Role of early life nutrition on regulating the hypothalamic–anterior pituitary–testicular axis of the bull

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Abstract

The objective of this study was to examine the effect of nutrition during the first 18 weeks of life on the physiological and transcriptional functionality of the hypothalamic (arcuate nucleus region), anterior pituitary and testes in Holstein–Friesian bull calves. Holstein–Friesian bull calves with a mean ($\pm$ S.D.) age and bodyweight of 19 ($\pm$ 8.2) days and 47.5 ($\pm$ 5.3) kg, respectively, were assigned to either a HIGH ($n=10$) or LOW ($n=10$) plane of nutrition, to achieve an overall target growth rate of 1.2 or 0.5 kg/day, respectively. At 126 ± 1.1 days of age, all calves were euthanised. Animal performance (weekly) and systemic concentrations of metabolic (monthly) and reproductive hormones (fortnightly) were assessed. Testicular histology, targeted gene and protein expression of the arcuate nucleus region, anterior pituitary and testes were also assessed using qPCR and immunohistochemistry, respectively. The expression of candidate genes in testicular tissue from post pubertal 19-month-old Holstein–Friesian bulls ($n=10$) was compared to that of the 18-week-old calves. Metabolite and metabolic hormone profiles generally reflected the improved metabolic status of the calves on the HIGH ($P<0.001$). Calves offered a HIGH plane of nutrition were heavier at slaughter ($P<0.001$), had larger testes ($P<0.001$), larger seminiferous tubule diameter ($P<0.001$), more mature spermatogenic cells ($P<0.001$) and more Sertoli cells ($P<0.05$) in accordance with both morphological and transcriptional data. Overall, testicular gene expression profiles suggested a more mature stage of development in HIGH compared with LOW and were more closely aligned to that of mature bulls. Ghrelin receptor was the only differentially expressed gene between LOW and HIGH calves in either the anterior pituitary ($P<0.05$) or arcuate nucleus region of the hypothalamus ($P<0.10$) and was upregulated in LOW for both tissues. This study indicates that an enhanced plane of nutrition during early calthood favourably alters the biochemical regulation of the hypothalamus–anterior pituitary–testicular axis, advancing testicular development and hastening spermatogenesis.

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Introduction

Following the inception of genomically assisted selection, young genetically elite bulls are now being identified within weeks of birth as potential sires for use in artificial insemination programmes. However, these bulls cannot produce ejaculates of sufficient quality for cryopreservation until they are greater than 1 year of age (Murphy *et al.* 2018) and demand for their semen often far exceeds supply. Hastening the onset of puberty and subsequent sexual maturation would therefore benefit the industry and make high-quality semen available at a younger age (Harstine *et al.* 2015).

The hypothalamic–anterior pituitary–testicular axis (HPT) is a cohesive biological system that controls the secretion of male hormones and in turn spermatogenesis (Ramaswamy & Weinbauer 2014). The hypothalamus is acknowledged as the homeostatic regulator of the body

(Morton *et al.* 2014). Signals from hormones including leptin, ghrelin, insulin and insulin-like growth factor 1 (IGF-1) are received by hypothalamic neurons located in the arcuate nucleus region (Amstalden *et al.* 2011) and signal nutritional status to the preoptic nuclei region that are largely involved in the regulation of reproduction (Lechan & Toni 2000). The gonadotropin-releasing hormone (GnRH) nerve projections, originating from the preoptic nuclei region, attach to the median eminence, where they secrete GnRH into the hypophyseal portal system leading to the anterior pituitary (Fujioka *et al.* 2007).

Bull calves experience a transient rise in anterior pituitary-derived systemic luteinising hormone (LH) from approximately 8 to 20 weeks of age (Evans *et al.* 1996). Furthermore, studies have shown that bull calves offered a high plane of nutrition during the first 6 months of life

had an earlier and greater rise of LH (Thundathil *et al.* 2016). Enhanced nutrition during this critical period has a direct effect on hypothalamic GnRH pulsatility, ultimately leading to enhanced LH pulsatility as well as testosterone synthesis and release, leading in turn to advanced testicular development and pubertal onset (Dance *et al.* 2015, Byrne *et al.* 2018). Additionally any delay in the timing of puberty as a consequence of undernutrition during calthood cannot be mitigated by dietary augmentation thereafter (Brito *et al.* 2007a, Byrne *et al.* 2018).

While the reproductive axis has the capacity to respond to changing metabolic signals (Hill *et al.* 2008), and it is clear that a high plane of nutrition in early calthood can advance puberty in both male and female cattle (Kenny *et al.* 2018), the precise molecular mechanisms by which this is mediated have not been elucidated to date in the bull. Thus, the objective of this study was to characterise the effects of a high compared to a low plane of nutrition during the first 18 weeks of life on aspects of physiological and transcriptional functionality of the hypothalamic (arcuate nucleus region), anterior pituitary and testes in Holstein–Friesian bull calves.

Materials and methods

All procedures involving animals were approved by the Teagasc Animal Ethics Committee (TAEC30/2013); licensed by the Irish Health Products Regulatory Authority (licence number AE19132/P013) in accordance with the European Union Directive 2010/36/EU.

Experimental design and animal management

Twenty Holstein–Friesian bull calves with a mean ($\pm$ S.D.) age and bodyweight of 19 ($\pm$ 8.2) days and 47.5 ($\pm$ 5.3) kg respectively, were sourced from three high health status commercial dairy farms blocked on age, sire ($n=4$), liveweight and farm of origin. After a 5-day acclimatisation during which the calves feed allowance was increased daily to reach their target growth rate, calves were assigned to either a HIGH or LOW plane of nutrition. Calves were individually fed milk replacer and concentrates (Tables 1 and 2) using an electronic feeding system (Forster-Technik Vario; Engen, Germany). Calves on HIGH received 1200 g of milk replacer in 8 L of water daily, together with concentrate *ad libitum*. Calves on LOW were allocated 500 g of milk replacer in 4 L of water plus a maximum of 1 kg of concentrates daily. Diets were designed based on National Research Council guidelines (NRC 2001). Calves were weaned when consuming a minimum of 1 kg of concentrate for 3 consecutive days, at a mean age ($\pm$ S.D.) of 82 ($\pm$ 3.9) days. Following weaning, HIGH were offered concentrates *ad libitum*, while LOW calves received 1 kg of concentrate daily. All calves had daily access to fresh water and approximately 0.5 kg of hay. Calves were weighed weekly. At a mean age ($\pm$ S.D.) of 126 ($\pm$ 1.1) days of age, all calves were euthanised following intravenous administration of sodium pentobarbitone (1 mL/1.4 kg bodyweight; Euthatal,

Table 1 Chemical composition of milk replacer offered to Holstein–Friesian bull calves from 2 to 12 weeks of age.

Chemical composition (g/kg)	Values
ADF	12.0 $\pm$ 1.98
Crude ash	65.7 $\pm$ 2.22
CP	216.3 $\pm$ 1.24
DM (%)	96.7 $\pm$ 0.15
NDF	5.1 $\pm$ 1.00
Oil B	235.0 $\pm$ 44.10

ADF, acid detergent fibre; CP, crude protein; DM, dry matter; NDF, neutral detergent fibre; Oil B, acid hydrolysis.

Merial S.A.S, Toulouse, France). Death was confirmed by lack of ocular response and was followed by exsanguination and decapitation.

The head was removed from the carcass and the skullcap was opened within 10 min of death. The brain was then removed from the skull by severing the infundibulum, optic nerves and brain stem. The region of the hypothalamus containing the arcuate nucleus tissue was dissected according to Komatsu *et al.* (2012). Two small triangular sections were harvested from either side of the bottom of the third ventricle of the hypothalamus, which contains the arcuate nucleus. The pituitary gland was removed from the sella turcica following which anterior and posterior sections of the pituitary gland were separated. The testes were also excised, the tunica albuginea, epididymides and any excess connective tissue removed and the testes were weighed. Two portions of the testicular parenchyma were dissected from each testis. One section of each tissue was fixed in 10% neutral buffered formalin and then prepared for histological sectioning. The second section was snap-frozen in liquid nitrogen, and subsequently stored at -80°C for long-term storage pending further processing.

Table 2 Diet and chemical composition of concentrate diet offered to Holstein–Friesian bull calves from 2 to 18 weeks of age.

Concentrate	Values
Diet composition (%)	
Rolled Barley	26.5
Soya bean meal	25
Maize	15
Beet pulp	12.5
Soya hulls	12.5
Molasses	5
Mineral and vitamins	2.5 ^a
Vegetable oil	1
Chemical composition (g/kg)	
ADF	103.1 $\pm$ 6.76
Crude ash	68.8 $\pm$ 0.91
CP	167.9 $\pm$ 1.86
DM (%)	88.9 $\pm$ 0.66
NDF	204.3 $\pm$ 18.2
Oil B	30.8 $\pm$ 0.72

Pre-weaning: 2–12 weeks of age; post-weaning: 12–18 weeks of age.

^aMineral and vitamin composition: vitamin A (10 IU/kg), vitamin D₃ (2 IU/kg), vitamin E (40 mg/kg), iodine (8 mg/kg), cobalt (40 mg/kg), copper (88 mg/kg), manganese (81 mg/kg), zinc (139 mg/kg) and selenium (11 mg/kg).

Oil B, acid hydrolysis.

For comparative purposes, Holstein–Friesian bulls ($n=10$) finished on a standard diet (17.9 g/kg DM crude protein; 16.5 MJ/kg DM gross energy) were slaughtered at a mean ($\pm$ s.d.) age and bodyweight of 586 ($\pm$ 50) days and 532 ($\pm$ 48) kg respectively. Sections of testicular parenchyma were snap-frozen in liquid nitrogen at slaughter and subsequently stored at -80°C pending further processing. These mature bulls were of a similar genetic background to that of the calves described earlier and reared under similar conditions, at the same research facility. Their purpose was to act as a 'positive control' in which to evaluate the effects of nutritional augmentation on sexual precocity in the bull calf.

Blood sampling

Blood samples were taken at 2, 6, 10, 14 and 18 weeks of age. Blood samples were collected an hour after morning feeding via jugular venepuncture to determine systemic concentrations of IGF-1, insulin, leptin, adiponectin, albumin, urea, total protein, triglycerides, beta hydroxybutyrate (BHB), creatinine, globulin, glucose, non-esterified fatty acids (NEFA) as well as LH, follicle-stimulating hormone (FSH) and testosterone. The protocol for serum and plasma collection processing has been described by [Byrne et al. \(2017b\)](#).

Metabolite assays

Concentrations of albumin, urea, total protein, triglycerides, BHB, creatinine, globulin, glucose and NEFA were analysed using commercial biochemical assay (Olympus Diagnostics and Randox Laboratories LTD, Co. Antrim, Northern Ireland) on a Beckmann Coulter AU 400 clinical analyser (Olympus Diagnostics) as described previously by [Lawrence et al. \(2011\)](#). Intra-assay CVs were as follows for glucose (3.03, 1.88 and 2.48%), urea (5.59, 2.09 and 1.96%), BHB (1.58, 0.88 and 0.72%), triglycerides (0.005, 0.77 and 0.52%), protein (2.34, 1.55 and 2.3%), albumin (2.44, 1.03 and 1.88%) and creatinine (3.02, 2.01 and 2.01%) for low, medium and high standards, respectively. Globulin concentration was calculated as the difference between total protein and albumin concentrations. All samples, within each metabolite, were analysed on a single assay.

Metabolic hormones

Insulin-like growth factor 1 was quantified by radioimmunoassay (RIA) after an acid–ethanol extraction and Tris neutralisation procedure, as described previously by [Beltman et al. \(2010\)](#). The inter- and intra-assay CVs for the low, medium and high IGF-1 were 10.8, 4.36 and 4.05% and 6.7, 1.57 and 2.77% respectively. The sensitivity of IGF-1 assay was 4 ng/mL. Insulin concentrations were measured by immunoradiometric assay (IRMA; INS-Irma, DAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium) as described by [Ochocińska et al. \(2016\)](#). The inter- and intra-assay CVs for the low, medium and high insulin standards were for 0.09, 7.16 and 2.93% and 6.58, 9.23 and 6.62% respectively. The sensitivity of insulin assays was 1 ng/mL. Leptin concentration was quantified using a competitive enzyme immunoassay as described by [Sauerwein et al. \(2004\)](#).

The range of the assay was between 0.6 and 9.0 ng/mL. The intra-assay CV was 5% as all samples were analysed on a single assay. Adiponectin concentrations were analysed using an enzyme immunoassay described by [Heinz et al. \(2015\)](#). The range of the assay was between 0.03 and 7.0 ng/mL and the intra-assay CV was 5%.

Reproductive hormones

Serum samples were analysed for testosterone concentration using an RIA (Testo-RIA-CT, DAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium) and assays were performed according to the manufacturer's instructions. The sensitivity of the assay was 0.1 ng/mL. Intra-assay CV for testosterone was 4.71, 8.05 and 4.87% for low, medium and high testosterone quality controls, respectively, as testosterone was analysed on a single assay.

LH was quantified by RIA as previously described by [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 diluted (Equitech-Bio, INC, Kerrville, TX, USA) 1:100 in 5% PEG. Assay tubes were incubated for 1 h at room temperature, centrifuged for 20 min at 1600 g, followed by separation of the free fraction by decanting the supernatant. The intra- and inter-assay CV for LH was 14.46% and 8.59% and 1.38% and 0.25% for low and high LH quality controls, respectively. The sensitivity of the assay was 0.05 ng/mL.

FSH was quantified using RIA as described by [Crowe et al. \(1997\)](#). The intra- and inter-assay CV for FSH was 3.3, 5.03 and 9.26% and 4.26, 4.31 and 1.05% for low, medium and high FSH quality controls, respectively. The sensitivity of the assay was 0.05 ng/mL.

Testicular histology

Histology was employed in the testes to assess outer seminiferous tubule diameter, stage of spermatogenesis, lumen development and nuclear volume density of Sertoli cells. Sections (5 μm thick) were stained using the periodic acid-Schiff method ([Bancroft 1996](#)). For each section, the outer seminiferous tubule diameters were measured using a calibrated eyepiece micrometer at $\times 400$ magnification using a bright field light microscope. Measurements were made on 20 different round tubules selected at random from each testis, and mean diameter was calculated. The histological sections were also examined to determine the most mature stages of spermatogenesis at $\times 1000$ magnification, using oil immersion. In the cross-sections of 20 seminiferous tubules per testis, the most mature spermatogenic cell type in the course of spermatogenesis was established using the methodology of [Curtis and Amann \(1981\)](#). The progression of seminiferous tubule development was classified using the method of lumen scoring reported by [Rode et al. \(2015\)](#) (Supplementary Table 4, see section on [supplementary data](#) given at the end of this article). Measurements were conducted on 20 different tubules selected at random from each testis.

Sections of testes (5 µm thick) were stained using haematoxylin and eosin (H&E). The point-counting method was carried out at ×1000 magnification by bright field light microscope using a 50 point ocular grid (1250 points were evaluated per testes) to determine the nuclear volume density of Sertoli cells as previously described by [Johnson and Nguyen \(1986\)](#) (Supplementary Fig. 1). The evaluator was blind to the treatments.

RNA isolation and purification

Total RNA was extracted using RNeasy Universal plus Kit (Qiagen). The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, Germany). RNA quality was assessed on the Agilent Bioanalyzer 2100 using the RNA 6000 Nano Lab Chip Kit (Agilent Technologies). RNA was purified using the RNA Clean & Concentrator (Zymo Research, Irvine, CA, USA) and RNA integrity values of greater than eight were deemed to be of high quality.

Complementary DNA synthesis

Total RNA (2 µg) was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using Multiscribe reverse transcriptase according to manufacturer's instructions. Samples were stored at −20°C pending further analysis.

Primer design and reference gene selection

All primers targeting reference and candidate genes were obtained from a commercial supplier (Sigma-Aldrich Ireland; Supplementary Tables 1, 2 and 3). Primer3 (<http://primer3.ut.ee/>) software was utilised to design primers ([Koressaar & Remm 2007](#)). Primer specificity was established using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The genes examined in the arcuate nucleus region of the hypothalamus included: kisspeptin (*KISS1*), G protein-coupled receptor (*GPR54*), gonadotropin-releasing hormone (*GnRH*), agouti-related protein (*AGRP*), neuropeptide Y (*NPY*), pro-opiomelanocortin precursor (*POMC*), melanocortin 4 receptor (*MC4R*), insulin-like growth factor 1 (*IGF-1*), insulin-like growth factor 1 receptor (*IGF-1R*), leptin receptor (*OBR*) and growth hormone secretagogue receptor (ghrelin receptor; *GHSR*). The genes examined in the anterior pituitary included: luteinising hormone beta (*LHB*), follicle-stimulating hormone beta (*FSHB*), *GHSR*, somatotropin precursor/growth hormone (*GHI*), gonadotropin-releasing hormone receptor (*GnRHR*), *IGF-1R* and *IGF-1*. The genes examined in the testes included: proliferation cell nuclear antigen (*PCNA*), Thy-1 cell surface antigen (*THY1*), tight junction protein 1/zonula occludens protein 1 (*ZO1*), GATA-binding protein 4 (*GATA4*), androgen receptor (*AR*), follicle-stimulating hormone receptor (*FSHR*), aquaporin-8 (*AQP8*), ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), anti-Müllerian hormone (*AMH*), claudin 11 (*CLDN11*) and luteinising hormone receptor (*LHR*). In the

current study, four reference genes were tested across all samples with RT-PCR, including ribosomal protein S9 (*RPS9*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*) and ubiquitin (*UBQ*). Data were analysed using GeNorm (GenEx 5.2.1.3; MultiD Analyses, Gothenburg, Sweden). GeNorm is a software package that measures the total stability of the tested reference genes by calculating the intra- and intergroup CV and combining both CVs to give a stability value (*M* value) ([Keogh et al. 2015](#)). A lower *M* value indicates a greater stability in gene expression across all samples. In the current study, the *M* value scores in the testicular tissue for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), ubiquitin (*UBQ*) and ribosomal protein S9 (*RSP9*) were 2.44, 1.23, 0.99 and 0.99 respectively. In anterior pituitary tissue, *M* value scores for *YWHAZ*, *GAPDH*, *UBQ* and *RSP9* were 0.95, 0.65, 0.50 and 0.50 respectively. The *M* value scores for the arcuate region for *GAPDH*, *YWHAZ*, *UBQ* and *RSP9* were 1.39, 0.50, 0.43 and 0.43 respectively. On this basis, *UBQ* and *RSP9* were selected as suitably stable housekeeping genes for each of the tissues, and results are presented relative to an average of both genes.

Gene expression

The RT-qPCR assays were performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) as per the protocol reported by [Keogh et al. \(2015\)](#). The formula $E = 10^{(-1/\text{slope}) - 1}$ was used where slope refers to the slope of the linear curve of CT values plotted against log dilution. Only primers with PCR efficiencies between 90 and 110% were used. Gene expression was analysed using GenEx software (www.multid.se/genex.html), which allowed for compensation of PCR efficiencies, before averaging for RT-PCR replicates. Each gene of interest was normalised using the geometric mean of the four reference genes.

Immunohistochemical analysis

Immunohistochemistry was used to assess cell proliferation in the arcuate nucleus by assessing Ki67 staining and to validate the specificity of the tissue using proteins KISS1 (Kisspeptin) and NPY (Neuropeptide Y) that are highly specific to the arcuate nucleus; while Sertoli cell number and developmental stage was assessed in the testes using transcription factor GATA-binding protein 4 (*GATA4*) and anti-Müllerian hormone (*AMH*). Antigen retrieval of the deparaffinised tissue sections was performed using a PT-Link module (Dako) at 95–99°C for 20 min in a citric acid buffer (0.01 M, pH 6.0). Slide staining was performed using a Dako autostainer Link 48 (Dako) according to the manufacturer's instructions. The primary antibodies used were anti-Ki67 as a marker of cellular marker for proliferation (Ready-to-use, Dako), anti-KISS1 (1:1000, ABIN672726, Antibodies-online) and anti-NPY (1:150, ABIN724475, Antibodies-online) on the arcuate sections and anti-GATA4 (1:1000, LS-C355535, Source Bioscience) and anti-AMH (1:75, ab84952, Abcam) on the testicular

sections. The secondary antibody was a rabbit-anti-goat antibody and slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Bovine kidney, human breast cancer and bovine cerebrum sections were used as positive controls for Ki67, KISS1 and NPY staining of the arcuate tissue respectively. Bovine heart and ovary sections were used as positive controls for GATA4 and AMH staining of the testes respectively.

All stained slides were analysed using the software Aperio ImageScope (Leica Biosystem, Dublin, Ireland). The cytoplasmic V2 algorithm was selected to calculate the number of cells containing stain within the nucleus and the cytoplasm per area (mm^2) and percentage stained vs unstained for GATA4 (Fig. 1). The colour deconvolution algorithm was selected to analyse AMH staining as it separated a stained tissue image into two colour channels, corresponding to the actual colours of the stains used and facilitated measurement of intensity (weak, moderate and strong intensity) and provision of the number of cells containing stain per area (mm^2 ; Fig. 1). The cytoplasmic V2 algorithm was selected to analyse DAB staining intensity (weak, moderate and strong intensity) and provide the number of cells containing stain within the nucleus and the cytoplasm per area (mm^2) for Ki67, NPY and KISS1 (Fig. 2).

Each section stained with GATA4 (two per calf; $n=10$) was examined at $400\times$ magnification and ten round seminiferous tubules were randomly selected and assessed (Harstine *et al.* 2017). The number of stained Sertoli cell nuclei present in a circular monolayer at the wall of a round seminiferous tubule was recorded (Harstine *et al.* 2017).

Figure 1 AMH (anti-Müllerian hormone; $\times 400$) and GATA4 (GATA-binding protein 4; $\times 100$) staining in the testes. The images illustrate different staining intensities; (A) negative control, (B) AMH weak staining, (C) AMH moderate staining, (D) AMH strong staining, (E) GATA4-negative staining and (F) GATA4-stained cells. Tissues for analysis were collected at slaughter (18 weeks of age).

Statistical analysis

All PCR data analysed using GenEx were log transformed to obtain normal distribution prior to subsequent statistical analysis. Area under the curve (AUC) for FSH, LH and testosterone was determined using Sigma Plot, version 11 (Systat Software, San Jose, CA, USA). All bodyweight, testicular measurements, histology, immunohistochemistry, PCR and monthly blood analysis data were analysed using the procedures of Statistical Analysis Software (SAS version 9.3). All data were tested for normality (UNIVARIATE procedure) and where appropriate, transformed to the power of lambda (TRANSREG procedure). Data were analysed using ANOVA (MIXED procedure). The covariance matrix (simple or compound symmetry) was determined for each variable by examining the Bayesian Information Criteria (BIC; smaller is better) value. Fixed effects included plane of nutrition, week of age and their interactions, where appropriate. Animal was the experimental unit. Sampling time was included in the statistical models as a repeated measure for weights and blood analysis. Differences were deemed statistically significant if $P < 0.05$, while P values > 0.05 and < 0.10 were deemed as indicating a tendency towards statistical significance. All results are presented as mean $\pm$ S.E.M.

Results

Animal performance

Pre-weaning average daily gain (ADG) was greater for calves on HIGH in comparison with LOW ($P < 0.001$; Table 3). The post-weaning ADG was also greater for HIGH calves ($P < 0.001$) compared to LOW, which resulted in pre-slaughter bodyweights being greater for HIGH calves compared with LOW ($P < 0.001$). Paired testicular weight at slaughter was also greater for the calves on HIGH compared with those on LOW ($P < 0.001$; Table 4). The MATURE bulls grew at 1.4 kg/day prior to slaughter.

Metabolic hormones and metabolites

Metabolite and metabolic hormone data are presented in Figs 3 and 4, respectively. There was a plane of nutrition $\times$ week interaction for albumin ($P < 0.001$); HIGH calves had greater albumin concentrations at 14 and 18 weeks of age. Total protein was greater in LOW compared to HIGH calves ($P < 0.01$) and total protein increased in both planes of nutrition between 2 and 18 weeks of age ($P < 0.01$). There was no plane of nutrition $\times$ week interaction or no effect of either plane of nutrition or week on triglyceride concentrations ($P > 0.05$). There was no effect of plane of nutrition on NEFA ($P > 0.05$); however, there was an effect of week ($P < 0.01$). There was a plane of nutrition $\times$ week interaction for glucose concentration ($P < 0.01$); glucose concentrations were greater in HIGH compared to LOW calves at 6, 14 and 18 weeks of age ($P < 0.05$) but not at other timepoints. There was a plane of nutrition $\times$ week

Figure 2 KI67, NPY (Neuropeptide Y) and KISS1 (Kisspeptin) nuclear staining in the arcuate tissue (×400). The images illustrate different staining intensities; (A) KI67-negative staining, (B) KI67 weak staining, (C) KI67 moderate staining and (D) KI67 strong staining, (E) NPY unstained cells, (F) NPY-stained cells, (G) KISS1 negative staining, (H) KISS1 weak staining, (I) KISS1 moderate staining and (J) KISS1 strong staining. Tissues for analysis were collected at slaughter (18 weeks of age).

interaction for globulin concentrations ($P=0.06$); as LOW calves had greater globulin concentrations from 10 to 18 weeks of age ($P<0.05$). There was a plane of nutrition×week interaction for creatinine ($P<0.01$). Creatinine was not different between the planes of nutrition from week 2 to 10 ($P>0.05$). At week 14, there was a tendency for creatinine to be greater on LOW compared to HIGH ($P=0.09$) with concentrations greater on LOW at week 18 ($P<0.05$). There was a plane of nutrition×week interaction for BHB ($P<0.001$). At weaning, BHB was lower on LOW ($P<0.001$). However, at week 14, LOW had a greater BHB concentration compared to HIGH calves ($P<0.001$).

There was no effect of plane of nutrition on adiponectin concentrations ($P>0.05$). Adiponectin increased for both planes of nutrition between 2 and 18 weeks of age ($P<0.001$). There was no effect of plane of nutrition on leptin ($P>0.05$), but leptin increased linearly between 2 and 18 weeks of age ($P<0.01$). There was a plane of nutrition×week interaction for IGF-1 ($P<0.001$). There was no difference between planes of nutrition in IGF-1

concentration at either weeks 2 or 6 ($P>0.05$). At all other timepoints HIGH had a greater concentration compared to LOW calves (week 10, 2.5-fold change in IGF-1 concentration; week 14, 5-fold change; week 18, 2.9-fold change). The calves on the low plane of nutrition maintained a constant IGF-1 concentration throughout the trial period. Insulin concentrations tended to be greater in HIGH compared to LOW calves ($P=0.06$). Insulin concentrations showed little change between 2 and 10 weeks of age; however, concentrations increased between 14 and 18 weeks of age for both planes of nutrition ($P<0.01$).

Reproductive hormones

There was no effect of plane of nutrition on mean concentrations of FSH or LH ($P>0.05$; Fig. 5) but there was an effect of week on both hormones ($P<0.001$). There was a tendency towards a plane of nutrition×week of age interaction for mean concentration of testosterone ($P=0.09$) as testosterone was greater in HIGH compared to LOW calves at 14 and 18 weeks of age.

In contrast to mean concentrations, calves offered a HIGH a plane of nutrition had greater ($P<0.01$) LH AUC compared to LOW calves.

Table 3 Effect of a HIGH compared to a LOW plane of nutrition (mean±S.E.M.) on the pre-weaning average daily gain, post-weaning average daily gain, total average daily gain and live weight at the time of slaughter (18 weeks of age) of Holstein–Friesian bull calves.

	HIGH	LOW	Significance
Pre-weaning ADG (kg)	0.73±0.02	0.43±0.02	***
Post-weaning ADG (kg)	1.46±0.03	0.57±0.04	***
Overall ADG (kg)	1.08±0.03	0.50±0.03	***
Bodyweight at slaughter (kg)	160.9±3.98	107.1±3.19	***

Pre-weaning: 2–12 weeks of age; post-weaning: 12–18 weeks of age.
*** $P<0.001$.
ADG, average daily gain.

Testicular histology

Seminiferous tubule diameter was greater for HIGH compared to LOW calves ($P<0.001$; Table 4). There was a greater percentage of seminal chords with germ cells and prespermatogonia in the LOW compared to the HIGH calves ($P<0.001$). Conversely, there were a greater

Table 4 Effect of a HIGH compared to a LOW plane of nutrition (mean $\pm$ S.E.M.) on the paired testes weight and on morphological properties including seminiferous tubule diameter and stage of spermatogenesis.

	HIGH	LOW	Significance
Paired testes weight (g)	55.4 $\pm$ 3.24	31.4 $\pm$ 2.57	***
Seminiferous tubule diameter (μ m)	85.4 $\pm$ 2.09	72.5 $\pm$ 1.43	***
% Gonocyte and Prespermatogonia	31.5 $\pm$ 2.11	57 $\pm$ 1.20	***
% Spermatogonia	68.5 $\pm$ 2.11	43 $\pm$ 1.20	***
No. of Sertoli cells	28 $\pm$ 0.7	24 $\pm$ 0.6	*
Volume density of Sertoli cells	9.4 $\pm$ 0.41	8.4 $\pm$ 0.27	***

Pre-weaning: 2–12 weeks of age; post-weaning: 12–18 weeks of age. Tissue samples collected at slaughter at 18 weeks of age.

*** P <0.001; * P <0.05.

S.E.M., standard error of the mean.

percentage of seminal chords with spermatogonia in the HIGH calves compared to the LOW (P <0.001).

The lumen scores for seminiferous tubules for both planes of nutrition ranged between 1 and 4 (Fig. 6). Number of tubules with a lumen score of 1 or 2 did not differ between planes of nutrition (P >0.05). However, LOW calves had a greater number of seminiferous tubules with a score of 3 (P <0.001), but less tubules with a score of 4 (P <0.001) compared to HIGH calves.

Calves assigned to a LOW plane of nutrition had a lower number of Sertoli cells in the testes (P <0.001; Fig. 7 and Table 4) and also a lower volume density of Sertoli cells compared to HIGH calves (P <0.05; Table 4) when testes weight was included as a covariate (P <0.001).

Gene expression

Arcuate RT-PCR data highlight a tendency for an effect of plane of nutrition on *GHSR* mRNA expression (P =0.09), which was greater for LOW compared to HIGH calves. There was no effect of plane of nutrition on relative transcript abundance *KISS1*, *GPR54*, *GnRH*, *AGRP*, *NPY*, *POMC*, *MC4R*, *IGF-1*, *IGF-1R* and *OBR* expression in the accurate tissue (P >0.05; Supplementary table 1).

Anterior pituitary RT-PCR data are presented in Table 5. There was an effect of plane of nutrition on *GHSR* mRNA expression (P <0.05), which was greater for LOW compared with HIGH calves. There was no effect of plane of nutrition on *FSH β* , *LH β* , *IGF-1*, *GH1*, *GnRHR* or *IGF-1R* (P >0.05; Table 5).

For testicular tissue, we compared relative transcript abundance (i) within the calves on the two planes of nutrition and (ii) between each of the two planes of nutrition and the mature bulls. In the testes, *PCNA* mRNA expression was greater in both prepubertal groups of calves when compared with the mature bulls (P <0.001; Table 6). *AR* mRNA expression was upregulated in the mature bulls compared to both the HIGH (P <0.01) and the LOW (P =0.06) calves. *FSHR* mRNA expression was upregulated in the HIGH calves compared to the mature bulls (P <0.01). *GATA4* mRNA expression was upregulated in both HIGH and LOW calves (P <0.001) compared with the mature bulls. *AMH* in the LOW

was upregulated compared with both the HIGH calves (P <0.05) and the mature bulls (P <0.001). *CLDN11* mRNA expression was upregulated in the HIGH calves (P <0.05) and the mature bulls (P <0.001) compared

Figure 3 Effect of a HIGH compared to a LOW plane of nutrition on the plasma concentrations of metabolites of Holstein-Friesian bull calves. Black full line: low plane of nutrition; Black dotted line: high plane of nutrition; T, plane of nutrition; W, week; T*W, plane of nutrition by week interaction; NS, non-significant; * P <0.05, ** P <0.01, *** P <0.001, error bars represent standard error of the mean. P =0.07 shows a tendency for significance between the high and low planes of nutrition at week 6 and 18 for total protein.

Figure 4 Effect of a HIGH compared to a LOW plane of nutrition on the plasma concentrations of metabolic hormones of Holstein-Friesian bull calves. Black full line: low plane of nutrition, Black dotted line: high plane of nutrition; T, plane of nutrition; W, week; T*W, plane of nutrition by week interaction; NS, non-significant; *P<0.05, **P<0.01, ***P<0.001, error bars represent standard error of the mean.

with the LOW. *LHR* mRNA expression was upregulated in both HIGH calves and LOW (P<0.01) in comparison with the mature bulls. There was no effect of plane of nutrition on the relative expression of *APQ8*, *THY1*, *UCHL1* or *ZO1* (P>0.05; Table 6).

Immunohistochemistry

The arcuate nucleus was stained using KI67, KISS1 and NPY. The staining for NPY was located cytoplasmically, and there was no difference in the number of protein-positive cells per unit of area between planes of nutrition (P>0.05). There were no differences in KI67 and KISS1 protein intensity or in the number of protein-positive cells per area between planes of nutrition (P>0.05).

The testicular parenchyma was stained for the presence of AMH and GATA4. There was a greater percentage of weak AMH staining in HIGH calves in comparison with LOW (P<0.05). There were no differences found between overall GATA4 expression within the testes (P>0.05).

Discussion

Calves offered a high plane of nutrition were heavier at slaughter, had larger testes, larger seminiferous tubule diameter, more mature spermatogenic cells and more Sertoli cells. The weights of the calves at slaughter are comparable to that of Holstein-Friesian bull calves employed in other studies on similar nutritional regimes (Dance *et al.* 2015, Byrne *et al.* 2017b). Larger testes would suggest greater spermatogenic potential and is thought to reflect a greater abundance of Sertoli cells,

Figure 5 Effect of a HIGH compared to a LOW plane of nutrition on the serum concentrations of reproductive hormones. Black full line: low plane of nutrition, Black dotted line: high plane of nutrition; T, plane of nutrition; W, week; T*W, plane of nutrition by week interaction; NS, non-significant; *P<0.05, **P<0.01, ***P<0.001, error bars represent standard error of the mean; P=0.08 shows a tendency for significance between the high and low planes of nutrition at week 10 for FSH.

and therefore, the capacity to support more sperm throughout the bull's lifetime (O'Donnell *et al.* 2000). These advancements in testicular morphology were validated by transcriptomic analysis. Additionally, systemic metabolites and metabolic and reproductive hormone profiles broadly reflected the plane of nutrition

Figure 6 Effect of a HIGH compared to a LOW plane of nutrition on the lumen development of the seminiferous tubules *** $P<0.001$; Lumen Score 1: Tubules lack a lumen (solid spermatogenic cords), Lumen Score 2: Tubules contain a single vacuole, Lumen Score 3: Tubules contain several independent vacuoles, Lumen Score 4: Tubules contain several aggregating vacuoles. Tissues for analysis were collected at slaughter (18 weeks of age).

offered (Byrne *et al.* 2017b). However, there were no clear differences between the two planes of nutrition with regard to targeted gene and protein expression in either the arcuate nucleus or anterior pituitary.

The systemic concentrations of the various metabolites measured in this study were within the normal range for calves and, in general, reflected the divergent metabolic status of the animals on the two contrasting planes of nutrition employed, as well as the switch-over from mainly liquid to solid feed post-weaning at 10 weeks. For example, BHB concentrations pre-weaning were low for both groups reflecting the relatively low quantity of concentrate consumed (Overvest *et al.* 2016). However, BHB concentrations increased as calves were transitioned to solid feed post weaning and were similar for both groups prior to slaughter, indicating normal rumen development (Khan *et al.* 2011). Glucose concentrations mapped,

Figure 7 Effect of a HIGH compared to a LOW plane of nutrition on GATA4 stained Sertoli cell number ($\times 400$); (A) high plane of nutrition and (B) low plane of nutrition. The number of stained Sertoli cell nuclei present in a circular monolayer at the wall of a round seminiferous tubule was recorded (Harstine *et al.* 2017). There were a greater number of Sertoli cells in the testes of HIGH calves in comparison with their contemporaries on LOW ($P<0.001$). Tissues for analysis were collected at slaughter (18 weeks of age).

Table 5 Effect of a HIGH compared to a LOW plane of nutrition (mean $\pm$ S.E.M.) on the expression of genes in the anterior pituitary.

Gene	Plane of nutrition		Significance
	HIGH	LOW	
<i>FSHβ</i>	10.0 $\pm$ 2.89	9.2 $\pm$ 1.93	NS
<i>LHβ</i>	2.1 $\pm$ 0.42	1.8 $\pm$ 0.20	NS
<i>GHSR</i>	2.4 $\pm$ 0.31	3.6 $\pm$ 0.35	$P<0.05$
<i>GH1</i>	1.9 $\pm$ 0.27	2.6 $\pm$ 0.42	NS
<i>GnRHR</i>	2.3 $\pm$ 0.33	2.7 $\pm$ 0.36	NS
<i>IGF-1R</i>	1.8 $\pm$ 0.21	2.0 $\pm$ 0.19	NS
<i>IGF-1</i>	1.9 $\pm$ 0.37	2.3 $\pm$ 0.37	NS

The results are relative to the average of the reference genes *UBQ* and *RSP9*. Pre-weaning: 2–12 weeks of age; post-weaning: 12–18 weeks of age. Tissue samples collected at slaughter at 18 weeks of age.

NS, non-significant; S.E.M., standard error of the mean.

in general, the prevailing plane of nutrition, with concentrations markedly greater for calves on the high plane of nutrition post weaning, reflecting their much greater concentrate intake. Such conclusions have been drawn in previous studies for calves on a similar feeding regime (Byrne *et al.* 2017a). Creatinine, decreased with age for both planes of nutrition, indicative of normal renal development and is consistent with other recent reports in the literature (Byrne *et al.* 2017a). Plasma concentrations of urea were greater in the HIGH calves, reflecting their greater dietary protein consumption (Bartlett *et al.* 2006). Similar results for globulin and total protein concentrations have been observed where calves were offered restricted compared with an unlimited milk allowance (Hammon *et al.* 2002). Several studies including a recent report by Schäff *et al.* (2016) have shown that systemic concentrations of albumin increase steadily in the weeks after birth. In our study, we observed a steady increase in concentrations of albumin over time in HIGH calves, with greater concentrations compared to their contemporaries on LOW during the post-weaning period.

Systemic concentrations of leptin increase proportionally with increases in body fat (Landry *et al.* 2013) and the hormone plays an important role in energy homeostasis (Ahima *et al.* 2000). However, in our study, we did not find any difference between planes of nutrition with respect to leptin. This is in agreement with other studies using both dairy bull calves (Dance *et al.* 2015, Byrne *et al.* 2017b) and may be due to the typical low abundance of subcutaneous fat in young growing calves. However, following RNASeq analysis of the subcutaneous adipose tissue collected at slaughter, calves on HIGH had >4.5 log fold greater *LEP* expression compared to their contemporaries on LOW (English *et al.* 2018). In contrast to leptin, the adipokine adiponectin is inversely related to body fat in cattle (Sauerwein & Häußler 2016) and thus we expected that calves on the high plane of nutrition would have lower concentrations. However, adiponectin concentrations in our study increased in all calves over time;

Table 6 Effect of a HIGH and a LOW plane of nutrition (mean $\pm$ S.E.M.) compared to mature bulls on the expression of genes in the parenchyma of the testes.

Gene	MATURE	HIGH	LOW	Significance		
				M-H	H-L	M-L
<i>AMH</i>	2.6 $\pm$ 0.12	5.2 $\pm$ 0.59	6.7 $\pm$ 0.31	***	*	***
<i>PCNA</i>	5.6 $\pm$ 0.29	1.4 $\pm$ 0.10	1.3 $\pm$ 0.07	***	NS	***
<i>GATA4</i>	14.6 $\pm$ 0.56	31.7 $\pm$ 1.96	27.1 $\pm$ 1.89	***	NS	***
<i>ZO1</i>	3.8 $\pm$ 0.09	3.7 $\pm$ 0.47	4.0 $\pm$ 0.15	*	NS	NS
<i>CLAUDIN11</i>	14.7 $\pm$ 0.11	15.3 $\pm$ 0.76	12.9 $\pm$ 0.48	NS	*	**
<i>THY1</i>	4.8 $\pm$ 0.23	5.1 $\pm$ 0.92	5.5 $\pm$ 0.41	NS	NS	NS
<i>AR</i>	0.6 $\pm$ 0.01	0.4 $\pm$ 0.06	0.5 $\pm$ 0.03	*	<i>P</i> =0.06	NS
<i>FSHR</i>	9.4 $\pm$ 0.12	11.5 $\pm$ 0.37	10.2 $\pm$ 1.18	**	NS	<i>P</i> =0.06
<i>AQP8</i>	12.4 $\pm$ 0.21	14.5 $\pm$ 1.70	12.2 $\pm$ 1.41	NS	NS	NS
<i>UCHL1</i>	4.8 $\pm$ 0.41	5.8 $\pm$ 0.57	5.6 $\pm$ 0.76	NS	NS	NS
<i>LHR</i>	0.7 $\pm$ 0.17	5.4 $\pm$ 1.55	2.6 $\pm$ 0.62	***	NS	**

The results are relative to the average of the reference genes *UBQ* and *RSP9*. Pre-weaning: 2–12 weeks of age; post-weaning: 12–18 weeks of age. Mature bulls offered a commercial ration and slaughtered at 19 months of age. Tissue samples collected at slaughter at 18 weeks of age.

P*<0.05; *P*<0.01; ****P*<0.001.

H, high; L, low; M, mature; NS, non-significant; S.E.M., standard error of the mean.

age-associated increases in adiponectin concentrations have been previously reported (Heinz *et al.* 2015). It has been widely suggested that IGF-1 is a mediator of the effect of nutrition on the functionality of the HPT axis (Brito *et al.* 2007b). Consistent with this, we observed 1.5–3.5 fold greater concentrations of IGF-1 in the systemic circulation of calves on the HIGH compared with the LOW, throughout the experimental period.

The GH secretagogue receptor also known as GHSR has been found to have the greatest expression in the anterior pituitary and hypothalamus relative to any other tissue in cattle (Komatsu *et al.* 2012). Thus, ghrelin via GHSR can stimulate the direct release of GH from the somatotrophic cells in the anterior pituitary or indirectly via the GHSR in the arcuate nucleus in the hypothalamus (Reichenbach *et al.* 2012). Ghrelin concentrations in the systemic circulation are regulated by food intake (Stanley *et al.* 2005). Our finding of an upregulation of *GHSR* in both the arcuate nucleus and anterior pituitary in 18-week-old bull calves on a low plane of nutrition is consistent with the inhibitory effect of ghrelin on GnRH pulsatility in heifers (Chouzouris *et al.* 2016). Despite the well-documented relationship between GHSR and GH secretion (Sun *et al.* 2004) as well as the aforementioned positive effect of a high plane of nutrition on systemic concentrations of IGF-1, transcript abundance for *GH* in the anterior pituitary was not affected by diet in the current study. The disparity in these findings may be due to the transitory nature of gene transcripts in the brain (Bondy & Lee 1993).

Low systemic concentrations of leptin stimulate NPY/AGRP release and feed intake and inhibit POMC/CART/MC4R activity and energy expenditure (Schwartz *et al.* 2000). NPY/AGRP have opposing roles to that of POMC/ α -MSH in the control of feeding and energy expenditure (Stanley *et al.* 2005). It has been reported that *NPY* and *AGRP* were downregulated and *POMC* and α -MSH were upregulated reported in heifer calves

fed a high concentrate diet in order to achieve rapid bodyweight gain from three to seven months of age compared to contemporaries offered a high forage diet (Allen *et al.* 2012). As previously outlined, we failed to observe any effect of plane of nutrition on systemic concentrations of leptin, consistent with the lack of difference also observed for transcript abundance of *NPY*, *AGRP*, *POMC* or *MC4R* between the two planes of nutrition. Additionally, the lack of difference at the transcript level for *NPY* levels was validated using immunohistochemistry.

It has been reported that hypothalamic expression of *KISS1* and *GPR54* transcripts are at their lowest prior to puberty with maximal expression occurring at puberty, for example, in both male and female rats (Navarro *et al.* 2005). This suggests that although *GPR54* expression may increase from pre to post puberty; the number of GnRH neurons remains unchanged (Ezzat Ahmed *et al.* 2009). Short-term fasting of prepubertal female and male rats resulted in a decline in *KISS1* mRNA expression and an increase in *GPR54* mRNA expression in the hypothalamus (Castellano *et al.* 2005). These studies are contradictory of the results from our study. In addition, there were no differences observed in expression of *GnRH* either in the arcuate region of either plane of nutrition or in the expression of *GnRHR*, *FSH β* and *LH β* in the anterior pituitary gland in the current study. The lack of differences in *KISS1* mRNA was further corroborated at the protein level by immunohistochemistry. The lack of a difference in systemic leptin may contribute to the similar *KISS1* mRNA expression in the current study as kisspeptin is hypothesised to play a mediatory role between leptin- and GnRH-secreting neurons in the hypothalamus. This in turn may also explain the lack of mRNA expression difference in the anterior pituitary (Clarke *et al.* 2015).

In the seminiferous tubules, fluid filled vacuoles multiply and aggregate to form the lumen, which is

not fully functional in bulls until after 20 weeks of age (Wrobel *et al.* 1986). While a previous study has been carried out on stallions (Rode *et al.* 2015), to the author's knowledge, this is the first study to evaluate the effect of plane of nutrition on seminiferous tubule lumen development in bull calves. The HIGH calves had a more mature stage of lumen development and while no fully formed lumens were observed, nonetheless our data suggest that a more favourable metabolic status in early calfhood advances testicular functionality. Curtis and Amann (1981) reported outer seminiferous tubule diameter of $79 \pm 2 \mu\text{m}$ and $110 \pm 5 \mu\text{m}$ for Holstein–Friesian bull calves at 16 weeks and 20 weeks of age respectively and the diameters measured in the HIGH calves in our study were consistent with this range. Consistent with more mature seminiferous tubule development, there was a greater percentage of spermatogonia cells observed in the HIGH calves compared with the LOW in the current study. This indicates that both treatment groups were within the stage of development appropriate for their age. Along with the advanced germ cell development, our results also show that a greater plane of nutrition had a positive influence on the rise of testosterone, which may stimulate an early onset of puberty (Chandolia *et al.* 1997). While directly related to spermatogenic potential (Johnson *et al.* 2000), there has been no information to-date on the effect of early life plane of nutrition on Sertoli cell number in calves. Harstine *et al.* (2017) demonstrated that Angus calves treated with FSH from 1 to 3 months of age had a greater number of Sertoli cells. Sertoli cells harbour a fixed number of germ cells and Sertoli cell number can only be influenced before puberty (Johnson *et al.* 2000). Thus, our data suggest that calves offered a high plane of nutrition during the first 4–5 months of life may have the capacity to produce more sperm during their life time. Nevertheless, recent data from our research group indicate that while a bull which has been subjected to an enhanced nutritional regimen in early life may not necessarily produce more sperm at a particular ejaculate during the early post-pubertal period hastening of puberty does lead to earlier production of high-quality saleable semen and thus such bulls will typically yield a greater overall quantity of semen over the first 6–8 months post puberty (Byrne *et al.* 2018). Consistent with this, we observed a tendency towards greater systemic concentrations of FSH in the HIGH calves at 10 weeks of age, which agrees with the positive relationship between Sertoli cell number and systemic FSH (Bagu *et al.* 2004). Brito (2014) summarised work carried out on Angus and Angus $\times$ Charolais bulls receiving adequate nutrition and reported that FSH concentrations are at their highest between 10 and 14 weeks of age. This is similar to the pattern observed in the current study.

It has been reported that AMH controls the recession of Müllerian ducts in the male foetus (Rota *et al.* 2002)

and is secreted from Sertoli cells from the time of prenatal sexual differentiation until puberty (Vigier *et al.* 1984). AMH secretion is at its maximum at birth, and it declines after 8 weeks of age, in bulls (Kitahara *et al.* 2016). This validates the results of the current study where transcript abundance for *AMH* in the LOW calves was upregulated compared with both HIGH calves and mature bulls and was greater, in turn, for HIGH calves compared to mature bulls. This again was corroborated by our immunohistochemical (IHC) work. Sertoli cell proliferation predominantly occurs in the early life of the bull (Rawlings *et al.* 2008) and *GATA4* is used as a specific immunological marker for Sertoli cell nuclei in bulls (McCoard *et al.* 2001). This is consistent with the significantly greater *GATA4* expression levels in both groups of calves in comparison with the mature bulls in the current study, which is possibly due to the crossover of presumptive Sertoli cells and mature Sertoli cells. As mentioned previously, there was a greater number of *GATA4*-stained Sertoli cells in HIGH compared with LOW calves. This is consistent with recent work carried out by Harstine *et al.* (2017) who found an increase in *GATA4* stained Sertoli cells in FSH treated animals compared with untreated contemporaries. However, no differences in systemic FSH concentrations were observed in our study.

Testicular expression of *FSHR* decreases with testes development in bulls (Dias & Reeves 1982) with the number of receptors decreasing quickly from birth to 2 months of age and from then decreasing at a slower rate to 2–5 years of age (Dias & Reeves 1982). This is consistent with the greater expression of *FSHR* observed for both groups of calves compared to the adult bulls in the current study. Other studies have reported a decline in *FSHR* levels in rat and mice testes as they age (Ketelslegers *et al.* 1978). However, this result may be affected by an increase in germ cell population, causing an overall dilution of *FSHR* (Faucette *et al.* 2014). Given the absence of an effect of plane of nutrition on systemic FSH concentration or indeed *FSH β* transcript abundance in the anterior pituitary, in our study, it is not entirely surprising that there was no effect on testicular *FSHR* expression.

We observed a tendency for greater systemic concentrations of LH in HIGH calves. Other studies have shown that circulating LH is highest in bull calves between 12 and 20 weeks of age, after which concentrations begin to decline (Rawlings *et al.* 2008) and our study corroborates these findings. Similarly, it has been reported that the concentration of LH receptors in Hereford $\times$ Charolais bulls was high postnatally but decreased from 13 to 25 weeks of age, which may be due to the decline in foetal Leydig and undifferentiated Leydig progenitor cell numbers (Bagu *et al.* 2006). These authors also found that testicular LHR number increased from 25 to 56 weeks in the same bulls, when the study concluded (Bagu *et al.* 2006). It is postulated that an

increase in LHR at this stage may be due to Leydig cell maturation or an increase in Leydig cell number (Amann 1983). In our study, we also observed greater LHR expression in the testicular tissue of calves when compared with adult bulls. Systemic concentrations of testosterone have been shown to increase in beef bred bull calves following the early LH rise, which typically occurs between 8 and 20 weeks (Amann 1983). Even though no significant differences were found in peripheral LH concentration between planes of nutrition, the tendency for greater concentrations in the high plane of nutrition may have a knock on positive influence on testosterone concentration. The greater AUC recorded in HIGH compared to LOW bull calves, for LH, is possibly as a result of greater LH pulsatility in HIGH. Such a finding was reported by Brito *et al.* (2007c) when Angus and Angus $\times$ Charolais bulls offered a high vs a low plane of nutrition had, on average, 3–4 more LH pulses during a 10-h period, between 14 and 18 weeks of age. The greater testosterone concentration in the HIGH calves is consistent with the aforementioned greater systemic concentrations of IGF-1 for these animals. Given the well-documented positive effects of IGF-1 on testosterone (Dance *et al.* 2015), it is possible that the lower concentrations of IGF-1 in the LOW group affected the peri-pubertal testosterone concentrations also, by influencing Leydig cells (Brito *et al.* 2007c). This may be a contributory factor for the lower rise in testosterone concentration in the LOW calves.

The expression of AR increases during postnatal and prepubertal phases of development in the testes of primates (McKinnell *et al.* 2001) and humans (Chemes *et al.* 2008). In addition, it has been reported that AR expression fluctuates cyclically in the Sertoli cells depending on the stage of spermatogenesis in the seminiferous tubule (Smith & Walker 2014). In part agreement with the aforementioned studies, we observed greater expression of AR in testicular tissue of adult bulls compared with the HIGH calves only.

A study by Wrobel (2000) divided postnatal Simmental bull calves into groups based on their seminiferous tubule diameter and found that animals with a seminiferous tubule of 50–80 μ m (5–15 weeks old) have increased PCNA staining at the pre-Sertoli stage; however, this decreased if the pre-Sertoli cells were differentiating. Whereas, in the 80–120 μ m (18–27 weeks old), the PCNA staining was restricted to all germ cells present as pre-Sertoli cells have fully transformed into adult cells and no longer have proliferative ability. In accordance with this work, the LOW calves should have had a downregulation of PCNA compared with the HIGH, due to having less mature seminiferous tubules. Despite this obvious difference in seminiferous tubule development; however, we failed to detect a difference in PCNA expression between the groups. This highlights the discrepancies that can exist between transcript

abundance, or lack thereof, at a point in time and biological function.

In the current study, the mature bulls and the HIGH calves had a similar level of testicular expression of *CLDN11* with both groups having greater transcript abundance compared to LOW calves and suggests a favourable developmental benefit to the HIGH calves. In agreement, *CLDN11* mRNA and protein levels were lower in the immature testes of rabbits (postnatal Day 10) in comparison with those of adults (postnatal Day 180) (Park *et al.* 2011). Zonula occludens protein 1 (ZO1), also referred to as tight junction protein 1, is involved in blood–testis barrier function. Expression of ZO1 was found to be decreased in undernourished mature rams (Guan *et al.* 2014, 2015). However, in contrast, in our calves, all of which were in positive energy balance, we did not observe an effect of either stage of maturity or plane of nutrition (within calves) on transcript abundance for ZO1.

Conclusion

In summary, this is the first published study to investigate the effect of early life plane of nutrition on the molecular control and cross-talk of the HPT axis in Holstein–Friesian bull calves. While there was no clear difference between the two planes of nutrition with respect to targeted gene and protein expression in the arcuate nucleus region of the hypothalamus or in the anterior pituitary *per se*, it is clear from the analyses conducted on testicular tissue that an improved metabolic state during early calthood advances testicular development.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0671>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Ahima RS, Saper CB, Flier JS & Elmquist JK 2000 Leptin regulation of neuroendocrine systems. *Frontiers in Neuroendocrinology* **21** 263–307. (<https://doi.org/10.1006/frne.2000.0197>)
- Allen CC, Alves BRC, Li X, Tedeschi LO, Zhou H, Paschal JC, Riggs PK, Braga-Neto UM, Keisler DH, Williams GL *et al.* 2012 Gene expression in the arcuate nucleus of heifers is affected by controlled intake of high- and low-concentrate diets. *Journal of Animal Science* **90** 2222–2232. (<https://doi.org/10.2527/jas.2011-4684>)
- Amann RP 1983 Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *Journal of Dairy Science* **66** 2606–2622. ([https://doi.org/10.3168/jds.S0022-0302\(83\)82135-3](https://doi.org/10.3168/jds.S0022-0302(83)82135-3))
- Amstalden M, Alves BR, Liu S, Cardoso RC & Williams GL 2011 Neuroendocrine pathways mediating nutritional acceleration of puberty: insights from ruminant models. *Frontiers in Endocrinology* **2** 109. (<https://doi.org/10.3389/fendo.2011.00109>)
- Bagu ET, Madgwick S, Duggavathi R, Bartlewski PM, Barrett DM, Huchkowsky S, Cook SJ & Rawlings NC 2004 Effects of treatment with LH or FSH from 4 to 8 weeks of age on the attainment of puberty in bull calves. *Theriogenology* **62** 861–873. (<https://doi.org/10.1016/j.theriogenology.2003.12.021>)
- Bagu ET, Cook S, Gratton CL & Rawlings NC 2006 Postnatal changes in testicular gonadotropin receptors, serum gonadotropin, and testosterone concentrations and functional development of the testes in bulls. *Reproduction* **132** 403–411. (<https://doi.org/10.1530/rep.1.00768>)
- Bancroft JD 1996 *Theory and Practice of Histological Technique*. New York: Churchill Livingstone Publications. pp 1–725
- Bartlett KS, McKeith FK, VandeHaar MJ, Dahl GE & Drackley JK 2006 Growth and body composition of dairy calves fed milk replacers containing different amounts of protein at two feeding rates. *Journal of Animal Science* **84** 1454–1467. (<https://doi.org/10.2527/2006.8461454x>)
- Beltman ME, Forde N, Furney P, Carter F, Roche JF, Lonergan P & Crowe MA 2010 Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on Day 7 after insemination. *Reproduction, Fertility and Development* **22** 987–999. (<https://doi.org/10.1071/RD09302>)
- Bondy CA & Lee WH 1993 Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. Functional implications. *Annals of the New York Academy of Sciences* **692** 33–43. (<https://doi.org/10.1111/j.1749-6632.1993.tb26203.x>)
- Brito LFC 2014 Endocrine control of testicular development and initiation of spermatogenesis in bulls. In *Bovine Reproduction*, pp 30–38. John Wiley & Sons Inc, Oxford, UK.
- Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Boisclair YR, Ehrhardt RA & Kastelic JP 2007a Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. *Journal of Reproduction and Fertility, Supplement* **134** 171–181. (<https://doi.org/10.1530/REP-06-0353>)
- Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS & Kastelic JP 2007b Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. *Domestic Animal Endocrinology* **33** 460–469. (<https://doi.org/10.1016/j.domaniend.2006.09.004>)
- Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS & Kastelic JP 2007c Effect of nutrition during calfhood and peripubertal period on serum metabolic hormones, gonadotropins and testosterone concentrations, and on sexual development in bulls. *Domestic Animal Endocrinology* **33** 1–18. (<https://doi.org/10.1016/j.domaniend.2006.04.001>)
- Byrne CJ, Fair S, English AM, Johnston D, Lonergan P & Kenny DA 2017a Effect of milk replacer and concentrate intake on growth rate, feeding behaviour and systemic metabolite concentrations of pre-weaned bull calves of two dairy breeds. *Animal* **20** 1–8. (<https://doi.org/10.1017/S1751731117000350>)
- Byrne CJ, Fair S, English AM, Urh C, Sauerwein H, Crowe MA, Lonergan P & Kenny DA 2017b Effect of breed, plane of nutrition and age on growth, scrotal development, metabolite concentrations and on systemic gonadotropin and testosterone concentrations following a GnRH challenge in young dairy bulls. *Theriogenology* **96** 58–68. (<https://doi.org/10.1016/j.theriogenology.2017.04.002>)
- Byrne CJ, Fair S, English AM, Cirot M, Staub C, Lonergan P & Kenny DA 2018 Plane of nutrition pre and post-six months of age in Holstein-Friesian bulls: I. Effects on performance, body composition, age at puberty and post-pubertal semen production. *Journal of Dairy Science* **101** 3447–3459. (<https://doi.org/10.3168/jds.2017-13719>)
- Castellano J, Navarro V, Fernandez-Fernandez R, Nogueiras R, Tovar S, Roa J, Vazquez M, Vigo E, Casanueva F & Aguilar E 2005 Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology* **146** 3917–3925. (<https://doi.org/10.1210/en.2005-0337>)
- Chandolia RK, Honaramooz A, Bartlewski PM, Beard AP & Rawlings NC 1997 Effects of treatment with LH releasing hormone before the early increase in LH secretion on endocrine and reproductive development in bull calves. *Journal of Reproduction and Infertility* **111** 41–50. (<https://doi.org/10.1530/jrf.0.1110041>)
- Chemes HE, Rey RA, Nistal M, Regadera J, Musse M, Gonzalez-Peramato P & Serrano A 2008 Physiological androgen insensitivity of the fetal, neonatal, and early infantile testis is explained by the ontogeny of the androgen receptor expression in Sertoli cells. *Journal of Clinical Endocrinology and Metabolism* **93** 4408–4412. (<https://doi.org/10.1210/jc.2008-0915>)
- Chouzouris TM, Dovolou E, Dafopoulos K, Georgoulas P, Vasileiou NG, Fthenakis GC, Anifandis G & Amiridis GS 2016 Ghrelin suppresses the GnRH-induced preovulatory gonadotropin surge in dairy heifers. *Theriogenology* **86** 1615–1621. (<https://doi.org/10.1016/j.theriogenology.2016.05.022>)
- Clarke H, Dhillo WS & Jayasena CN 2015 Comprehensive review on kisspeptin and its role in reproductive disorders. *Endocrinology and Metabolism* **30** 124–141. (<https://doi.org/10.3803/EnM.2015.30.2.124>)
- Cooke DJ, Crowe MA & Roche JF 1997 Circulating FSH isoform patterns during recurrent increases in FSH throughout the oestrous cycle of heifers. *Journal of Reproduction and Infertility* **110** 339–345. (<https://doi.org/10.1530/jrf.0.1100339>)
- Crowe MA, Padmanabhan V, Hynes N, Sunderland SJ, Enright WJ, Beitins IZ & Roche JF 1997 Validation of a sensitive radioimmunoassay to measure serum follicle-stimulating hormone in cattle: correlation with biological activity. *Animal Reproduction Science* **48** 123–136. ([https://doi.org/10.1016/S0378-4320\(97\)00022-5](https://doi.org/10.1016/S0378-4320(97)00022-5))
- Curtis SK & Amann RP 1981 Testicular development and establishment of spermatogenesis in Holstein bulls. *Journal of Animal Science* **53** 1645–1657. (<https://doi.org/10.2527/jas1982.5361645x>)
- Dance A, Thundathil J, Wilde R, Blondin P & Kastelic J 2015 Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. *Journal of Dairy Science* **98** 987–998. (<https://doi.org/10.3168/jds.2014-8564>)
- Dias JA & Reeves JJ 1982 Testicular FSH receptor numbers and affinity in bulls of various ages. *Journal of Reproduction and Infertility* **66** 39–45. (<https://doi.org/10.1530/jrf.0.0660039>)

- English AM, Waters SM, Cormican P, Byrne CJ, Fair S & Kenny DA 2018 Effect of early calf-hood nutrition on the transcriptomic profile of subcutaneous adipose tissue in Holstein-Friesian bulls. *BMC Genomics* **19** 281–293. (<https://doi.org/10.1186/s12864-018-4681-2>)
- Evans AC, Pierson RA, Garcia A, McDougall LM, Hrudka F & Rawlings NC 1996 Changes in circulating hormone concentrations, testes histology and testes ultrasonography during sexual maturation in beef bulls. *Theriogenology* **46** 345–357. ([https://doi.org/10.1016/0093-691X\(96\)00190-2](https://doi.org/10.1016/0093-691X(96)00190-2))
- Ezzat Ahmed A, Saito H, Sawada T, Yaegashi T, Yamashita T, Hirata T-I, Sawai K & Hashizume T 2009 Characteristics of the stimulatory effect of kisspeptin-10 on the secretion of luteinizing hormone, follicle-stimulating hormone and growth hormone in prepubertal male and female cattle. *Journal of Reproduction and Development* **55** 650–654. (<https://doi.org/10.1262/jrd.20255>)
- Faucette AN, Maher VA, Gutierrez MA, Jucker JM, Yates DC, Welsh TH, Amstalden M, Newton GR, Nuti LC, Forrest DW *et al.* 2014 Temporal changes in histomorphology and gene expression in goat testes during postnatal development. *Journal of Animal Science* **92** 4440–4448. (<https://doi.org/10.2527/jas.2014-7903>)
- Fujioka H, Yamanouchi K, Akema T & Nishihara M 2007 The effects of GABA on embryonic gonadotropin-releasing hormone neurons in rat hypothalamic primary culture. *Journal of Reproduction and Development* **53** 323–331. (<https://doi.org/10.1262/jrd.18103>)
- Guan Y, Liang G, Hawken PAR, Meachem SJ, Malecki IA, Ham S, Stewart T, Guan LL & Martin GB 2014 Nutrition affects Sertoli cell function but not Sertoli cell numbers in sexually mature male sheep. *Reproduction, Fertility and Development* **28** 1152–1163. (<https://doi.org/10.1071/RD14368>)
- Guan Y, Liang G, Hawken PA, Malecki IA, Cozens G, Vercoe PE, Martin GB & Guan LL 2015 Roles of small RNAs in the effects of nutrition on apoptosis and spermatogenesis in the adult testis. *Scientific Reports* **5** 10372. (<https://doi.org/10.1038/srep10372>)
- Hammon HM, Schiessler G, Nussbaum A & Blum JW 2002 Feed intake patterns, growth performance, and metabolic and endocrine traits in calves fed unlimited amounts of colostrum and milk by automate, starting in the neonatal period. *Journal of Dairy Science* **85** 3352–3362. ([https://doi.org/10.3168/jds.S0022-0302\(02\)74423-8](https://doi.org/10.3168/jds.S0022-0302(02)74423-8))
- Harstine BR, Maquivar M, Helsel LA, Utt MD, Premanandan C, Dejarnette JM & Day ML 2015 Effects of dietary energy on sexual maturation and sperm production in Holstein bulls. *Journal of Animal Science* **93** 2759–2766. (<https://doi.org/10.2527/jas.2015-8952>)
- Harstine BR, Cruppe LH, Abreu FM, Utt MD, Cipriano RS, Lemes A, Premanandan C, Dejarnette JM & Day ML 2017 Impact of a timed-release follicle-stimulating hormone treatment from one to three months of age on endocrine and testicular development of prepubertal bulls. *Journal of Animal Science* **95** 1669–1679. (<https://doi.org/10.2527/jas.2016.1067>)
- Heinz JFL, Singh SP, Janowitz U, Hoelker M, Tesfaye D, Schellander K & Sauerwein H 2015 Characterization of adiponectin concentrations and molecular weight forms in serum, seminal plasma, and ovarian follicular fluid from cattle. *Theriogenology* **83** 326–333. (<https://doi.org/10.1016/j.theriogenology.2014.06.030>)
- Hill JW, Elmquist JK & Elias CF 2008 Hypothalamic pathways linking energy balance and reproduction. *American Journal of Physiology: Endocrinology and Metabolism* **294** E827–E832. (<https://doi.org/10.1152/ajpendo.00670.2007>)
- Johnson L & Nguyen HB 1986 Annual cycle of the Sertoli cell population in adult stallions. *Journal of Reproduction and Infertility* **76** 311–316. (<https://doi.org/10.1530/jrf.0.0760311>)
- Johnson L, Varner DD, Roberts ME, Smith TL, Keillor GE & Scrutchfield WL 2000 Efficiency of spermatogenesis: a comparative approach. *Animal Reproduction Science* **60–61** 471–480. ([https://doi.org/10.1016/S0378-4320\(00\)00108-1](https://doi.org/10.1016/S0378-4320(00)00108-1))
- Kenny DA, Heslin J & Byrne CJ 2018 Early onset of puberty in cattle: implications for gamete quality and embryo survival. *Reproduction, Fertility and Development* **30** 101–117. (<https://doi.org/10.1071/RD17376>)
- Keogh K, Waters SM, Kelly AK, Wylie AR & Kenny DA 2015 Effect of feed restriction and subsequent re-alimentation on hormones and genes of the somatotrophic axis in cattle. *Physiological Genomics* **47** 264–273. (<https://doi.org/10.1152/physiolgenomics.00134.2014>)
- Ketelslegers JM, Hetzel WD, Sherins RJ & Catt KJ 1978 Developmental changes in testicular gonadotropin receptors: plasma gonadotropins and plasma testosterone in the rat. *Endocrinology* **103** 212–222. (<https://doi.org/10.1210/endo-103-1-212>)
- Khan MA, Weary DM & von Keyserlingk MA 2011 Hay intake improves performance and rumen development of calves fed higher quantities of milk. *Journal of Dairy Science* **94** 3547–3553. (<https://doi.org/10.3168/jds.2010-3871>)
- Kitahara G, Kamata R, Sasaki Y, El-Sheikh Ali H, Mido S, Kobayashi I, Hemmi K & Osawa T 2016 Changes in peripheral anti-Müllerian hormone concentration and their relationship with testicular structure in beef bull calves. *Domestic Animal Endocrinology* **57** 127–132. (<https://doi.org/10.1016/j.domaniend.2016.06.005>)
- Komatsu M, Kojima M, Okamura H, Nishio M, Kaneda M, Kojima T, Takeda H, Malau-Aduli AE & Takahashi H 2012 Age-related changes in gene expression of the growth hormone secretagogue and growth hormone-releasing hormone receptors in Holstein-Friesian cattle. *Domestic Animal Endocrinology* **42** 83–93. (<https://doi.org/10.1016/j.domaniend.2011.09.006>)
- Koressaar T & Remm M 2007 Enhancements and modifications of primer design program Primer3. *Bioinformatics* **23** 1289–1291. (<https://doi.org/10.1093/bioinformatics/btm091>)
- Landry D, Cloutier F & Martin LJ 2013 Implications of leptin in neuroendocrine regulation of male reproduction. *Reproductive Biology* **13** 1–14. (<https://doi.org/10.1016/j.repbio.2012.12.001>)
- Lawrence P, Kenny DA, Earley B, Crews DHJ & McGee M 2011 Grass silage intake, rumen and blood variables, ultrasonic and body measurements, feeding behavior and activity in pregnant beef heifers differing in phenotypic residual feed intake. *Journal of Animal Science* **89** 3248–3261. (<https://doi.org/10.2527/jas.2010-3774>)
- Lechman RM & Toni R 2000 Functional anatomy of the hypothalamus and pituitary. In *Endotext*. (available at: <https://www.ncbi.nlm.nih.gov/books/NBK279126/>)
- McCoard SA, Lunstra DD, Wise TH & Ford JJ 2001 Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. *Biology of Reproduction* **64** 689–695. (<https://doi.org/10.1095/biolreprod64.2.689>)
- McKinnell C, Saunders PT, Fraser HM, Kelnar CJ, Kivlin C, Morris KD & Sharpe RM 2001 Comparison of androgen receptor and oestrogen receptor beta immunoreactivity in the testes of the common marmoset (*Callithrix jacchus*) from birth to adulthood: low androgen receptor immunoreactivity in Sertoli cells during the neonatal increase in testosterone concentrations. *Reproduction* **122** 419–429. (<https://doi.org/10.1530/rep.0.1220419>)
- Morton GJ, Meek TH & Schwartz MW 2014 Neurobiology of food intake in health and disease. *Nature Reviews Neuroscience* **15** 367–378. (<https://doi.org/10.1038/nrn3745>)
- Murphy EM, Kelly AK, O'Meara C, Eivers B, Lonergan P & Fair S 2018 Influence of bull age, ejaculate number, and season of collection on semen production and sperm motility parameters in Holstein Friesian bulls in a commercial artificial insemination centre. *Journal of Animal Science* **96** 2408–2418. (<https://doi.org/10.1093/jas/sky130>)
- Navarro VM, Castellano JM, Fernandez-Fernandez R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C *et al.* 2005 Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology* **146** 1689–1697. (<https://doi.org/10.1210/en.2004-1353>)
- NRC 2001 *Nutrient Requirements of Dairy Cattle*. Washington D.C.: National Academy of Sciences.
- Ochocińska A, Śnitko R, Czekuć-Kryśkiewicz E, Kepka A, Szalecki M & Janas RM 2016 Evaluation of the immunoradiometric and electrochemiluminescence method for the measurement of serum insulin in children. *Journal of Immunoassay and Immunochemistry* **37** 243–250. (<https://doi.org/10.1080/15321819.2015.1126601>)
- O'Donnell L, Stanton P & de Kretser DM 2000 Endocrinology of the male reproductive system and spermatogenesis. In *Endotext*. (available at: <https://www.ncbi.nlm.nih.gov/books/NBK279031/>)
- Overvest MA, Bergeron R, Haley DB & DeVries TJ 2016 Effect of feed type and method of presentation on feeding behavior, intake, and growth of dairy calves fed a high level of milk. *Journal of Dairy Science* **99** 317–327. (<https://doi.org/10.3168/jds.2015-9997>)

- Park CJ, Lee JE, Oh YS, Shim S, Kim DM, Park NC, Park HJ & Gye MC 2011 Postnatal changes in the expression of claudin-11 in the testes and excurrent ducts of the domestic rabbit (*Oryctolagus cuniculus domesticus*). *Journal of Andrology* **32** 295–306. (<https://doi.org/10.2164/jandrol.110.010611>)
- Ramaswamy S & Weinbauer GF 2014 Endocrine control of spermatogenesis: role of FSH and LH/testosterone. *Spermatogenesis* **4** e996025. (<https://doi.org/10.1080/21565562.2014.996025>)
- Rawlings N, Evans ACO, Chandolia RK & Bagu ET 2008 Sexual maturation in the bull. *Reproduction in Domestic Animals* **43** (Supplement 2) 295–301. (<https://doi.org/10.1111/j.1439-0531.2008.01177.x>)
- Reichenbach A, Steyn FJ, Sleeman MW & Andrews ZB 2012 Ghrelin receptor expression and colocalization with anterior pituitary hormones using a GHSR-GFP mouse line. *Endocrinology* **153** 5452–5466. (<https://doi.org/10.1210/en.2012-1622>)
- Rode K, Sieme H, Richterich P & Brehm R 2015 Characterization of the equine blood-testis barrier during tubular development in normal and cryptorchid stallions. *Theriogenology* **84** 763–772. (<https://doi.org/10.1016/j.theriogenology.2015.05.009>)
- Rota A, Ballarin C, Vigier B, Cozzi B & Rey R 2002 Age dependent changes in plasma anti-Müllerian hormone concentrations in the bovine male, female, and freemartin from birth to puberty: relationship between testosterone production and influence on sex differentiation. *General and Comparative Endocrinology* **129** 39–44. ([https://doi.org/10.1016/S0016-6480\(02\)00514-2](https://doi.org/10.1016/S0016-6480(02)00514-2))
- Sauerwein H & Häußler S 2016 Endogenous and exogenous factors influencing the concentrations of adiponectin in body fluids and tissues in the bovine. *Domestic Animal Endocrinology* **56** (Supplement) S33–S43. (<https://doi.org/10.1016/j.domaniend.2015.11.007>)
- Sauerwein H, Heintges U, Hennies M, Selhorst T & Daxenberger A 2004 Growth hormone induced alterations of leptin serum concentrations in dairy cows as measured by a novel enzyme immunoassay. *Livestock Production Science* **87** 189–195. (<https://doi.org/10.1016/j.livprodsci.2003.08.001>)
- Schäff CT, Gruse J, Maciej J, Mielenz M, Wirthgen E, Hoeflich A, Schmicke M, Pfuhl R, Jawor P, Stefaniak T *et al.* 2016 Effects of feeding milk replacer ad libitum or in restricted amounts for the first five weeks of life on the growth, metabolic adaptation, and immune status of newborn calves. *PLoS ONE* **11** e0168974. (<https://doi.org/10.1371/journal.pone.0168974>)
- Schwartz MW, Woods SC, Porte D Jr, Seeley RJ & Baskin DG 2000 Central nervous system control of food intake. *Nature* **404** 661–671. (<https://doi.org/10.1038/35007534>)
- Smith LB & Walker WH 2014 The regulation of spermatogenesis by androgens. *Seminars in Cell and Developmental Biology* **30** 2–13. (<https://doi.org/10.1016/j.semcdb.2014.02.012>)
- Stanley S, Wynne K, McGowan B & Bloom S 2005 Hormonal regulation of food intake. *Physiological Reviews* **85** 1131–1158. (<https://doi.org/10.1152/physrev.00015.2004>)
- Sun Y, Wang P, Zheng H & Smith RG 2004 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *PNAS* **101** 4679–4684. (<https://doi.org/10.1073/pnas.0305930101>)
- Thundathil JC, Dance AL & Kastelic JP 2016 Fertility management of bulls to improve beef cattle productivity. *Theriogenology* **86** 397–405. (<https://doi.org/10.1016/j.theriogenology.2016.04.054>)
- Vigier B, Picard JY, Tran D, Legeai L & Jossio N 1984 Production of anti-müllerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* **114** 1315–1320. (<https://doi.org/10.1210/endo-114-4-1315>)
- Wrobel KH 2000 Prespermatogenesis and spermatogoniogenesis in the bovine testis. *Anatomy and Embryology* **202** 209–222. (<https://doi.org/10.1007/s004290000111>)
- Wrobel KH, Schilling E & Zwack M 1986 Postnatal development of the connexion between tubulus seminiferous and tubulus rectus in the bovine testis. *Cell and Tissue Research* **246** 387–400. (<https://doi.org/10.1007/BF00215902>)

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