Effect of feed restriction during calthood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls

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Abstract

The objective of the present study was to evaluate the effects of feed restriction during calthood on serum concentrations of metabolic hormones, gonadotropins, and testosterone, and on sexual development in bulls. Eight beef bull calves received a control diet from 10 to 70 weeks of age. An additional 16 calves had restricted feed (75% of control) from 10 to 26 weeks of age (calthood), followed by either control or high nutrition ($n=8/\text{group}$) during the peripubertal period until 70 weeks of age. Restricted feed during calthood inhibited the hypothalamic GnRH pulse generator, reduced the pituitary response to GnRH, impaired testicular steroidogenesis, delayed puberty, and reduced testicular weight at 70 weeks of age, regardless of the nutrition during the peripubertal period. Restricted feed reduced serum IGF-I concentrations, but concentrations of leptin, insulin, and GH were not affected. In conclusion, restricted feed during calthood impaired sexual development in bulls due to adverse effects on every level of the hypothalamus–pituitary–gonad axis and these effects were not overcome by supplemental feeding during the peripubertal period. Furthermore, based on temporal associations, the effects of restricted feed on the hypothalamus–pituitary–gonad axis might be mediated by serum IGF-I concentrations. These results supported the hypotheses that the pattern of LH secretion during the early gonadotropin rise during calthood is the main determinant of age of puberty in bulls and that gonadotropin-independent mechanisms involved in testicular growth during the peripubertal period are affected by previous LH exposure.

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Introduction

The hypothalamus dictates the timing of sexual development through gonadotrophin-releasing hormone (GnRH) secretion in bulls. Circulating gonadotropin concentrations increase at 2–3 months of age in bulls (early gonadotropin rise), triggering the cellular events that lead to testicular growth and establishment of spermatogenesis (Rawlings *et al.* 1978, Amann & Walker 1983). Luteinizing hormone (LH) secretion during the period of the early gonadotropin rise seems to be the main determinant of age at puberty in bulls. Whereas increased LH pulse frequency and mean concentration during the early rise were associated with a reduced age at puberty (Evans *et al.* 1995, Aravindakshan *et al.* 2000), inhibiting LH secretion during the early gonadotropin rise delayed puberty in bulls (Chandolia *et al.* 1997b). In bulls, nutrition during calthood had direct effects on the

GnRH pulse generator in the hypothalamus; low nutrition decreased LH secretion, whereas high nutrition increased secretion (Brito *et al.* 2007a, 2007b). Therefore, restricted feed during calthood may affect the early gonadotropin rise and age at puberty in bulls, regardless of the nutrition provided after this critical period of development.

The portion of the central system involved in monitoring body metabolism and energy, the ‘metabolic sensor’, provides signals to the GnRH pulse generator in the hypothalamus and is a central link between nutrition and reproduction (Blache *et al.* 2002, 2003, Schneider 2004). The metabolic sensor translates signals provided by circulating (peripheral) concentrations of certain hormones into neuronal signs that ultimately regulate gonadotropin secretion. Identification of hormonal links between nutritional status and the metabolic sensor is

essential for elucidation of the mechanisms by which nutrition affects reproduction. Metabolic hormones such as leptin, insulin, growth hormone (GH), and insulin-like growth factor-I (IGF-I) are involved in controlling appetite and metabolism and may also be involved in regulating gonadotropin secretion. Temporal associations suggested that IGF-I might be involved in regulating LH secretion in bulls receiving varying levels of nutrition during calthood (Brito *et al.* 2007a, 2007b).

After the period of the early gonadotropin rise, gonadotropin concentrations decreased and remained largely unchanged thereafter (Rawlings *et al.* 1978, Amann & Walker 1983). However, testicular growth was accelerated from 6 to 16 months of age in bulls (peripubertal period; Coulter 1986, Barth & Ominski 2000), indicating that gonadotropin-independent mechanisms are involved in regulating testicular development. These mechanisms are largely unknown, but might involve metabolic hormones, since receptors for these hormones were detected in various cell types in the testes and these hormones affected testicular cell multiplication/differentiation and Leydig cell steroidogenesis (Cailleau *et al.* 1990, Spiteri-Grech & Nieschlag 1992, Lin 1995, El-Hefnawy *et al.* 2000, Wang & Hardy 2004). Circulating concentrations of metabolic hormones were associated with testes size in developing bulls (Brito *et al.* 2007a, 2007c), but differences in testes weight in the absence of differences in metabolic hormone concentrations during the peripubertal period in bulls receiving varying levels of nutrition suggested that the gonadotropin-independent mechanisms involved in testicular growth are dependent on previous exposure to gonadotropins during calthood (Brito *et al.* 2007a, 2007b). Therefore, altered gonadotropin secretion during calthood due to restricted feed may affect mature testes size in bulls.

The objective of the present study was to evaluate the effects of feed restriction during calthood on serum metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. We hypothesized that restricted feed during calthood impairs LH secretion during the early gonadotropin rise, with long-term effects on age at puberty and testes size that cannot be reverted by improved nutrition during the peripubertal period. We also hypothesized that metabolic hormones are involved in mediating the effects of restricted feed on sexual development.

Materials and Methods

Bulls and treatments

Twenty-four Angus and Angus X Charolais bull calves (from Angus and Charolais heifer dams) were weaned at 8 weeks of age (± 12 days) and block randomized (according to genotype and age) into two groups to receive control ($n=8$) or restricted ($n=16$) diets from 10

to 26 weeks of age. The control diet consisted of barley silage supplemented with 8.5% barley grain and 5.5% canola meal (as fed) and was fed *ad libitum*. This diet contained 13.2% crude protein, 1.66 Mcal/kg net energy of maintenance, and 1.05 Mcal/kg net energy of gain (dry matter basis). Bulls in the restricted feed group were fed 75% of the amount consumed by the control group. After 26 weeks of age, bulls in the control group continued to receive the same diet (control/control) and the bulls previously fed the restricted diet received either the same diet as the control group (restricted/control) or a high-nutrition diet (restricted/high; $n=8$ /group). The high-nutrition diet consisted of barley silage supplemented with 22% barley grain and 15% canola meal and was fed *ad libitum*. This diet contained 14.4% crude protein, 1.75 Mcal/kg net energy of maintenance, and 1.13 Mcal/kg net energy of gain. All diets contained 0.5% molasses and 0.5% mineral–vitamin premix.

Sexual development

The bulls were examined every 4 weeks from 14 to 70 weeks of age. Body weight was recorded (starting at 10 weeks of age) and backfat was measured between the 12th and 13th ribs over the longissimus muscle using an A-mode ultrasound (Krautkramer USK 7; Krautkramer Inc., Lewistown, PA, USA). Scrotal circumference was determined with a Coulter Scrotal Tape (Trueman Manufacturing; Edmonton, AB, Canada). The width and length of the testes were measured with calipers and paired testes volume was calculated using a formula ($\text{volume} = 0.5236 \times \text{length} \times \text{width}^2$; Bailey *et al.* 1998). To determine age at puberty, attempts to collect semen (by electroejaculation) were initiated once scrotal circumference (SC) reached 26 cm and were done at 2-week intervals. Sperm concentration was determined with a hemocytometer and the percentage of progressively motile sperm was estimated with phase contrast microscopy under $400\times$ magnification. Puberty was defined as an ejaculate containing ≥ 50 million spermatozoa with $\geq 10\%$ motile spermatozoa (Wolf *et al.* 1965). Bulls were sent to slaughter at 70 weeks of age and the testes were recovered and weighed.

Blood sampling and RIA

During the period of feed restriction, eight bulls were randomly chosen from the restricted feed group for blood sampling. Intensive blood sampling (sampling every 15 min for 10 h) was performed every 4 weeks from 14 to 34 weeks of age. Gonadotropin-releasing hormone (Fertagyl; Intervet Canada Ltd., Whitby, ON, Canada) was administered ($0.04 \mu\text{g/kg}$, i.v.) after the first 10 h and blood samples were collected every 15 min for another 90 min. Additionally, single blood samples were collected before feeding every 4 weeks until 66 weeks of

age. Blood samples were allowed to clot at room temperature overnight and centrifuged to separate serum, which was stored at -20°C before analysis. Serum concentrations of leptin, insulin, GH, IGF-I, follicle-stimulating hormone (FSH), and testosterone were determined in pooled samples from intensive samplings. LH concentrations were determined in serum samples obtained during the entire intensive sampling period, including after GnRH treatment. FSH and testosterone concentrations were also determined in serum samples obtained after GnRH treatment. Serum GH concentrations were determined only until 34 weeks of age. Serum concentrations of leptin, insulin, GH, IGF-I, LH, and FSH were determined by double-antibody RIA, whereas testosterone concentrations were determined by solid-phase RIA using a commercial kit. Tracer leptin was labeled using iodo-gen (Ehrhardt *et al.* 2000), whereas other tracer hormones were labeled with ^{125}I by the Chloramine-T method (Greenwood *et al.* 1963). Intra- and inter-assay CVs were $<10\%$ for all hormones.

Purified recombinant bovine leptin (Ehrhardt *et al.* 2000) was used for preparation of standards and for tracer labeling. Leptin concentrations were determined using rabbit bovine-leptin antiserum, as previously described in cattle (Ehrhardt *et al.* 2000); the sensitivity of the assay was 0.1 ng/ml . Insulin, GH, and IGF-I concentrations were determined as previously described in ruminants (Van Kessel *et al.* 1990). Purified recombinant bovine insulin (Lilly Research Laboratories, Indianapolis, IN, USA) was used for the preparation of standards and for tracer labeling. Insulin concentrations were determined using guinea pig bovine-insulin antiserum (Rutter & Manns 1987); the sensitivity of the assay was 0.1 ng/ml . Purified recombinant bovine GH AFP-11182B (National Hormone & Pituitary Program (NHPP); Torrance, CA, USA) was used for the preparation of standards and for tracer labeling. GH concentrations were determined using monkey bovine-GH antiserum (Anti-bGH AFPB55; NHPP); the sensitivity of the assay was 0.05 ng/ml . Purified recombinant human IGF-I (Ciba Geigy Animal Health, St Aubin, Switzerland) was used for the preparation of standards and for tracer labeling. IGF-I concentrations were determined using mouse human-IGF-I MAB (Kerr *et al.* 1990). Samples were submitted to acid-ethanol (87.5% ethanol, 12.5% 2 mol HCl; v/v) extraction prior to analysis; the sensitivity of the assay was 1.0 ng/ml .

Concentrations of gonadotropins and testosterone were determined as previously described in bulls (Evans *et al.* 1995). LH NIH-bLH-B4 (NHPP) was used for the preparation of standards and USDA-bLH-I-1 (NHPP) was used for tracer labeling. LH concentrations were determined using rabbit bovine-LH antiserum (Rawlings *et al.* 1984); the sensitivity of the assay was 0.05 ng/ml . FSH USDA-bFSH-I-1 (NHPP) was used for the preparation of standards and AFP-5332B (NHPP) was used for tracer

labeling. FSH concentrations were determined using rabbit ovine-FSH antiserum (NIDDK-anti-oFSH-1; NHPP); the sensitivity of the assay was 0.1 ng/ml . Testosterone concentrations were determined using a total testosterone RIA kit (Diagnostic Products Corporation, Los Angeles, CA, USA), but testosterone standards were prepared with purified hormone (Sigma Chemical Co.); the sensitivity of the assay was 0.04 ng/ml .

Statistical analyses

Characteristics of LH secretion were determined with the PC-pulsar software (J Gitzen and V Ramirez, University of Illinois, Chicago, IL, USA) and included pulse frequency and amplitude, total secretion (area under the curve), and basal, mean, and peak concentrations. Mean concentration and total secretion of LH, FSH, and testosterone after GnRH challenge were also evaluated. Statistical analyses were conducted using Statistical Analysis System (SAS Institute; Cary, NC, USA) and Statistix (Analytical Software; Tallahassee, FL, USA). Analysis was divided into two periods; period 1 was the period of feed restriction (10–26 weeks of age) and period 2 was the period after feed restriction (30–66 weeks of age). Mixed model analysis with Tukey's test was used to determine and locate the effects of nutrition (restricted and control during period 1; control/control, restricted/control, and restricted/high during period 2), age, and the nutrition-by-age interaction on body weight, backfat, SC, paired testes volume, and serum hormone concentrations. The covariate structure that best fitted each end point was used (Littell *et al.* 1998). One-way ANOVA (and an LSD test) was used to detect and locate the effects of nutrition on age at puberty and paired testes weight.

Results

Body weight at 10 weeks of age was not different between restricted and control groups ($108.0 \pm 2.3\text{ kg}$; mean $\pm$ S.E.M.). During period 1, there were effects of nutrition, age, and a nutrition-by-age interaction ($P < 0.05$) on body weight, SC, and paired testes volume. There were also effects of nutrition and age on backfat ($P < 0.001$; Fig. 1). Body weight, backfat, SC, and paired testes volume increased continuously with age in all groups; overall, these were lower in bulls in the restricted feed group than in the control group. Although there were significant nutrition-by-age interactions, there were no significant differences between groups within age in body weight and SC. There were effects of age and nutrition-by-age interactions ($P < 0.05$) on body weight, backfat, SC, and paired testes volume during period 2 (Fig. 1). Body weight, SC, and paired testes volume increased continuously with age in all groups, whereas backfat increased until 58, 62, and 66 weeks of age in control/control, restricted/control, and restricted/high

Figure 1 Mean ($\pm$ s.e.m.) body weight, backfat, scrotal circumference (SC), and paired testes volume (PTV) in bulls receiving control nutrition or restricted feed ($n=8$ /group) from 10 to 26 weeks of age (period 1) and receiving control or high nutrition (control/control, restricted/high, and restricted/control; $n=8$ /group) after the period of feed restriction (period 2). Body weight, SC, and paired testes volume increased ($P<0.05$) continuously with age. N, nutrition effect; A, age effect; N*A, nutrition-by-age interaction effect. *Overall changes ($P<0.05$) with age. ^{a,b}Differences ($P<0.05$) between groups within age. *Changes ($P<0.05$) with age within group.

nutrition groups respectively; there were no significant differences between groups within age.

There were age effects ($P<0.0001$) on serum leptin and insulin concentrations, and effects of nutrition, age, and a nutrition-by-age interaction ($P<0.01$) on IGF-I concentrations during period 1 (Fig. 2). Serum leptin and insulin concentrations increased ($P<0.05$) after 18 and 26 weeks of age respectively. Serum IGF-I concentrations remained unchanged in bulls in the restricted feed group and IGF-I concentrations were lower ($P<0.05$) in bulls in this group than in those in the control group after 18 weeks of age. There was also an age effect ($P<0.01$) on serum GH concentrations during period 1; concentrations decreased from ~ 10 ng/ml between 14 and 22 weeks, to ~ 7 ng/ml at 26 weeks of age (data not shown). There were age and nutrition by

age effects ($P<0.01$) on serum leptin, insulin, and IGF-I concentrations, and nutrition effects on insulin and IGF-I concentrations during period 2 (Fig. 2). Leptin concentrations increased in the restricted/high and control/control nutrition groups, but did not change significantly in the restricted/control nutrition group. Bulls in the restricted/high nutrition group had a more sustained increase in insulin concentrations than bulls in the other two groups. In the restricted/control and restricted/high nutrition groups, serum IGF-I concentrations increased until 54 weeks of age and decreased thereafter. Overall, bulls in the restricted/high nutrition group had greater ($P\leq 0.07$) insulin concentrations than those in the other two groups, and greater ($P<0.05$) IGF-I concentrations than those in the restricted/control nutrition group. Serum GH concentrations were not

Figure 2 Mean ($\pm$ S.E.M.) serum leptin, insulin, and IGF-I concentrations in bulls receiving control nutrition or restricted feed ($n=8$ /group) from 10 to 26 weeks of age (period 1) and receiving control or high nutrition (control/control, restricted/high, and restricted/control; $n=8$ /group) after the period of feed restriction (period 2). N, nutrition effect; A, age effect; N*A, nutrition-by-age interaction effect. *Overall changes ($P<0.05$) with age. ^{a,b}Differences ($P<0.05$) between groups within age. *Changes ($P<0.05$) with age within group.

affected by nutrition or age after period 1 (concentrations from 30 to 34 weeks of age were ~ 8 ng/ml; data not shown).

There were nutrition effects ($P\leq 0.07$) on LH pulse frequency, peak concentration, and total secretion, and age effects ($P<0.005$) on LH pulse frequency and total secretion during period 1 (Fig. 3). Overall, LH pulse frequency, peak concentration, and total secretion were lower in bulls in the restricted feed group than in those in the control group and LH pulse frequency and total secretion decreased ($P<0.05$) after 22 weeks of age. There was an age effect ($P<0.01$) on LH total secretion during period 2 (Fig. 3); total secretion increased from 30 to 34 weeks of age. Neither nutrition nor age affected LH basal concentrations or LH pulse amplitude during periods 1 and 2 (data not shown).

There were nutrition effects ($P<0.05$) on mean serum LH and testosterone concentrations and age effects ($P<0.005$) on mean serum LH, FSH, and testosterone concentrations during period 1 (Fig. 4). Overall, LH and testosterone concentrations were lower ($P<0.05$) in bulls in the restricted feed group than in bulls in the control

group. Gonadotropin concentrations decreased ($P<0.05$) after 18 weeks of age, whereas testosterone concentrations increased ($P<0.05$) after this age. There were age effects ($P<0.0005$) on serum LH, FSH, and testosterone concentrations, and a nutrition-by-age interaction ($P<0.0001$) on LH concentrations during period 2 (Fig. 4). Serum LH concentrations increased ($P<0.05$) at 50 and 66 weeks of age only in bulls in the restricted/high nutrition group. Overall, FSH concentrations increased ($P<0.05$) after 54 weeks and testosterone concentrations increased ($P<0.05$) after 38 weeks of age.

There were nutrition and age effects ($P<0.01$) on mean LH concentrations and total secretion during period 1 (Fig. 5). Both GnRH-induced mean LH concentrations and total LH secretion were lower ($P<0.01$) in bulls in the restricted feed group than in those in the control group. Mean LH concentrations and total secretion increased ($P<0.05$) at 18 weeks of age. Neither nutrition nor age affected GnRH-induced LH secretion during period 2 (Fig. 5). There were age effects ($P<0.0001$) on mean FSH concentrations and total secretion after GnRH treatment during period 1 (both decreased after 18 weeks of age), but no nutrition or age effect during period 2 (data not shown).

Figure 3 Mean ($\pm$ S.E.M.) number of LH pulses, serum peak concentration, and total secretion in 10 h in bulls receiving control nutrition or restricted feed ($n=8$ /group) from 10 to 26 weeks of age (period 1) and receiving control or high nutrition (control/control, restricted/high, and restricted/control; $n=8$ /group) after the period of feed restriction (period 2). N, nutrition effect; A, age effect. *Overall changes ($P<0.05$) with age.

There were nutrition and age effects ($P<0.05$) on mean testosterone concentrations and total secretion after GnRH treatment during period 1 (Fig. 6). Overall, GnRH-induced mean testosterone concentrations and total secretion increased ($P<0.05$) with age and was lower ($P<0.05$) in bulls in the restricted feed group than in those in the control group. There was an effect of age and a nutrition-by-age interaction ($P<0.005$) on mean testosterone concentrations and total secretion after GnRH treatment during period 2 (Fig. 6). GnRH-induced mean testosterone concentrations and total secretion increased ($P<0.05$) from 30 to 34 weeks of age only in bulls in the restricted/control group.

Bulls in the control/control nutrition group were younger ($P<0.05$) at puberty and had greater ($P<0.05$) paired testes weight at 70 weeks of age than those in the restricted/control nutrition group (292.6 ± 10.6 vs 330.5 ± 9.0 days and 600.0 ± 12.1 vs 528.3 ± 26.2 g respectively; mean $\pm$ S.E.M.). Age at puberty and paired testes weight for bulls in the restricted/high nutrition

group were intermediate (312.7 ± 12.5 days and 552.9 ± 24.9 g respectively).

Discussion

Bulls that received restricted feed during calfhood were older at puberty and had lighter testes at 70 weeks of age. Therefore, restricted feed during calfhood adversely affected sexual development and these effects were not compensated by nutritional supplementation during the peripubertal period. The adverse effects of restricted feed on sexual development were exerted on all levels of the hypothalamus–pituitary–gonad axis and may have been mediated by IGF-I.

Secretion of LH was reduced during the period of the early gonadotropin rise during calfhood in bulls receiving restricted feed. Puberty was delayed in these bulls, despite the lack of major differences in metabolic hormones, gonadotropins, or testosterone concentrations after diets were changed in the peripubertal

Figure 4 Mean ($\pm$ S.E.M.) serum LH, FSH, and testosterone concentrations in bulls receiving control nutrition or restricted feed ($n=8$ /group) from 10 to 26 weeks of age (period 1) and receiving control or high nutrition (control/control, restricted/high, and restricted/control; $n=8$ /group) after the period of feed restriction (period 2). N, nutrition effect; A, age effect; N*A, nutrition-by-age interaction effect. *Overall changes ($P < 0.05$) with age. *Changes ($P < 0.05$) with age within group.

period. This observation supported the hypothesis that the pattern of LH secretion during the early gonadotropin rise during calthood is the main determinant of age of puberty in bulls. In that regard, late-maturing bulls had lower LH secretion than early maturing bulls during the early gonadotropin rise (Evans *et al.* 1995, Aravindakshan *et al.* 2000). Moreover, bulls receiving a low-nutrition diet had reduced LH pulse frequency during the early gonadotropin rise and were older at puberty than those receiving medium or high nutrition (Brito *et al.* 2007a).

Although differences in LH pulse frequency observed between control and restricted feed groups during calthood were not significant at $P < 0.05$ level, an important consideration is that intensive samplings were performed for only 10 h due to practical constraints. It seems reasonable to assume that differences in LH secretion would only increase had samplings been performed for longer periods. Therefore, the tendency ($P = 0.07$) of greater LH pulse frequency in the control group should be considered of biological significance. Lower LH pulse frequency in bulls receiving restricted feed indicated that nutrition affected the GnRH pulse generator in the hypothalamus during the period of

the early gonadotropin rise. The GnRH pulse generator is the primary regulator of reproductive function and is the primary locus of inhibition by restricted nutrition in males and females (Schneider 2004).

Metabolic signals, hormonal mediators and modulators, and neuropeptides are involved in an intricate mechanism by which circulating (peripheral) signals are translated within the brain and ultimately regulate the function of GnRH neurons (Blache *et al.* 2003, Schneider 2004). Although temporal associations do not necessarily reflect cause-and-effect relationships, decreased IGF-I concentrations during calthood in bulls receiving restricted feed suggested that IGF-I may serve as a signal translating nutritional status to the hypothalamus, since the differences in IGF-I concentrations during calthood coincided with differences in LH pulse secretion. In support of this possible regulatory role, IGF-I receptors were present in the brain in rodents and in immortalized GnRH-secreting neurons (GTI-7 cell line), GnRH neurons expressed IGF-I receptors, and IGF-I stimulated GnRH secretion from GTI-7 cells and female rat brain *in vitro* (Hiney *et al.* 1991, Anderson *et al.* 1999, Miller & Gore 2001, Daftary & Gore 2004). Control of GnRH secretion by IGF-I may also involve reducing

Figure 5 Mean ($\pm$ S.E.M.) serum LH concentration and total secretion after GnRH challenge (0.04 μ g/kg, i.v.) in bulls receiving control nutrition or restricted feed ($n=8$ /group) from 10 to 26 weeks of age (period 1) and control or high nutrition (control/control, restricted/high, and restricted/control; $n=8$ /group) after the period of feed restriction (period 2). N, nutrition effect; A, age effect. *Overall changes ($P<0.05$) with age.

hypothalamic hypersensitivity to negative steroid feedback during pubertal development (Wilson 1995). Moreover, in previous studies, LH secretion was also reduced concomitantly with low serum IGF-I concentrations in bulls receiving low nutrition during calfhood, whereas LH secretion was augmented in bulls receiving high nutrition concomitantly with high serum IGF-I concentrations (Brito *et al.* 2007a, 2007b).

The suppression of LH secretion in the present study was more pronounced than in a previous study conducted to evaluate the effects of diet on gonadotropins secretion in young bulls (Brito *et al.* 2007a). In that study, low nutrition during calfhood resulted in reduced LH pulse frequency, but mean and peak LH concentrations and total LH secretion were not affected as in the present study. Moreover, in contrast to the previous study, GnRH-stimulated LH secretion was reduced in bulls receiving restricted feed in the present study, indicating that restricted feed also affected pituitary function. One explanation for the apparent contrast between these studies is the difference in experimental design. In the previous study, the group of bulls receiving low nutrition received a diet of plain forage (no concentrate), but intake was not restricted in contrast with the present experiment (intake was limited in the restricted feed group). Therefore, we inferred that LH secretion is regulated by not only the availability of nutrients but also by the central center responsible for the sensations of hunger and satiety. The inhibitory

effects of limited availability of nutrients on LH secretion were exerted only on the hypothalamus (Brito *et al.* 2007a). In contrast, the combination of limited availability of nutrients with the hunger sensation experienced by bulls with restricted intake in the present study affected both hypothalamic and pituitary function, producing much more severe inhibition of LH secretion. IGF-I receptors were detected in the pituitary and IGF-I stimulates gonadotropin secretion from rat pituitary *in vitro* (Rosenfeld *et al.* 1984, Soldani *et al.* 1995), indicating a possible regulatory role for IGF-I on pituitary function. However, circulating IGF-I concentrations during calfhood in the present study were not substantially different from that observed in the previous study in which low nutrition did not affect pituitary function (Brito *et al.* 2007a).

Restricted feed did not significantly reduce serum concentrations of leptin, insulin, and GH. The large differences in LH secretion without differences in concentrations of these metabolic hormones between restricted feed and control groups in the present study supported the notion that these hormones have only a permissive effect on gonadotropin secretion. Feed restriction in ewes decreased LH secretion concomitantly with decreased leptin and insulin concentrations, but leptin and insulin concentrations returned to control values at the end of the feed restriction period without any rebound in LH secretion (Recabarren *et al.* 2004). Moreover, differences in leptin, insulin, and GH

Figure 6 Mean ($\pm$ S.E.M.) serum testosterone concentrations and total secretion after GnRH challenge ($0.04 \mu\text{g/kg}$, i.v.) in bulls receiving control nutrition or restricted feed ($n=8/\text{group}$) from 10 to 26 weeks of age (period 1) and receiving control or high nutrition (control/control, restricted/high, and restricted/control; $n=8/\text{group}$) after the period of feed restriction (period 2). N, nutrition effect; A, age effect; N*A, nutrition-by-age interaction effect. *Overall changes ($P<0.05$) with age. *Changes ($P<0.05$) with age within group.

concentrations were not associated with differences in LH secretion in young bulls receiving either low or high nutrition during calfhood (Brito *et al.* 2007a, 2007b). The results of the present experiment indicated that feed intake restricted to 75% of the normal *ad libitum* intake resulted in circulating leptin, insulin, and GH concentrations that were not involved in altering the early gonadotropin rise in young bulls. Therefore, reduced gonadotropin secretion due to reduced concentrations of these metabolic hormones must result from severe undernutrition. Alternatively, short-term mechanisms involving leptin and insulin may also be important, as a drastic decrease in circulating concentrations of these hormones promoted by fasting also inhibited LH secretion in cattle (Amstalden *et al.* 2000, 2002).

Restricted feed during calfhood also reduced both physiological and GnRH-stimulated testosterone secretion. Therefore, nutrition also had direct effects on Leydig cell number or function (or both). These effects could be mediated by IGF-I, since receptors have been identified in Leydig cells in several species and IGF-I increased proliferation and differentiation of Leydig cells (Spiteri-Grech & Nieschlag 1992, Lin 1995). Leydig cell numbers and expression of steroidogenic enzymes were decreased in IGF-I-null mice and IGF-I treatments in combination with LH were necessary for normal Leydig cell development in these animals (Wang & Hardy 2004).

In vitro, IGF-I up-regulated Leydig cell LH receptors and increased both basal and LH-stimulated testosterone secretion (Spiteri-Grech & Nieschlag 1992, Lin 1995). Leydig cells also produced IGF-I, indicating the existence of a paracrine/autocrine mechanism of testicular regulation involving the IGF-I system (Rouiller-Fabre *et al.* 1998). Interestingly, IGF-I stimulated testosterone secretion and testosterone in turn up-regulated IGF-I receptors and IGF-I production by Leydig cells (Cailleau *et al.* 1990). Therefore, circulating IGF-I may have initially up-regulated testosterone production, which in turn stimulated IGF-I secretion and established a positive feedback loop between IGF-I and testosterone secretion in Leydig cells in peripubertal bulls. In that regard, IGF-I treatment increased gonadal sensitivity to gonadotropins and hastened puberty in female monkeys (Wilson 1998).

In rams, improved nutrition had positive effects on testicular growth that were maintained beyond the period of the gonadotropins rise promoted by improved nutrition (Blache *et al.* 2002). In bulls, testicular growth rate was maximal during the peripubertal period (6–16 months of age), coincident with decreasing gonadotropin concentrations (Rawlings *et al.* 1978, Amann & Walker 1983). These observations indicated the existence of gonadotropin-independent mechanisms regulating testicular development in male ruminants. Circulating metabolic hormones may be involved in regulating testicular growth

during the peripubertal period in bulls (Brito *et al.* 2007a, 2007c). However, although circulating concentrations of leptin, insulin, IGF-I, and testosterone after improved nutrition during the peripubertal period in bulls that had received restricted feed were either similar or greater than in control bulls, testes weight at 70 weeks of age was still less in bulls that had received restricted feed. These observations supported the hypothesis that gonadotropin-independent mechanisms of testicular development which may involve metabolic hormones are dependent on previous exposure to gonadotropins. The pattern of LH secretion during calthood may 'prime' testicular development and dictate maximum adult testicular size in bulls. Accordingly, suppressing LH secretion with prolonged GnRH treatment during calthood reduced paired testes weight at 50 weeks of age (Chandolia *et al.* 1997a). Moreover, high nutrition during calthood resulted in either greater LH pulse frequency or a more sustained increase in pulse frequency during the early gonadotropin rise with greater testes weight at 16 months of age when compared with control nutrition, in the absence of differences in metabolic hormone concentrations during the peripubertal period in bulls (Brito *et al.* 2007a, 2007b).

In conclusion, restricted feed during calthood affected sexual development in bulls as a result of adverse effects on every level of the hypothalamus–pituitary–gonad axis. Restricted feed inhibited the hypothalamic GnRH pulse generator, reduced the pituitary response to GnRH, and adversely affected testicular steroidogenesis. Temporal associations indicated that the effects of restricted feed on the HPG axis might be mediated by circulating IGF-I concentrations. Bulls receiving restricted feed had lower LH secretion during calthood, delayed puberty, and lessened testicular weight at 16 months of age, independent of the increased circulating concentrations of metabolic hormones and testosterone after improved nutrition during the peripubertal period. These observations supported the hypotheses that the pattern of LH secretion during the early gonadotropin rise during calthood is the main determinant of age of puberty in bulls and that gonadotropin-independent mechanisms involved in testicular growth during the peripubertal period are affected by previous LH exposure.

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