeptide Y (NPY) signaling, this peptide increases dramatically in cerebrospinal fluid during undernutrition and negatively modulates secretion of luteinizing hormone (LH) when centrally infused into cattle (Gazal *et al.*, 1998). Given the inhibitory role of NPY on sexual maturation, leptin-mediated suppression of *NPY* expression in the arcuate nucleus is probably important in controlling pubertal development (Pedrazzini *et al.*, 2003).

The action of NPY in the hypothalamus is modulated by a family of G-protein coupled receptors, consisting of at

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least five distinct members (Y1, Y2, Y4, Y5 and Y6) (Blomqvist and Herzog, 1997). The products of *NPY-Y1* and *NPY-Y4* mediate the detrimental effects of NPY on the gonadotrope axis through knockout models (Sainsbury *et al.*, 2002; Pedrazzini, 2004). Our hypothesis was that late-maturing *B. indicus* heifers have less *LEP* expression in adipose tissue and/or less sensitivity to leptin signaling in the hypothalamus. It was our objective to test whether early-maturing *B. indicus* heifers had greater amounts of leptin mRNA in adipose tissues, and altered expression of hypothalamic genes related to leptin signaling.

Material and Methods

Animals and treatments

In November 2003, 100 pastured *Bos indicus* heifers were selected based on breed attributes (Nellore), month of birth (November-2001), and body weight (between 280 kg and 300 kg), from a population of 500 heifers in a herd at the Hildergard Georgina Von Pritzelwitz experimental station managed by the University of São Paulo in Londrina, Paraná, Brazil. These 100 heifers were submitted to rectal palpation and scored as prepubertal or postpubertal according to the presence or absence of a palpable corpus luteum (CL) and ovary size. After scoring, 15 prepubertal heifers and 15 postpubertal heifers, born in the same month with similar body weight and body condition score, were selected and given a prostaglandin ($PGF_2\alpha$) injection to induce estrus (Ciosin, Coopers, Brazil). The occurrence of estrus was visually monitored in these 30 heifers on the third and fourth day after the $PGF_2\alpha$ injection, and on the fourth day all 15 heifers that were previously scored as prepubertal and showed no sign of estrus after $PGF_2\alpha$ injection were weighed and submitted to rectal palpation to confirm an absence of CL. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

Six heifers that had palpable CL and showed estrus after $PGF_2\alpha$ injection (early-maturing), and six heifers that had no CL or signs of estrus (late-maturing) were selected for the experiment and slaughtered on the fifth day after $PGF_2\alpha$ injection. The prepubertal or postpubertal conditions of the heifers were confirmed at slaughter by dissection of the ovaries. At slaughter, samples from the subcutaneous, omental and perirenal adipose tissues, and hypothalamus were collected, frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis. The optical chiasm and mammilary bodies were used as anatomical markers for collecting the hypothalamus. For statistical purposes, the treatments were defined as the two heifer groups, early-maturing or late-maturing heifers.

Total RNA from tissue samples was isolated using TRIzol® Reagent (Invitrogen, USA) according to Chomczynski and Saachi (1987). The quality of isolated RNA was determined by measuring the absorbance at 260 and 280 nm and its integrity was verified as mainly 18S and 28S rRNA by electrophoresis in 1% (w/v) agarose gel.

Reverse Transcription (RT) PCR

We used 5 µg of total RNA from each tissue sample for cDNA synthesis. After denaturing at 70 °C for 10 min, half of the sample (2.5 µg) was reverse-transcribed into cDNA with 0.5 µg of oligo thymidine and 200 units of Superscript II reverse transcriptase (RT) (Invitrogen) in a final volume of 20 µL, for 60 min at 42 °C. The other half was incubated without reverse transcriptase and used as a negative control in polymerase chain reactions (PCR) to confirm the absence of residual genomic DNA contamination.

Oligonucleotide primer pairs specific for *LEP*, *Ob-Rb*, suppressor of cytokine signaling 3 (*SOCS-3*), *NPY*, *NPY-Y1* and *NPY-Y4* were designed based on bovine GenBank sequences (Table 1). One µL of each RT reaction was used as template for PCR reactions in a final volume of 50 µL with 1.5 mM $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate (Invitrogen), 0.25 µM of each primer (Invitrogen), 4 units of Taq polymerase (Invitrogen), and 1× PCR buffer (Invitrogen). The following amplification conditions were used: 95 °C for 5 min followed by 40 cycles at 95 °C for 45 s, 55 °C for 60 s and 72 °C for 1 min. After the last cycle, the reactions were held at 72 °C for 10 min.

Primers specific for the ribosomal protein gene *RP-L19* (also known as *L19*) were used as positive controls for all samples to verify the success of the RT reactions, and primers specific for the NK2 homeobox 1 hypothalamic marker gene *NKX2-1* (also known as TITF1) were used to verify possible sampling variations when dissecting the hypothalamus. In addition to incubating the RNA without reverse transcriptase, negative control reactions were performed similarly without addition of a template from the RT reaction. Amplified cDNA were visualized by agarose gel electrophoresis and staining with ethidium bromide.

Quantification of gene expression

Quantification of gene expression was performed using the LightCycler Real-Time PCR System (Roche Diagnostics; Switzerland) based on the second derivative maximum method. Samples without cDNA were used as a negative control. With this method a second derivative maximum within the exponential phase of the amplification curve is linearly related to the starting concentration of template cDNA molecules.

A master-mix of the reaction components was prepared with 1.1 mM $MgCl_2$, 110 nM forward primer, 110 nM reverse primer, 222 µM dNTP, 278 mg/L bovine serum albumin, 0.08 units/µL of Taq Platinum DNA poly-

Table 1 - Oligonucleotide primer pairs designed for use in polymerase chain reaction (PCR) amplification

Genes	Oligonucleotide primers: 5' → 3'	GenBank accession number	PCR insert size (bp)
RP-L19	F-GAAATCGCCAATGCCAAC R-GAGCCTTGTCTGCCTTCA	NM000981	361
NKX2.1	F-TGGGGGACGTGAGCAAGAATATG R-CAAGGTTTGCCGTCTTTCACCAG	XM874978	370
Leptin	F-TGTCTTACGTGGAGGCTGTG R-GCCGCAACATGTCCTGTAGT	U43943	432
Ob-Rb	F-TTTTGGAGCCTGAAACCATT R-TGGTGGAGAATTGTTGCTCA	NM001012285	329
SOCS 3	F- TTCAGCTCCAAGAGCGAGTACC R- ACTGGATGCGCAGGTTCTTG	NM174466	217
NPY	F- CTTGGCCAGATACTACTCAGCG R-AAAGAGGCAGAGACTGGAGAGC	AY491054	200
NPY-Y1	F- TGATGCCTTCAAGGACAAATACG R- GGACAGCAGCATGATGTTGATTC	XM580988	235
NPY-Y4	F- ACCCTGCTTATTGCCAACCTGG R- TGGATTGGTGATGAGCTGATGC	XM582253	210

merase (Invitrogen) and 45,450-fold diluted nucleic acid stain Sybr Green I (Invitrogen). LightCycler mastermix (18 μL) was filled in the LightCycler glass capillaries, and 2 μL of cDNA added as PCR template. The capillaries were closed, centrifuged for 5 s at 700 x g and placed into the LightCycler rotor. The following LightCycler experimental run protocol was used: denaturation program (95 °C for 5 min), amplification and quantification program repeated 40 times (95 °C for 10 s, 55 °C for 15 s, 72 °C for 20 s), melting curve program (75-95 °C with a heating rate of 0.1 °C per second and a continuous fluorescence measurement) and a final cooling step to 40 °C.

Statistical analysis

To calculate gene expression, a relative quantification method was used (Yuan *et al.*, 2006):

$$Ratio = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}}}{(E_{\text{RP-L19}})^{\Delta Ct_{\text{RP-L19}}}}$$

where E is the amplification efficiency of each gene, Ct the threshold cycle and ΔCt the late-maturing (LM) threshold cycle minus the early-maturing threshold cycle ($\Delta Ct = Ct_{\text{LM}} - Ct_{\text{EM}}$).

The relative expression method was used to minimize possible variations due to the efficiency of reverse transcription and quantity of template utilized. A dilution curve with a series of cDNA concentrations was calculated for each gene to obtain the amplification efficiency. The SAS procedure Proc Mixed (SAS, 2000) was used to perform simple linear regression for each group based on the model described by Yuan *et al.* (2006). The amplification efficiency (E) was calculated as $E = 2^{(-1/\text{slope})}$. If the E values were not different than 2, then the gene expression ratio (R) was calculated using the equation $R = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta Ct_{RP\text{-}L19} - \Delta Ct_{\text{target}}$. When the E values were different than 2 we adjusted $\Delta\Delta Ct$ using the percentage amplification efficiency (PE) to give $\Delta\Delta Ct\_adj = PE_{RP\text{-}L19} * \Delta Ct_{RP\text{-}L19} - PE_{\text{target}} * \Delta Ct_{\text{target}}$ and the gene expression ratio was calculated $R = 2^{-\Delta\Delta Ct\_adj}$. Data were analyzed using analysis of covariance (ANCOVA) considering the fixed effects of treatment, gene and the treatment × gene interaction, as well as the random effects of the animals (treatment) and gene × treatment (animal). Significance was assumed to be $p < 0.05$ and tendency was assumed to be $p < 0.1$.

Results

When testing the efficiency of gene amplification, regression analysis resulted in regression coefficient (R^2) values above 0.96, indicating a linear relationship between starting cDNA concentration and Ct. The efficiency of amplification for *LEP*, *RP-L19* and *NPY-Y1* indicated a slope significantly different than -1, therefore the ratio of gene expression was calculated considering the calculated efficiency of amplification (Table 2). For the other genes, efficiency was considered to be equal to 2.

Heifers that attained puberty earlier had greater leptin gene expression in adipose tissue ($p < 0.05$, Table 3), and there was a significant treatment by tissue interaction ($p < 0.05$, Table 3). After adjusting for differences in the housekeeping gene *RP-L19* expression, *LEP* expression was detected by real-time PCR at 2.1 cycles earlier in the postpubertal heifers than in the prepubertal heifers ($\Delta\Delta CT = -2.1$, Table 3), which represents an average increase of 4.3-fold in *LEP* expression among the three adipose tissue depots (Figure 1). Early-maturing heifers had greater *LEP* expression in omental fat depot (10-fold increase, $p = 0.05$) and subcutaneous fat depot (6.9-fold increase, $p = 0.01$), while there was no effect on *LEP* ex-

Table 2 - Efficiency of gene amplification using real-time polymerase chain reaction amplification.

Gene	Slope coefficient	95% confidence interval		Amplification efficiency $E = 2^{(-1/\text{slope})}$	Percentage amplification efficiency (*PE*)
		Minimum	Maximum		
Leptin	-1.82	-2.02	-1.65	1.5	0.55
Ob-Rb	-0.98	-1.09	-0.86	2.0	1.02
NPYY1	-1.44	-1.82	-1.05	1.6	0.70
RPL19	-1.38	-1.72	-1.04	1.7	0.73
NPY	-0.99	-1.12	-0.87	2.0	1.01
NPYY4	-0.96	-1.08	-0.85	2.1	1.04
SOCS3	-1.19	-1.48	-0.90	1.8	0.84

Table 3 - Quantification of gene expression in the adipose tissue depots of early-maturing (EM) and late-maturing (LM) *Bos indicus* Nellore heifers.

Adipose tissue	Leptin_{EM}	Leptin_{LM}	$\textit{RP-L19}_{EM}$	$\textit{RP-L19}_{LM}$	Adjusted threshold cycle*	SEM†	p-value
Omental	28.5	36.3	18.1	19.4	-3.34	1.3	0.05
Perirenal	24.6	24.4	18.8	18.0	-0.49	0.9	0.60
Subcutaneous	29.0	34.9	19.6	20.3	-2.78	0.8	0.01

*$\Delta\Delta Ct\_adj = PE_{RP\text{-}L19} \times \Delta Ct_{RP\text{-}L19} - PE_{\text{target}} \times \Delta Ct_{\text{target}}$, where *Ct* is the threshold cycle, *RP-L19* is the ribosomal protein gene, *PE* is the percentage amplification efficiency, and $\Delta Ct = Ct_{LM} - Ct_{EM}$. †SEM = Standard Error of the Mean.

pression in the perirenal fat depot (1.4-fold increase, $p > 0.60$) (Figure 1).

Hypothalamic expression of the long isoform of leptin receptor (*Ob-Rb*) was not different between heifer groups ($p > 0.50$, Table 4). Dissection of the hypothalamus was based on anatomical markers and there should have been no difference between the two groups of heifers in the area of the brain that was sampled. This was checked by analyzing the expression of the NK2 homeobox 1 hypothalamic marker gene *NKX2-1* (also known as *TITF-1*) which is only expressed in the hypothalamus and is not involved in reproductive events (Suzuki *et al.*, 1998). We found that *NKX2-1* expression was not affected by treatment ($p > 0.90$, data not shown), demonstrating that there was no difference between the heifer groups in the area of the brain sampled. There was also no treatment effect on *SOCS-3* expression by the hypothalamus ($p > 0.80$, Table 4). The expression of *NPY* in the hypothalamus was not statistically different ($p > 0.7$) between heifer groups (Table 4). However, for heifers that reached puberty earlier there was a tendency ($p = 0.1$) for less expression of *NPY-Y1* (8.3-fold less) and *NPY-Y4* (14.3-fold less), as shown in Table 4 and Figure 2. When the data for both NPY receptors, *NPY-Y1* and *NPY-Y4* were analyzed together, there was a statistically significant ($p = 0.03$) 11-fold reduction in expression in early-maturing heifers (data not shown).

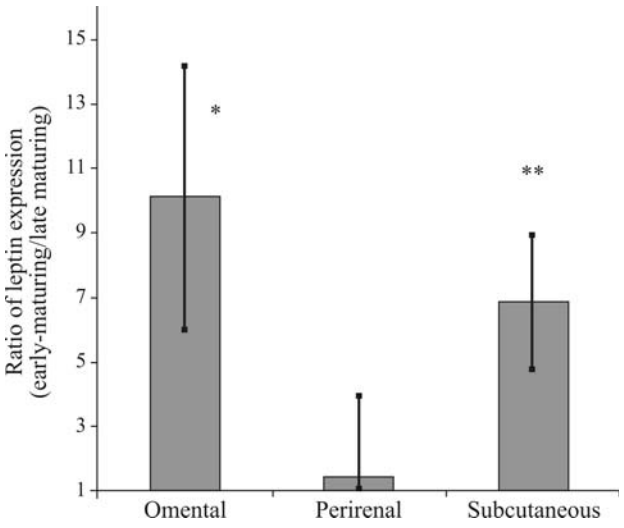

Figure 1 - Ratio of leptin gene expression in the adipose tissue depots of early-maturing and late-maturing *Bos indicus* Nellore heifers. Lines on bars are standard errors. *Ratio different from 1, $p < 0.05$. **Ratio different from 1, $p < 0.01$.

Discussion

The increased *LEP* expression in adipose tissue of early-maturing heifers supports the idea that the circulating concentration of leptin is an important signal for the initiation of pubertal processes (Ahima *et al.*, 1997), and suggests that heifers with greater adipose tissue *LEP* expression could attain puberty earlier and at lighter body weight. Although well adapted to tropical grazing conditions, *B. indicus* cattle reach puberty at a much later age than most *B. taurus* breeds, even when both breeds are raised under similar environmental and nutritional conditions (Rodrigues *et al.*, 2002). Later puberty results in a later age at first calving and reduces the slaughter rate and

Table 4 - Quantification of hypothalamic gene expression in early-maturing and late-maturing *Bos indicus* Nellore heifers.

Gene	$Target_{EM}$	$Target_{LM}$	$RP\text{-}L19_{EM}$	$RP\text{-}L19_{LM}$	Adjusted threshold cycle*	SEM†	p-value
Ob-Rb	31.6	35.1	19.1	22.1	-0.53	-0.77	0.51
SOCS3	32.4	34.5	19.1	22.1	0.85	3.14	0.81
NPY	25.2	28.8	19.1	22.1	-0.68	-2.23	0.77
NPYY1	27.3	27.2	19.1	22.1	-3.14	1.73	0.11
NPYY4	26.7	25.9	19.1	22.1	-3.77	1.91	0.08

*$\Delta\Delta Ct\_adj = PE_{RP\text{-}L19} \times \Delta Ct_{RP\text{-}L19} - PE_{target} \times \Delta Ct_{target}$, where Ct is the threshold cycle, *RP-L19* is the ribosomal protein gene, PE is the percentage amplification fficiency, and $\Delta Ct = Ct_{LM} - Ct_{EM}$.
†SEM = Standard Error of the Mean.

overall efficiency of cow-calf operations using *B. indicus* breeds in grazing systems.

In both *B. indicus* and *B. taurus* heifers, early stage of sexual maturation is regulated by the maturation of the hypothalamus (Evans *et al.*, 1994; Rodrigues *et al.*, 2002). The physiological regulation for maturation of the hypothalamus leading to puberty is not well understood, but clearly both body weight and chronological age influence the onset of puberty (Moran *et al.*, 1989; Kinder *et al.*, 1995). Nutrition is also an important element determining reproductive status in cattle and other mammals. A complex and controversial relationship between body fatness and the control of the reproductive axis was proposed by Frisch *et al.* (1980).

More recently, it has been postulated that leptin could explain the link between body fat, nutrition and control of the reproductive axis, with plasma concentrations of leptin being permissive to puberty in rodents (Barash *et al.*, 1996). Exogenous administration of leptin in feed restricted beef heifers is able to increase LH concentrations and gonadotropin-releasing hormone (GnRH) stimulated LH secretion by the pituitary (Amstalden *et al.*, 2002; Maciel *et al.*, 2004a; Zieba *et al.*, 2004). However, leptin administration for 40 days in well fed heifers was not able to advance puberty or increase LH secretion (Maciel *et al.*, 2004b; Zieba *et al.*, 2004). These and other studies suggest that acute or chronic feed restriction can sensitize the reproductive axis to leptin (Dyer *et al.*, 1997; Nagatani *et al.*, 2000). Taken together, these results indicate that in *B. indicus* heifers raised on pasture, with less body fat, averaging 300 kg at age 36 months, as is very common in grazing systems, the circulating concentrations of leptin should be limiting GnRH secretion and the onset of puberty. Therefore, those heifers with greater adipose tissue *LEP* expression could reach puberty earlier at a lower body weight.

Our results agree with the findings of other workers showing that amount of leptin mRNA is less in subcutaneous tissue than in perirenal adipose tissue (Kim *et al.*, 2000; Yang *et al.*, 2003). The reason for the greater *LEP* expression in perirenal fat could be explained by a greater adipocyte diameter (Yang *et al.*, 2003). Fasting-induced decrease of *LEP* expression occurs predominantly in the subcutaneous fat, while there is no effect in either perirenal or omental fat depots (Kim *et al.*, 2000). In our study, attainment of puberty was related to *LEP* expression in the subcutaneous and omental fat depots, but not in the perirenal fat depot.

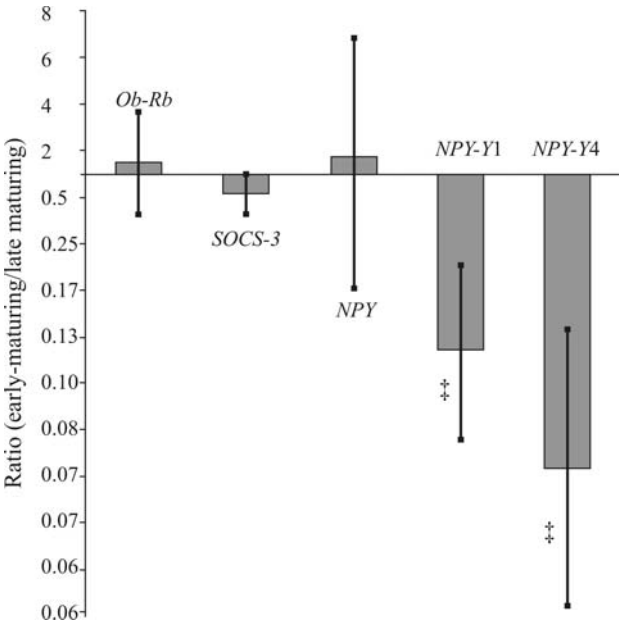

Figure 2 - Ratio of hypothalamic gene expression in early-maturing and late-maturing *Bos indicus* Nellore heifers. Lines on bars are standard errors. Tendencies for treatment differences ($p < 0.10$) between means are indicated by the symbol, ‡ bellow the appropriate bars.

In humans and rodents, leptin signaling in the hypothalamus is essential for sexual maturation and leptin receptor deficient rodents cannot attain puberty (Huszar *et al.*, 1997). This suggests that it is possible that early-maturing heifers could have a greater *Ob-Rb* expression in the hypothalamus and, therefore, reach puberty with less circulating leptin. However, our results do not support this hypothesis because *B. indicus* heifers that reached puberty earlier had greater *LEP* expression but equal *Ob-Rb* expression in the hypothalamus. In another study with cattle, *Ob-Rb* expression in the hypothalamus was not different in Holstein and Charolais bulls (both *B. taurus*) at 18 months of age, although Holstein cattle reached puberty on average

at an earlier age than Charolais cattle (Ren *et al.*, 2002). However, *Ob-Rb* expression in the hypothalamus of rams was altered by photoperiod, with rams subjected to a long day-length photoperiod being sexually inactive, had similar circulating leptin but greater *Ob-Rb* expression than rams exposed to a short-day photoperiod (Clarke *et al.*, 2003). The expression of *NPY* was also higher in the hypothalamus of rams subjected to a long-day photoperiod (Clarke *et al.*, 2003). Because NPY is a potent inhibitor of GnRH secretion by the hypothalamus, this could explain why rams under a long-day photoperiod were sexually inactive although they had similar plasma leptin concentration and greater *Ob-Rb* expression than sexually active rams under a short-day photoperiod (Clarke *et al.*, 2003).

In mammalian cell lines, SOCS-3 acts as an inhibitor of leptin signaling and leptin administration is capable of increasing *SOCS-3* mRNA in the hypothalamus of *ob/ob* mouse and other mammals (Bjorbaek *et al.*, 1998; Eyckerman *et al.*, 2000). It is thought that there is a feedback mechanism, by which an increase of leptin induces *SOCS-3* expression and directly inhibits leptin receptor signaling, which would be responsible for creating leptin resistance (Bjorbaek *et al.*, 1998). In our study, although there was greater adipose tissue *LEP* expression in heifers that attained puberty earlier *SOCS-3* expression in the hypothalamus was not altered.

The reproductive axis of cattle is very sensitive to leptin when the cattle are malnourished (Maciel *et al.*, 2004a) but leptin fails to stimulate the hypothalamic-adenohypophyseal axis of well-nourished sheep and cattle, suggesting that physiological resistance to leptin may occur in animals that are in neutral or positive energy balance (Amstalden *et al.*, 2005). It was thought that well-fed animals could be resistant to leptin due to increased *SOC-3* expression in the hypothalamus and adenohypophysis, however fasting increased and did not decrease the amounts of *SOCS-3* mRNA in the adenohypophysis of cows (Amstalden *et al.*, 2005). Taken together, these results suggest that *SOCS-3* expression is not related to control of the reproductive axis in cattle.

Neurons from the arcuate hypothalamus nucleus produce NPY, one of the most abundant peptides in the hypothalamus (Friedman and Halaas, 1998; Williams *et al.*, 2000). The various NPY functions are mediated by a family of NPY receptors (Y1, Y2, Y4, Y5 and Y6) (Gehlert, 1999) and NPY signaling in the hypothalamus regulates the effects of leptin on reproductive activity in rodents and primates (Aubert *et al.*, 1998; Sainsbury *et al.*, 2002). In *ob/ob* rats, a lack of leptin inhibition leads to chronically elevated *NPY* expression in the hypothalamus, while treatment with leptin reduces NPY and restores fertility (Sainsbury *et al.*, 2002). Also, when centrally administered, NPY greatly inhibits sexual function in rats (Pierroz *et al.*, 1996). Based on these observations it is possible that changes in hypothalamic NPY signaling could be involved in sexual maturation of heifers. In our study, although hypothalamic expression of NPY was the same in early-maturing or late-maturing heifers there was a tendency for less expression of the *NPY-Y1* and *NPY-Y4* receptors in the hypothalamus of early-maturing heifers.

The importance of NPY-Y1 signaling for the continuous maturation of the prepubertal hypothalamus has been demonstrated in knock-out models. Animals deficient in the Y1 receptor have increased pituitary LH and increased seminal vesicle size after 48 h of starvation (Pedrazzini *et al.*, 1998). Daily injections of leptin into juvenile *Y1*$^{-/-}$ female mice causes an advancement in puberty compared to wild-type mice, which is accompanied by an increase in uterus weight. An improved function of the gonadotropic axis is also seen in *Y1*$^{-/-}$, *ob/ob* double knockout mutant mice, which suggests that the permissive role of leptin in attainment of puberty is likely mediated by NPY and NPY-Y1 signaling (Pralong *et al.*, 2002). Ablation of the Y4 receptor in *ob/ob* mice also restores fertility to 100% in male mice and improves fertility in female double knockout mice by 50% (Sainsbury *et al.*, 2002).

In conclusion, *LEP* expression was greater in adipose tissues and expression of NPY receptor genes was less in the hypothalamus of early-maturing heifers. These results suggest that, because of the lower expression of NPY receptor genes, the hypothalamus of heifers reaching puberty earlier could be less sensitive to NPY inhibition. So, there are two detected pathways that could be responsible for hastened puberty in these heifers, greater *LEP* expression by the adipose tissue, and lesser expression of NPY receptor genes in the hypothalamus.

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