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Plane of nutrition before and after 6 months of age in Holstein-Friesian bulls: II. Effects on metabolic and reproductive endocrinology and identification of physiological markers of puberty and sexual maturation

C. J. Byrne,*† S. Fair,‡ A. M. English,*‡ C. Urh,§ H. Sauerwein,§ M. A. Crowe,# P. Lonergan,† and D. A. Kenny*†[1]

*Animal and Bioscience Department, Teagasc, Dunsany, Co. Meath, C15 PW93, Ireland
†School of Agriculture and Food Science, University College Dublin, Belfield, Dublin, D04 F6X4, Ireland
‡Laboratory of Animal Reproduction, Department of Biological Sciences, University of Limerick, Limerick, V94 T9PX, Ireland
§Institute for Animal Science, Physiology and Hygiene Unit, University of Bonn, 53115 Bonn, Germany
#School of Veterinary Medicine, University College Dublin, Belfield, Dublin, D04 F6X4, Ireland

ABSTRACT

The aim of this study was (1) to examine the effect of plane of nutrition during the first and second 6 mo of life on systemic concentrations of reproductive hormones and metabolites in Holstein-Friesian dairy bulls, and (2) to establish relationships with age at puberty and postpubertal semen production potential. Holstein-Friesian bull calves (n = 83) with a mean (standard deviation) age and body weight of 17 (4.4) d and 52 (6.2) kg, respectively, were assigned to a high or low plane of nutrition for the first 6 mo of life. At 24 wk of age, bulls were reassigned, within treatment, either to remain on the same diet or to switch to the opposite diet until puberty, resulting in 4 treatment groups: high-high, high-low, low-low, and low-high. Monthly blood samples were analyzed for metabolites (albumin, urea, total protein, β-hydroxybutyrate, glucose, non-esterified fatty acid, triglycerides and creatinine), insulin, insulin-like growth factor-1, leptin, adiponectin, FSH, and testosterone. A GnRH challenge was carried out at 16 and 32 wk of age (n = 9 bulls per treatment). Blood was collected at 15-min intervals for 165 min, with GnRH administered (0.05 mg/kg of body weight, i.v.) immediately after the third blood sample. Blood samples were subsequently analyzed for LH, FSH, and testosterone. Stepwise regression was used to detect growth and blood measurements to identify putative predictors of age at puberty and subsequent semen quality traits. Metabolic hormones and metabolites, in general, reflected metabolic status of bulls. Although FSH was unaffected by diet, it decreased with age both in monthly samples and following GnRH administration. Testosterone was greater in bulls on the high diet before and after 6 mo of age. Testosterone concentrations increased dramatically after 6 mo of age. Luteinizing hormone was unaffected by diet following GnRH administration but basal serum LH was greater in bulls on a high diet before 6 mo of age. In conclusion, the plane of nutrition offered before 6 mo of age influenced metabolic profiles, which are important for promoting GnRH pulsatility, in young bulls.

Key words: gonadotropin, follicle-stimulating hormone, luteinizing hormone, testosterone

INTRODUCTION

Based on the published literature, the average age at puberty across *Bos taurus* beef and dairy-bred bulls is approximately 320 d, ranging between 287 and 369 d of age (Brito et al., 2007b; Dance et al., 2015; Harstine et al., 2015). The onset of puberty relies on the initiation of the GnRH pulse generator, which signals the anterior pituitary to secrete FSH and LH (Duittoz et al., 2016). These hormones are also important for Leydig and Sertoli cell proliferation and differentiation in the testes. The timing and intensity of the early transient LH rise, which typically occurs from approximately 8 to 20 wk of age, is an important determinant of age at puberty in the bull (Evans et al., 1995).

Secretion of GnRH is regulated by metabolic hormones in response to the metabolic status of the animal (Schneider, 2004; Clarke and Arbabi, 2016). Metabolites such as glucose have been shown to play an important role within the arcuate nucleus region of the hypothalamus by exciting pro-opiomelanocortin neurons and inhibiting appetite-promoting neuropeptide-Y, thereby promoting GnRH secretion (Burdakov et al., 2005). Insulin-like growth factor-1 and insulin are important for signaling metabolic status to the hypothalamus, and

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[1]Corresponding author: david.kenny@teagasc.ie

an increase in LH secretion between 12 and 18 wk of age has been reported in bulls, associated with a concomitant increase in IGF-1 (Brito et al., 2007a; Dance et al., 2015). Although neither of the aforementioned studies reported an effect of nutrition on systemic leptin before 31 wk of age, kisspeptin, a mediator of metabolic status, has neurons that are anatomically linked to pro-opiomelanocortin and neuropeptide-Y neurons (De Bond and Smith, 2014). These kisspeptin neurons have been shown to have leptin receptors, suggesting that kisspeptin may mediate metabolic signaling to the hypothalamus (Sanchez-Garrido and Tena-Sempere, 2013). Pulsatility of LH is not detected in bull calves before 8 wk of age (Rodriguez and Wise, 1989); thus, if appreciable increases are to be attained in LH secretion, nutritional programming should be commenced before and continued beyond 8 wk of age.

In a companion study (Byrne et al., 2018), we reported that age at puberty in Holstein-Friesian bulls is regulated by diet pre-6 mo of age, irrespective of the diet post-6 mo of age. Although characteristic changes in gonadotropins (LH and FSH) in response to age and diet have been reported in prepubertal dairy bulls (Dance et al., 2015), prepubertal metabolites have not been characterized in the same way. In prepubertal Holstein-Friesian bulls, the metabolic and endocrinological response to an increase or decrease in plane of nutrition in later calfhood remains unclear. Characterization of how nutrition and nutritional changes in the prepubertal period affect age at puberty and subsequent semen production in bulls is necessary.

We hypothesized that a high plane of nutrition before 6 mo of age would increase blood concentrations of gonadotropins and metabolites consistent with an improved metabolic status, thus promoting earlier sexual and testicular development. The specific objective of the study was to examine the effect of growth rate (consistent vs. interrupted) on key reproductive and metabolic hormones that regulate reproductive and sexual development and to identify physiological markers indicative of age at puberty and subsequent sexual maturation and semen quality in Holstein-Friesian bulls.

MATERIALS AND METHODS

All animal procedures performed were conducted under experimental license from the Irish Department of Health and Children (license number B100/4516). Protocols were developed in accordance with the *Cruelty to Animals Act* (Ireland 1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC.

Animals and Management

The experimental design and animals used in the current study have been described previously (Byrne et al., 2018). Briefly, autumn-born Holstein-Friesian bull calves (n = 83) with a mean (±SD) age and BW of 17 (4.4) d and 52 (6.2) kg, respectively, were blocked by age, BW, sire, and farm of origin and assigned to a high (**HPN**) or low (**LPN**) plane of nutrition for the first 6 mo of life. Bulls assigned to HPN (n = 37) and LPN (n = 46) received 1,200 and 450 g of milk replacer, respectively. Bulls on HPN were fed concentrate ad libitum, whereas LPN bulls received a maximum of 1 kg of concentrates daily. All bulls were offered hay as a source of roughage and had ad libitum access to water. Bulls were individually fed using an electronic feeding system (Vario, Forster-Technik, Engen, Germany) before weaning and thereafter were penned according to treatment until turnout to pasture at 24 wk of age. Bulls were rotationally grazed in their respective treatment groups until the onset of puberty, at which time they were rehoused in slatted-floor pens. Bulls were offered these diets for a minimum of 56 d and weaned once they were consuming 1 kg of concentrates for 3 consecutive days. After weaning, HPN bulls were offered ad libitum concentrate and LPN bulls received 1 kg of concentrate daily; both groups were offered hay to appetite. At 24 wk of age, bulls were reassigned, within treatment, either to remain on the same diet or to change to the opposite diet until onset of puberty. This resulted in 4 groups: HPN-HPN (**Hi-Hi**), HPN-LPN (**Hi-Lo**), LPN-LPN (**Lo-Lo**), and LPN-HPN (**Lo-Hi**), with n = 19, 18, 22, and 24 bulls, respectively. Bulls were turned out to pasture at 26 wk of age, where Hi-Hi and Lo-Hi bulls received grass and concentrate ad libitum, and Lo-Lo and Hi-Lo bulls received grass to appetite plus 0.5 kg of concentrate daily. Puberty was deemed to have been reached when an ejaculate containing a minimum of 50×10^6 sperm with >10% progressive linear motility (**PLM**) was collected (Wolf et al., 1965). After puberty, semen was collected monthly. Sexual maturity was deemed to have been reached when a bull produced an ejaculate containing ≥30% PLM and ≥70% normal morphology (Brito et al., 2004). All semen was collected via electro-ejaculation.

Blood Sampling

Monthly blood samples were collected via jugular venipuncture, until puberty was attained. On each occasion, blood was collected into a 9-mL evacuated tube containing lithium heparin (Greiner Vacuette; Cruinn Diagnostics, Dublin, Ireland) and subsequently

analyzed for adiponectin, IGF-1, metabolites [albumin, urea, total protein, BHB, glucose, nonesterified fatty acids (**NEFA**), triglycerides, and creatinine] and leptin. For insulin analysis, blood was collected into a 6-mL K_3-EDTA (Vacuette, Cruinn Diagnostics) tube. Blood was centrifuged at 1,750 × *g* for 15 min, and plasma was collected and stored at −20°C before analysis. Blood samples were also collected into a 9-mL evacuated serum separator tube (Becton Dickinson, Dublin, Ireland) at the same time points. Blood was subsequently allowed to clot overnight and then centrifuged at 800 × *g* for 10 min; serum was harvested and stored at −20°C pending analysis, outlined below.

IGF-1. Concentrations of IGF-1 were determined using a RIA following acid-ethanol extraction, using the method previously described by Beltman et al. (2010). The intra- and interassay coefficients of variation (**CV**) were determined by replicating a low, normal, and high reference sample at the beginning, middle, and end of each assay. Intra- and interassay CV for IGF-1 were 12.5, 6.6, and 5.1% and 13.7, 8.4, and 9.6% for low, medium, and high, respectively. The sensitivity of the assay, defined as the lowest concentration detectable, was 4 ng/mL.

Insulin. Concentrations of insulin (n = 12 bulls per treatment) were determined using insulin immunoradiometric assay (INS-IRMA) kits (DIAsource Immunoassays, Louvain-la-Neuve, Belgium) as previously used by Adrien et al. (2012). Intra- and interassay CV for insulin were 5.4, 4.0, and 4.3% and 4.9, 6.5, and 6.3% for low, medium, and high, respectively. The sensitivity of the assay, defined as the lowest concentration detectable, was 1 ng/mL.

Leptin and Adiponectin. Concentrations of leptin were determined using an enzyme immunoassay, as previously described by Sauerwein et al. (2004). Intra- and interassay CV were 5 and 9%, respectively. Adiponectin concentrations were determined using enzyme immunoassay, as previously described by Mielenz et al. (2013). Intra- and interassay CV were 5 and 10%, respectively. The sensitivity of the leptin and adiponectin assays, defined as the lowest concentration detectable, were 0.6 and 0.03 ng/mL, respectively.

Plasma Metabolites. Concentrations of albumin, urea, total protein, BHB, glucose, NEFA, triglycerides, and creatinine were determined as described in Lawrence et al. (2011). All metabolite concentrations were measured on an automatic analyzer (AU 400; Olympus, Tokyo, Japan). The interassay CV for low, medium, and high were <10% for all metabolites. The sensitivities of the assays, defined as the lowest concentration detectable, were as follows: glucose: 0.02 mmol/L, urea 0.9: mmol/L, BHB: 0.1 mmol/L, NEFA: 0.072 mmol/L, triglycerides: 0.004 mmol/L, total protein: 0.8 g/L, albumin: 0.1 g/L, creatinine: 2.3 μmol/L. Globulin concentration was calculated as the difference between total protein and albumin concentrations.

FSH, LH, and Testosterone. Monthly serum concentrations of FSH (n = 10 bulls per treatment) were determined using the method of Crowe et al. (1997). The sensitivity of the assay was 0.05 ng/mL. Intra- and interassay CV were 8.7, 7.2, and 9.3% and 15.8, 19.7, and 9.5% for low, medium, and high, respectively. Monthly serum concentrations of testosterone (**TT**; n = 16 bulls per treatment) were determined using solid-phase RIA kits (DIAsource Immunoassays) as previously described by Collodel et al. (2014). The sensitivity of the assay was 0.1 ng/mL. Intra- and interassay CV were 12.1, 10.0, and 9.0% and 12.5, 8.2, and 9.5% for low, medium, and high, respectively. Monthly serum concentrations of LH (n = 16 bulls per treatment) were determined using the method of Cooke et al. (1997), modified so that the separation step (second antibody) used a polyethylene glycol (PEG) method. Briefly, after assay incubation with primary antibody, 100 μL of 1% normal mouse serum in assay buffer was added to assay tubes. This was followed by 1 mL of goat-anti-mouse antibody (Equitech-Bio Inc., Kerrville, TX) diluted 1:100 in 5% PEG. Assay tubes were incubated for 1 h at room temperature and centrifuged for 20 min at 1,600 × *g*; then, the free fraction was separated by decanting the supernatant. The sensitivity of the assay was 0.05 ng/mL. Intra- and interassay CV were 9.1, 18.2, and 7.2% and 2.5, 4.6, and 2.4% for low, medium, and high, respectively.

GnRH Challenge

To quantitatively characterize anterior pituitary function, a GnRH challenge was carried out at 16 and 32 wk of age (n = 9 bulls per treatment). Intensive blood sampling (every 15 min for 165 min) was conducted. A GnRH challenge was chosen instead of a longer, non-stimulated, diurnal-intensive blood sampling regimen, based on the results of Brito et al. (2007a). In that study, similar results were reported between the longer intensive blood sampling and the shorter GnRH challenge. Intravenous catheters were inserted 12 h before sampling commenced. At each challenge, a GnRH agonist (Buserelin Receptal; Intervet Ireland Ltd., Dublin, Ireland) was administered (0.05 mg/kg of BW, i.v.) immediately after the third blood sample was taken. Blood samples (n = 12 per animal) were collected at 15-min intervals for gonadotropins (LH, FSH) and TT. Blood was processed as described above to obtain serum. Concentrations of FSH and TT were analyzed using RIA as

described above. Intra- and interassay CV for FSH and TT were as outlined above. Concentrations of LH were determined using the method of Cooke et al. (1997). The sensitivity of the assay was 0.05 ng/mL. Intra- and interassay CV for LH were 12.1, 7.1, and 8.0% and 9.0, 11.1, and 9.5% for low, medium, and high, respectively.

Statistical Analysis

Area under the curve for FSH, LH, and TT between 0 and 135 min relative to GnRH administration was determined using Sigma Plot (version 11, Systat Software, San Jose, CA). Basal serum concentration (mean of concentrations at 30 and 15 min before GnRH administration) for each animal was included as a covariate in the statistical analysis. Both monthly and GnRH challenge data were analyzed using appropriate procedures of SAS software (version 9.3, SAS Institute Inc., Cary, NC). Data were tested for normality of distribution (UNIVARIATE procedure) and, where appropriate, transformed to the power of lambda (TRANSREG procedure). Data were then subjected to a repeated-measures ANOVA (MIXED procedure). Differences in individual least squares means were evaluated using the Tukey-Kramer adjustment. Diet pre- and post-6 mo of age, block, age, and their interactions were included in the model. If not statistically significant ($P > 0.05$), the interaction term was subsequently excluded from the final model. Multiple regression analysis was used (REG and STEPWISE procedure, SAS version 9.3) to identify statistically significant predictor variables for age at puberty, age at sexual maturation, scrotal circumference (**SC**) at puberty, average postpubertal sperm output, PLM, and semen volume (Byrne et al., 2018). Average daily gain measurements, concentrations of reproductive and metabolic hormones and metabolites, as well as physical and ultrasound measurements were used as independent variables. Data on growth-related traits and systemic concentrations of reproductive and metabolic hormones and metabolites were used as independent variables. All results are presented as mean ± standard errors of the mean, unless stated otherwise. All potential explanatory variables were initially tested using Pearson correlation analysis. Groups or pairs of variables with a correlation coefficient >0.40 were tested against each other in a regression model. For correlated variables, the variable contributing most to the model coefficient of determination were included in the final regression models. The fixed effect of dietary treatment was also included in the model. An α-level of <0.15 was set as the criterion for retention of a particular variable in the multivariate regression model.

RESULTS

The effect of treatment on plasma concentrations of metabolites is presented in Figure 1 and Supplemental Figure S1 (https://doi.org/10.3168/jds.2017-13720). We detected a pre- × post-6 mo diet interaction for plasma albumin ($P < 0.05$), such that the Hi-Hi bulls had greater albumin than Hi-Lo and Lo-Lo bulls ($P < 0.01$) and tended to have greater albumin than Lo-Hi bulls ($P = 0.07$). We also observed a post-6 mo diet × age interaction for albumin ($P < 0.01$). Albumin was unaffected by diet offered during the post-6 mo period until 30 wk of age when Hi had greater albumin than Lo bulls; the difference remained at 34 and 38 wk of age but there was no difference between diets at 42 wk of age. Diet offered before 6 mo had no effect on albumin. A pre-6 mo diet × age interaction for BHB was detected ($P < 0.001$). At 8, 12, and 16 wk of age, bulls on Lo had greater BHB than bulls on Hi, although no differences were detected at subsequent samplings. We detected a tendency for a pre- × post-6 mo diet interaction for creatinine ($P = 0.08$). Bulls on the Lo-Hi diet had greater creatinine concentrations than bulls on either Hi-Hi ($P < 0.01$; Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13720) or Hi-Lo ($P < 0.05$) and tended to be greater than those on the Lo-Lo diet ($P = 0.07$). We also observed a pre-6 mo diet × age interaction for creatinine ($P < 0.001$). Creatinine was greater at 12, 30, 34, and 38 wk of age in bulls on a Hi versus a Lo diet before 6 mo of age ($P < 0.05$).

We detected no interactions among the main effects for globulin. Globulin tended to be greater in bulls offered a Hi diet post-6 mo ($P = 0.06$). Age had a quadratic effect on globulin ($P < 0.001$), increasing linearly up to 16 wk of age and then plateauing for the remainder of the feeding period. There was a pre-6 mo diet × age interaction for glucose ($P < 0.001$). Bulls on Hi pre-6 mo had greater glucose at 16 and 20 wk of age ($P < 0.001$), with no further differences detected at other sampling points. Bulls on Hi post-6 mo had greater glucose than bulls on Lo ($P < 0.001$). A post-6 mo diet × age interaction was evident for NEFA ($P < 0.001$). Concentrations of NEFA were greater in bulls on a Lo diet post-6 mo at 34 and 42 wk of age. Before 6 mo of age, bulls on Hi had greater NEFA than bulls on Lo ($P < 0.05$).

We detected a pre-6 mo diet × age interaction for total protein ($P < 0.001$); at 30 wk of age, bulls on Hi pre-6 mo had greater total protein than bulls on Lo during the same period. No differences were observed at any other time point. A post-6 mo diet × age interaction was evident, with bulls on Hi post-6 mo having greater total protein at 34 wk of age than bulls on

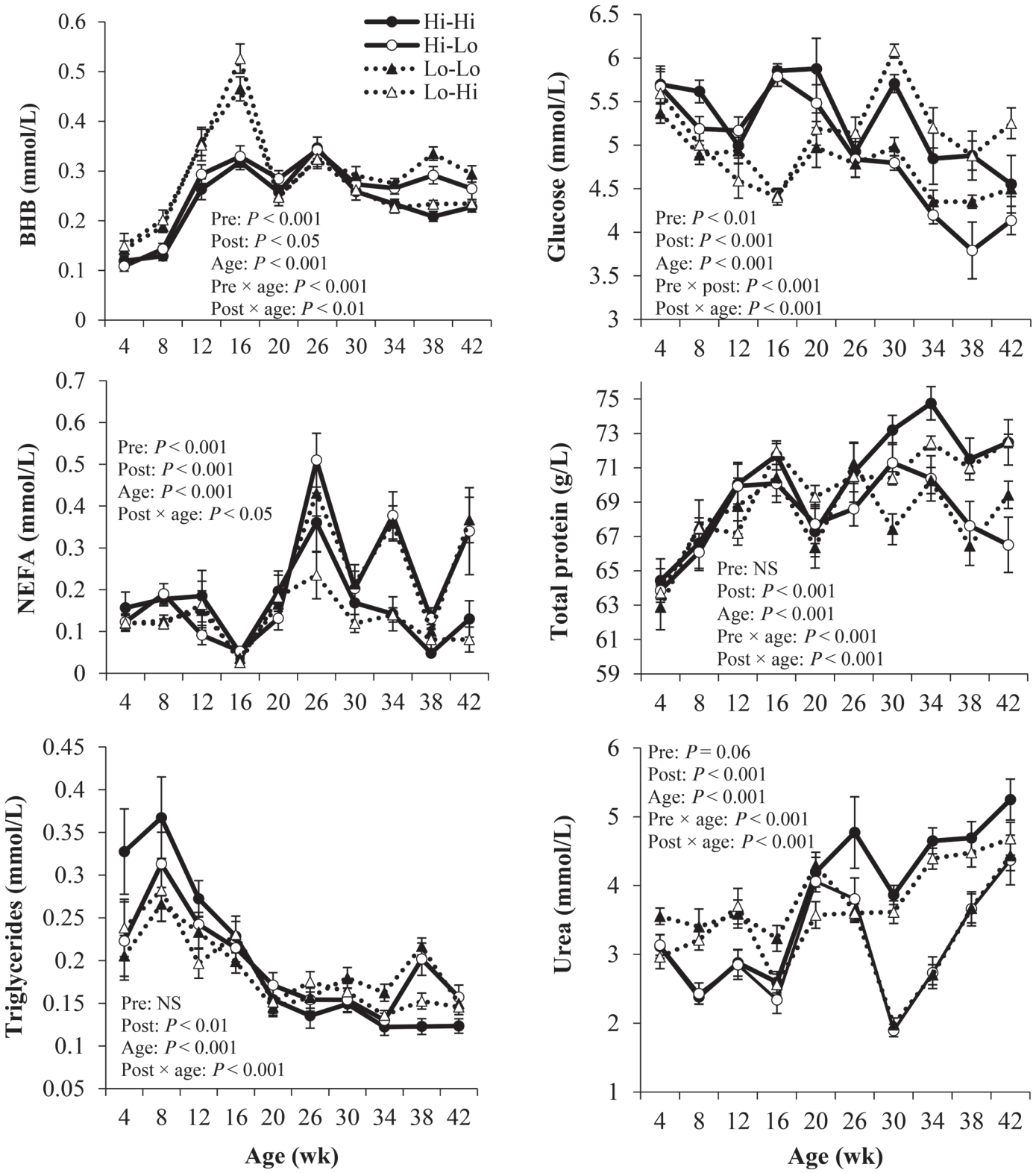

Figure 1. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls on BHB, glucose (upper panel), nonesterified fatty acids (NEFA), total protein (middle panel), triglycerides, urea (bottom panel) taken at monthly intervals (n = Hi-Hi: 19; Hi-Lo: 18; Lo-Lo: 22; Lo-Hi: 24). Values are means ± SEM.

Lo ($P < 0.001$). A post-6 mo diet × age interaction was detected for triglycerides ($P < 0.05$), with greater concentrations at 38 wk of age in bulls on Lo post-6 mo than on Hi. Differences in triglyceride concentrations were not observed at any other time point. A pre-6 mo diet × age interaction for urea ($P < 0.001$) was detected. Bulls on a Lo diet pre-6 mo had greater urea at 8 wk of age compared with bulls on Hi. We also detected a post-6 mo diet × age interaction for urea ($P < 0.001$), manifested as bulls on a Hi diet post-6 mo having greater urea concentrations than bulls on Lo at 30, 34, and 38 wk of age.

There was a pre-6 mo diet × age interaction for IGF-1 ($P < 0.001$; Figure 2), such that bulls on a Hi diet pre-6 mo had greater IGF-1 than bulls on Lo from 12 to 20 wk of age but not at other sampling time points. We detected a post-6 mo diet × age interaction for IGF-1 ($P < 0.001$). A greater concentration of IGF-1 was evident for bulls on Hi post-6 mo of age from 26 to 42 wk of age. Before 26 wk of age, IGF-1 was unaffected by diet during the post-6 mo period. A pre-6 mo diet × age interaction was observed for insulin ($P < 0.001$; Figure 2), which manifested in the same manner as that for IGF-1. There was a post-6 mo diet × age interaction for insulin ($P < 0.001$; Figure 2). Diet post-6 mo had no effect on insulin until 30 wk of age, when concentrations on Hi were greater than on Lo, which persisted for the remainder of the differential feeding period.

We detected a post-6 mo diet × age interaction for leptin ($P < 0.001$; Figure 2). Bulls on Hi post-6 mo had greater leptin at 38 wk of age than bulls on Lo. The diet offered before 6 mo of age did not affect leptin. We observed a tendency toward a pre- × post-6 mo diet interaction for adiponectin ($P = 0.05$; Figure 2): the Hi-Hi diet had lower adiponectin than Lo-Hi but neither was different from Hi-Lo or Lo-Lo. Adiponectin increased between 8 and 20 wk of age ($P < 0.05$), following a decrease at 26 and 30 wk ($P < 0.001$); it increased again at 34 wk ($P < 0.05$).

Monthly serum FSH concentrations were unaffected by diet (Figure 3) but were affected by age ($P < 0.001$). Circulating FSH increased between 4 and 8 wk of age ($P < 0.05$) and remained elevated until 20 wk of age. The concentration of FSH tended to decrease ($P = 0.09$) between 20 and 26 wk of age, with no further changes thereafter. There was a tendency toward a pre-6 mo diet × age interaction for monthly concentration of TT ($P = 0.08$). Bulls on Hi had greater TT compared with bulls on Lo at 16 wk of age, but not at other time points. We also observed a post-6 mo diet × age interaction for monthly concentrations of TT ($P < 0.01$), where bulls on Hi post-6 mo had greater TT at 30 wk of age ($P < 0.05$) and tended to have greater TT at 34 wk of age ($P = 0.06$) compared with bulls on Lo. Diet did not affect monthly serum concentrations of LH ($P > 0.05$; Figure 3). There was an effect of age ($P < 0.01$), with a tendency for LH to be greater at 16 than at 34 wk of age ($P = 0.07$) and greater at 38 than at 34 wk of age ($P = 0.09$).

Regarding intensive blood sampling at 16 and 32 wk of age, for statistical analysis, concentrations of each hormone are represented by areas under the curve (Figures 4 to 6). We found no diet × age interactions for concentrations of either FSH or LH (Figures 4 and 5). Concentrations of FSH were not affected by diet but tended to be greater at 16 than at 32 wk of age ($P = 0.07$). There was no effect of diet either pre- or post-6 mo of age on LH (Figure 5); LH concentrations were greater at 32 than at 16 wk of age ($P < 0.05$). In contrast, mean LH concentrations before GnRH administration (average of −30, −15, and 0 min) were greater for bulls on a Hi diet pre-6 mo ($P < 0.05$; Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13720). There was a post-6 mo diet × age interaction for TT ($P < 0.001$; Figure 6), such that bulls on Hi post-6 mo had greater TT than bulls on Lo at 32 but not 16 wk of age. At 16 wk of age, bulls on Hi pre-6 mo had greater TT than those on Lo ($P < 0.01$).

Stepwise regression models, using various combinations of data on growth characteristics (Byrne et al., 2018) and both metabolic and reproductive hormones as independent variables, accounted for 29, 19, 67, 66, and 15% of the variation in age at puberty, age at sexual maturity, sperm output post-puberty, semen volume post-puberty, and PLM of sperm post-puberty, respectively (Table 1).

Plasma total protein concentration at 9 mo of age accounted for the greatest proportion (29%) of variability in age at puberty. There were also statistically significant, though weak, further contributions from ADG from 2 wk of age to puberty (9%) and urea concentration at 4 mo of age (7%). The variation in age at sexual maturity could be explained by a combination of ADG from beginning of the experiment to puberty (9%) and urea concentration at 4 mo of age (10%). Overall, 20% of the variability in postpubertal total sperm output was explained by total protein concentration at 5 mo of age, with BW at 10 wk of age explaining a further 15% of the variation in total sperm output. Rump fat depth at 8 mo of age and NEFA concentration at 3 mo of age both explained 10% of total sperm output. A combined total of 11% of total sperm output post-puberty was explained by IGF-1 concentration at 1 mo of age (6%) and NEFA at 4 mo of age (5%). Concentration of BHB at 6 mo of age explained 26% of the variation in semen volume post-puberty, whereas urea at 7 mo of age, NEFA at 3 mo of age, urea at 3 mo of age, and leptin at 1 mo of age explained 20, 15, 12, and 9% of the

variation, respectively. Small amounts of the variation in PLM post-puberty were explained by albumin at 3 mo of age (9%) and glucose at 3 mo of age (6%).

DISCUSSION

Not all metabolites and metabolic hormones are affected by prevailing diet. Although IGF-1 and insulin exhibited an immediate response to diet, the responses of leptin and adiponectin were observed over a longer period. A high plane of nutrition increased the TT-producing potential of these bulls, as evident from samples collected monthly and following the GnRH challenge. This study also showed that prepubertal plane of nutrition, as indicated by plasma protein and urea concentrations, might help explain the variation

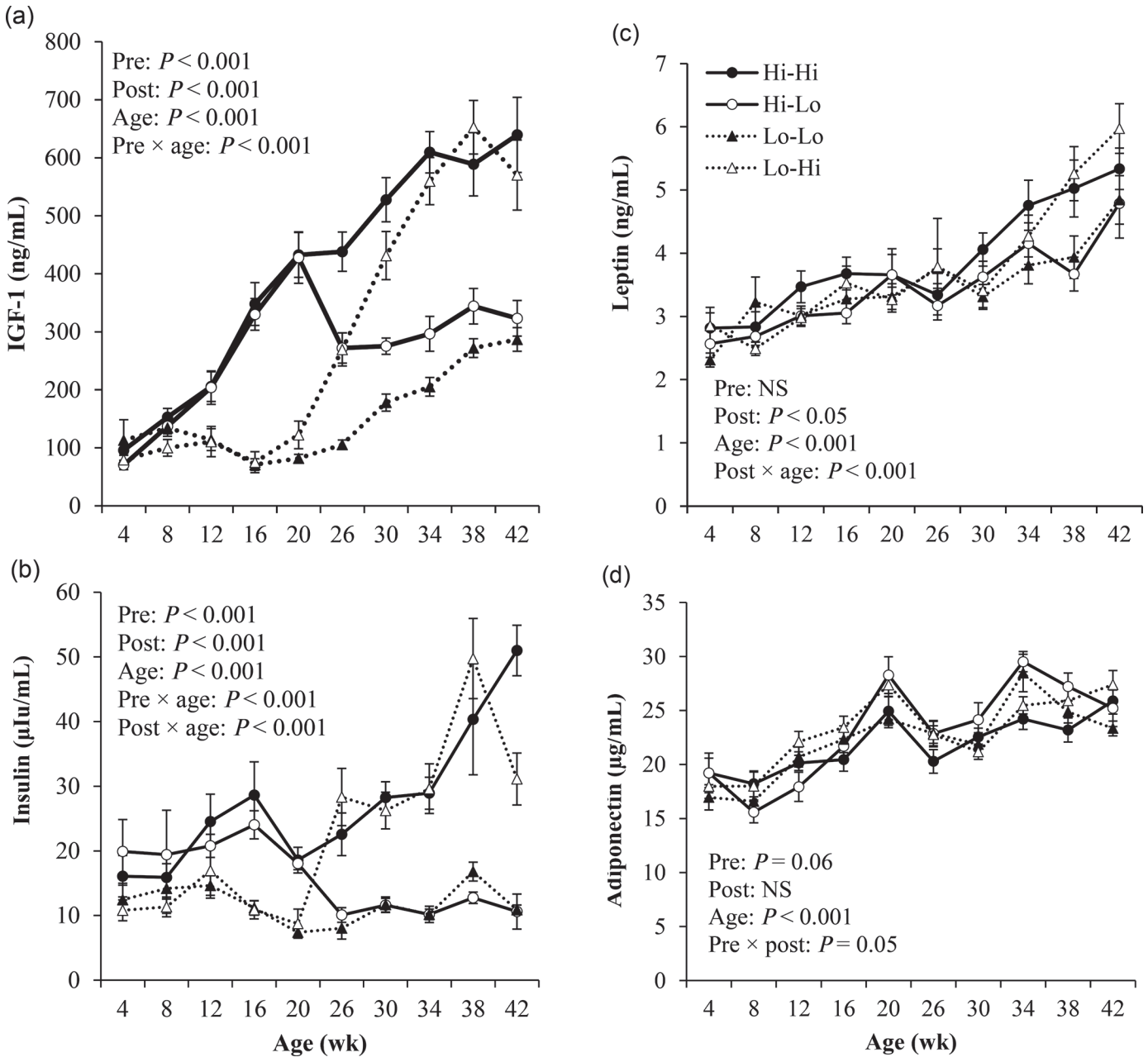

Figure 2. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls on plasma concentrations of IGF-1 (n = Hi-Hi: 19; Hi-Lo: 18; Lo-Lo: 22; Lo-Hi: 24; panel a), insulin (n = 10 per group, panel b), leptin (panel c), and adiponectin (n = Hi-Hi: 19; Hi-Lo: 18; Lo-Lo: 22; Lo-Hi: 24; panel d) taken at monthly intervals. Values are means ± SEM.

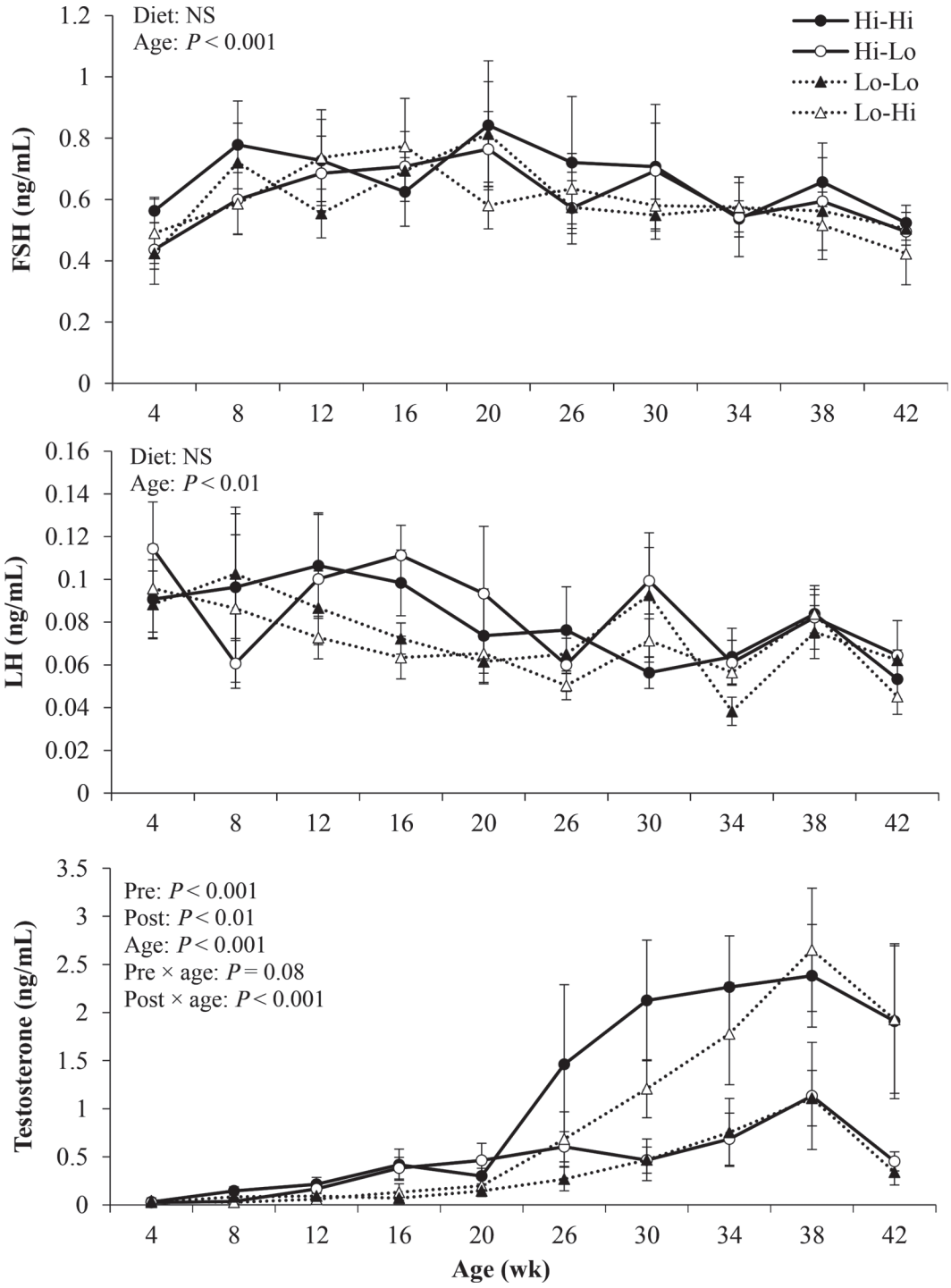

Figure 3. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls on serum concentrations of FSH (n = 12 per group, top panel), LH (n = 16 per group; middle panel), and testosterone (n = 16 per group; bottom panel) taken at monthly intervals. Values are means ± SEM.

around age at puberty and postpubertal semen volume, traits that are important when considering the lifetime reproductive potential of a young bull.

The greater concentration of albumin, as a result of a higher plane of nutrition, is consistent with previous reports (Tolleson et al., 2012). Latent effects of

a high pre-6 mo diet were found at wk 34 for total protein, whereas a prevailing high diet in the post-6 mo period tended to yield greater globulin. Similar results have been reported in dairy-bred ewes of different BCS, as a result of differing planes of nutrition (Caldeira et al., 2007). Systemic concentrations of BHB typically increase as the rumen develops in calves, due to a concomitant increase in concentrate consumption (Baldwin and Jesse, 1992.). Thus, it is likely that the increased BHB in Lo calves between 8 and 16 wk of age (periweaning period) was due to sustained greater concentrate consumption. We recently reported an increase in BHB concentration in Holstein-Friesian calves offered a low compared with a high level of milk replacer in the immediate preweaning period (9 to 10 wk of age), consistent with increased concentrate intake (Byrne et al., 2017a). Creatinine concentrations are normally greater in heavier calves with greater muscle

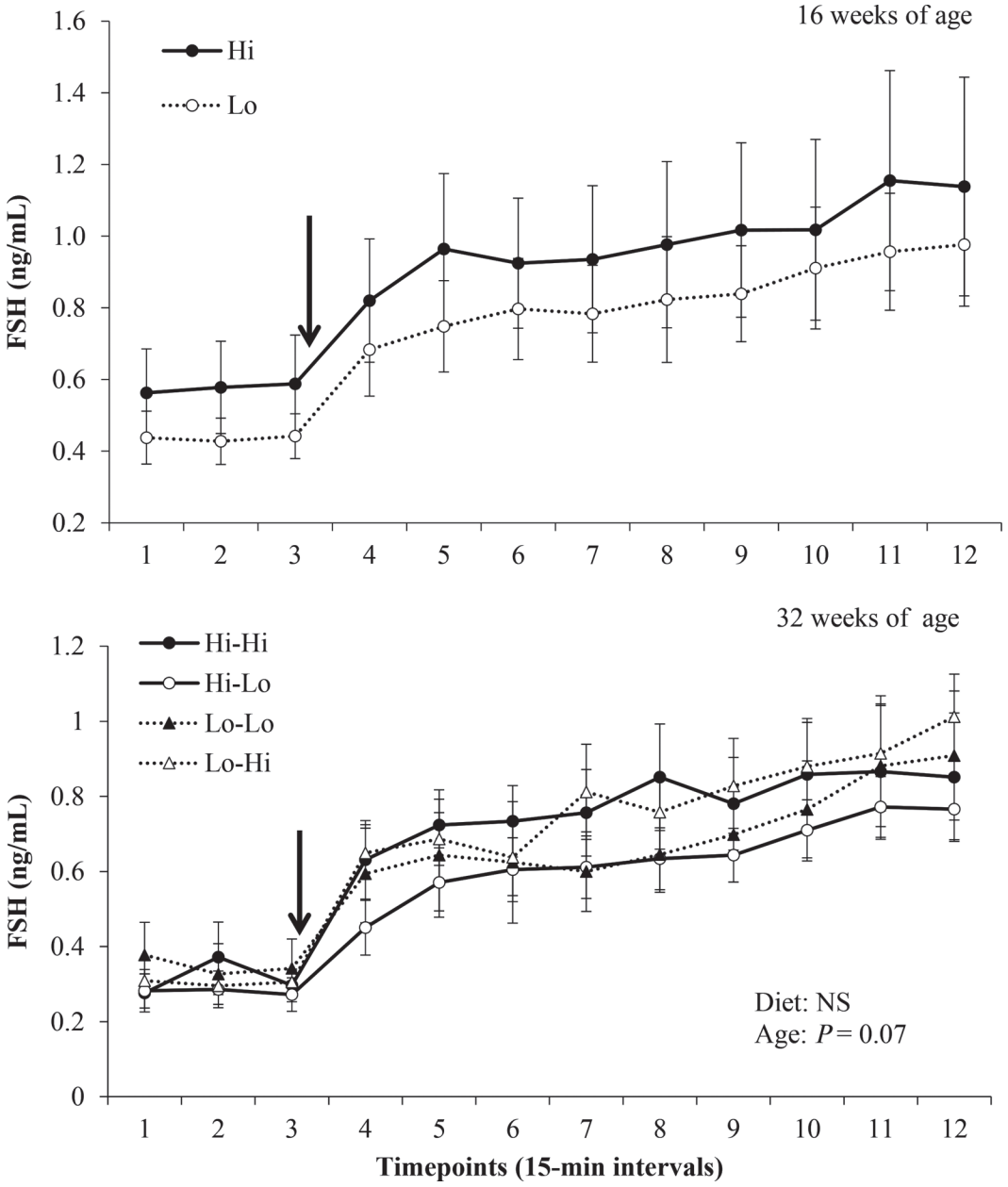

Figure 4. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls (n = Hi-Hi: 8; Hi-Lo: 9; Lo-Lo: 9; Lo-Hi: 8) on serum concentrations of FSH following a GnRH challenge at 16 wk (top panel) and 32 wk (bottom panel) of age. Administration of GnRH indicated by arrow. Values are means ± SEM.

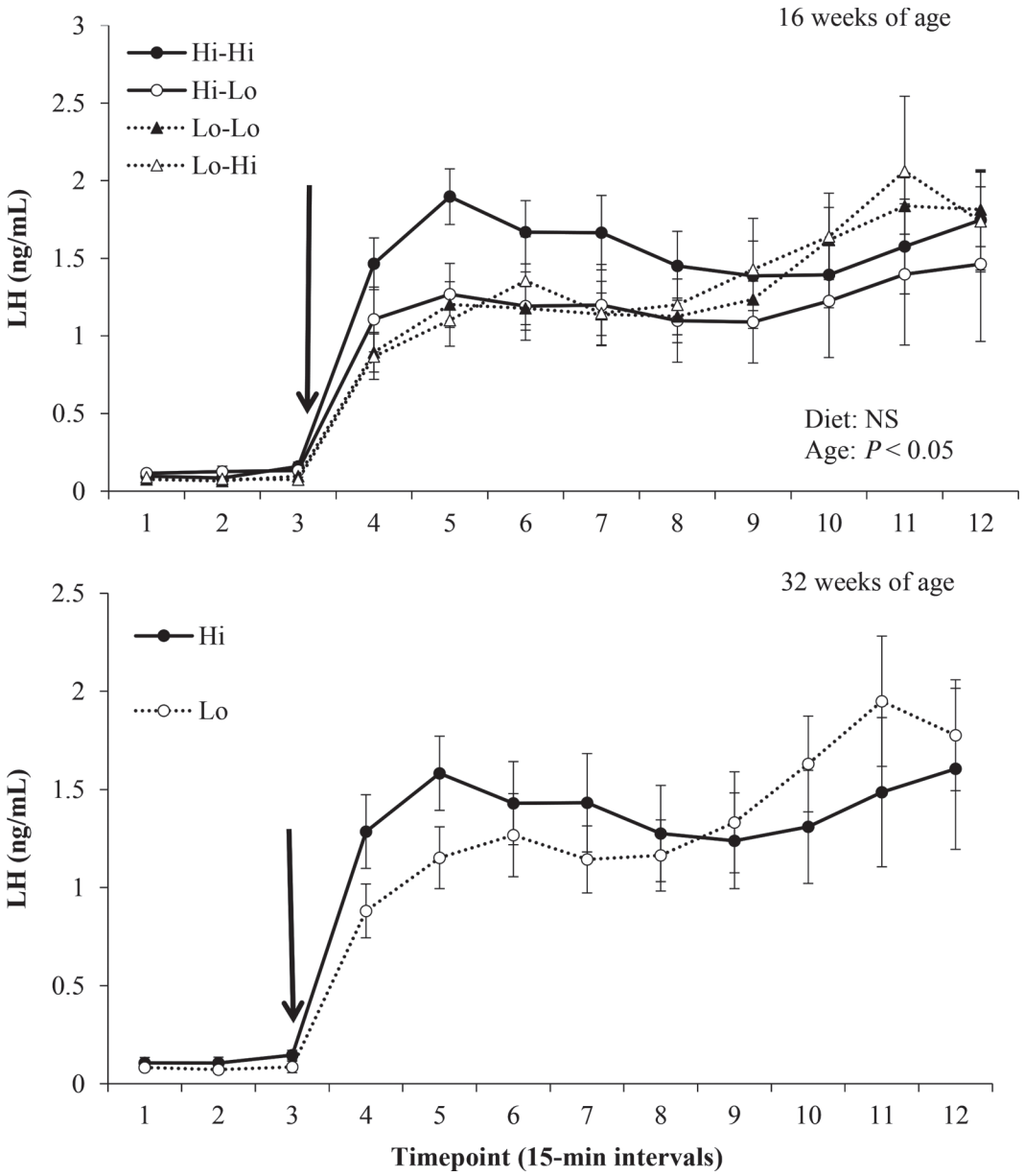

Figure 5. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls (n = Hi-Hi: 8; Hi-Lo: 9; Lo-Lo: 9; Lo-Hi: 8) on serum concentrations of LH following a GnRH challenge at 16 wk (top panel) and 32 wk (bottom panel) of age. Administration of GnRH indicated by arrow. Values are means ± SEM.

mass (Williams et al., 1987). In contrast, bulls on the Lo-Hi diet had greater creatinine than those on the Hi-Hi or Hi-Lo diet and concentrations tended to be greater than for Lo-Lo bulls. Creatinine was greater at 12, 30, 34, and 38 wk of age in bulls offered a Lo diet before 6 mo than in bulls offered a Hi diet before 6 mo, indicating some latent effects of diet. More recently, we have shown that creatinine was unaffected when the same diets were offered to either Holstein-Friesian or Jersey calves up to weaning (Byrne et al., 2017a).

Bulls on the Hi diet post-6 mo of age tended to have greater globulin concentrations than bulls on the Lo diet, possibly due to the increased protein intake on an ad libitum concentrate diet (Bertoni et al., 2008). Concomitant with an increase in insulin, glucose was greater at 16 and 20 wk of age in bulls offered a Hi diet before 6 mo. We have previously shown greater systemic concentrations of glucose when bulls were offered concentrate ad libitum compared with on a restricted basis (Keogh et al., 2015; Byrne et al., 2017b). As negative

energy balance leads to excess lipolysis resulting in an increase of NEFA (Jorritsma et al., 2003), the greater NEFA at 34 and 42 wk of age in bulls on a Lo versus a Hi diet post-6 mo is in agreement with the expected physiological response. The greater NEFA concentrations observed on the Hi diet pre-6 mo in the current study are surprising; nonetheless, the concentrations were low and do not indicate excess fat mobilization.

Total protein concentrations were greater in bulls offered a Lo diet pre-6 mo than bulls offered a Hi diet; however, by 34 wk of age this had reversed and bulls offered a Hi diet post-6 mo had greater total protein concentrations than bulls offered a Lo diet post-6 mo, indicating some residual effects of the pre-6 mo diet. In Holstein-Friesian bulls offered an ad libitum or restricted diet, it took 25 d of re-alimentation before previously

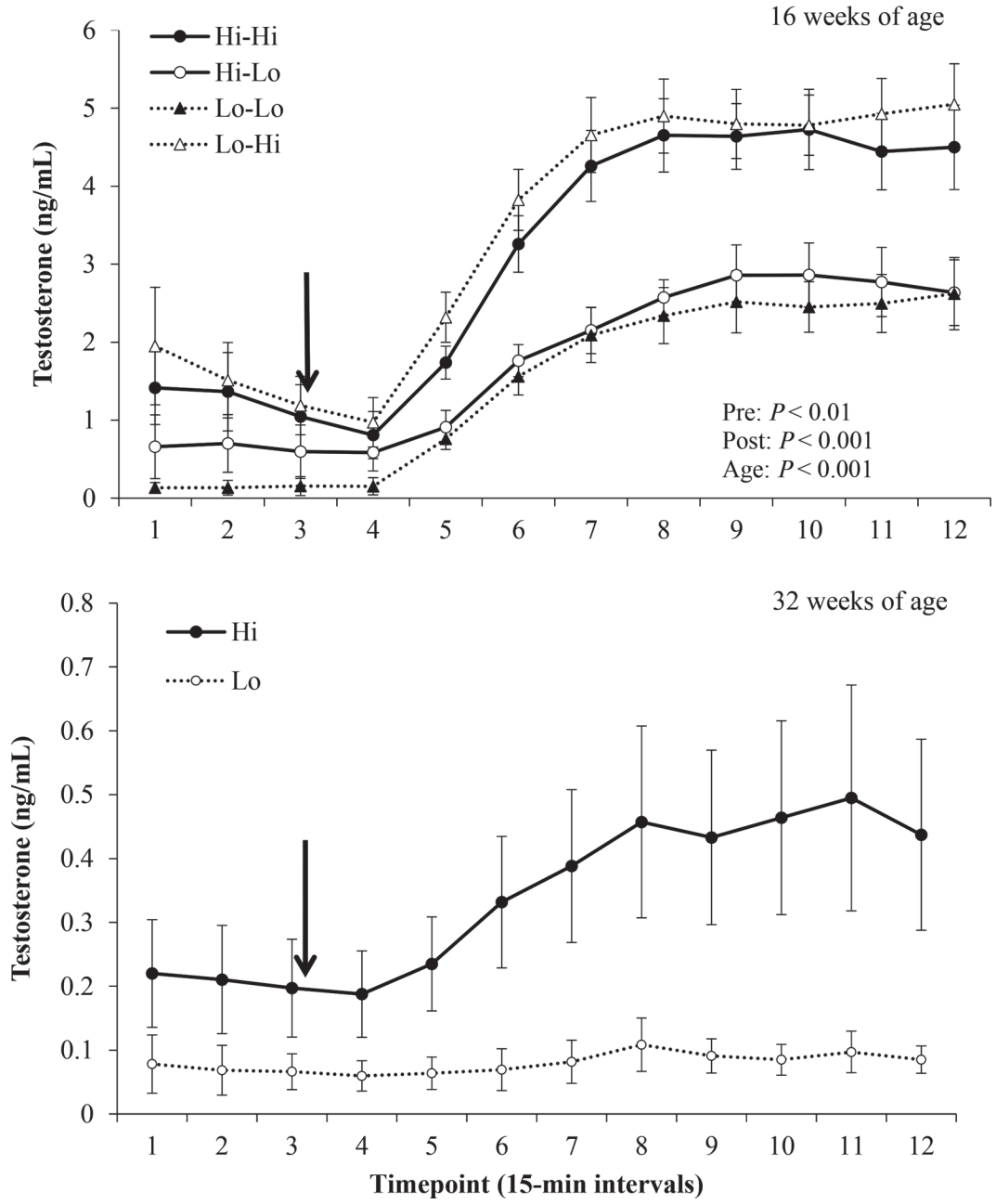

Figure 6. Effect of a high (Hi) versus a low (Lo) diet during the pre- and post-6 mo period in prepubertal Holstein-Friesian bulls (n = Hi-Hi: 8; Hi-Lo: 9; Lo-Lo: 9; Lo-Hi: 8) on serum concentrations of testosterone following a GnRH challenge at 16 wk (top panel) and 32 wk (bottom panel) of age. Administration of GnRH indicated by arrow. Values are means ± SEM.

restricted bulls had total protein concentrations similar to that of the unrestricted bulls (Keogh et al., 2015). In that study, total protein was reduced at the beginning of the re-alimentation period, in agreement with Gonzaga Neto et al. (2011), who suggested that this reduction could be due to increased nitrogen utilization or retention of protein in tissues of cattle that had previously been diet-restricted. Triglyceride concentrations were greater in bulls offered a Lo diet post-6 mo of age, in agreement with previous reports from our group (Byrne et al., 2017a, 2018) when Holstein-Friesian bulls were offered similar contrasting diets to those used here up to 8 mo of age. Although there is a lack of published literature regarding the effects of diet offered to calves on triglyceride concentrations, the effects of plane of nutrition do not appear to be in line with the expected outcome. It has been suggested that triglycerides are typically mobilized in response to negative energy balance–induced lipolysis (Van Soest, 1982). It has also been suggested that systemic triglyceride and leptin concentrations are positively related (Geary et al., 2003). However, in the current study, these 2 analytes appear to be in opposition to each other. Given the antilipolytic characteristics of leptin (Chilliard et al., 1998), the findings of the current study also make sense because all bulls were in positive energy balance. The greater urea concentrations in bulls on the Hi compared with the Lo diet at 8 wk of age are in contrast to the reported association between increased concentrate consumption and urea concentrations (Berends et al., 2014). However, the calves on the Hi diet were consuming a high amount of milk replacer with 22% CP which may explain the observed difference. As the bulls on the Hi diet were on 8 L of milk replacer, they would have had a reduced concentrate intake compared with the Lo bulls being offered only 4 L of milk replacer (Byrne et al., 2017a). The greater urea concentrations in Hi bulls between 30 and 38 wk of age compared with Lo bulls are in agreement with Gleghorn et al. (2004), who reported that an increased CP intake is associated with an increase in blood urea concentrations in steers.

Systemic concentrations of IGF-1 reflected the prevailing dietary plane of nutrition but only after 12 wk of age. Nutritional effects on IGF-1 have been reported at 11 wk of age in Holstein-Friesian bulls, where calves on an HPN (ad libitum concentrate and forage) had greater IGF-1 than those on an LPN (same intake as Hi but with forage only; Dance et al., 2015). Receptors for IGF-1 have been detected in the pre-optic area of the hypothalamus, suggesting a stimulatory role in GnRH secretion (Daftary and Gore, 2005). Indeed, an increase in LH responsiveness to GnRH was detected when rat anterior pituitary cell lines were incubated with IGF-1 (Soldani et al., 1995), highlighting the potentially important direct association between IGF-1 and gonadotropin secretion. An effect of prevailing

Table 1. Stepwise regression for age at puberty, age at sexual maturation, and average total sperm output and average volume after puberty

Regression	Slope	SE	R^2	*P*-value
Age at puberty[1] ($\Sigma R^2 = 0.29$; γ-intercept = 150.4)				
Total protein at 36 wk of age	2.0	0.62	0.13	<0.01
Overall ADG	77.5	29.09	0.09	<0.05
Urea at 16 wk of age	8.0	3.34	0.07	<0.05
Age at sexual maturation[1,2] ($\Sigma R^2 = 0.19$; γ-intercept = 317.0)				
Urea at 16 wk of age	9.2	3.27	0.10	<0.01
Overall ADG	40.1	15.58	0.09	<0.05
Average total sperm output after puberty[3] ($\Sigma R^2 = 0.67$; γ-intercept = −9.7)				
Total protein at 20 wk of age	0.3	0.07	0.20	<0.001
Weight at 10 wk of age	−0.1	0.02	0.15	<0.01
Nonesterified fatty acids (NEFA) at 12 wk of age	−2.8	1.03	0.10	<0.05
Rump fat depth at 32 wk of age	2.1	0.80	0.10	<0.05
Adiponectin at 28 wk of age	−0.1	0.07	0.06	0.05
Globulin at 24 wk of age	0.1	0.05	0.05	0.06
Average volume after puberty[3] ($\Sigma R^2 = 0.83$; γ-intercept = 5.2)				
BHB at 24 wk of age	−13.0	3.70	0.27	<0.01
Urea at 28 wk of age	1.25	0.41	0.20	<0.01
NEFA at 12 wk of age	−2.7	1.04	0.15	<0.05
Urea at 12 wk of age	0.73	0.31	0.12	<0.05
Leptin at 4 wk of age	1.0	0.47	0.09	0.06
Average PLM after puberty[3] ($\Sigma R^2 = 0.15$; γ-intercept = 89.0)				
Albumin at 12 wk of age	−0.6	0.23	0.09	<0.05
Glucose at 36 wk of age	1.6	0.76	0.06	0.05

[1]Age at puberty = when bulls first produced an ejaculate containing ≥50 million sperm cells and ≥10% progressive linear motility (PLM). Age at sexual maturation = when bulls first produced an ejaculate containing ≥70% normal morphology sperm cells and ≥30% PLM.

[2]Age at puberty excluded.

[3]Average from mo 1 to 5 post-puberty.

plane of nutrition on IGF-1 following a change in diet after 26 wk of age has been reported in beef bulls (Brito et al., 2007a); however, in contrast to our results, the response of IGF-1 to nutrition was slower in beef bulls than in dairy bulls (24 vs. 8 wk). Insulin followed the same pattern as IGF-1 pre-6 mo of age. Insulin was greater in Hi bulls from 30 to 42 wk of age. In heifers, a decrease in insulin, induced by fasting, has been associated with a reduction in LH pulsatility (Amstalden et al., 2000). In the current study, a Hi diet led to greater systemic insulin concentrations between 12 and 20 wk of age; this period is considered most crucial for stimulation of LH pulsatility in bulls (Rodriguez and Wise, 1989). Bulls on the Lo diet did not display significantly suppressed concentrations of insulin, most likely because all bulls were in positive energy balance. In this instance, insulin would be available to regulate blood glucose concentrations allowing maintenance of glucose homeostasis (Aronoff et al., 2004).

Leptin was unaffected by diet offered before 6 mo of age. Leptin was greater at 38 wk of age in bulls on the Hi diet post-6 mo of age compared with bulls on Lo. Given that backfat in bulls offered a Hi diet post-6 mo was greater from 32 to 40 wk of age (Byrne et al., 2018), it is surprising that differences in leptin were only found at 38 wk of age, as animals with greater adiposity have been shown to have greater concentrations of leptin (Geary et al., 2003). It has previously been shown, however, that plane of nutrition does not affect leptin concentrations in bulls before 6 mo of age (Brito et al., 2007a; Dance et al., 2015), perhaps due to the low deposition of subcutaneous fat in these young growing animals. In contrast to our findings, neither of the aforementioned studies reported an effect of diet on leptin before puberty. Adiponectin was lower in bulls on Hi-Hi than Lo-Hi but not the other 2 diets, with fluctuations over time. Adiponectin is reportedly inversely related to body fat mass in cattle (Sauerwein and Häußler, 2016); therefore, it would be expected that bulls with a higher potential for fat deposition (HPN vs. LPN) would have lower adiponectin concentrations. Increased adiponectin concentrations have been associated with disturbances in GnRH pulsatility in men (Lanfranco et al., 2004), indicating that adiponectin has an opposing effect to that of leptin on GnRH pulsatility.

Consistent with previous reports in young bulls, FSH was unaffected by prevailing nutritional status (Brito et al., 2007a; Dance et al., 2015). The increase in FSH between 4 and 8 wk of age in the current study is uncharacteristic in calves compared with the literature, where it has been reported that FSH is typically high postnatally (Evans et al., 1995). In Hereford and Charolais bulls, the period of elevated FSH is similar to that in the current study; however, the decline in FSH began at 15 wk of age in the current study compared with 20 wk in a previous study (Evans et al., 1996). In our study, the decline was observed in samples collected monthly and at the time of the GnRH challenges. The period of elevated FSH concentrations has been shown to coincide with a period of rapid proliferation of Sertoli cells (Rawlings et al., 2008). In addition, a negative feedback of androgens on gonadotropins has been reported (Rawlings and Evans, 1995), meaning that once TT increases, the pulsatile release of LH and FSH decreases and remains low. In vitro, it has been shown that culturing immature Sertoli cells from 8-wk-old calves in combination with both IGF-1 and FSH can increase Sertoli cell proliferation (Dance et al., 2017).

Although LH concentrations following a GnRH challenge were unaffected by diet, basal LH (mean of 3 samples before GnRH injection) was greater in bulls offered a Hi diet pre-6 mo. Previously, a tendency for total LH production during a 10-h period to be greater in beef and dairy bulls offered a Hi diet has been reported (Brito et al., 2007a; Dance et al., 2015). In the current study, mean LH concentration over 45 min was greater when dairy bulls were offered a high compared with a low plane of nutrition. The age-associated decrease in mean LH before GnRH administration has been reported in early- versus late-maturing beef-bred bulls (Evans et al., 1995) and, as such, has been linked to age at puberty. In the current study, LH concentrations after GnRH administration were greater at 32 wk than at 16 wk of age. It is possible that the response of the anterior pituitary to exogenous GnRH in the form of a bolus dose is different from that of the regular pulsatile GnRH release. In that regard, if bulls had a larger anterior pituitary at an older age they may have a larger LH store for release. Indeed, in heifers, it has been shown that pituitary size increases with age (Desjardins and Hafs, 1968); therefore, the response to exogenous GnRH would likely be greater, as has been shown in Holstein-Friesian bulls (Dance et al., 2015), where GnRH induced LH concentrations were greater as the bulls approached 31 wk of age. Nevertheless, basal LH concentrations in samples collected at monthly intervals were not different between diets. In contrast, Brito et al. (2007a) reported that Angus and Charolais bulls offered a control diet had greater basal LH concentrations from 14 to 22 wk of age than contemporaries offered a restricted plane of nutrition. In agreement with our findings, Brito et al. (2007a) reported no effect of plane of nutrition between 26 and 42 wk of age, in a similar model to that used in the current study where, from 26 wk of age, control bulls remained on the same diet and restricted bulls had their plane of nutrition increased to either a control or high plane of nutrition until 70 wk of age.

The greater concentration of TT at 16 wk of age in bulls on a Hi diet pre-6 mo indicates that these bulls had more mature testes, likely due to increased LH stimulation of the Leydig cells. A greater concentration of TT at 18 wk as a result of a higher plane of nutrition has been reported in Angus and Charolais bulls (Brito et al., 2007a) and similarly, at 15 wk of age, following a 10-h intensive blood sampling in Holstein-Friesian bulls (Dance et al., 2015). The increase in TT concentration from samples collected monthly and during the GnRH challenges coincides with an increase in SC in these bulls (Byrne et al., 2018). The greater concentration of TT in Lo-Hi bulls between 30 and 34 wk of age is in contrast to Brito et al. (2007a), who reported that beef bulls restricted until 26 wk of age and then supplemented with concentrate to achieve an increased ADG do not have greater TT concentrations post-6 mo (Brito et al., 2007a). This may be due a smaller difference in SC observed in the beef bull study than in the current study.

Almost 30% of the variation in age at puberty is explained by variables associated with growth and protein intake, supporting the hypothesis that age at puberty is dictated by performance during the prepubertal period (Evans et al., 1995; Brito et al., 2007b). Temporal associations between IGF-1, LH, and puberty have been reported (Dance et al., 2015) with IGF-1 concentration being dictated by prevailing plane of nutrition and thus ADG. Similarly, the variation in age at sexual maturity was accounted for by overall ADG (9%) and urea concentration at 4 mo of age (10%). Growth rate and SC have been previously associated with sexual maturity in beef bulls (Brito et al., 2012). Variation in postpubertal semen production and quality was explained by systemic concentrations of metabolic hormones and metabolites. These results highlight the importance of prepubertal plane of nutrition on subsequent postpubertal fertility in bulls. Over 60% of the variation in both sperm output and semen volume produced was explained by variables characterizing the metabolic status of prepubertal bulls. As demand for semen from young bulls, such as those in this study, is very high in the immediate postpubertal period, every effort should be made to ensure an enhanced plane of nutrition is offered prepuberty to ensure optimum postpubertal semen production.

CONCLUSIONS

Metabolic hormone and metabolite profiles generally reflected the prevailing diet offered. Although systemic indicators of positive metabolic status such as insulin, IGF-1, and glucose were all elevated during the gonadotropin rise and were consistent with advanced puberty in bulls offered a high dietary allowance during this critical window of development, our data suggest that leptin does not appear to be directly involved in the early-life nutritional advancement of puberty. Stepwise logistic regression analysis revealed that when all the various physiological and endocrinological measurements were examined holistically in this study, total protein concentration at 9 mo of age explained the greatest proportion of the interanimal variation in age at onset of puberty. Furthermore, a small and significant degree of the variance in postpubertal sperm output was explained by measuring systemic concentrations of total protein at 9 mo of age, again highlighting the importance of enhanced nutrition in the prepubertal period. There was also an indication that urea concentration at 7 mo of age may influence the volume of semen produced later in life, although further work is required to investigate this relationship.

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