The Molecular Control of the Hypothalamic-Pituitary-Testicular Axis in the Bull

Submitted in accordance with academic requirements to the Department of Biological Sciences, Faculty of Science and Engineering, University of Limerick in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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Abstract

The objectives of this thesis were: 1) to understand the effect of early calf nutrition on the transcriptome of subcutaneous adipose tissue and their downstream effects on the hypothalamic-pituitary-testicular (HPT) axis in Holstein Friesian bulls, 2) to characterise the physiological, cellular and transcriptional response of the HPT to contrasting planes of nutrition during early calf-hood in Holstein Friesian bulls and 3) to describe key pathways in the HPT axis which are under nutritional control. Holstein Friesian bull calves with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, respectively, were assigned to either a high (n=10) or low (n=10) plane of nutrition. Diets were designed in order to achieve a growth rate of 1.2 and 0.5 kg/day for the high and low planes of nutrition, respectively. At 126±1.1 days of age, the calves were euthanized. In the first study, animal performance and systemic concentrations of metabolic hormones and metabolites were measured. Testicular histology, targeted gene and protein expression of the arcuate nucleus, anterior pituitary and testes were also measured. Testes from adult bulls that were reared under similar conditions were also excised for gene expression comparison. The metabolites and metabolic hormones generally reflected the improved metabolic status of the calves on the high plane of nutrition (P<0.001). Only ghrelin receptor was upregulated in the anterior pituitary (P<0.05) and ARC (P<0.10) in the low plane of nutrition compared to high plane of nutrition. 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 in accordance with both morphological and transcriptional data. This study suggests that a high plane of nutrition during the early calf-hood period alters gene expression within the HPT and testicular morphology which hastens spermatogenesis and testicular development. In the second study, arcuate nucleus from the hypothalamus, anterior pituitary and testes samples were harvested and RNASeq analysis was performed. There were 0, 49 and 1,346 genes differentially expressed in the arcuate nucleus, anterior pituitary and testicular tissue of bulls on the low relative to the high plane of nutrition, respectively (P<0.05; False Discovery Rate<0.05). Plane of nutrition had no effect on gene expression in the arcuate nucleus or anterior pituitary tissue with regard to the early onset of puberty. Low plane of nutrition down regulated the expression of genes involved in cholesterol and androgen biosynthesis in the testes. A low plane of nutrition also resulted in up-regulation of genes known to positively influence male fertility and formation of gonadal cells. This study aids in the detection of molecular based biomarkers for the selection of cattle with early pubertal onset. In the third study, subcutaneous adipose tissue samples were harvested and RNASeq analysis was performed. There were 674 genes differentially expressed in adipose tissue of calves on the low compared with the high plane of nutrition (P<0.05; FDR<0.05; fold change >2.0). Plane of nutrition altered the expression of genes across an array of putative biological processes but the most dominant cellular processes affected were cellular energy production and branched chain amino acid degradation. A high plane of nutrition caused upregulation of genes such as leptin (LEP) and adiponectin (ADIPQ), which are known to directly affect reproductive function. These results provide an insight into the effect of augmenting the plane of nutrition of Holstein-Friesian bull calves in the prepubertal period on the transcriptome of adipose tissue.
Declaration

I, hereby, declare that I am the sole author of this dissertation and that it has not been submitted to any other university or higher education institution, or for any other academic award within this University. Reference and acknowledgements have been made, where necessary, to identify the work of others.

Signature: ___________________________    Date: ______________

Anne-Marie English, B.Sc.Ed.
Dedication

To my parents

Without your love and support, I would never have believed I was capable of such a feat.
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<td>A</td>
<td>Spermatogonia A</td>
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<tr>
<td>ABAT</td>
<td>(S)-3-amino-2-methylpropionate transaminase</td>
</tr>
<tr>
<td>ACACA</td>
<td>Acetyl-CoA carboxylase-α</td>
</tr>
<tr>
<td>ACADSB</td>
<td>2-methylacyl-CoA dehydrogenase</td>
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<tr>
<td>ACAT1</td>
<td>Acetyl-CoA C-acetyltransferase</td>
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<td>Acetyl CoA</td>
<td>Acetyl coenzyme A</td>
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<td>ACO1</td>
<td>Aconitase 1</td>
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<tr>
<td>ACO2</td>
<td>Aconitase 2</td>
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<tr>
<td>ADF</td>
<td>Acid detergent fibre</td>
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<td>ADG</td>
<td>Average daily gain</td>
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<td>ALDH6A1</td>
<td>Methylmalonate-semialdehyde dehydrogenase</td>
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<td>AdipoR1</td>
<td>Adiponectin receptor 1</td>
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<td>AdipoR2</td>
<td>Adiponectin receptor 2</td>
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<td>AgRP</td>
<td>Agouti-related protein</td>
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<td>AI</td>
<td>Artificial Insemination</td>
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<td>AMH</td>
<td>Anti-Müllerian hormone</td>
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<td>α-MSH</td>
<td>Alpha melanocortin-stimulating hormone</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>AQP-8</td>
<td>Aquaporin-8</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<td>ARC</td>
<td>Arcuate nucleus</td>
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ATP
Adenosine triphosphate

AUC
Area under the curve

B
Spermatogonia B

BAT
Brown adipose tissue

BCAA
Branched chain amino acids

BCAT2
Branched chain amino acid transaminase 2

BCS
Body condition score

BCV
Biological Coefficient of Variation.

BDNF
Brain-derived neurotropic factor

bFGF
Basic fibroblast growth factor

BHB
Betahydroxybutyrate

BIC
Bayesian information criteria

BLAST
Basic Local Alignment Search Tool

BMP
Bone morphogenetic proteins

cAMP
Cyclic adenosine monophosphate

CART
Cocaine and amphetamine regulated transcript

cDNA
Complimentary Deoxyribonucleic acid

CLDN11
Claudin 11

CP
Crude protein

CRF
Corticotrophin-releasing factor

CRH
Corticotropin-releasing hormone

CS
Citrate synthase

CV
Coefficients of variations
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>D</td>
<td>Diplotene</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially expressed genes</td>
</tr>
<tr>
<td>DGAT2</td>
<td>Diacylglycerol acyltransferase 2</td>
</tr>
<tr>
<td>DHCR24</td>
<td>D24 sterol reductase</td>
</tr>
<tr>
<td>DHCR7</td>
<td>7- dehydrocholesterol reductase</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsomedial nucleus</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>Dyn</td>
<td>Dynorphin A</td>
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<tr>
<td>EBI</td>
<td>Economic Breeding Index</td>
</tr>
<tr>
<td>EBP</td>
<td>Cholesterol D-isomerase</td>
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<tr>
<td>ECHS1</td>
<td>Enoyl-CoA hydratase</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetate</td>
</tr>
<tr>
<td>EHHADH</td>
<td>Enoyl-CoA hydratase</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FABP4</td>
<td>Fatty acid-binding protein 4</td>
</tr>
<tr>
<td>FADH2</td>
<td>Reduced flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthetase</td>
</tr>
<tr>
<td>F DPS</td>
<td>Dimethylallytranstransferase</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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FGF1  Fibroblast growth factor 1
FGF2  Fibroblast growth factor 2
FH  Fumarase
FSH  Follicle stimulating hormone
FSHβ  Follicle stimulating hormone β
FSHR  Follicle stimulating hormone receptor
g  Gram
L  Grams per litre
GABA_A  Gamma-amino butyric acid type A
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GATA2  GATA binding protein 2
GATA4  Transcription factor GATA-binding protein 4
GDF-10  Growth differentiation factor 10
GH (1)  Growth hormone
GHRH  Growth hormone receptor hormone
GHSR  Ghrelin receptor
GHS-R1a  Ghrelin receptor-1a
GLP-1  Glucagon-like peptide-1
GnIH  Gonadotropin inhibiting hormone
GnRH  Gonadotropin releasing hormone
GnRHR  Gonadotropin releasing hormone receptors
GPR54  G protein-coupled receptor 54
h  Hours
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>HADHA</td>
<td>Enoyl-CoA hydratase</td>
</tr>
<tr>
<td>HADHB</td>
<td>Enoyl-CoA hydratase</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HF</td>
<td>Holstein Friesian</td>
</tr>
<tr>
<td>HH</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HIBCH</td>
<td>3-hydroxyisobutyryl-CoA hydrolase</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HPT</td>
<td>Hypothalamic-Pituitary-Testicular</td>
</tr>
<tr>
<td>Hsd17b10</td>
<td>3-hydroxy-2-methylbutyryl-CoA dehydrogenase</td>
</tr>
<tr>
<td>ICBF</td>
<td>Irish Cattle Breeding Federation</td>
</tr>
<tr>
<td>IDH3B</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL17</td>
<td>Interleukin 17</td>
</tr>
<tr>
<td>In</td>
<td>Intermediate spermatogonia</td>
</tr>
<tr>
<td>INSIG-1</td>
<td>Insulin-induced gene 1</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>IRMA</td>
<td>Immunoradiometric assay</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilodalton</td>
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<tr>
<td>KEGG</td>
<td>Kyoto Encyclopaedia of Genes and Genomes</td>
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</table>
Kg  
KISS1  
KNDy  
KO  
Kp10  
Kp13  
Kp14  
Kp54  
l  
L  
LDLR  
LEP  
LH  
LHβ  
LHR  
LSS  
LXR  
MAP  
MC4R  
MDH1  
MDS  
Min  
ml
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<th>Term</th>
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<tr>
<td>MLXIPL</td>
<td>MLX interacting protein-like</td>
</tr>
<tr>
<td>mm²</td>
<td>Millimetres squared</td>
</tr>
<tr>
<td>MR</td>
<td>Milk replacer</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MVD</td>
<td>Disphosphomevalonate decarboxylase</td>
</tr>
<tr>
<td>MVK</td>
<td>Mevalonate kinase</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>Ob/LEP</td>
<td>Leptin</td>
</tr>
<tr>
<td>Ob-R</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>Ob-Rb</td>
<td>Leptin receptor B</td>
</tr>
<tr>
<td>Oil B</td>
<td>Acid hydrolysis</td>
</tr>
<tr>
<td>P</td>
<td>Pachytene</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly-ethylene glycol</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PLIN1</td>
<td>Perilipin 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMVK</td>
<td>Phosphomevalonate</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PPARGC1A/PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha</td>
</tr>
<tr>
<td>PTM</td>
<td>Peritubular myoid</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>P450scc</td>
<td>Cytochrome P450 side chain cleavage enzyme</td>
</tr>
<tr>
<td>RBI</td>
<td>Relative Breeding Index</td>
</tr>
<tr>
<td>RLN3</td>
<td>Relaxin-3</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNASeq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>RP</td>
<td>Rathke’s pouch</td>
</tr>
<tr>
<td>RPS9</td>
<td>Ribosomal protein S9</td>
</tr>
<tr>
<td>RFRP</td>
<td>RF-amide related peptide</td>
</tr>
<tr>
<td>RFRP-3</td>
<td>RF-amide related peptide-3</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>Sa</td>
<td>Spermatids type Sa</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical analysis software</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>Sb1</td>
<td>Spermatids type Sb1</td>
</tr>
</tbody>
</table>
Sb2  Spermatids type Sb2
Sc   Spermatids type Sc
SCARB1 Scavenger receptor B1
SCC  Somatic cell count
S.D. Standard Deviation
Sd1  Spermatids type Sd1
Sd2  Sperm
s.e.m. Standard error of the mean
SHBG Sex hormone binding globulin
SREBF1 Sterol regulatory element binding factor 1
SREBP-c Sterol regulatory element binding protein-1c
SS   Secondary spermatocytes
STAR Spliced Transcripts Alignment to a Reference
SUCLG1 Succinyl-CoA synthetase 1
SUCLG2 Succinyl-CoA synthetase 2
SVF  Stromal vascular fraction
TCA  Tricarboxylic acid
TDN  Total digestible nutrients
TGFβ Transforming growth factor β
THY1 Thy-1 cell surface antigen
TM7SF2 D14 sterol reductase
TRH  Thyrotropin-releasing hormone
UBQ  Ubiquitin
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tr>
<td>UCHL1</td>
<td>Ubiquitin carboxyl-terminal esterase L1</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WNT2B</td>
<td>Wnt family member 2B</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventromedial nucleus</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta</td>
</tr>
<tr>
<td>Z</td>
<td>Zygotene</td>
</tr>
<tr>
<td>ZO1</td>
<td>Zonula occludens protein 1</td>
</tr>
<tr>
<td>2D</td>
<td>2-dimensional</td>
</tr>
<tr>
<td>Δ-4</td>
<td>Delta-4</td>
</tr>
<tr>
<td>Δ-5</td>
<td>Delta-5</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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CHAPTER 1: Literature Review
1.1 Introduction

The Agricultural food and drink sector is Ireland’s largest indigenous industry with an annual output of over €10.8 billion (BordBia, 2016). The dairy industry is the most profitable farming enterprise with total dairy and ingredients exports in 2015 of €3.24 billion (BordBia, 2016) and the Food Wise 2025 report has set an ambitious target of a 60% increase in primary production to €10 billion in Ireland by the year 2025 (DAFM, 2015). To achieve this in the dairy sector, the industry requires an annual productivity improvement, mainly through better genetics, of 1.5 to 2 per cent per year (DAFM, 2011).

In 2002, the Irish Cattle Breeding Federation (ICBF) introduced the Economic Breeding Index (EBI), a single figure profit index which aids in the identification of the most elite and profitable bulls to breed replacement heifers, in response to a decline in fertility in the national dairy herd over the previous two decades (Mee, 2004). The EBI replaced the Relative Breeding Index (RBI) which valued animals based on milk production traits only such as fat and protein yield (Berry, 2007). The EBI is comprised of seven sub-indexes related to profitable milk production; production (fat and protein content), fertility (calving interval and survival), calving performance (direct calving difficulty, maternal calving difficulty, gestation length and calf mortality), beef carcass (cull cow weight, carcass weight, carcass conformation and carcass fat), maintenance, management (milking time and milking temperament) and health (lameness, somatic cell count (SCC) and mastitis). Through its use the next generation of replacement heifers will be genetically superior to their parents in terms of their increased ability to produce, from a mainly forage based diet, more milk solids (protein and fat) per cow per year while maintaining a high rate of fertility.
In 2009, Ireland began to select dairy bulls for use in artificial insemination (AI) programmes using genomic assisted selection technologies which has augmented the annual increase in genetic gain of the national dairy herd. Genomic selection increases the reliability of the breeding value for individual animals. The most elite bulls can now be identified, from their DNA (deoxyribonucleic acid) profile, within days/weeks of birth and are now entering AI stations as soon as they reach puberty (Schefers and Weigel, 2012). The age at which these young bulls attain puberty can range from 8-11 months (Rawlings et al., 2008), although this is influenced by management. These young bulls invariably yield a low volume of semen with a high proportion of abnormal sperm (Lunstra and Echternkamp, 1982). For example, a 10-12 month old bull typically produces 50-150 frozen insemination doses per ejaculate (compared to 500-1000 for a mature bull) and in addition, semen can normally only be collected once per week, compared to 3 times per week for a mature bull (R. Monaghan, National Cattle Breeding Centre, Enfield, Co. Meath, Ireland; personal communication). This represents up to a 60 fold reduction in output from a young compared to a mature bull and given that these young bulls have the highest genetic merit, demand for semen far exceeds supply thus limiting genetic improvement (Ball and Peters, 2004) of the national dairy herd. Ireland’s dairy industry operate in a seasonal system to ensure compact calving to coincide with grass growth and therefore these young bulls are often only in high demand during this season. Therefore, it is imperative that we gain a greater understanding of the effect of early calf-hood nutrition on the molecular pathways controlling the hypothalamic-pituitary-testicular axis. This will advance the knowledge of the onset of puberty in Holstein-Friesian bulls and the factors that affect sperm production in order to design the best rearing protocols to be used in specialised rearing units for these elite and valuable
bulls so as to hasten puberty and facilitate the collection of high volumes of good quality semen.

1.2 Anatomy and Physiology of the Hypothalamus and Pituitary Gland

1.2.1 Hypothalamus

The hypothalamus consists of a ten populations of neurons called nuclei (Figure 1.1). The hypothalamus is acknowledged as the homeostatic regulator of the body (Kandel and Schwartz, 1991), as it receives information related to factors including salt and water levels and blood pressure. It also regulates metabolism through regulating glucose, free fatty acids and amino acids, circulating peptides such as as insulin like growth factor-1 (IGF-I), growth hormone (GH), leptin and insulin and gastro-intestinal hormones such as ghrelin and makes compensatory alterations designed to keep them within desirable parameters (Schwartz et al., 2000, Segal, 2006, Cota et al., 2006, Morton et al., 2006). The hypothalamus communicates for the initiation and control of all bodily functions by signalling via axons to distal brain sites and by directing hormone release from the pituitary, via the infundibulum (Pocock et al., 2013).

When the body is in a severe energy deficit, metabolic signals in the form of hormones emanating from the gastrointestinal tract, adipose tissue and other peripheral organs are sent from hypothalamic neurons involved in satiety and energy homeostasis namely the arcuate nuclei (ARC) to the preoptic nuclei that stimulate gonadotropin release. The metabolic signals have a crucial effect on the reproductive axis as it has the capacity to respond to changing concentrations of metabolic signals (Hill et al., 2008). Therefore, the ARC and preoptic nuclei in the hypothalamus manipulate the timing of sexual development via the regulation of gonadotropin-releasing hormone (GnRH) secretion in bulls (Brito et al., 2007a).
Figure 1.1: Diagrammatic representation of the nuclei arrangement in the hypothalamus and the relationship between the hypothalamus and the pituitary gland. The region encompassed by the curly brackets is the hypothalamus (BiologicalDiscussion.com, 2006).

1.2.2 Arcuate Nuclei

The ARC is located adjacent to the third ventricle and the median eminence which lacks a blood-brain barrier and its main function is in the regulation of both the metabolic and reproductive functions by communicating peripheral weight regulating signals to other regions of the brain (Filby et al., 2008, Allen et al., 2012, Schwartz et al., 2000). The ARC consists of metabolic sensing neurons and reproductive related neurons (Redmond et al., 2011). Neuropeptide Y (NPY), agouti-related protein (AgRP) and proopiomelanocortin (POMC) neurons are known to respond to changes in metabolism (McShane et al., 1993, Stanley et al., 2005) and mediate nutritional effects on reproduction in heifers (Amstalden et al., 2014). There are no leptin receptors on GnRH neurons and therefore, the effects of leptin are mediated via kisspeptin (KISS1). KISS1 is
mainly secreted in the ARC (Gottsch et al., 2004, Estrada et al., 2006) and has been shown to stimulate GnRH secretion in the hypothalamus. The ARC is known as the primary hypothalamic site of action for peripheral hormones and transmits these signals to secondary hypothalamic such as the preoptic nuclei area (POA), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and paraventricular nucleus (PVN) (Stanley et al., 2005).

1.2.3 Pre-optic Nuclei

The POA contains the majority of the cell bodies of GnRH secretory cells in the hypothalamus and thus has the most control of the reproductive axis by integrating both nutritional and hormonal responses (Maruska and Fernald, 2011). The GnRH nerve projections originating from the POA attach to the median eminence, where they secrete GnRH hormones into the hypophyseal portal system (Fujioka et al., 2007). Atrial natriuretic peptide and other natriuretic peptides that contain oestrogen receptors are in abundance in the POA of the female rat and play a major role in stimulating the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from anterior pituitary cells (Watson et al., 1994). Kisspeptin neurons are also found in POA and mediate the metabolic signals effect on GnRH (Hassaneen et al., 2016).

1.2.4 Other Hypothalamic Nuclei

The VMN also plays a critical role in energy homeostasis. It comprises the neuron: gamma-amino butyric acid type A (GABA_A) and anorexigenic brain-derived neurotropic factor (BDNF; Roth and Sathyanarayana, 2012). It has been reported that BDNF interacts with leptin to aid in the regulation of appetite (Komori et al., 2006) and has been implicated in regulating energy homeostasis via the melanocortin system (Stanley et al., 2005b, 2005). The VMN obtains NPY, AgRP and alpha melanocortin-
stimulating hormone (α-MSH) projections from the ARC and the VMN transmits these signals via projections to the DMN (Stanley et al., 2005).

It has been found that NPY, AgRP and α-MSH projections from the ARC influence two neuron populations in the PVN (Stanley et al., 2005). Paraventricular nucleus has two types of cells that respond to NPY, AgRP and α-MSH which are tonically firing neurosecretory cells and preautonomic cells (Roth and Sathyanarayana, 2012). The PVN releases corticotrophin-releasing factor (CRF) and thyrotropin-releasing hormone (TRH), both appetite suppressing peptides (Morton et al., 2006, Berthoud and Morrison, 2008).

1.2.5 Pituitary Gland

This small gland is the master gland of the brain, situated beneath the hypothalamus in the sella turcica. The pituitary gland is segregated into two regions; the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis). The anterior and posterior lobes are divided by an intermediate lobe (Figure 1.1).

1.2.6 Anterior Pituitary Gland

The organogenesis of the anterior pituitary in the bovine has not been defined, whereas, the differentiation and development of the mouse anterior pituitary has. At embryonic Day 8.5, cells in the oral ectoderm form the embryonic pituitary, otherwise known as the Rathke’s pouch (RP; Figure 1.2; Treier et al., 1998). By embryonic Day 16.5, all RP cells have been assigned a cell type and are expressing the specific mRNA (messenger ribonucleic acid) for that cell type (Denef, 2003). The assignment of RP cells to a specific anterior pituitary cell type occurs immediately after the cells emerge from proliferation (Treier et al., 1998, Ericson et al., 1998). However, they will not progress to functioning
anterior pituitary cells until they have been activated or inhibited by various transcription factors (Lamolet et al., 2001, Zhao et al., 2001).

These anterior pituitary cell types included corticotropes, thyrotropes, somatotropes, lactotropes and gonadotropes; with each cell type secreting specific hormones, in a pulsatile manner (Yeung et al., 2006; Figure 1.2). The hormones secreted by the hypothalamus (TRH, GnRH, growth hormone-releasing hormone (GHRH), corticotropin-releasing hormone (CRH), somatostatin and dopamine) travel through the hypothalamic-hypophyseal portal system and act on G protein-coupled receptors. The level of responsiveness of the anterior pituitary cells to GnRH can be altered by GnRH level, negative testosterone feedback from the testes and circadian rhythm (Yeung et al., 2006). The gonadotrophic cells in the anterior pituitary are responsible for the regulation of testicular function through secretion of gonadotropins, LH and FSH (Schanbacher, 1982).

Figure 1.2: (A) The development of the pituitary gland. (B) The hypophyseal portal system connecting the hypothalamus and the anterior pituitary (Yeung et al., 2006).
1.3 The Effects of Nutrition on the Brain and Neuroplasticity

Undernutrition and over nutrition can cause temporary or critical effects to all organs especially the central nervous system depending on its duration of effect. The neurons in the central nervous system have the capability to disassemble and reassemble in response to changes in the physiological environment such as stress, malnutrition, sleep, hormones and drugs (Sodhi and Sanders-Bush, 2004). These adaptations include alterations in gene expression, changes in neurotransmitter release or changes in phenotype or behaviour (Sodhi and Sanders-Bush, 2004). This action of adaptation is referred to as brain plasticity, which is present as two forms; neuronal plasticity and synaptic plasticity. There are two critical or vulnerable periods in brain development (Calikoglu et al., 2001); the initial development of the foetus and early in postnatal life; length depending on species. For examples in rodents, the first 3 weeks of postnatal life are the most crucial to brain development and neuroplasticity (Bedi and Warren, 1988, Balasz et al., 1986, Dobbing, 1981). As the brain is developing it is thought that at this stage it overdevelops by producing more connections and cells than necessary. Unused connections are trimmed according to their level of activity which reflects the principle “use it or lose it” (Shors et al., 2012).

1.4 Male Reproductive Anatomy and Physiology

The reproductive tract of the bull consists of the testes, the secondary sex organs and the accessory sex glands. All the organs work in tandem for the formation, maturation and transportation of sperm. The secondary sex organs include the penis, the epididymis and the vas deferens, the urethra and the accessory glands include the ampullary glands, the seminal vesicles, the prostate gland and the bulbourethral or Cowper’s gland. In the bull
only the testes remain external to the body. The penis remains in the body protected by the prepuce until erection.

### 1.4.1 Testes

The main functions of the testes are the biosynthesis of androgens by the Leydig cells; and sperm production by the seminiferous tubules (Schanbacher, 1982). The testes vary in size, location and shape between species. In the bull, the testicles are located near the sigmoid flexure and are held exterior and perpendicular to the body in the scrotum. Bovine testes must be kept between 4-5 °C below core body temperature for effective spermatogenesis (Thundathil et al., 2012). If the temperature of the testes drops below this defined temperature, the testes can be pulled closer to the body by the dartos tunic muscle at the bottom of the scrotum and/or by the cremaster muscle within the spermatic chord and vice versa in warm weather.

Each of the testes consists of convoluted seminiferous tubules which are divided into lobes by the septa or trabeculae. Within each lobule, there are between 1 to 4 seminiferous tubules embedded in tissue containing blood vessels, nerves and Leydig cells. The lobules are encapsulated by a layer of connective tissue called the tunica albuginea which itself is covered by a visceral and a parietal layer of tunica vaginalis. The seminiferous tubules consist of three cell types: peritubular myoid (PTM) cells, Sertoli cells and germ cells (Smith and Walker, 2014). The Sertoli, Leydig and germ cells will be discussed later. The PTM cells which surround the external wall of the seminiferous tubule in the testes of the bull resemble myofibroblasts and contract to transport sperm through the network of ducts in the testes. Adult bovine testis vary in size depending on breed but typically each testis weighs between 300-400g (Bearden et al., 2004).
1.4.2 Secondary Sex Organs

Once sperm are formed in the seminiferous tubules, they must make their way through the rete testis to the epididymis (Figure 1.3). The epididymis is attached to one side of the testicle and is divided into three regions, the caput (head), corpus (body) and cauda (tail). The epididymis is purported to have four major functions including the transport of sperm from the testes to the vas deferens, the maturation of developing sperm, storage of viable sperm cells and the concentration of sperm by surplus fluid reabsorption (Whittier, 1993). Immature sperm undergo a period of maturation in the epididymis, which involves the completion of differentiation and attainment of motility, lasting between 10-15 days before passing into the vas deferens.

Figure 1.3: Diagram of the anatomy of the testis of the bull (Hafez and Hafez, 2000)
1.4.3 Accessory Glands

The seminal vesicles are lobulated glands that lie laterally to the ends of both the vas deferens (Figure 1.4). The seminal vesicles are comprised of alveoli which are covered with secretory epithelium (Roberts, 2010) and provide approximately 70% of the fluid volume of the ejaculate. The secretions from the seminal vesicles are composed of fructose, prostaglandins and numerous androgen-dependent secretory proteins that are needed for clotting of the ejaculate and protection of the sperm (Roberts, 2010).

The prostate gland is located at the neck of the bladder where it empties into the urethra. It consists of lobes that originate from the primitive urethra and these lobes are composed of alveoli; constructed similar to those of the seminal vesicles (Roberts, 2010). It can be divided into two portions; the external portions (corpus prostatae) and the internal portion (pars disseminate prostatae) (Dellman and Wrobel, 1976). The prostate is poorly developed in the bull and its secretions are low in volume.

The third accessory gland, the Cowper’s glands or bulbourethral glands are small, firm glands located on either side of the urethra. It is believed that one of the chief functions of their secretion is to cleanse the urethra of any residue of urine which might be harmful to sperm (Deutscher, 1980). The clear secretion that often drips from the penis during sexual excitement prior to service is largely produced by these glands (Deutscher, 1980).
1.4.4 Sertoli Cells

Sertoli cells are somatic cells located in the seminiferous tubules (Tripathi et al., 2014). Their cytoplasm, which holds the developing germ cells, extends from the lamina propria to the lumen of the seminiferous tubule (Amann and Schanbacher, 1983). Each Sertoli cell is bound together via tight junctions. In the bull in early postnatal life, undifferentiated Sertoli cells form and by approximately 30-40 weeks the adult Sertoli cells are in place (Rawlings et al., 2008). Sertoli cell number is fixed at puberty (Sharpe et al., 2003, Buzzard et al., 2003) and is determined prior to the formation of the fully functional blood-testis barrier at puberty (Orth, 1993, Bardin et al., 1994) and Sertoli cell number does not change post pubertally. Proliferation of Sertoli cells during the peripubertal period have been linked with an increase in concentrations of LH, testosterone (Amann and Walker, 1983) and FSH (Allan et al., 2004). Sertoli cells control the communication across the blood-testis barrier which provides a favourable environment for the development of the germ cells to sperm.
The most critical role that these cells play is in the hormonal control of spermatogenesis which is induced by FSH. They secrete both inhibin and activin which feedback to the hypothalamus. These cytokines stimulate the Leydig cells to secrete testosterone via an autocrine pathway (Yao et al., 2011). Sertoli cells can also inhibit testosterone synthesis by releasing oestrogen and transforming growth factor-\(\alpha\), in response to follicle-stimulating hormone (Yao et al., 2011).

### 1.4.5 Leydig Cells

Leydig cells are large, polyhedral cells with spherical nuclei and are located in the testes adjacent to the seminiferous tubules (Mendis-Handagama and Siril Ariyaratne, 2005). Leydig cells form from mesenchymal cells and differentiate from fetal to mature Leydig cells (Chamindrani Mendis-Handagama and Siril Ariyaratne, 2001). Fetal Leydig cell number in bull calves declines during the early postnatal period (Hooker, 1970). The initiation of differentiation and maturation of adult Leydig cells in bulls begins around 12 to 16 weeks and lasts until 28 weeks of age (Amann and Schanbacher, 1983). Their main function is in the production of androgenic steroids, such as testosterone, androstenedione and dihydrotestosterone in males as well as oestrogen in the boar (Dellman and Wrobel, 1976). The anterior pituitary’s secretion of LH is critical to sustain the function and structure of the Leydig cells in the postnatal testis. The cytoplasm in Leydig cells contains smooth endoplasmic reticulum, mitochondria and peroxisomes. The metabolism of testosterone takes place in the mitochondria for the first step, which involves the cytochrome P450 enzyme, CYP17A1 (Amann and Schanbacher, 1983) and the second step takes place in the smooth endoplasmic reticulum (van der Molen, 1981). Testosterone is then secreted by the smooth endoplasmic reticulum of the Leydig cells.
1.5 Adipose

Adipose tissue can be categorised into white and brown adipose tissue. White adipose tissue (WAT) can be divided into two classes; visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). The SAT can be located around the gluteofemoral region (buttock and thigh). The omental region (deposit near the stomach and spleen and can expand into the ventral abdomen), mesenteric region (deposit is attached in a web-form to the intestine), retroperitoneal region (deposit are found along the dorsal wall of the abdomen and surrounding the kidney), pericardial (a regional fat deposit that surrounds the heart) and surrounding the gonads are all categorised as VAT (Bjorndal et al., 2011). Brown adipose tissue (BAT) is only located above the clavicle (collarbone) and in the subscapular (shoulder blade) region (Bjorndal et al., 2011). Both tissue types have different functions; BAT is critical in the process of transference of energy to heat through thermogenesis mediated by mitochondrial UCP1 (Cannon and Nedergaard, 2004), while WAT holds the energy reserve by storing triacylglycerol during periods of excess and mobilising these stores during periods of deprivation (Gregoire et al., 1998). However, WAT also plays a major role as an endocrine organ. The function of WAT in metabolic and reproductive processes is complex. However, new reports have been published indicating a new type of recruitable brownish adipocytes named beige adipocytes, which are found among white adipocytes, especially in SAT (Wu et al., 2012). Beige adipocytes are recruited after a short-term cold experience (Jiang et al., 2017). They resemble WAT in having extremely low basal expression of UCP1, but like BAT, they respond to cyclic AMP stimulation with high UCP1 expression and respiration rates (Wu et al., 2012).
Adipocytes are acutely sensitive to changes in nutritional status and therefore, are under the regulation of hormones such as insulin (Bjorndal et al., 2011). Hormones such as leptin and adiponectin (AdipoQ) which are secreted from adipocytes within WAT are understood to play a part in the regulation of reproduction. Leptin increases proportionally in line with an increase in body fat and signals energy abundance (Landry et al., 2013) and high AdipoQ levels has been found to negatively affect the reproductive network (Campos et al., 2008).

There are several cell types that encompass WAT which can be divided into adipocytes and stromal vascular fraction (SVF) which includes: pre-adipocytes, fibroblasts, macrophages and vascular cells (Sorisky et al., 2013). Mesenchymal stem cells (MSC) are pluripotent and have the capacity to differentiate into adipocytes, osteoblast, chondrocytes and myocytes (Lowe et al., 2011). In the correct environment and gene expression signals, MSC can differentiate into preadipocytes (Scott et al., 2011). There are many signals that influence MSC to form adipocytes, such as extracellular factors including bone morphogenetic proteins (BMP) (Huang et al., 2009), Transforming growth factor β (TGFβ) (Zamani and Brown, 2011), IGF-I (Kawai and Rosen, 2010), interleukin 17 (IL17) (Zuniga et al., 2010), fibroblast growth factor 1 and 2 (FGF1, FGF2; (Widberg et al., 2009, Xiao et al., 2010), activin (Zaragosi et al., 2010) and WNT and hedgehog (HH) (Marion et al., 2009). Once preadipocytes are committed to the path of adipocyte differentiation, it is critical for these committed cells to go through a period of growth arrest (Gregoire et al., 1998). The correct environmental gene expression cues are then necessary for post confluent mitosis and clonal expression to take place and the subsequent terminal differentiation into a mature adipocyte (Gregoire et al., 1998).
However, it must be noted that different precursors are needed for the development of WAT and BAT.

Cellular hypertrophy (an increase in cell size) and hyperplasia (an increase in cell number) are both critical for adipose growth. Growth patterns of adipose depend on species, physiological or nutritional factors. Studies in Hereford x Angus and Holstein steers found that by 14 months of age hyperplasia was completed in all but the intermuscular adipose tissue (Hood and Allen, 1973). Nutritional restriction in Friesian bull calves from birth to 95 days of age resulted in a 40 % reduction in growth rate, a 68 % decrease in lipid deposition, a reduction in adipose cell hypertrophy but no effect on adipose cell number (Robelin and Chilliard, 1989). This demonstrates that nutritional restriction in early calf hood negatively effects adipose cell size but not number.

1.6 Metabolic Control of Reproduction

Even though, reproduction is essential for the survival of any species, it can be perturbed, if sufficient energy resources are not available for this energy demanding function (Roa and Tena-Sempere, 2014). There is no doubt that the fulfilment of the reproductive function, including oestrous cycle, pregnancy, milk production and nourishment of offspring is far more energy demanding for the female than the fulfilment of the reproductive function for the male. However, premating interactions such as searching for females in oestrus, fighting for territory and dominance over other males, and repeated copulations are energy consuming for the male. Energy costs of mating could be considerable, particularly during the breeding season for males of wild species, as reflected by a decline in time spent feeding resulting in weight loss. The reproductive axis has the ability to respond to shifting metabolic concentrations caused by restricted growth and body weight gain (Hill et al., 2008). This response is evident for not only the
deprivation of energy stores but also for consistent energy surpluses, such as obesity (Roa and Tena-Sempere, 2014).

Nutritional restriction during the postnatal period has the biggest impact on the timing of reproductive maturity by inhibiting GnRH release and therefore the release of LH (Day et al., 1986). Malnutrition constitutes a greater stress to spermatogenesis in prepubertal males than in post pubertal males (Brito et al., 2007b). Restricted feed during calf hood has been shown to impede the hypothalamic GnRH pulse generator and through its interaction with the anterior pituitary gland, impair steroidogenesis in the testes, delay puberty and decrease testicular weight at 70 weeks of age (Brito et al., 2007a). Studies have shown that a high plane of nutrition before 31 weeks of age leads to an earlier onset of puberty in Holstein-Friesian bulls (Dance et al., 2015, Byrne et al., 2017b). However, restricted feed during the prepubertal period (prior to 26 weeks) affects the early gonadotropin rise (which begins between 10-12 weeks) and age at which bulls reach puberty, regardless of the plane of nutrition fed from 27 weeks to 70 weeks of age (Brito et al., 2007a). There was a greater testosterone concentration at 16 weeks of age in Holstein Friesian bulls on a high diet pre-six months which coincided with increase in scrotal circumference growth in these bulls (Byrne et al., 2017b). Holstein bulls fed a low, medium and high diet from 1 to 80 weeks of age reached puberty at 57, 49 and 43 weeks of age, respectively (Bratton et al., 1956).

1.7 Spermatogenesis

Spermatogenesis comprises of a series of cellular divisions and differentiations that result in a haploid male germ cell. The three stages of spermatogenesis are spermatocytogenesis, meiosis and spermiogenesis (Figure 1.5) and combined have a duration of approximately
61 days in the bull (Amann and Schanbacher, 1983, Amann, 1983). These stages take approximately 21, 23 and 17 days, respectively (Johnson et al., 2000).

Proliferation of the spermatogonia is carried out via mitosis. During the 21 days of spermatocytogenesis, spermatogonia A undergo mitotic divisions to produce intermediate (In), spermatogonia B (Johnson et al., 2000). Tight junctions form behind the cells and the junctions relax in front of them so that they can progress through the compartments and not compromise the integrity of the blood-testes barrier (Figure 1.6). For the next 23 days, the primary leptotene spermatocytes go through stages of meiosis differentiating through zygotene (Z), pachytene (P) and diplotene (D) before going through the first meiotic division to form secondary spermatocytes (Johnson et al., 2000). In the final stage, spermiogenesis, which takes 17 days in the bull, the secondary spermatocytes go through a second meiotic division to produce spermatids. Spermatids mature into round spermatids and then progress onto long spermatids. Finally, long spermatids begin to develop flagella and acrosomes which denote them as mature sperm (Bagu et al., 2006, Curtis and Amann, 1981, Wrobel, 2000, Rawlings et al., 2008, Abdel-Raouf, 1960).
Figure 1.5: Stages of spermatogenesis. The letters indicate the developmental step, and the numbers associated with each germ cell step indicate the developmental age of each cell type in the middle of each spermatogenic stage. A spermatogonia (A), intermediate (In), B spermatogonia (B), leptotene primary spermatocytes (L), zygotene (Z), pachytene (P), diplotene (D), secondary spermatocytes (SS), spermatids type Sa (Sa), spermatids type Sb1 (Sb1), spermatids type Sb2 (Sb2), spermatids type Sc (Sc), spermatids type Sd1 (Sd1), sperm (Sd2) (Johnson et al., 2000)
Figure 1.6: Illustration of the seminiferous tubule showing the development of the germ cells and relationship to the adjacent Sertoli cells (Amann and Schanbacher, 1983)

1.8 Puberty

There are a series of changes in the reproductive system from the new-born and infantile stage, to the eventual onset of puberty and the movement into adulthood (Fink, 2000, Tena-Sempere and Huhtaniemi, 2003). The definition of puberty is defined as the age that a bull first produces an ejaculate yielding at least $50 \times 10^6$ spermatozoa with a minimum of 10% motility (Wolf et al., 1965).
As the spermatogonia progress and mature they move out from the basal membrane, towards the lumen of the tubule. Once the spermatogonia enter the adluminal compartment, spermatogonia B develop into leptotene primary spermatocytes at approximately 20 weeks of age (Abdel-Raouf, 1960, Curtis and Amann, 1981, Bagu et al., 2006). In Holstein bulls, the spermatogonia stage of development predominates at approximately 16 weeks (Evans et al., 1996) and primary spermatogonia increase in concentration up until 44 weeks (Abdel-Raouf, 1960). Secondary spermatocytes numbers increase between 20-30 weeks of age (Abdel-Raouf, 1960, Curtis and Amann, 1981, Bagu et al., 2006). Primordial germ cells also known as gonocytes, the precursors of sperm can be found from birth up to approximately 30 weeks of age in the basal compartment of the seminiferous tubules (Bagu et al., 2006, Curtis and Amann, 1981, Wrobel, 2000, Rawlings et al., 2008). In the final stage, the secondary spermatocytes mature into round spermatids which look like typical cells from 25-30 weeks of age and then progress onto long spermatids. Long spermatids begin to develop flagella and acrosomes which denote them as mature spermatozoa or sperm between 32 and 40 weeks of age (Abdel-Raouf, 1960, Curtis and Amann, 1981, Bagu et al., 2006).

Breeds differ in age and body weight at puberty. It is known that dairy bulls such as Holstein bulls reach puberty earlier than beef breeds such as Aberdeen Angus and considerably earlier than Charolais (Jainudeen and Hafez, 2000). Crossbred beef animals reach puberty at an earlier age than purebred equivalents (Jainudeen and Hafez, 2000). Well-fed Holstein bulls achieved puberty at a mean age of 39 to 41 week (Amann, 1983). Holstein-Friesian bulls on a high diet pre-six months reached puberty approximately 30 days earlier than bulls on a low diet (283 ± 5.6 vs. 323 ± 6.5 days of age), respectively (Byrne et al., 2017b).
1.9 Physiological Mechanisms controlling Pubertal Development

There are a number of key pathways that aid in the regulation of development of the reproductive axis. Some of the hormones involved will be discussed below.

1.9.1 Gonadotropin Releasing Hormone

Gonadotropin-releasing hormone is a vital peptide that mediates the neural control of reproductive physiology and fertility (Cohen-Tannoudji et al., 2012, Constantin, 2011, Anjum et al., 2012). The structure of GnRH consists of a linear sequence of pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Agr-Pro-Gly-amide (Schally et al., 1971), which is common across mammalian species (Schally et al., 1971, Conn et al., 1987); however, differences in this structure has being found in non-mammalian species such as the lamprey, chicken and salmon (Sherwood et al., 1986). Across the vertebrate species there are currently 23 identified GnRH analogues (Millar, 2005).

The main role of GnRH is in the modulation of the neuroendocrine control of the reproductive system by means of an episodic, pulsatile secretion stimulated by the hypothalamus; known as the “hypothalamic GnRH pulse-generator” (Chu et al., 2012). From the hypothalamus, GnRH travels via neurons down the infundibulum to the anterior pituitary where it stimulates the synthesis and release of the gonadotropins, FSH and LH; these hormones act on the Sertoli and Leydig cells, respectively, to stimulate spermatogenesis and androgen production (Filby et al., 2008, Cohen-Tannoudji et al., 2012).

The secretion of GnRH in bull calves is minimal in the juvenile period, however, pulsatile secretion begins at approximately 2 weeks of age, as shown when analysing blood from the hypophyseal portal system (Rodriguez and Wise, 1989).
1.9.2 Luteinising Hormone and Follicle Stimulating Hormone

The hormone GnRH binds to its receptor (GnRHR) on the surface of gonadotrophic cells within the anterior pituitary; which results in the production and secretion of LH and FSH (Wolfe et al., 2014). Both hormones comprise of a mutual α subunit and a unique β subunit (Morgan et al., 1975). The β subunits give LH and FSH their functions.

Receptors on Leydig cells are bound by LH to stimulate androgen secretion (Jin and Yang, 2014) and to activate adenyl cyclase. This enzyme catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), which serves as a secondary messenger to activate a cytoplasmic protein kinase which aids in the conversion of cholesterol to testosterone (Neaves, 1975). The discharge of LH is not continuous but occurs in pulses, that occur three to eight times throughout the day (Amann and Schanbacher, 1983). In bull calves, during birth and 6 weeks of age, mean serum LH concentrations are low (Aravindakshan et al., 2000) at a concentration of approximately 0.1 ng/mL (Evans et al., 1996). There is a transient rise of LH which occurs between 8 and 20 weeks of age, with a peak at 12-15 weeks (0.8 ng/mL; Evans et al., 1996), declines between 20 and 24 weeks of age and reaches a nadir between 25 and 35 weeks of age (Evans et al., 1996; Figure 1.7). Subsequently, LH concentrations rise coinciding with puberty at approximately 42 weeks (Evans et al., 1996). The LH pulses are at a high frequency after 4 weeks of age and lead to differentiation and maturation of adult Leydig cells and a switch from androstanediolone to testosterone secretion around 12 to 16 weeks of age, which gradually reaches peak testosterone concentration at approximately 42 weeks (Amann, 1983).
The FSH binds to its receptor on Sertoli cells to stimulate spermatogenesis. In the male, FSH is required early in life for the determination of Sertoli cell number, and for induction and maintenance of normal sperm production post puberty (Simoni et al., 1999). FSH levels are elevated postnatally and decrease to 25 weeks where they remain constant and begin to rise again after 40 weeks (Evans et al., 1995, Evans et al., 1996). However, another study has found a postnatal decline between 12-20 weeks of age and unchanging concentrations of FSH prepuberty (Bagu et al., 2006).

**Figure 1.7:** Mean (± s.e.m.) concentrations of luteinising hormone (LH) and follicle stimulating hormone (FSH) in blood samples collected from young Hereford x Charolais bulls (Evans et al., 1996)

### 1.9.3 Testosterone

Testosterone is an androgenic steroid secreted by the smooth endoplasmic reticulum of the Leydig cells and is largely known for its role in the development of sex organs as well

The metabolism of testosterone is a multi-step process stimulated by LH (Figure 1.8). The first step takes place in the mitochondria and comprises of the formation of pregnenolone; which involves the cytochrome P450 enzyme, CYP17A1 (Amann and Schanbacher, 1983). In a series of reactions, pregnenolone is converted to testosterone via either delta-4 (Δ-4) or delta-5 (Δ-5) pathway in the smooth endoplasmic reticulum (van der Molen, 1981). The Δ-4 pathway produces testosterone via the intermediates 17-alpha-hydroxypregnenolone, dehydroepiandrosterone and 4-androstene-3,17-dione (Yamazaki and Shimada, 1997). In the alternative pathway Δ-5, pregnenolone is catalysed by P450 enzyme CYP17A1 to produce testosterone via the intermediates progesterone, 17-alpha-hydroxyprogesterone and 4-androstene-3,17-dione (Rose et al., 1997).

Testosterone and oestrogen are carried in the blood bound to Sex Hormone Binding Globulin (SHBG). In a healthy functioning adult, only a small concentration of the hormone is free in the blood to bind with the androgen receptor (Maruyama et al., 1987). A decrease in SHBG binding capacity results in lower testosterone levels (Caminos et al., 2008, Isidori et al., 1999, Caprio et al., 2003, Giagulli et al., 1994).
Testosterone is secreted in pulses; the timing and pattern of which are specific to each individual animal and are affected by age, reproductive status, health and external and environmental factors (Schanbacher and Ford, 1977). An early transient rise in serum LH concentrations occurs during 8 to 20 weeks of age (Evans et al., 1996) and concomitantly mean testosterone concentrations are low; however, testosterone increases markedly after 28 weeks of age and reaches adult concentrations (peak concentration: 9 ng/mL) by approximately 40 weeks of age (Amann and Walker, 1983; Evans et al., 1996; Figure 1.9). The increase of testosterone concentrations observed in bulls (Figure 1.9) coincides with a decrease in LH and FSH secretion at the same time-points (Rawlings and Evans, 1995) where androgens were shown to have a negative feedback on gonadotrophin release in bulls.
The pituitary of rams exposed to excess testosterone during their prenatal development had increased sensitivity to GnRH and therefore, increased LH secretion (Recabarren et al., 2013). However, in the same animals there was a decrease in testicular sensitivity to LH secretion and consequently, no change in testosterone production. This manifested in a reduction in sperm concentration and motility as well as scrotal circumference (Recabarren et al., 2008) and an increase in the number of Sertoli cells and FSHR (follicle stimulating hormone receptor)(Rojas-Garcia et al., 2010). There is no clear explanation for this response; however, one possibility is that the LH secreted in reaction to the GnRH stimulus may be a different isoform as was the case in females (Perera-Marin et al., 2007, Olivares et al., 2009). Another contributory factor may be the reduction in LHR expression in testosterone exposed males.

1.9.4 Kisspeptin (KISS1) and G Protein – Coupled Receptor (GPR54) System

Kisspeptin otherwise known as metastin is an excitatory neuropeptide that stimulates the release of gonadotropins in numerous species including cattle (Messager et al., 2005). Kisspeptin has a long chain 54 amino acid (Kp54) and can be cleaved into three short kisspeptins (Kp10, Kp13, Kp14); which are all biologically active and have a high affinity
ligand for G protein-coupled receptor 54 (GPR54) (Muir et al., 2001, Kotani et al., 2001, Ohtaki et al., 2001, Clements et al., 2001). KISS1 neurons are found in the ARC and the preoptic nucleus of cattle (Hassaneen et al., 2016). The KISS1 neurons in the ARC are co-expressed with two other neuropeptides, neurokinin B (NKB) and dynorphin A (Dyn) which are collectively known as Kisspeptin, Neurokinin B and dynorphin A neurons (KNDy) neurons (Navarro et al., 2009, Hassaneen et al., 2016). Studies have shown that KNDy neurons control the KISS1 fibres in the median eminence that make direct contact with GnRH nerve terminals and control the release of GnRH in cattle (Hassaneen et al., 2016). A decrease in KISS1 neurotransmission is thought to add to the age-related LH surge abnormalities (Lederman et al., 2010). Studies conducted in a variety of species and in both sexes e.g. male rats, ewes and men showed that administering of KISS1 centrally, by the ventricles in the brain or via the circulatory system led to an increase in both LH and FSH in peripheral circulation. KISS1 neurons relay metabolic status via its receptor GPR54 on the GnRH neurons (Castellano et al., 2010, Kalamatianos et al., 2008, Quennell et al., 2011, Wahab et al., 2011). Approximately 40% of kisspeptin neurons present in the mouse ARC were shown to express the active leptin receptor (Ob-Rb) mRNA, demonstrating that KISS1 neurons are direct targets for regulation by leptin (Smith et al., 2006). Administration of IGF-I activates the KISS1 gene in the anteroventral periventricular and ARC of the hypothalamus in prepubertal female rats (Hiney et al., 2009). Studies also have shown that KISS1 stimulates the secretion of GH from prepubertal Holstein heifers (Kadokawa et al., 2008).

It has been shown that GPR54 is essential for LH and FSH secretion to stimulate the onset of puberty (Seminara et al., 2003) and is located mainly in the hypothalamus and basal ganglia but also in the placenta, pituitary, spinal cord, pancreas, heart, muscle, kidney,
liver, intestines, thymus, lung and testis (Lee et al., 1999, Muir et al., 2001, Stafford et al., 2002). There is a major cluster of \textit{GPR54} mRNA in the ARC, the lateral hypothalamic area and the dorsomedial nucleus in the rat (Lee et al., 1999).

\textbf{1.9.5 Gonadotropin-Inhibitory Hormone (GnIH)}

This hormone was first shown to inhibit GnRH in quails (Tsutsui et al., 2000). It has since being shown to exist in many avian and mammal species and regulates the synthesis and secretion of GnRH via GPR147 (Tsutsui, 2009, Tsutsui et al., 2010a, Tsutsui et al., 2010b). The hormone GnIH and RF-amide related peptides (RFRPs) have been characterised in brain tissue of various mammals (Ukena et al., 2002, Ubuka et al., 2009, Yoshida et al., 2003, Fukusumi et al., 2001). The importance of GnIH was demonstrated by the administration of GnIH and RF-amide related peptide-3 (RFRP-3) centrally and peripherally which inhibited LH secretion in both sexes of adult hamsters (Kriegsfeld et al., 2006), male adult rats (Johnson et al., 2007, Murakami et al., 2008) and adult ewes (Clarke et al., 2008). It has been reported that GnIH immunostaining was increased in Leydig cells in mice during the pubertal period and in sperm post puberty, which suggests a role in the regulation of steroid synthesis in the testes and in sperm maturation (Anjum et al., 2012). It may also be involved in the regulation of germ cell differentiation in the testes as GnIH has been shown to be expressed in spermatocytes and spermatids (Bentley et al., 2008).

\textbf{1.9.6 Inhibin and Activin}

Inhibin B is a glycoprotein that is synthesised in the Sertoli cells and its target is the gonadotrophic cells in the anterior pituitary, where it functions as a negative feedback regulator of FSH in bulls (Kaneko et al., 2001). The production of inhibin from the testes peaks at or before puberty and declines to adult levels; it is suggested that this is due to
the establishment of Sertoli cell number (Kaneko et al., 2001, Matsuzaki et al., 2001). Also at puberty, the ram pituitary gland has sensitivity to feedback from inhibin (Tilbrook et al., 1999). Activin A is a member of the TGFβ family and its role is to stimulate the synthesis of FSH by the gonadotrophic cells and therefore, regulate spermatogenesis (Hedger and Winnall, 2012). It has been found that activin A regulates spermatogenic cell and Sertoli cell proliferation and differentiation in the fetal and post-natal testes (Itman et al., 2006).

1.10 Nutritional Influences on Puberty through Metabolic Signalling Peptides and Metabolic Hormones

Metabolic signalling molecules mediate the secretion of GnRH by altering the afferent inputs to the GnRH neurons (Amstalden et al., 2014). Some of these peptides and hormones will be discussed below.

1.10.1 Neuropeptide Y (NPY) and Agouti-related Protein (AGRP)

Neuropeptide Y (NPY) is located on chromosome four in cattle (Thue and Buchanan, 2004) and codes for a strong orexigenic (appetite stimulator) and highly conserved peptide consisting of 36 amino acids (Bahar and Sweeney, 2008). Several biological pathways that regulate feeding behaviour, appetite and energy homeostasis in animals and humans are influenced by NPY (Tatemoto et al., 1982, Wynne et al., 2005, Arora and Anubhuti, 2006, White, 1993). It also promotes the secretion of growth hormone in cattle (Thomas et al., 1999), a rise in leptin expression in sheep (Chilliard et al., 2001) and the reduction in fatty acid secretion in adipocytes (Bradley et al., 2005). In bulls, NPY has been found to be abundant in the ARC and the cerebral and cerebellar regions of the brain, whereas, subcutaneous adipose tissue and small intestine have lower amounts (Bahar and Sweeney, 2008). There was an increase in NPY expression (McShane et al., 1992) along with NPY immunoreactivity (Chaillou et al., 2002) in the ARC of sheep that
were on restricted diets. In the prenatal stage of life, NPY is the dominant signal in stimulating positive energy balance to promote growth; however, in later development, NPY levels decrease and melanocortin levels increase (Roth and Sathyanarayana, 2012). This indicates that it works as a “developmental switch” in the monitoring of energy metabolism (Melnick et al., 2007). Studies have shown AGRP is composed of approximately 132 amino acids and is co-expressed with NPY in neurons found in the ARC (Hahn et al., 1998). It has been shown that AGRP is not as potent as NPY; however, its effects last longer (Hagan et al., 2000). It has been reported that NPY act directly on GnRH neurons and this may also be the case for AGRP as melanocortin agonists have direct action on GnRH neurons (Roa and Herbison, 2012). It has been reported that NPY has been linked to the mediation of leptin (Amstalden et al., 2000) as the administration of NPY stimulated appetite, via the inhibition of leptin (Barb et al., 2006). The maintenance of high levels of NPY suppresses pubertal development in heifers (Amstalden et al., 2014). Leptin receptor mRNA and NPY neurons are colocalised in the ARC of ewes (Williams et al., 1999). Low leptin levels stimulate NPY and AGRP release, feed intake and inhibit POMC and Cocaine and amphetamine regulated transcript (CART) activity and energy expenditure (Schwartz et al., 2000).

1.10.2 Pro-opiomelanocortin (POMC) and Alpha melanocortin stimulating hormone (α-MSH)

Alpha melanocortin stimulating hormone (α-MSH) is a hormone formed following post transcriptional cleavage of the hypothalamic POMC. Food intake can be inhibited by α-MSH via the activation of melanocortin-4-receptor (MC4R) (McMinn et al., 2000). The POMC neurons are primarily located in the ARC and are colocalised with AGRP, a POMC and α-MSH antagonist and leptin receptor (Ob-R) neurons (Hahn et al., 1998).
NPY/AGRP have opposing roles to that of POMC/ α-MSH in the control of feeding and energy expenditure (Stanley et al., 2005).

1.10.3 Ghrelin

Ghrelin is a 28 amino-acid peptide which is secreted mainly from the fundic region of the stomach (Valassi et al., 2008; Figure 1.10). As well as being an orexigenic peptide, ghrelin also controls growth hormone, adrenocorticotropic hormone and prolactin secretion, metabolism function, heart function, sleep and reproduction (Valassi et al., 2008). Circulating ghrelin concentration decreases after food intake and when levels of nutrients such as glucose increase (Tschop et al., 2000, Sakata et al., 2002). It has been reported that ghrelin decreases in both plasma and gastric fluid after consumption of both fat and carbohydrate, however, carbohydrates suppress ghrelin expression longer than fat (Sanchez et al., 2004). Ghrelin receptor (GHS-R1a) has been shown to be located on the NPY and GHRH neurons in the hypothalamus (Lucidi et al., 2005). In the anterior pituitary, ghrelin has been found to inhibit LH pulse frequency in rams (Harrison et al., 2008) and men (Lanfranco et al., 2008). Ghrelin has been shown to down regulate KISS1 expression in the medial preoptic area (Forbes et al., 2009), which delays pubertal onset in male rats (Fernandez-Fernandez et al., 2005).
Figure 1.10: Schematic representation of the effect of ghrelin on the hypothalamic-pituitary-gonadal axis (Dupont et al., 2010)

1.10.4 Leptin

Leptin is a 167 amino acid hormone product of the ob gene and is secreted by white adipose tissue (Williams et al., 2002). Although it is prominent in adipocytes it is also found in lesser intensities in other sites which are species-specific (Ingvartsen and Boisclair, 2001), such as rat gastric epithelium (Bado et al., 1998), human placenta (Masuzaki et al., 1997) and chicken liver (Taouis et al., 1998). It functions via endocrine, paracrine and autocrine means in homeostasis, metabolism, immune function and reproduction (Williams et al., 2002). Leptin production increases proportionally in line with an increase in WAT (Landry et al., 2013); evidently then, it is a signal of energy sufficiency (Sanchez-Garrido and Tena-Sempere, 2013). It signals energy abundance by
informing the different bodily systems including the reproductive axis of the status of energy reserves the body has via circulating leptin levels (Ahima et al., 2000).

Leptin is essential for the onset of puberty; however, leptin alone cannot activate puberty (Barash et al., 1996, Castellano et al., 2009, Cheung et al., 1997, Roa and Tena-Sempere, 2010). Leptin has been shown to increase LH secretion in the anterior pituitary and to increase GnRH in the hypothalamus of the pig (Barb et al., 2004). Although it may seem counterintuitive, high levels of leptin associated with obesity directly inhibit gonadal development via sex steroid secretion (Tena-Sempere et al., 1999, Caprio et al., 2001). The normalization of body weight in ob/ob male mice due to leptin treatment has the ability to reverse the effects of overeating and body weight gain and to re-establish reproductive development (Chehab et al., 1996, Mounzih et al., 1997, Farooqi et al., 1999). The decrease in LH from rats that were fasting for 48 h was prevented by leptin treatment. This effect was also recorded in underfed, post pubertal sheep (Morrison et al., 2002). In humans, males exhibit a rise in leptin secretion during approximately 5-10 years of age followed by a steady decline (Tena-Sempere and Barreiro, 2002). Inherited leptin deficiency causes early onset of obesity, hyperphagia (abnormally increased appetite), hypogonadotropic hypogonadism (decreased functionality of the testes due to a GnRH deficiency) and delayed onset of puberty (Strobel et al., 1998, Ozata et al., 1999, Clement et al., 1998). Numerous studies on the effect of high and low plane of nutrition in bulls prior to six months of age found no difference in blood leptin concentration (Dance et al., 2015, Brito et al., 2007c, Byrne et al., 2017c); however, plasma leptin concentration was found to be higher during the pre-pubertal period (6-7 months of age) in comparison to during puberty and post-puberty in Holstein bulls (Gholami et al., 2010). It has been
reported in ruminants that severe undernutrition is needed for leptin to influence gonadotrophin secretion (Zieba et al., 2005)

Leptin receptors (Ob-R) play the role of mediator of the biological effects of leptin (Abavisani et al., 2011). There are many different isoforms of leptin receptor; one isoform Ob-Rb is expressed principally in the ventromedial nuclei, ventral premammillary nuclei and the ARC in the rodent brain (Guan et al., 1997, Mercer et al., 1996). The effect of leptin on food intake and energy homeostasis are mediated by a system of orexigenic neuropeptides including NPY, galanin, galanin-like peptide, melanin-concentrating hormone and AgRP and anorexigenic neuropeptides such as CRH, POMC, α-MSH and CART (Ingvartsen and Boisclair, 2001). Approximately 60% of cells containing NPY also expressed leptin receptor (Ob-Rb) in the sheep hypothalamus (Iqbal et al., 2001).

1.10.5 Adiponectin (AdipoQ)

Adiponectin is an adipokine which predominantly secreted by adipocytes (Arita et al., 1999). Its function is to regulate lipid and glucose metabolism, insulin sensitivity as well as inflammation (Kadowaki et al., 2006, Ahima and Lazar, 2008, Galic et al., 2010). The protein decreases in cases of insulin resistance, diabetes, atherosclerosis and coronary artery disease (Kawano and Arora, 2009). Plasma adiponectin concentrations are inversely correlated with the adipose tissue reservoir (Yamauchi et al., 2014). It has been reported that concentrations of adiponectin in the plasma were significantly lower in males than those in females in humans and rodents; which may be due to oestradiol and testosterone regulation of the plasma adiponectin concentration (Nishizawa et al., 2002). Both ADIPOR1 and ADIPOR2, receptors of AdipoQ, are expressed in the anterior pituitary and the ARC in the hypothalamus of cows (Tabandeh et al., 2011). Feeding inhibits AdipoQ expression in WAT and therefore, inhibits adiponectin serum
concentration (Steinberg and Kemp, 2007). AdipoQ has been found to inhibit the secretion of GnRH via the inhibition of KISS1 and also can bypass this network and inhibit LH secretion directly (Wen et al., 2008). It has also been found in sperm along with its receptors, ADIPOR1 and ADIPOR2 in rams (Kadivar et al., 2016). Studies by the same group have found that serum adiponectin concentration, sperm AdipoQ and its receptors mRNA abundances were correlated with fertility (sperm structure, function and capacitation and steroidogenesis) in Holstein Friesian bulls (Kasimanickam et al., 2013).

1.10.6 Insulin / Insulin like growth factor 1 (IGF-I)

Insulin like growth factor, formally known as Somatomedin C, is a small peptide produced mainly in the liver but also in the hypothalamus (Noguchi et al., 1987, Daftary and Gore, 2003), which is similar to insulin both in structure and function. It plays a role in signalling the metabolic status to the GnRH pulse generator (Brito, 2006). In rats, the administration of IGF-I caused an increase in GnRH and LH secretion (Hiney et al., 2004). Dairy bulls on a high level of nutrition from birth had an increased concentration of IGF-I at an earlier life stage than those fed a low plane of nutrition (Dance et al., 2014, Byrne et al., 2017c). This result was also observed in beef bulls (Brito et al., 2007c, Brito et al., 2007b, Brito et al., 2007a). There is a relationship between IGF-I concentrations, LH concentrations and testicular LH receptor number, which highlights the role of IGF-I in the regulation of the early gonadotropin rise and reproductive development (Dance et al., 2014). The concentration of GH was reduced with increased IGF-I during the sexual development in bulls (Brito, 2006). It appears that IGF-I is a mediator of the effect of nutrition on reproduction via the Hypothalamic-Pituitary-Testicular (HPT) axis (Brito et al., 2007b). IGF-I signals nutritional status to the hypothalamus via receptors in the NPY and POMC nuclei of the arcuate (Amstalden et al., 2014). Receptors for IGF-I have also been found in the pre-optic area of the hypothalamus, suggesting a stimulatory role in
GnRH secretion (Daftary and Gore, 2003). Systemic IGF-I is produced in the liver as a consequence of GH binding to its specific receptors on the surface of the hepatocytes (Breier et al., 1988). However, in nutritionally restricted animals, the liver becomes unaffected by the influence of GH which results in lower concentrations in IGF-I and greater concentrations of GH due to hypothalamic implementation of negative feedback on GnRH (Breier and Gluckman, 1991). There are six insulin-like growth factor binding proteins that regulate IGF-I activity by controlling the availability of IGF-I to their receptors (Clemmons, 2016).

Insulin is a polypeptide hormone produced by the β cells in the islets of Langerhans in the pancreas (Loomis et al., 1983, Squires, 2003). Insulin is a metabolically active hormone responsible for anabolic processes promoting growth, development, and nutrient homeostasis (Taniguchi et al., 2006, Cheng et al., 2010). Insulin regulates blood glucose concentrations by increasing the uptake of glucose into tissues to be stored as glycogen or lipid. Insulin acts as a metabolic signal that influences the release of GnRH from the hypothalamus. Insulin has been shown to act on hypothalamic neurons that express NPY and AGRP, as well as those expressing POMC (Könner et al., 2007). Peripheral insulin administration suppresses the release of NPY in the paraventricular nucleus in rats (Sahu et al., 1995) and central administration decreases both NPY expression and NPY concentrations in the hypothalamus. Insulin plays a role in regulating the synthesis and secretion of leptin as WAT cultured with insulin increased the synthesis and secretion of leptin (Barr et al., 1997). However, leptin negatively affects the synthesis and secretion of insulin (Fehmann et al., 1997).

1.11 Evolution of Technologies used to study Transcriptomics

First developed in 1983 by Karl Mullis, polymerase chain reaction (PCR) allows the exponential amplification of fragments of DNA. This technique formed the cornerstone of
the human genome project and its fundamental to many molecular biology laboratory protocols (Mullis et al., 1986, Powledge, 2004, Saiki et al., 1985, Valasek and Repa, 2005, Whitney et al., 2004). Further discoveries were made to improve PCR and this led to the development of the next generation PCR, real-time PCR (RT-PCR) in 1996. This methodology enables the amplification and subsequent detection and quantification of one or more specific sequences in a DNA sample (Valasek and Repa, 2005, Kubista et al., 2006). The fundamental difference between the two technologies was that PCR detects the DNA at the endpoint of the reaction, while RT-PCR allowed detection as the reaction progressed in real time.

In 1995, the first report on application of DNA microarrays to analyse gene expression was published (Brown et al., 1995). A microarray is a 2D array consisting of a collection of thousands of micro spots, each containing a specific RNA or DNA sequences, which are attached to a small glass or silicon slide (Gabig and Wegrzyn, 2001, Kapranov et al., 2003). Microarrays can be used to measure DNA, through comparative genome hybridisation or RNA, via expression profiling using complimentary deoxyribonucleic acid (cDNA) as the template (Hegde et al., 2000, Schena et al., 1995). Microarrays can be broadly divided into two main types based on the nature of the probe attached to the array surface, cDNA arrays and oligonucleotide arrays (Hegde et al., 2000, Schena et al., 1995). Two colour or cDNA microarrays are less commonly used nowadays due to gene bias and poor labelling efficiency (Rosenzweig et al., 2004). In contrast to cDNA arrays, oligonucleotide arrays or single-channel microarrays are hybridised with only one sample (Jaluria et al., 2007), thus to obtain data of differential expression between test and control samples, two separate hybridisations, one for each chip, have to be performed. Like RT-
qPCR, microarrays provide us with a plethora of uses in the fields of basic biological sciences, translation medicine and diagnostics.

The RNA sequencing (RNASeq) approach to transcriptome profiling uses deep-sequencing technologies and has thus far revolutionised our view of the extent and complexity of the transcriptome (Wang et al., 2009b). RNASeq offers many novel advantages over earlier technologies, such as the identification of alternative splicing. It is not limited to detecting transcripts and also sequence variation on bases can be easily revealed (Oshlack and Wakefield, 2009). The main advantage of RNASeq is that the expression of thousands of genes can be detected simultaneously and reveal differentially expressed genes that may not be expected; whereas using RT-PCR, specific genes must be selected and analysed separately.

However, a change in gene expression in a given gene does not automatically mean that there is a change in the protein expression. Proteins carry out work in the cell, while mRNA is only a vehicle that transfers genetic information from the gene to be translated into a protein. Therefore, immunohistochemistry is an important tool that can be used to validate gene expression data outputted by techniques such as RT-PCR and RNASeq. Immunohistochemistry (IHC) is the application of monoclonal or polyclonal antibodies to detect and quantify specific antigens in tissue sections (Duraiyan et al., 2012).

1.12 Rationale for this Thesis

Research recently carried out by our research group found bulls offered a high plane of nutrition pre-six months were younger at puberty and sexual maturation (Byrne et al., 2017b). Effects of dietary restriction in early life cannot be mitigated by enhancing dietary intake post six-months of age nor will post six-month dietary restriction reduce the
advantages obtained from a high pre six-month diet. This therefore affects the transient rise of LH which occurs between 8 and 20 weeks of age (Evans et al., 1996). Our rationale was that sacrificing bull calves on two divergent planes of nutrition at 18 weeks of age, which is still within the (arbitrary) 8-20 week window of influence, allowed the animals’ sufficient time to express their respective growth performance potential. This allowed us the opportunity to characterise the response of the HPT axis at a point in time within this relevant window. Our hypothesis was that early calf nutrition would positively impact the transcriptome of the HPT axis. This thesis will investigate holistically the effect of early life enhanced nutritional status of bull calves on the molecular functionality of the HPT axis. Data from this study will enable us to identify the key genes controlling early development of the HPT axis. Ultimately, the results of this work, following appropriate validation, will facilitate the targeting of genes and variants which could be harnessed within the context of a genomic breeding programme.

1.13 Objectives

The objectives of this thesis were:

1. To understand the effect of early calf nutrition on the transcriptome of subcutaneous adipose tissue and their downstream effects on the HPT axis.

2. To characterise the physiological, cellular and transcriptional response of the HPT axis to contrasting planes of nutrition during early calf-hood in Holstein Friesian bulls.

3. To describe key pathways in the HPT axis which are under nutritional control.
CHAPTER 2: Role of Early Life Nutrition on Regulating the Hypothalamic-Anterior Pituitary–Testicular Axis of the Bull

2.1 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 (ARC region), anterior pituitary and testes in Holstein Friesian bull calves. Holstein Friesian bull calves with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, respectively, were assigned to either a high (n=10) or low (n=10) plane of nutrition. At 126±1.1 days of age, the calves were euthanized. Animal performance and systemic concentrations of metabolic and reproductive hormones were assessed. Testicular histology, targeted gene and protein expression of the ARC, anterior pituitary and testes were also assessed using qPCR and immunohistochemistry, respectively. Testicular tissue from post pubertal 19 month old Holstein Friesian bulls (n=10) that were reared under similar conditions was also available for comparative gene expression analysis. The metabolites and metabolic hormones generally reflected the improved metabolic status of the calves on the high plane of nutrition (P<0.001). Only ghrelin receptor was upregulated in the anterior pituitary (P<0.05) and ARC (P<0.10) in the low plane of nutrition compared to high. 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 in accordance with both morphological and transcriptional data. This study indicates that a high plane of nutrition during early calf-hood alters gene expression which advances testicular development and hastens spermatogenesis.

Keywords: bull calves, plane of nutrition, HPT axis, reproduction, puberty

2.2 Introduction

Due to DNA based marker 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 approximately 9 to 10 months of age 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 semen available at a younger age (Harstine et al., 2015).

The HPT is a cohesive biological system that controls the secretion of male hormones and in turn spermatogenesis (Ramaswamy and Weinbauer, 2014). The hypothalamus is acknowledged as the homeostatic regulator of the body (Kandel and Schwartz, 1991). Signals from hormones including leptin, ghrelin, insulin and IGF-I are received by hypothalamic neurons located in the ARC region (Amstalden et al., 2011) and signal nutritional status to the preoptic nuclei region that are largely involved in the regulation of reproduction (Lechan and Toni, 2000). The 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 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 six months 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 (Byrne et al., 2017c, Brito et al., 2007b), which leads to larger testes and attainment of puberty at a younger age (Dance et al., 2015, Brito et al., 2007b). The negative effects of feed restriction during the prepubertal period cannot be mitigated in the peripubertal or pubertal period, regardless of the nutrition provided after this crucial period of development (Brito et al., 2007b, Byrne et al., 2017b).
The reproductive axis has the capacity to respond to changing concentrations of metabolic signals (Hill et al., 2008) and it is clear that a high plane of nutrition in the early calf hood period can advance puberty (Byrne, et al. 2016). However, the molecular mechanism by which this is mediated has not been investigated to-date in the bull. The objective of this study was to characterise the effect of nutrition during the first 18 weeks of life on aspects of physiological and transcriptional functionality of the hypothalamic (ARC region), anterior pituitary and testes in Holstein Friesian bull calves.

2.3 Material 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.

2.3.1 Experimental design and animal management

Twenty Holstein-Friesian bull calves with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, respectively, were sourced from commercial dairy farms in spring time. These calves were assigned to two treatment groups (high plane of nutrition and low plane of nutrition) by blocking the calves on age, sire, live weight and farm of origin. Calves were group-housed indoors on sawdust-floored pens with a space allowance of 1.6 m²/calf as per EU regulations (S.I. No. 311 of 2010). Calves were individually fed milk replacer and concentrate (Table 2.1 and 2.2) using an electronic feeding system (Förster-Technik, Vario, Engen, Germany); individual identification was facilitated by a neck collar incorporating a transponder on each calf. After five days acclimatisation, calves were assigned to either high or low plane of nutrition. Calves on the high plane of nutrition received 1200 g of milk replacer in 8 L of water daily, together with concentrate *ad libitum*. Calves on the low plane of nutrition 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 (±S.D.) of 82 (±3.9) days. Following weaning, high calves were offered concentrates ad libitum, while the low plane of nutrition calves received 1 kg of concentrate daily. All calves had daily access to fresh water and approximately 0.5 kg of hay throughout the trial period. Calves were weighed weekly. At a mean age (±S.D.) of 126 (±1.1) days of age, all calves were euthanized following intravenous administration of sodium pentobarbitone (1mL/1.4kg bodyweight; Euthatal, Merial S.A.S, Toulouse, France). Death was confirmed by lack of ocular response and was followed by exsanguination and decapitation. Calf health was monitored throughout the trial and vaccination for BVD and pneumonia.

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 ARC 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 ARC. 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, epididymes 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.

For comparison purposes, sections of the parenchyma were taken from post-pubertal HF bulls (n=10) that were slaughtered at a mean (±S.D.) age and bodyweight of 586 (±50) days and
532 (±48) kg, respectively. These mature bulls were reared under similar conditions at the same research facility as that of the high plane of nutrition calves described above.

### 2.3.2 Blood sampling

Blood samples were taken at 2, 6, 10, 14, and 18 weeks of age an hour after feeding. Blood samples were collected via jugular venepuncture to determine systemic concentrations of IGF-I, insulin, leptin, adiponectin, albumin, urea, total protein, triglycerides, beta hydroxybutyrate (BHB), creatinine, globulin, glucose, non-esterified fatty acids (NEFA) as well as LH, FSH and testosterone. Blood was collected into a 9-mL evacuated tube containing lithium heparin as an anticoagulant (Greiner Vacuette, Cruinn Diagnostics, Dublin, Ireland) for albumin, urea, total protein, BHB, glucose, NEFA, triglycerides, creatinine, adiponectin, IGF-I and leptin. Blood was collected into a 6-mL K3 ethylenediaminetetraacetic acid (K3 EDTA; Vacuette, Cruinn Diagnostic) for insulin. Blood was centrifuged at 1750 g for 15 min; plasma was separated and stored at -20°C until analysis. Blood samples for FSH, LH and testosterone were collected into a 9-mL evacuated serum separator tube (Becton Dickinson, Dublin, Ireland.). Blood was allowed to clot overnight and then centrifuged at 800 g for 10 min and serum was collected and stored at -20°C before analysis.

### 2.3.3 Metabolite assays

Concentrations of albumin, urea, total protein, triglycerides, BHB, creatinine, globulin, glucose and NEFA were analysed using commercial biochemical assay kits (Olympus Diagnostics, Tokyo, Japan, and Randox Laboratories LTD, Co. Antrim, Northern Ireland) on a Beckmann Coulter AU 400 clinical analyser (Olympus Diagnostics, Tokyo, Japan). 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.

2.3.4 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-I were 10.8%, 4.36% and 4.05% and 6.7%, 1.57% and 2.77%, respectively. The sensitivity of IGF-I assay was 4 ng/mL. Insulin concentrations were measured by Immunoradiometric assay (IRMA; INS-Irma, DIAsource 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 ELISA was validated with this set of samples. The range of the assay was between 0.6-9.0 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 0.03-7.0 ng/mL and the intra-assay CV was 5%.

2.3.5 Reproductive hormones

Serum samples were analysed for testosterone concentration using an RIA (Testo-RIA-CT, DIAsource 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, 100µl 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, Texas, US) 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%, 9.96% 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.

2.3.6 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 x400 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 x1000 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 a modified version of the lumen scoring system reported by Rode (2015; **Table 2.3**). Measurements were conducted on 20 different tubules selected at random from each testis.

Sections (5-µm thick) were stained using Haematoxylin and Eosin (H&E). The point-counting method was carried out at x1000 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 (1985; Supplementary **Figure 2.1**). The evaluator was blind to the treatments.

**2.3.7 RNA isolation and purification**

Total RNA was extracted using RNeasy Universal plus Kit (Qiagen, Manchester, UK). 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, Cork, Ireland). RNA was purified using the RNA Clean & Concentrator (Zymo Research, Irvine, US) and RNA integrity values of greater than 8 were deemed to be of high quality.

**2.3.8 Complementary DNA synthesis**

Total RNA (2µg) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, US) using Multiscribe reverse transcriptase according to manufacturer’s instructions. Samples were stored at -20°C pending further analysis.
2.3.9 Primer design and reference gene selection

All primers targeting reference and candidate genes were obtained from a commercial supplier (Sigma-Aldrich Ireland, Dublin, Ireland; Supplementary Table 2.2-2.4). Primer3 (http://primer3.ut.ee/) software were utilized to design primers (Koressaar and 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 ARC region of the hypothalamus included: KISS1, GPR54, GnRH, AGRP, NPY, POMC, melanocortin 4, IGF-I, IGF-IR, leptin receptor (OBR) and growth hormone secretagogue receptor (ghrelin receptor; GHSR); The genes examined in the anterior pituitary included: LHB, FHSB, GHSR, somatotropin precursor/growth hormone (GH1), GnRHR, IGF-IR and IGF-I. 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), FSHR, aquaporin-8 (AQP8), ubiquitin carboxyl-terminal esterase L1 (UCHL1), anti-müllerian hormone (AMH), claudin 11 (CLDN11) and 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 indicated 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 0.9482, 0.6544, 0.5034 and 0.5034, respectively. The M value scores for the ARC 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.

2.3.10 Gene expression

The RT-PCR assays were performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) as per the protocol reported in 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 cycle threshold (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. A normalisation factor, calculated based on the geometric mean of the four reference genes, was used to normalise the expression of each gene of interest.

2.3.11 Immunohistochemical (IHC) analysis

Immunohistochemistry was used to assess cell proliferation in the ARC by assessing KI67 staining and to validate the specificity of the tissue using proteins KISS1 and NPY that are highly specific to the ARC; while Sertoli cell number and developmental stage was assessed in the testes using GATA4 and AMH. Antigen retrieval of the deparaffinised tissue sections was performed using a PT-Link module (DAKO, Santa Clara, US) at 95°–99°C for 20 min in a citric acid buffer (0.01M, pH 6.0). Slide staining was performed using a DAKO autostainer Link 48 (DAKO, Santa Clara, US) 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; mouse polyclonal), anti-KISS1 (1:1000, ABIN672726, Antibodies-online; rabbit polyclonal) and anti-NPY (1:150, ABIN724475, Antibodies-online; rabbit polyclonal) on the ARC sections and anti-GATA4 (1:1000, LS-C355535, Source Bioscience; rabbit polyclonal) and anti-AMH (1:75, ab84952, Abcam; rabbit polyclonal) 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 ARC tissue, respectively. Bovine heart and ovary sections were used as positive controls for GATA4 and AMH staining of the testes, respectively. All procedures were performed at the Research Pathology Core Facility, of University College Dublin, Ireland (http://www.ucd.ie/conway/research/coretechnologies/researchpathologycore/).

All stained slides were analysed using the software Aperio ImageScope (Leica Biosystems, Dublin, Ireland). The cytoplasmic V2 algorithm was selected to calculate the number of cells containing stain within the nucleus and the cytoplasm per area (mm²) for GATA4 (Figure 2.2). 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 allowed to measure intensity (weak, moderate and strong intensity) and provide the number of cells containing stain per area (mm²; Figure 2.2). 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 (mm²) for KI67, NPY and KISS1 (Figure 2.3).

Each section stained with GATA4 (two per calf; n=10) was also examined at x 400 magnification and 10 round seminiferous tubules were randomly selected and assessed
(Harstine et al., 2017b). 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., 2017b).

### 2.3.12 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). 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, Cary, NC, USA). 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 was determined for each variable by examining the Bayesian Information Criteria (BIC; smaller is better) value. Animal was the experimental unit. Sampling time was included in the statistical models as a repeated measure for weights and monthly blood analysis. All results are presented as mean ± s.e.m.

### 2.4 Results

#### 2.4.1 Animal performance

The pre-weaning average daily gain (ADG) was higher for calves on the high plane of nutrition in comparison with the low plane of nutrition (P<0.001; **Table 2.4**). The post weaning ADG was also higher for the high plane of nutrition (P<0.001) compared to the low plane of nutrition which resulted in pre-slaughter bodyweights being higher for the high plane of nutrition compared with the low plane of nutrition (P<0.001). Paired testicular weight at slaughter was also higher for the calves on the high plane of nutrition compared with those on the low plane of nutrition (P<0.001; **Table 2.5**).
2.4.2 Metabolic hormones and metabolites

Metabolic hormones and metabolite data are presented in Figures 2.3 and 2.4, respectively. Overall, systemic concentrations of hormones and metabolites reflected the divergence in metabolic status of the animals. There was a treatment x week interaction for albumin (P<0.0001). There was an effect of treatment (P<0.01) and week (P<0.01) for total protein. There was no treatment x week interaction or no effect of either treatment or week on triglyceride concentrations (P>0.05). There was no effect of treatment on NEFA (P>0.05); however, there was an effect of week (P<0.01). There was a treatment x week interaction for glucose concentration (P<0.01). There was a tendency for a treatment x week interaction for globulin concentrations (P=0.06). There was no difference between treatments in globulin concentration at week 2 (P>0.05); subsequently calves on the low plane of nutrition had higher globulin for the remainder of the trial period (P<0.05). There was a treatment x week interaction for creatinine (P<0.01). Creatinine was not different between treatment groups from week 2 to 10 (P>0.05). At week 14, there was a tendency for creatinine to be higher on the low plane of nutrition (P=0.09); creatinine was higher in the low plane of nutrition at week 18 (P<0.05). There was a treatment x week interaction for BHB (P<0.001). At weaning, BHB was lower on the low plane of nutrition (P<0.001). However, at week 14 the low plane of nutrition had a higher BHB concentration in comparison with the high plane of nutrition (P<0.001). Thereafter, there was no difference between treatments (P>0.05).

There was both an effect of treatment and week represented by an increase in adiponectin over time (P<0.001). Adiponectin concentration were greater on the low plane of nutrition at week 2 (P=0.05). There was no difference in concentration (P>0.05) between treatments at any sampling thereafter, with the exception of week 10 (weaning), where there was a tendency for the low plane of nutrition to be greater than the high plane of nutrition (P=0.06). There was no effect of treatment on leptin (P>0.05) but there was an effect of week. There
was a treatment x week interaction for IGF-I (P<0.001). There was no difference between treatments in IGF-I concentration at week 2 (P>0.05). Subsequently, calves on the high plane of nutrition had a greater concentration than the low plane of nutrition throughout the rest of the trial (week 6, 1.8 fold change; week 10, 2.5 fold change; week 14, 5 fold change; week 18, 2.9 fold change). The animals on the low plane of nutrition maintained a constant concentration throughout the trial. There was an effect of treatment (P<0.01) and an effect of week (P<0.05) for insulin. There was no difference in circulating concentrations of insulin during weeks 2, 6 or 10 (P>0.05). Insulin concentrations increased in the high plane of nutrition calves throughout the rest of the trial period (week 14, P<0.05; week 18, P<0.05).

2.4.3 Reproductive hormones

There was no effect of treatment on FSH, LH or testosterone (P>0.05; Figure 2.5) but there was an effect of week on all three (P<0.0001). There was a difference in AUC on LH as the high plane of nutrition had a greater area than the low plane of nutrition (P<0.01).

2.4.4 Testicular histology

Seminiferous tubule diameter was greater in the calves on the high plane of nutrition in comparison with low plane of nutrition (P<0.001; Table 2.5). There were only two groups found in the seminal chords namely, germ cells and prespermatogonia and spermatogonia. There was a greater percentage of seminal chords with germ cells and prespermatogonia in the low plane of nutrition than the high plane of nutrition (P<0.001). Conversely, there were a greater percentage of seminal chords with spermatogonia in the high plane of nutrition than the low plane of nutrition (P<0.001).

The lumen scores for seminiferous tubules for both treatment groups ranged between 1 and 4 (Figure 2.6). There was no difference between the treatment groups in the number of tubules that had a lumen score of either 1 or 2 (P>0.05). There was a difference between treatments
for seminiferous tubules with a lumen score of 3 (P<0.001) as the calves on the low plane of nutrition had a greater number than those on the high plane of nutrition (P<0.001). There was a greater number of the calves on the high plane of nutrition with seminiferous tubules with a lumen score of 4 than those on the low plane of nutrition (P<0.001).

There was a greater number of Sertoli cells in the testes of the high plane of nutrition in comparison with their contemporaries on the low plane of nutrition (P<0.001; Table 2.5 and Figure 2.7). There was a greater volume density of Sertoli cells in the low plane of nutrition compared with the high (P<0.05; Table 2.5); however, there was no difference in volume density of Sertoli cells between either plane of nutrition when testes weight was taken into account.

2.4.5 Gene expression

The ARC RT-PCR data are presented in Table 2.6. There was a tendency for an effect of treatment on GHSR (P=0.09), which was upregulated in the low plane of nutrition in comparison with the high plane of nutrition. There was no effect of plane of nutrition on KISS1, GPR54, GnRH, AGRP, NPY, POMC, MC4R, IGF-I, IGF-IR and OBR expression in the arcuate tissue (P>0.05; Table 2.6).

Anterior Pituitary RT-PCR data are presented in Table 2.7. There was an effect of plane of nutrition on GHSR (P<0.05), which was upregulated in the low plane of nutrition in comparison with the high plane of nutrition. There was no effect of plane of nutrition on FSHB, LHB, IGF-I, GH1, GnRHR or IGF-IR (P>0.05; Table 2.7).

In the testes, PCNA was upregulated in both groups of calves when compared with the mature bulls (P<0.001; Table 2.8). AR was upregulated in the mature bulls in comparison to both the high plane of nutrition (P<0.01) and the low plane of nutrition (P=0.06). FSHR was upregulated in the high plane of nutrition compared to the mature bulls (P<0.01). GATA4 was
upregulated in both the high and the low plane of nutrition (P<0.001) compared with the mature bulls. AMH in the low plane of nutrition was upregulated compared with both the high plane of nutrition (P<0.05) and the mature bulls (P<0.001). CLDN11 expression was upregulated in the high plane of nutrition (P<0.05) and the mature bulls (P<0.001) compared with the low plane of nutrition. Luteinising hormone receptor (LHR) expression was upregulated in both the high and the low plane of nutrition (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 2.8).

2.4.6 Immunohistochemistry

The ARC 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 area between treatments (P>0.05; Table 2.9). There were no differences in KI67 and KISS1 protein intensity or in the number of protein positive cells per area between treatments (P>0.05; Table 2.9).

The testicular parenchyma was stained using AMH and GATA4. There was a higher percentage of weak AMH staining in the high plane of nutrition in comparison with the low plane of nutrition (P<0.05; Table 2.9). There were no differences found between overall GATA4 expression within the testes (P>0.05; Table 2.9).

2.5 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 (Byrne et al., 2017b, Dance et al., 2015). Larger testes would suggest greater spermatogenic potential and is thought to reflect a
greater abundance of Sertoli cells and therefore, the capacity to support more sperm (O’Donnell et al., 2000). These testicular morphological differences were validated by transcriptomic analysis and metabolites, metabolic as well as reproductive hormones broadly reflected the plane of nutrition 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 the ARC and 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, in general, the prevailing plane of nutrition, with concentrations markedly higher in the high plane of nutrition calves post-weaning, reflecting their much greater concentrate intake. Such conclusions have been drawn in previous studies in calves on a similar feeding regime (Byrne et al., 2017a). Creatinine, decreased with age for both treatments, indicative of normal renal development and is consistent other recent reports in the literature (Byrne et al., 2017a). Plasma concentrations of urea were higher in the high plane of nutrition, reflecting their greater dietary protein consumption (Blome et al., 2003, Bartlett et al., 2006). Similar results for globulin and total protein concentrations have been observed where calves were offered restricted compared with unlimited milk (Hammon et al., 2002). A number of 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 calves on the high plane of nutrition, with concentrations higher than their contemporaries on the low plane of nutrition during the post-weaning period.

Systemic concentrations of leptin production increased proportionally with increases in body fat (Landry et al., 2013) and play an important role in energy homeostasis (Ahima et al., 2000). However, in our study we did not find any difference between treatments with respect to leptin. This is in agreement with other studies using both dairy and beef bull calves (Dance et al., 2015, Brito et al., 2007b, Byrne et al., 2017c) and may be due to the low levels of subcutaneous fat in young growing calves. However, the subcutaneous adipose tissue from the high and low plane of nutrition was analysed using RNA sequencing technology and found that calves on the high plane of nutrition in our study had >4.5 log fold higher LEP expression compared with animals the low animals (Chapter 4). AdipoQ in contrast to leptin is inversely related to body fat in cattle (Sauerwein and Häußler, 2016) and thus we expected that calves on the high plane of nutrition would have lower concentrations. However, AdipoQ concentrations in our study increased in all calves over time with the exception of a tendency towards a higher concentration in the low plane of nutrition calves at 10 weeks of age. It has been widely suggested that IGF-I 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 to 3.5 fold higher concentrations of IGF-I in calves on the high plane of nutrition compared with the low plane of nutrition, 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 somatotropic cells in the anterior pituitary or indirectly via the GHSR in the ARC 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 ARC 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-I, transcript abundance for GH in the anterior pituitary was not affected by diet in the current study.

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 down regulated and POMC and α-MSH were upregulated in heifer calves fed on a high concentrate diet to achieve rapid bodyweight gain compared to contemporaries offered a high forage diet from three-seven months of age (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 dietary treatment groups. 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 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). A later study carried out by the same group found that
rats raised in large litters postnatally had reduced levels of KISS1 (Castellano et al., 2011). These studies are contradictory of the results from our study. In addition, there were no differences observed in expression of GnRH either in the ARC region of either treatment group or in the expression of GnRHR, FSHB and LHB 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.

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 previous studies have been carried out on stallions (Rode et al., 2015; Heninger et al., 2006), 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 plane of nutrition had a more mature stage of lumen development and suggests that a more favourable metabolic status in early calfhood advances testicular functionality. Curtis and Amann (1981) reported outer seminiferous tubule diameter of 79 ± 2 μm and 110 ± 5 μm for Holstein Friesian bull calves at 16 weeks and 20 weeks of age, respectively and the diameters measured in the high plane of nutrition in our study were consistent with this range. Consistent with more mature seminiferous tubule development, there was a greater ratio of spermatogonia cells in the high plane of nutrition compared with the low plane of nutrition 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 higher 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. (2017b) demonstrated that Angus calves treated with FSH from 1-3 months of age had a greater number of Sertoli
cell. In another study published by this group on the effect of FSH treatment on calves from 2-6 months of age, they reported an increase in activin A which is beneficial to spermatogenesis (Harstine et al., 2017a). Sertoli cells harbour a fixed number of germ cells and Sertoli cell number can only be influenced pre-pubertally (Johnson et al., 2000). Thus, our data, for the first time, indicates that calves offered a high plane of nutrition during the first four to five months of life may have the capacity to produce more sperm during their lifetime. Consistent with this we observed a strong tendency towards higher systemic concentrations of FSH on the high plane of nutrition 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) summarized work carried out on Angus and Angus x Charolais bulls receiving adequate nutrition and 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 sexual differentiation until puberty (Vigier et al., 1984). AMH secretion is at its maximum at birth and it declines thereafter, until puberty which results from the activation of the HPT axis, which gives rise to an increase in testicular testosterone and onset of spermatogenesis in mice and boys (Hero et al., 2012, Tan et al., 2005). This validates the results of the current study where transcript abundance for AMH in the low plane of nutrition calves was upregulated compared with both the high plane of nutrition and mature bulls and was higher, in turn, for the high plane of nutrition calves than mature bulls. This again was corroborated by our IHC work. Sertoli cells proliferate until 6 months of age (Ortavant et al., 1977) and GATA4 is used as a specific marker for Sertoli cell nuclei in bulls (McCoard et al., 2001) using IHC. This is consistent with the significantly higher GATA4 expression levels in the high and low plane of nutrition 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 the high compared with the low plane of nutrition. This reflects the work carried out by Harstine et al. (2017b) who found an increase in GATA4 stained Sertoli cells in FSH treated animals versus a control. However, no change in FSH concentration was observed in our study.

Testicular expression of *FSHR* has been found to decrease with testes development in bulls (Dias and Reeves, 1982). The number of receptors have been shown to decrease quickly from birth to 2 months of age and from then decrease at a slower rate to 2-5 years of age in bulls (Dias and Reeves, 1982). This is consistent with the significantly higher expression observed in the high and low plane of nutrition in comparison with the adult bulls in the current study. Other studies have shown that there is a decline in FSHR levels in rats 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 *FSHB* transcript abundance in the anterior pituitary, in our study, it is not entirely surprising that there was no effect on testicular *FSHR* expression.

We did not observe any effect of diet on systemic concentrations of LH. 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 (Evans et al., 1996, Rawlings et al., 2008) and our study corroborates these findings. Similarly, it has been reported that the concentration of LH receptors in Hereford x Charolais bulls is high postnatally but decreases from 13 to 25 weeks of age; which maybe be due to the decline in fetal 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, while no effect of diet was evident, we did observe greater LHR expression in the testicular tissue of calves when compared with adult bulls. Blood concentrations of testosterone have been shown to increase in beef bred bull calves following the early LH rise, which typically occurs between 10 and 18 weeks (Amann, 1983, Rawlings and Evans, 1995). Even though no significant differences were found in LH concentration between planes of nutrition, the numerically higher concentration at its peak at 12 weeks in the high plane of nutrition may have a knock on positive influence on testosterone concentration. Also there was a greater area under the curve for high plane of nutrition compared to the low for LH. The greater testosterone concentration in the high plane of nutrition is consistent with the aforementioned higher systemic concentrations of IGF-I in the high plane of nutrition. Given the well documented positive effects of IGF-I on testosterone (Dance et al., 2015), it is possible that the lower concentrations of IGF-I in the low plane of nutrition effected 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 bulls. Ideally an intensive bleed would have been carried out to get more specific data on LH pulsatility; however, as these calves were very young and only on trial for a short period of time it was decided to take weekly blood samples to avoid any major stress that could have a long term effect on the calves.

It has been reported that AR are expressed at high levels in the peritubular cells from the fetal period onwards (Shapiro et al., 2005, Berensztein et al., 2006, Chemes et al., 2008, Boukari et al., 2009). In the human and rodent adult male, AR are maintained at a high level in Leydig and peritubular cells of the testes (Rey et al., 2009). While Sertoli cells, do not express AR during fetal and/or neonatal life (You and Sar, 1998), expression increases during postnatal and prepubertal phases of development in the testes of rodents (You and Sar, 1998, Al-Attar
et al., 1997, Tan et al., 2005), 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 stage of spermatogenesis in the seminiferous tubule (Smith and Walker, 2014). In part agreement with the aforementioned studies, we observed significant difference in expression of AR in testicular tissue of adult bulls compared with the high plane of nutrition only.

There was a higher expression of PCNA in the testes of mature bulls compared with the 18 month old calves in the current study. PCNA is required during DNA replication (Jaskulski et al., 1988), nucleotide excision repair (Nichols and Sancar, 1992) and cell cycling (Xiong et al., 1992). 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 µm (5-15 weeks old) have an increased in PCNA staining at the pre-Sertoli stage, however, this decreased if the pre-Sertoli cells were differentiating. Whereas, in the 80-120 µm (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 plane of nutrition should have had a down regulation 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 there-off, at a point in time and biological function.

In the current study, the mature bulls and the high plane of nutrition had a similar level of testicular expression of CLDN11 with both groups having greater transcript abundance than low calves and could confer an important developmental benefit to the high plane of nutrition 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). Undernutrition of mature rams led to increased expression of CLDN11 and disorganisation of CLDN11 protein localisation in the testes (Guan et al., 2014) indicating a disruption of Sertoli cell tight junctions. 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, Guan et al., 2015). However, in contrast, we did not observe an effect of either stage of maturity or plane of nutrition (within calves) on transcript abundance for ZO1 in the current study.

The lack of differences in protein and gene expression could be due to the unavoidable differences in age of calves at the start. Calves had to be purchased from outside farms as there were no animals in calf to dairy calves on farm. Also it was important that the calves received adequate colostrum. This avoided any major health problems with the calves during the trial. Another point to consider is the mixed populations of cells in both the arcuate nucleus and the anterior pituitary. Further work is needed to isolate specific neurons and cell types in both tissues.

### 2.6 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 bulls. While there was no clear difference between the two planes of nutrition with respect to targeted gene and protein expression in the ARC 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 calf hood induces precocious testicular development. This is consistent with the advancements in the age at puberty onset observed by our group and others where a similar nutritional augmentation strategy was applied. The findings of this study will increase our
understanding of the early calf-hood nutritional control of the timing of pubertal onset in bulls; provide a platform for a more extensive investigation on the genes controlling puberty in bulls.
### Table 2.1 Chemical composition of milk replacer

<table>
<thead>
<tr>
<th>Chemical composition (g/kg)</th>
<th>Milk Replacer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF</td>
<td>12.0 ±1.98</td>
</tr>
<tr>
<td>Crude ash</td>
<td>65.7 ±2.22</td>
</tr>
<tr>
<td>CP</td>
<td>216.3 ±1.24</td>
</tr>
<tr>
<td>DM (%)</td>
<td>96.7 ±0.15</td>
</tr>
<tr>
<td>NDF</td>
<td>5.1 ±1.00</td>
</tr>
<tr>
<td>Oil B</td>
<td>235.0 ±44.10</td>
</tr>
</tbody>
</table>

ADF: Acid Detergent Fibre; CP: Crude Protein; DM: Dry Matter; NDF: Neutral Detergent Fibre; Oil B: Acid hydrolysis.
### Table 2.2 Diet and chemical composition of concentrate diet offered

<table>
<thead>
<tr>
<th>Diet composition (%)</th>
<th>Concentrate</th>
<th>Chemical composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADF</td>
</tr>
<tr>
<td>Rolled Barley</td>
<td>26.5</td>
<td>103.1 ±6.76</td>
</tr>
<tr>
<td>Soya bean meal</td>
<td>25</td>
<td>68.8 ±0.91</td>
</tr>
<tr>
<td>Maize</td>
<td>15</td>
<td>167.9 ±1.86</td>
</tr>
<tr>
<td>Beet pulp</td>
<td>12.5</td>
<td>88.9 ±0.66</td>
</tr>
<tr>
<td>Soya hulls</td>
<td>12.5</td>
<td>204.3 ±18.2</td>
</tr>
<tr>
<td>Molasses</td>
<td>5</td>
<td>30.8 ±0.72</td>
</tr>
<tr>
<td>Mineral and vitamins</td>
<td>2.5¹</td>
<td></td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Mineral and vitamin composition: vitamin A (10 mIU/kg), vitamin D3, (2 mIU/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). Mineral and vitamin composition for concentrate post puberty is the same as above with the exception of copper (80 mg/kg). Oil B = Acid hydrolysis
Table 2.3 Classification of seminiferous tubule development. Adapted from Rode et al., (2015)

<table>
<thead>
<tr>
<th>Lumen Score</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tubes lack a lumen (solid spermatic chords)</td>
</tr>
<tr>
<td>2</td>
<td>Tubules contain a single vacuole</td>
</tr>
<tr>
<td>3</td>
<td>Tubules contain several independent vacuoles</td>
</tr>
<tr>
<td>4</td>
<td>Tubules contain several aggregating vacuoles</td>
</tr>
<tr>
<td>5</td>
<td>Tubules have an open lumen but the seminiferous epithelium has only the height of one cell layer</td>
</tr>
<tr>
<td>6</td>
<td>Tubules have an open lumen – Germ cell population is still incomplete</td>
</tr>
<tr>
<td>7</td>
<td>Tubules have an open lumen – Full population of germ cells</td>
</tr>
</tbody>
</table>
**Table 2.4** Effect of a high compared to a low plane of nutrition on the pre-weaning average daily gain, post weaning average daily gain, total average daily gain and live weight at time of slaughter of Holstein Friesian bull calves.

<table>
<thead>
<tr>
<th></th>
<th>HIGH</th>
<th>LOW</th>
<th>SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-weaning ADG (kg)</td>
<td>0.73</td>
<td>0.43</td>
<td>0.02</td>
<td>***</td>
</tr>
<tr>
<td>Post -weaning ADG (kg)</td>
<td>1.46</td>
<td>0.57</td>
<td>0.07</td>
<td>***</td>
</tr>
<tr>
<td>Overall ADG to weaning (kg)</td>
<td>1.08</td>
<td>0.50</td>
<td>0.03</td>
<td>***</td>
</tr>
<tr>
<td>Bodyweight at slaughter (kg)</td>
<td>160.9</td>
<td>107.1</td>
<td>3.59</td>
<td>***</td>
</tr>
</tbody>
</table>

ADG: Average Daily Gain, ***P<0.001, SEM = standard error of the mean.
Table 2.5 Effect of a high compared to a low plane of nutrition on the paired testes weight and on morphological properties including seminiferous tubule diameter and stage of spermatogenesis

<table>
<thead>
<tr>
<th></th>
<th>HIGH</th>
<th>LOW</th>
<th>SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired testes weight (g)</td>
<td>55.4</td>
<td>31.4</td>
<td>2.91</td>
<td>***</td>
</tr>
<tr>
<td>Seminiferous Tubule Diameter (µm)</td>
<td>85.4</td>
<td>72.5</td>
<td>1.76</td>
<td>***</td>
</tr>
<tr>
<td>% Gonocyte and Prespermatogonia</td>
<td>31.5</td>
<td>57</td>
<td>1.66</td>
<td>***</td>
</tr>
<tr>
<td>% Spermatogonia</td>
<td>68.5</td>
<td>43</td>
<td>1.66</td>
<td>***</td>
</tr>
<tr>
<td>No. of Sertoli cells</td>
<td>28</td>
<td>24</td>
<td>0.65</td>
<td>*</td>
</tr>
<tr>
<td>Volume Density of Sertoli cells</td>
<td>9.4</td>
<td>8.4</td>
<td>0.35</td>
<td>***</td>
</tr>
</tbody>
</table>

***P<0.001, *P<0.05, SEM = standard error of the mean.
Table 2.6 Effect of a high compared to a low plane of nutrition on the expression of selected genes in the arcuate nucleus of the hypothalamus. The results are relative to the average of the reference genes *UBQ* and *RSP9*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>HIGH</th>
<th>LOW</th>
<th>SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>KISSI</td>
<td>3.8</td>
<td>3.2</td>
<td>1.5</td>
<td>NS</td>
</tr>
<tr>
<td>GPR54</td>
<td>1.1</td>
<td>1.3</td>
<td>0.45</td>
<td>NS</td>
</tr>
<tr>
<td>GnRH</td>
<td>1.4</td>
<td>1.7</td>
<td>0.57</td>
<td>NS</td>
</tr>
<tr>
<td>AGRP</td>
<td>4.5</td>
<td>4.8</td>
<td>1.60</td>
<td>NS</td>
</tr>
<tr>
<td>NPY</td>
<td>2.9</td>
<td>3.0</td>
<td>1.18</td>
<td>NS</td>
</tr>
<tr>
<td>POMC</td>
<td>4.6</td>
<td>4.5</td>
<td>0.94</td>
<td>NS</td>
</tr>
<tr>
<td>MC4R</td>
<td>3.8</td>
<td>4.8</td>
<td>0.82</td>
<td>NS</td>
</tr>
<tr>
<td>IGF-I</td>
<td>2.0</td>
<td>1.7</td>
<td>0.46</td>
<td>NS</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>7.8</td>
<td>7.9</td>
<td>0.95</td>
<td>NS</td>
</tr>
<tr>
<td>OBR</td>
<td>4.0</td>
<td>3.3</td>
<td>1.20</td>
<td>NS</td>
</tr>
<tr>
<td>GHSR</td>
<td>4.3</td>
<td>5.5</td>
<td>1.04</td>
<td>P=0.09</td>
</tr>
</tbody>
</table>

NS = Non significant, SEM = standard error of the mean
Table 2.7 Effect of a high compared to a low plane of nutrition on the expression of genes in the anterior pituitary. The results are relative to the average of the reference genes *UBQ* and *RSP9*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>HIGH</th>
<th>LOW</th>
<th>SEM</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FSHB</em></td>
<td>10.0</td>
<td>9.2</td>
<td>2.41</td>
<td>NS</td>
</tr>
<tr>
<td><em>LHB</em></td>
<td>2.1</td>
<td>1.8</td>
<td>0.31</td>
<td>NS</td>
</tr>
<tr>
<td><em>GHSR</em></td>
<td>2.4</td>
<td>3.6</td>
<td>0.33</td>
<td><em>P &lt;0.05</em></td>
</tr>
<tr>
<td><em>GHI</em></td>
<td>1.9</td>
<td>2.6</td>
<td>0.34</td>
<td>NS</td>
</tr>
<tr>
<td><em>GnRHR</em></td>
<td>2.3</td>
<td>2.7</td>
<td>0.35</td>
<td>NS</td>
</tr>
<tr>
<td><em>IGF-IR</em></td>
<td>1.8</td>
<td>2.0</td>
<td>0.20</td>
<td>NS</td>
</tr>
<tr>
<td><em>IGF-I</em></td>
<td>1.9</td>
<td>2.3</td>
<td>0.37</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Non significant, SEM = standard error of the mean
**Table 2.8** Effect of a high and a low plane of nutrition compared to mature bulls on the expression of genes in the parenchyma of the testes. The results are relative to the average of the reference genes *UBQ* and *RSP9*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MATURE</td>
<td>HIGH</td>
</tr>
<tr>
<td>AMH</td>
<td>2.6</td>
<td>5.2</td>
</tr>
<tr>
<td>PCNA</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>GATA4</td>
<td>14.6</td>
<td>31.7</td>
</tr>
<tr>
<td>ZO1</td>
<td>3.8</td>
<td>3.7</td>
</tr>
<tr>
<td>CLAUDIN11</td>
<td>14.7</td>
<td>15.3</td>
</tr>
<tr>
<td>THY1</td>
<td>4.8</td>
<td>5.1</td>
</tr>
<tr>
<td>AR</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>FSHR</td>
<td>9.4</td>
<td>11.5</td>
</tr>
<tr>
<td>AQP8</td>
<td>12.4</td>
<td>14.5</td>
</tr>
<tr>
<td>UCHL1</td>
<td>4.8</td>
<td>5.8</td>
</tr>
<tr>
<td>LHR</td>
<td>0.7</td>
<td>5.4</td>
</tr>
</tbody>
</table>

M = Mature, H = High, L = Low, NS = Non significant, * P<0.05, ** P<0.01, *** P<0.001, SEM = standard error of the mean
Figure 2.1 AMH (x400) and GATA4 (x100) 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 unstained cells and (F) GATA4 stained cells. In slide B, C and D the blue stained cells in a monolayer around the seminiferous tubules are Sertoli cells and any cells inside this monolayer are germ cells. The cytoplasm is expressing AMH, In slide F the dark brown cells are Sertoli cells that express GATA4.
Figure 2.2 KI67, NPY and KISS1 nuclear staining in the arcuate tissue (x400). 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. The brown stained cells in slide B, C, and D indicate proliferating cells. In slide F the brown stained cells are NPY expressing cells. In slide H, I and J the cells with the brown cytoplasm and blue/ brown nucleus are KISS1 expressing cells.
Table 2.9 Effect of low plane of nutrition and high plane of nutrition on protein concentrations on the arcuate nucleus (NPY, KISS1 and KI67) and the testes (AMH and GATA4).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein</th>
<th>Treatment</th>
<th>No. of cells containing stain per area (mm²)</th>
<th>% Weak Positive</th>
<th>% Moderate Positive</th>
<th>% Strong Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcuate Nucleus</td>
<td>NPY</td>
<td>LOW</td>
<td>928±228.1a</td>
<td>25.3±10.96a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
<td>1156±176.1a</td>
<td>28.8±7.99a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>KISS1</td>
<td>LOW</td>
<td>26920±4537.0a</td>
<td>23.1±8.10a</td>
<td>7±3.19a</td>
<td>2.1±1.36a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
<td>27895±5331.8a</td>
<td>30.9±6.14a</td>
<td>11.9±3.58a</td>
<td>4.7±2.98a</td>
</tr>
<tr>
<td></td>
<td>KI67</td>
<td>LOW</td>
<td>819±467.6a</td>
<td>3.9±2.89a</td>
<td>0.1±0.05a</td>
<td>0.01±0.007a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
<td>517±218.6a</td>
<td>1.5±0.51a</td>
<td>0.2±0.07a</td>
<td>0.1±0.05a</td>
</tr>
<tr>
<td>Testes</td>
<td>AMH</td>
<td>LOW</td>
<td>161±23.8a</td>
<td>29.1 ± 2.12a</td>
<td>22.5 ± 2.07a</td>
<td>13.4 ± 2.71a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
<td>114±27.4a</td>
<td>35.6 ± 1.14b</td>
<td>22.7 ± 3.18a</td>
<td>11.2 ± 2.75a</td>
</tr>
<tr>
<td></td>
<td>GATA4</td>
<td>LOW</td>
<td>1638±430.7a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIGH</td>
<td>1339±457.0a</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

AMH % Weak Positive was lower in the low plane of nutrition than the high (P<0.05).
Figure 2.3 Effect of a high compared to a low plane of nutrition on the systemic concentrations of metabolites of Holstein Friesian bull calves. Black full line: low plane of nutrition, Black dotted line: high plane of nutrition; T= Treatment, W=Week, T*W= Treatment 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 for total protein.
Figure 2.4 Effect of a high compared to a low plane of nutrition on the systemic concentrations of metabolic hormones of Holstein Friesian bull calves. Black full line: low plane of nutrition, Black dotted line: high plane of nutrition; T= Treatment, W=Week, T*W= Treatment 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 10 for adiponectin
Figure 2.5 Effect of high plane of nutrition versus low plane of nutrition on the systemic concentrations of reproductive hormones. Black full line: low plane of nutrition, Black dotted line: high plane of nutrition; T= Treatment, W=Week, T*W= Treatment 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.
Figure 2.6 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: Tubes lack a lumen (solid spermatic chords), Lumen Score 2: Tubules contain a single vacuole, Lumen Score 3: Tubules contain several independent vacuoles, Lumen Score 4: Tubules contain several aggregating vacuoles.
Figure 2.7 Effect of a high compared to a low plane of nutrition on GATA4 stained Sertoli cell number (x400); 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., 2017b). There was a greater number of Sertoli cells in the testes of HIGH in comparison with their contemporaries on LOW (P<0.001). Any brown stained cells which are inside the monolayer are spermatogonia.
Supplementary Figure 2.1 Method used to calculate the volume density of seminiferous tubules. Sections (5-µm thick) were stained using Haematoxylin and Eosin (H&E). The point-counting method was carried out at x1000 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 (1985). The red circles indicate a Sertoli cell hitting a point on the grid.
### Supplementary Table 2.1 Gene ID, sequence and accession number for all arcuate genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Accession number</th>
</tr>
</thead>
</table>
| KISS1  | Forward: TCCGCCTACAACCTGGAAACTC  
|        | Reverse: TTCGGGACACAAATTCGAGG            | AB466319.1       |
| GPR54  | Forward: GCCAGTTTCATTGGCCCATTA  
|        | Reverse: GCCCTGAGTATCATTGGTGCG          | GU289736.1       |
| GnRH   | Forward: AAGGACGCTCTGGGAAAGTCT  
|        | Reverse: CCCAGTACGAGCACTTCCAT           | NM_001078137.1   |
| AGRP   | Forward: GGCTGCATGAATCCTGTCTG  
|        | Reverse: CAGAGGTCTCGTGTTGTTGAC          | NM_173983.1      |
| NPY    | Forward: CGTGCGACACTAACATGAAT  
|        | Reverse: TTCCCGTGCTTTTCTCTCAT           | NM_001014845.3   |
| POMC   | Forward: AAGGACTCGGGGCTTATAAA  
|        | Reverse: GTGGGCGTCTTGTGATGATG           | NM_174151.1      |
| MC4R   | Forward: TTCTTTGGGGCAAGTCAAAG  
|        | Reverse: GGGGCTGGGTAGAGTTCATT          | NM_174110.1      |
| IGF-I  | Forward: TGACATTTCAGAGCAATGGG  
|        | Reverse: GAGGAGGATGTGATGGGCAT           | NM_001077828.1   |
| IGF-I-R| Forward: GACAACCAGAACTTTGACGCA  
|        | Reverse: CCCCAGTGCTTTCCGCACTTT          | NM_001244612.1   |
| OBR    | Forward: AACTGAGGAGGCAAGGAC  
|        | Reverse: AGAACGGAAGGTGTGTGAA            | NM_001012285.2   |
| GHSR   | Forward: TGTGTTCTGCTCTACGTGC  
|        | Reverse: ACCACTACAGCCACGATGCTTT         | NM_001143736.2   |
| GAPDH  | Forward: CTCGACCTGAGCACTGATA  
|        | Reverse: GGGCAAGTGCTGCTCCATT            | NM_001034034.2   |
| UBQ    | Forward: CTCGGAGTAGGCTGAGAAG  
|        | Reverse: CTCCAGCAGAGTGGCTTCC            | NM_001101152.2   |
| RPS9   | Forward: GCATCCACAGACTATTTCC  
|        | Reverse: GCAAAAGAATGACAGACCA           | GU817014.1       |

KISS1, kisspeptin, GPR54, G Protein – Coupled Receptor, GnRH, gonadotropin releasing hormone; AGRP, agouti-related Protein; NPY, neuropeptide Y; POMC, pro-opiomelanocortin precursor; MC4R, melanocortin 4 receptor; IGF-I, insulin-like growth factor 1, IGF-I-R, insulin-like growth factor 1 receptor; OBR, leptin receptor; GHSR, growth hormone secretagogue receptor (ghrelin receptor); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UBO, ubiquitin; RPS9, ribosomal protein S9; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,
### Supplementary Table 2.2 Gene ID, sequence and accession number for all anterior pituitary genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’ to 3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHB</td>
<td><strong>Forward</strong>: AGCTGTCACTGTGGATCACCTG&lt;br&gt;<strong>Reverse</strong>: GAAGGGAGGGGAAGAGAGTC</td>
<td>M_10077.1</td>
</tr>
<tr>
<td>FSHB</td>
<td><strong>Forward</strong>: CCAGACTCAAGAGCCCTCAA&lt;br&gt;<strong>Reverse</strong>: GCAGCTCACGAGATAGCTG</td>
<td>NM_174060.1</td>
</tr>
<tr>
<td>GHSR</td>
<td><strong>Forward</strong>: TGTTTCTGCTACGTGTC&lt;br&gt;<strong>Reverse</strong>: AGAGGGATGCTGCTGTTGAT</td>
<td>XM_592014</td>
</tr>
<tr>
<td>GH1</td>
<td><strong>Forward</strong>: ACTCCATCCAGAACACCCAG&lt;br&gt;<strong>Reverse</strong>: GCTGTTGGTGAAGACTCTG</td>
<td>NM_180996.1</td>
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<tr>
<td>GnRHR</td>
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</tr>
<tr>
<td>IGF-IR</td>
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<tr>
<td>GAPDH</td>
<td><strong>Forward</strong>: CCTGCCCGTTCGCAGATATA&lt;br&gt;<strong>Reverse</strong>: GGCGACGATGTCCACTT</td>
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</tr>
<tr>
<td>UBQ</td>
<td><strong>Forward</strong>: TACACAGTGGTGATGACG&lt;br&gt;<strong>Reverse</strong>: GAAGAGGATGCTGATGAG</td>
<td>NM_001015555.1</td>
</tr>
<tr>
<td>RPS9</td>
<td><strong>Forward</strong>: CCTCGAGCAAGAGCAGATAG&lt;br&gt;<strong>Reverse</strong>: GCAGAGTGGTGAAGACT</td>
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<tr>
<td>YWAHZ</td>
<td><strong>Forward</strong>: GCATCCACAGACTATTTCC&lt;br&gt;<strong>Reverse</strong>: GCAGAGTGGTGAAGACT</td>
<td>GU_817014.1</td>
</tr>
</tbody>
</table>

LHB, luteinising hormone beta; FSHB, follicle stimulating hormone beta; GHSR, growth hormone secretagogue receptor (ghrelin receptor); GH1, somatotropin precursor/growth hormone; GnRHR, gonadotropin releasing hormone receptor; IGF-IR, insulin-like growth factor 1 receptor; IGF-1, insulin-like growth factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UBQ, ubiquitin; RPS9, ribosomal protein S9; YWAHZ, tyrosine 3-monooxygenase/trypophan 5-monooxygenase activation protein, zeta;
### Supplementary Table 2.3 Gene ID, sequence and accession number for testes genes examined in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5' to 3')</th>
<th>Accession number</th>
</tr>
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<tr>
<td>PCNA</td>
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<td>ZO1</td>
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<td>NM_001206607.3</td>
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</tr>
</tbody>
</table>

PCNA, proliferation cell nuclear antigen; THY1, Thy-1 cell surface antigen; ZO1, tight junction protein 1/zonula occludens protein 1; GATA4, GATA binding protein 4; AR, androgen receptor; FSHR, follicle stimulating hormone receptor; AQP8, aquaporin-8; UCHL1, ubiquitin carboxyl-terminal esterase L1; AMH, anti-müllerian hormone; CLDN11, claudin 11; LHR, luteinising hormone receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UBQ, ubiquitin; RPS9, ribosomal protein S9; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.
CHAPTER 3: Effect of Early Calf-Hood Nutrition on the Transcriptional Regulation of the Hypothalamic-Pituitary-Testicular axis in Holstein-Friesian Bull Calves

3.1 Abstract

The aim of this study was to investigate the effect of divergent early calf-hood nutrition on the transcriptomic profile of the arcuate nucleus region of the hypothalamus, anterior pituitary and testes in Holstein-Friesian bulls. Holstein-Friesian bull calves with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, respectively, were assigned to either a high (n=10) or low (n=10) plane of nutrition. At 126 (±3) days of age, calves were euthanized, tissues samples were harvested and RNASeq analysis was performed. There were 0, 49 and 1,346 genes differentially expressed in the arcuate nucleus, anterior pituitary and testicular tissue of bull calves on the low relative to the high plane of nutrition, respectively (P<0.05; False Discovery Rate <0.05). Plane of nutrition had no effect on gene expression in the arcuate nucleus. Cell cycle processes such as mitotic roles of polo-like kinase and cell cycle:G2/M DNA damage checkpoint regulation were down regulated in the low relative to the high plane of nutrition in the anterior pituitary; however there was no evidence for differential expression of genes with a known function in the modulation of reproductive processes. The expression of genes involved in cholesterol and androgen biosynthesis in the testes were down regulated in animals on the low plane of nutrition. This study provides insight into the role of early life metabolic status in regulating the HPT axis and may provide putative candidate molecular biomarkers for early onset of puberty in the bull.

Keywords: Gene Expression, RNASeq, Androgen Biosynthesis, Spermatogenesis, Puberty
3.2 Introduction

Dairy bulls are now selected as potential AI sires within weeks of birth using genomic selection (Schefers and Weigel, 2012). There is increasing evidence for a positive effect of the plane of nutrition during calf hood on the early onset of puberty in the bull (Byrne et al., 2017b, Brito et al., 2007c, Dance et al., 2015). It is desirable not only for such bulls to reach puberty earlier but also that they have the capability of producing high volumes of good quality semen early in life, particularly within the context of seasonal dairy production systems. It is critically important, therefore, to gain a better understanding of the effect of early calf-hood nutrition on the biochemical pathways and key factors affecting sperm production to facilitate the design of improved rearing protocols for such genetically elite and valuable bulls.

Enhanced nutrition in the early calf-hood period has been shown to positively impact the hypothalamic GnRH pulse generator and its action on the anterior pituitary gland; thereby advancing the age of onset of puberty in bulls (Brito et al., 2007b). The hypothalamus is widely acknowledged as the homeostatic regulator of the body (Coll and Yeo, 2013). Metabolic signals are sent from organs such as the liver (IGF-1), pancreas (insulin) and adipose tissue (leptin, adiponectin) and received by metabolic sensing neurons involved in satiety and energy homeostasis within the ARC. Such biochemical signals are mediated by neurons including NPY, AGRP (Stanley et al., 2005b, McShane et al., 1993, Stanley et al., 2005) as well as KISS1 (Gottsch et al., 2004, Estrada et al., 2006). These neurons stimulate GnRH release thereby affecting reproductive function.

The anterior pituitary is the principal regulator for growth, metabolism and reproduction via the synthesis and/or release of an array of hormones that control these functions in multiple peripheral organs (Musumeci et al., 2015). The gonadotrophic cells in the anterior pituitary are characterised by the expression of GnRH receptors; which are responsible for the
regulation of testicular function through secretion of gonadotropins, LH and FSH (Schanbacher, 1982). The age at which bulls of dairy breeds attain puberty can range from 8-11 months (Rawlings et al., 2008), although this is influenced by management. There is a transient rise of LH which occurs between 8 and 20 weeks of age, with a peak at 12-15 weeks, declines between 20 and 24 weeks of age (Evans et al., 1996). Restricted nutrition during calf hood has been reported to affect steroidogenesis in the testes via inhibition of the magnitude of the hypothalamic GnRH pulse and therefore, the response of the anterior pituitary (Brito et al., 2007b).

The effect of level of nutrition offered to calves’ during the first six months of life on the early gonadotropin rise and the age at which puberty is reached cannot be rectified by the level of nutrition received thereafter (Dance et al., 2015, Byrne et al., 2017b, Brito et al., 2007b). Recent work by our group has demonstrated that Holstein-Friesian bulls fed a high plane of nutrition for the first six months of life reached puberty approximately one month earlier than bulls on a lower plane irrespective of their plane of nutrition during the subsequent months (Byrne et al., 2017b).

Studies have been carried out in heifers using microarray, in situ hybridisation and immunohistochemical technologies to investigate the effect of early life nutrition on the molecular control of the hypothalamus and its knock on effects on age at onset of puberty but there is a lack of information on the nutritional influence on the molecular control of the HPT axis of the bull calf (Allen et al., 2012, Alves et al., 2017, Cardoso et al., 2015). Recent advances in deep-sequencing technology provides the opportunity for in-depth insight into the global transcriptome of key biologically important tissues. The hypothesis under investigation in this study was that the global transcriptomic profiles of hypothalamic-pituitary-testicular tissues would be affected by plane of nutrition during the early calf-hood period in Holstein-Friesian bulls.
3.2 Material and Methods

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

3.2.1 Animal Model

This experiment was conducted as part of a larger study designed to examine the effect of early calf hood nutrition on the molecular control of the HPT axis. The animal model and management has previously been described in Chapter 2. Briefly, Holstein-Friesian bull calves (n = 20) were assigned to either a high or low plane of nutrition at a mean live weight (± S.D.) of 47.5 (± 5.3) kg and an age of 19 (± 8.2) days. Calves on the high and low planes of nutrition were individually fed milk replacer and concentrates. After five days acclimatisation, calves on the high plane of nutrition were offered 1,200 g of milk replacer in 8 L of water daily, together with concentrate *ad libitum*, while those on the low plane of nutrition received 500 g of milk replacer in 4 L of water plus a maximum of 1 kg of concentrates daily. Calves on both treatments were weaned when consuming a minimum of 1 kg of concentrate for three consecutive days, at a mean age of 82 (± 3.9) days. Following weaning, the high plane of nutrition group was offered *ad libitum* concentrates, while the low plane of nutrition group was offered 1 kg of concentrate, daily. All calves had daily access to approximately 0.5 kg of straw each and a constant supply of fresh water throughout the trial.

3.2.2 Tissue Collection

The calves were euthanized at a mean age of 126 (±1.1) days of age, using an intravenous overdose of sodium pentobarbitone. The timing of slaughter was chosen as all calves at this stage would have been expected to have experienced an endogenous transient LH rise (Rawlings et al., 2008) and the timing and magnitude of this rise affects would have affected
testicular testosterone synthesis. Death was confirmed by exsanguination followed by decapitation. The skullcap was opened and the brain was removed from the skull by severing the infundibulum, optic nerves and brain stem. Tissue enclosing the ARC nucleus region of the hypothalamus was dissected according to Komatsu et al. (2012). Two small triangular sections containing the arcuate nucleus were taken from either side of the bottom of the third ventricle. The pituitary gland was removed from the sella turcica and anterior and posterior sections of the pituitary gland were separated. The testes were excised and the tunica albuginea, epididymides and any excess connective tissue removed. Two sections of the parenchyma were dissected from the middle region of each testis. All samples were washed in sterile Dulbecco's phosphate-buffered saline (DPBS), snap-frozen in liquid nitrogen, and subsequently stored at −80°C pending further processing.

3.2.3 RNA isolation and purification

Total RNA was extracted from each tissue sample using RNeasy Universal plus Kit (Qiagen, Manchester, UK). The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, Delaware, US). RNA quality was assessed on the Agilent Bioanalyzer 2100 using the RNA 6000 Nano Lab Chip kit (Agilent Technologies, Ireland). RNA was purified using the RNA Clean and Concentrator kit (Zymo Research, Irvine, CA) and the RNA integrity number (RIN) for all samples was greater than 8.

3.2.4 RNA-Seq library preparation and sequencing

Sixty (20 calves x 3 tissues) cDNA libraries were prepared from high quality RNA using an Illumina TruSeq RNA Sample Preparation kit v2 following the manufacturer’s instructions (Illumina, San Diego, CA, USA). For each sample, 1μg of total RNA was used for cDNA preparation. All libraries were validated on the Agilent Bioanalyzer 2100 using the DNA
1000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd, Dublin, Ireland). Individual RNASEq libraries were pooled based on their respective sample-specific-6 bp adaptors and sequenced at 100 bp/sequence single-end reads using an Illumina HiSeq 2500 sequencer.

### 3.2.5 RNASeq data analyses

Raw sequence reads were checked for quality using FASTQC software (version 0.10.0) ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Two testes samples from the low plane of nutrition and one anterior pituitary sample from the high plane of nutrition were removed from the study due to low sequence reads. All reads passed the quality statistics and input reads were then aligned to the bovine reference genome (UMD4.0) using Spliced Transcripts Alignment to a Reference (STAR) aligner. Reads were only aligned if they contained fewer than two mismatches with the reference genome and uniquely mapped to the reference genome. The software package HTSeq (v0.5.4p5) was used to calculate the number of sequence reads overlapping all protein coding genes from the ENSEMBLv74 annotation of the bovine genome. The number of read counts mapping to each annotated gene from HTSeq was then collated into a single file and used for calculation of subsequent differential gene expression. Genes with low read counts across all libraries were excluded from subsequent analysis. Reads were filtered out if they contained fewer than five counts per million in nine samples out of the total sample number. Only uniquely mapped reads were used for subsequent differential gene expression analysis. The R (v 3.01) Bioconductor package EdgeR (v3.2.4), was applied to identify statistically significant differentially expressed genes (DEGs).

### 3.2.6 Pathway analysis

Biological pathways were identified using GOSeq software (v1.14.0)(Young et al., 2010) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway annotations (Kanehisa and
Goto, 2000) were used to identify biological pathways that were over-expressed among DEGs. GOSeq is an application for executing gene ontology analysis on RNASeq data while accounting for biases (Young et al., 2010). The online tool BioMart (www.ensembl.org/biomart/martview) was used to convert the bovine gene IDs, extracted from GOSeq, into human orthologs. These human orthologs were inputted into KEGG (http://www.genome.jp/kegg/pathway.html). To examine the molecular functions and biological pathways, the RNASeq data were also analysed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com).

3.3 Results

3.3.1 Animal Performance

Weight gain and other indicators of animal performance were outlined previously. In brief, calves on a high plane of nutrition had larger testes, greater seminiferous tubule diameter, more mature spermatogenic cells and more Sertoli cells. The benefits of high plane of nutrition on the testes were validated transcriptionally in this study.

3.3.2 Differential gene expression

The RNASeq data have been deposited in NCBI’s Gene Expression Omnibus and are accessible via GEO series accession number GSE97673. The average number of raw reads for arcuate, anterior pituitary and testes samples were 16.3 million (mean ± SD; 16,319,326 ± 7,160,418), 16.3 million (16,346,430 ± 5,094,647) and 18.3 million (18,324,101 ± 7,135,544), respectively. There were 0, 49 and 1,346 DEG identified in ARC, anterior pituitary and testes tissues between animals on the two divergent planes of nutrition (P<0.05; False Discovery Rate<0.05), respectively. A multi-dimensional scaling (MDS) plot was created in Edge-R which measured the similarity between samples from the two planes of nutrition the high and low. The MDS plot showed a lack of separation between samples from
the two planes of nutrition with regard to the arcuate nucleus and anterior pituitary tissue but generally good separation between samples from animals on the high compared with the low planes of nutrition in the testes tissue (Figure 3.1).

3.3.3 Pathway Analysis

There were 45 DEG out of the original 49 anterior pituitary genes (seven genes with increased expression and 38 genes with decreased expression in the low compared to the high plane of nutrition groups; Supplementary Table 1) and 1,315 DEGs out of the original 1,346 testes genes (1,049 genes with increased expression and 266 genes with decreased expression in the low compared to the high plane of nutrition; Supplementary Table 2) that mapped successfully to a molecular/biological pathway using IPA. These DEG were then analysed and allocated to a biological function within IPA. The pathways and processes most affected within anterior pituitary tissue of the calves on the low, compared to the high plane of nutrition were all related to cycle cell processes such as mitotic roles of polo-like kinase (P<0.0001) and cell cycle: G2/M DNA damage checkpoint regulation (P<0.0001). Those pathways and processes identified as most differentially expressed within testicular tissue were super pathway of cholesterol biosynthesis (P<0.0001) and androgen biosynthesis (P<0.0001). Information on the effect of plane of nutrition on the molecular and cellular functions and on the biochemical pathways of the anterior pituitary and testes are presented in Figures 3.2-3.5, respectively. Gametogenesis was predicted to be increased (z-score: 2.279) in testicular tissue of the low compared to the high plane of nutrition calves (Table 3.1). Male infertility function was predicted to be decreased (z-score: -2.236) in the low compared to the high plane of nutrition group.
3.4 Discussion

This is the first study to investigate the effect of plane of nutrition during the early calf-hood period on the transcriptomic profile of the HPT axis in young bulls. Despite large differences in animal growth rate, metabolic status and testicular development between the nutritional treatments groups employed in the study, no DEG were detectable within tissue from the arcuate nucleus region of the hypothalamus. Offering calves a low plane of nutrition caused down regulation of processes involved in cell cycle in the anterior pituitary. The high plane of nutrition induced an increase in DEG in the testes, affecting processes involved in hormone production including androgen and cholesterol biosynthesis in comparison to the low plane of nutrition. Surprisingly, the function of ‘gametogenesis’ was predicted to be upregulated in testicular tissue of calves on the low compared with the high plane of nutrition.

3.4.1 Cholesterol Biosynthesis

Cholesterol is the precursor for all steroid hormones and its availability is vital for their optimal production (Hu et al., 2010). Additionally, cholesterol is essential for sperm cell membrane as it regulates membrane fluidity, aiding in its stabilization (Keber et al., 2013).

Cholesterol can be synthesised de novo from acetate, cholesterol ester stores in intracellular lipid droplets or the uptake of cholesterol from low density lipoprotein receptors (LDLR) (Miller, 2007). There are five major stages in the process of cholesterol synthesis. Firstly, two acetyl CoA molecules are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and HMG-CoA is converted to mevalonate. Mevalonate is converted to isopentenyl pyrophosphate (IPP), which is then converted to squalene and the final step is the transformation of squalene to cholesterol. Genes encoding for the enzymes catalysing the biosynthesis of cholesterol including mevalonate kinase (MVK), phosphomevalonate (PMVK), disphosphomevalonate decarboxylase (MVD), dimethylallyltranstransferase (FDPS),
lanosterol synthase (*LSS*), D24 sterol reductase (*DHCR24*), D14 sterol reductase (*TM7SF2*), cholesterol D-isomerase (*EBP*) and 7-dehydrocholesterol reductase (*DHCR7*) were all found to be downregulated in the low compared to the high plane of nutrition group in testicular tissue. This is of interest as under normal physiological conditions, *de novo* cholesterol synthesis replenishes cholesterol stores in Leydig cells (Hu et al., 2010).

The low plane of nutrition had a negative effect on genes that mediate the transport of cholesterol. Interestingly, the uptake of high density lipoprotein (HDL) cholesteryl esters via binding Apolipoprotein E (HDL-ApoE) (Fofana et al., 2000), the selective uptake pathway of HDL cholesterol to the testes via SCARBI (Acton et al., 1996) and the LDLR pathway (Miller, 2007) were downregulated in calves on the low plane of nutrition. Liver X receptor (*LXR*) has been reported to be crucial for maintaining cholesterol homeostasis in murine Sertoli cells (Robertson et al., 2005). In our study plane of nutrition had no effect on *LXR* gene expression; however, the low plane of nutrition did affect its target genes such as Apolipoprotein E (*APOE*; log fold expression: -2.274) which is expressed in both Sertoli cells and germ cells of sexually immature rats and the Leydig cells of sexually mature rats (Olson et al., 1994) and Scavenger receptor B1 (*SCARBI*; log fold expression: -1.816) which is expressed in murine Leydig cells (Casado et al., 2012). The expression of LDLR was downregulated (log fold expression: -1.789) in calves on the low compared to the high plane of nutrition, indicating that this source of cholesterol inhibited testosterone synthesis as testicular Leydig cells under certain conditions obtain plasma lipoprotein-derived cholesterol for steroid synthesis (Azhar and Reaven, 2002). In an associated study we analysed blood from the animals employed here and found greater serum testosterone concentrations in the calves on the high compared to the low plane of nutrition (Chapter 2). Therefore, down regulation of cholesterol biosynthesis will likely negatively affect androgen biosynthesis.
3.4.2 Androgen Biosynthesis

Androgens are crucial for optimal male reproductive function, playing important roles in maintaining spermatogenesis and sexual function. The rate of testosterone and oestradiol production is controlled by gonadotropins, LH and FSH (Hu et al., 2010). In our study we observed that FSHR (follicle stimulating hormone receptor) was down regulated in the high compared to the low plane of nutrition in the testes (log fold expression: -1.021). In the male, FSH stimulates Sertoli cell proliferation, as well as the induction and maintenance of normal spermatogenesis (Simoni et al., 1999). Expression of FSHR mRNA and number of FSH receptors has been found to decrease with testes development as bulls mature (Dias and Reeves, 1982). It has been reported that steroidogenic acute regulatory protein (StAR) is the primary determinant in the process of steroid synthesis (Christenson and Strauss, 2001). The expression of StAR mRNA has previously been shown to be expressed in bull testes (Pilon et al., 1997) and it regulates the transport of cholesterol from the outer into the inner mitochondrial membrane. Treating bulls with GnRH agonist caused increased testosterone biosynthesis and StAR protein in the testes (Aspden et al., 1998). In our study, calves offered the low plane of nutrition had a lower expression of StAR (log fold change: -1.914) compared to their contemporaries on the high plane of nutrition. The expression of StAR has been reported to be stimulated by IGF-1 in fetal and adult mice Leydig cells (Manna et al., 2006, Villalpando and Lopez-Olmos, 2003). This is consistent with previous reports from our group showing greater plasma IGF-1 concentrations when the same animals in this study were offered a high compared with a low plane of nutrition (Chapter 2).

There are two pathways involved in the conversion of pregnenolone to testosterone in the Leydig cells, Δ4 or Δ5. In the Δ5 pathway, pregnenolone is converted to 17α-hydroxyprogrenolone then to dehydroepiandrosterone and finally to testosterone through
either androstenediol or androstenedione. Our data show that this pathway was negatively affected by the low plane of nutrition. The conversion of cholesterol to pregnenolone is catalysed by cytochrome P450 side chain cleavage enzyme (P450scc) located in the inner mitochondrial membrane (Stocco, 2001). The transcription of CYP11A1 gene encoding P450scc regulates the quantity of P450scc and therefore, the steroidogenic function (Miller, 2007). In the current study, CYP11A1 was down regulated in the low compared to the high plane of nutrition. Genes encoding for the enzymes catalysing the formation of testosterone including 3-β-hydroxysteroid dehydrogenase (HSD3B7 and HSD3B2) and steroid D-isomerase (HSD3B2 and EBP) were down regulated in the low compared to the high plane of nutrition, which led to decreased production of testosterone. However, reducing dietary nutrient intake did not down regulate all genes related to testosterone production as 17β-HSD, which codes for the enzyme responsible for the conversion of androstenedione to testosterone, was not altered by plane of nutrition.

3.4.3 Gametogenesis

There were 11 of 87 genes displaying upregulated expression in the low plane of nutrition animals consistent with a predicted increase in gametogenesis (spermatogenesis). These genes included SIAHI, RNF2, MCM9, CTCFL, FSHR, AGFG1, DDX25, MCM8 and KDM3A, which have all been reported to be involved in aiding gametogenesis (Garikapati et al., 2017, Lindgren et al., 2012, Kang-Decker et al., 2001). This was surprising and may be due to the low plane of nutrition calves experiencing a late spurt of early stage gametogenesis such as the development of spermatogonia whereas the high plane of nutrition calves could be at a more advance stage of spermatogenesis as indicated in the morphological data in Chapter 2. Male infertility function was predicted to be decreased in the low plane of nutrition compared to the high. There were five genes out of 11 genes which had an
expression consistent with an increase in male infertility. These genes including \textit{KMT2E} (\textit{MLL5}), \textit{SETX}, \textit{GMCL1}, \textit{SIRT1} and \textit{TDRD5}; which have all been reported to be involved in aiding spermatogenesis in adult mice and humans (Yap et al., 2011, Becherel et al., 2013, Kleiman et al., 2003, Bell et al., 2014, Yabuta et al., 2011). Gonadotropin regulated testicular RNA helicase (GRTH/DDX25) is regulated by gonadotropin in Leydig cells and germ cells and is necessary for completion of spermatogenesis (Tsai-Morris et al., 2012). In Leydig cells, GRTH is a negative regulator of the expression of genes involved in cholesterol synthesis and transfer (SREBP2, HMG-CoA and StAR) and therefore, exhibiting control over androgen synthesis (Fukushima et al., 2011). However, as referred to earlier, we found that testosterone concentrations were significantly higher for the calves on the high compared with the low plane of nutrition from 10 weeks of age until their slaughter at 18 weeks of age and the high calves had larger testes (Brito, 2014), a greater seminiferous tubule diameter, a greater number of more spermatogenic cells and a greater number of Sertoli cells. Our research group has also reported on the effect of plane of nutrition on systemic concentrations of testosterone in Holstein Friesian bulls calves at 16 and 32 weeks of age, following an exogenous GnRH challenge (Byrne et al., 2017b). Additionally we have shown Holstein Friesian bulls calves offered a high plane of nutrition pre six months to reach puberty (the age that a bull first produces an ejaculate yielding at least 50 x 106 spermatozoa with a minimum of 10% motility) approximately 30 days earlier that their counterparts on a lower dietary allowance (Byrne et al., 2017b). Although both functions were predicted to be upregulated in the calves on the low compared to the high treatment, the testes from the high plane of nutrition calves were at a more mature stage with regard to testes weight, seminiferous tubule diameter, stage of spermatogenesis and Sertoli cell number and lumen development at slaughter.
Differences in metabolic function in the ARC using microarray technologies were reported in heifer calves fed on a high concentrate diet to achieve rapid bodyweight gain, compared to contemporaries offered a high forage diet, from three-seven months of age (Allen et al., 2012). This study found that key genes involved in satiety such as NPY and AGRP were down regulated and POMC and α-MSH were upregulated in the high concentrate heifers. The genes NPY/AGRP are well documented in have opposing roles to that of POMC/α-MSH in the control of feeding and energy expenditure (Stanley et al., 2005b (Stanley et al., 2005). It has been reported that NPY acts directly on GnRH neurons and that this may also be the case for AGRP as melanocortin agonists have direct action on GnRH neurons (Roa and Herbison, 2012). A study also carried out by the same research group using in-situ hybridisation and immunohistochemical technologies reported that increased rates of growth during the juvenile period in heifers alter the methylation pattern of genomic DNA from the ARC and such alterations may be linked to advanced age at puberty (Alves et al., 2017). Our study however, found no DEG within ARC tissue between treatments. However, the studies reported above involved animals of a different sex, age, nutritional treatment and analytical technologies compared to our study which may have contributed to the difference in findings between studies. Our study is the first to date to utilize next generation sequencing technology to examine the effect of early calf hood nutrition on the global transcriptome of HPT tissue in Holstein-Friesian bull calves. The transcriptional profile of the ARC nucleus and anterior pituitary found in this study does not reflect the phenotype, in that the only DEG found in the anterior pituitary related to cell cycle and not specifically to metabolism or reproductive processes, as might be expected. This may be due to the transitory nature of gene transcripts in the brain (Bondy and Lee, 1993). However, while we did not detect DEG in either ARC or the anterior pituitary tissues which were specific to reproductive processes, gene expression profiles for testicular tissue were indicative of prior influence of plane of
nutrition on the functionality of the hypothalamic-anterior pituitary axis, upon which testicular development depends. This lack of effect on the high plane of nutrition on the arcuate nucleus and anterior pituitary gene expression may be related to the fact that the relevant neurons and cell types represent a minority of the total cell population found in this areas.

3.5 Conclusions

In conclusion, this study has demonstrated that plane of nutrition offered to young dairy bulls during the first 18 weeks of life has a significant effect on the transcriptional functionality of the testes. A low plane of nutrition during early calf hood led to a down regulation of the expression of genes affecting production of precursor cholesterol (systemic cholesterol was not measured in this study) and also the transport of both high and low density lipoprotein cholesterol transportation to the inner mitochondria from androgen biosynthesis. Surprisingly, genes purported to be involved in gametogenesis were significantly upregulated in the low plane of nutrition in comparison to that of the high. This directly contradicts our observations on the phenotype of the low calves that had small testes, a lower number of Sertoli cells and less mature spermatogenic cells than their counterparts offered the high plane of nutrition. Overall, our data contribute to the understanding of the molecular responsiveness of the HPT axis to early life plane of nutrition which undoubtedly affects the timing of puberty. The molecular signatures observed here, particularly in relation to testicular transcripts, could aid in the future identification of biomarkers of precocious puberty in young bulls.
Figure 3.1 Multidimensional scaling plot which shows the measured similarity of the samples in 2-dimensions. The samples labelled in yellow are the Holstein-Friesian dairy bulls fed on a low plane of nutrition and slaughtered at 18 weeks of age and those labelled in red are the Holstein-Friesian dairy bulls fed on a high plane of nutrition and slaughtered at 18 weeks of age. Arc=Arcuate nucleus, Pit=Anterior Pituitary, Tes=Testes. BCV = Biological Coefficient of Variation.
**Figure 3.2** The top biochemical pathway significantly enriched in the anterior pituitary of Holstein-Friesian dairy bulls fed on a low plane of nutrition in comparison to a high plane of nutrition, slaughtered at 18 weeks of age. Greens bars represent genes down regulated and red bars up regulated genes as percentages of the overall number of genes in each pathway. The significance of each pathway is represented by the yellow line describing $-\log(p$-value$)$. The P-value is calculated by the number of genes from our dataset of differentially expressed genes that participate in a particular pathway and dividing it by the total number of genes in the Canonical Pathway in IPA analysis.
Figure 3.3: The top molecular and cellular function of differentially expressed genes of the anterior pituitary of Holstein-Friesian dairy bulls fed on a low versus high plane of nutrition and slaughtered at 18 weeks of age. The bars indicate the likelihood [-log (P-value)] that the specific molecular and cellular function was affected by a high plane of nutrition. The threshold line in the bar chart represents a p-value of 0.05.
Figure 3.4: The top biochemical pathway significantly enriched in the testes of Holstein-Friesian dairy bulls fed on a low plane of nutrition in comparison to a high plane of nutrition, slaughtered at 18 weeks of age. Greens bars represent genes down regulated and red bars up regulated genes as percentages of the overall number of genes in each pathway. The significance of each pathway is represented by the yellow line describing $-\log(p\text{-value})$. The P-value is calculated by the number of genes from our dataset of differentially expressed genes that participate in a particular pathway and dividing it by the total number of genes in the Canonical Pathway in IPA analysis.
Figure 3.5 The top molecular and cellular function of differentially expressed genes of the anterior pituitary of Holstein-Friesian dairy bulls fed on a low versus high plane of nutrition and slaughtered at 18 weeks of age. The bars indicate the likelihood [-log (P-value)] that the specific molecular and cellular function was affected by a high plane of nutrition. The threshold line in the bar chart represents a p-value of 0.05.
Table 3.1a Gametogenesis genes differentially expressed in the testes tissue of the Holstein-Friesian dairy bulls feed on a low plane of nutrition in comparison to the high plane of nutrition and slaughtered at 18 weeks of age

<table>
<thead>
<tr>
<th>Genes</th>
<th>Predicted affect</th>
<th>Expr Log Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIAH1</td>
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</tr>
<tr>
<td>RNF2</td>
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<td>0.662</td>
</tr>
<tr>
<td>MCM9</td>
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<td>0.49</td>
</tr>
<tr>
<td>CTCFL</td>
<td>Increased</td>
<td>0.937</td>
</tr>
<tr>
<td>NUP153</td>
<td>Increased</td>
<td>0.48</td>
</tr>
<tr>
<td>COIL</td>
<td>Increased</td>
<td>0.616</td>
</tr>
<tr>
<td>FSHR</td>
<td>Increased</td>
<td>1.021</td>
</tr>
<tr>
<td>AGFG1</td>
<td>Increased</td>
<td>0.503</td>
</tr>
<tr>
<td>DDX25</td>
<td>Increased</td>
<td>0.781</td>
</tr>
<tr>
<td>MCM8</td>
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<td>KDM3A</td>
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<td>FADS2</td>
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<td>MNS1</td>
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**Table 3.1b** Continuation of gametogenesis genes differentially expressed in the testes tissue of the Holstein-Friesian dairy bulls feed on a low plane of nutrition in comparison to the high plane of nutrition and slaughtered at 18 weeks of age

<table>
<thead>
<tr>
<th>Genes</th>
<th>Predicted affect</th>
<th>Expr Log Ratio</th>
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<td>ADGRG2</td>
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<td>SCARB1</td>
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<tr>
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<td>ACVR2A</td>
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<tr>
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CHAPTER 4: Effect of Early Calf-Hood Nutrition on the Transcriptomic Profile of Subcutaneous Adipose Tissue in Holstein-Friesian Bulls

4.1 Abstract

Adipose tissue is a major endocrine organ and is thought to play a central role in the metabolic control of reproductive function in cattle. Plane of nutrition during early life has been shown to influence the timing of puberty in both male and female cattle, though the exact biological mechanisms involved are currently unknown. The aim of this study was to investigate the effect of early calf-hood nutrition on the transcriptomic profile of subcutaneous adipose tissue in Holstein-Friesian bulls to identify possible downstream effects on reproductive physiology. Holstein-Friesian bull calves with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, respectively, were assigned to either a high (n=10) or low (n=10) plane of nutrition. At 126 days of age, the bulls were euthanized, subcutaneous adipose tissue samples were harvested and RNASeq analysis was performed. There were 674 genes differentially expressed in adipose tissue of calves on the low compared with the high plane of nutrition (P<0.05; FDR<0.05; fold change >2.0). High plane of nutrition positively altered the expression of genes across an array of putative biological processes but the most dominant cellular processes affected were cellular energy production and branched chain amino acid degradation. A high plane of nutrition caused upregulation of genes such as leptin (LEP) and AdipoQ, which are known to directly affect reproductive function. These results provide an insight into the effect of augmenting the plane of nutrition of Holstein-Friesian bull calves in the prepubertal period on the transcriptome of adipose tissue.

Keywords: Gene Expression, Reproduction, Adipogenesis, Amino Acids, Mitochondrial Dysfunction, Puberty
4.2 Introduction

The advent of genomic selection in the dairy industry has resulted in the identification of sires for use in artificial insemination within weeks of birth and semen from these young bulls is in high demand once they reach puberty. Studies have shown that plane of nutrition in early calf-hood plays a critical role in the timing of the onset of puberty in bulls (Brito et al., 2007b, Dance et al., 2015, Byrne et al., 2017b). Recently, our research group has demonstrated that a high plane of nutrition prior to, but not after six months of age can accelerate puberty in Holstein-Friesian bulls by approximately one month (Byrne et al., 2017b). This has been associated with the advancement of a transient rise in systemic concentrations of LH, which normally occurs between approximately 8 and 20 weeks of age (Rawlings et al., 2008). There are many organs implicated in signalling nutritional status to the hypothalamus and anterior pituitary to stimulate GnRH and LH secretion, respectively, with white adipose tissue (WAT) key amongst these.

White adipose tissue has traditionally been known for its role in energy storage and release when energy expenditure is greater than energy intake; however, WAT is metabolically active and can therefore influence many bodily systems. The function of WAT in metabolic and reproductive processes is complex but it has been postulated that there is cross-talk between adipokines and the HPT axis. Adipogenesis and fat development are influenced by genetics, diet, body depot location and age (Jin et al., 2012, Romao et al., 2014). There is evidence that offering calves high starch diet during early calf hood induces precocious adipogenesis and lipid filling (Graugnard et al., 2010). It is postulated that the period of potential for stem cells to differentiate into adipocytes is very limited during early life development (Wang et al., 2009a). Adipose hormones such as leptin production increases proportionally in line with an increase in body fat (Landry et al., 2013); evidently then, it is a signal of energy sufficiency (Sanchez-Garrido and Tena-SEMPere, 2013). It has been reported
that subcutaneous adipose tissue has a higher concentration of both leptin and AdipoQ protein than that of visceral adipose (Ouchi et al., 2011). Leptin signals various bodily systems, including the HPT axis, of metabolic energy status and reserves (Ahima et al., 2000). Studies have shown that while there is an absence of leptin receptors on some key reproductively related cells such as GnRH neurons (Quennell et al., 2009); the effects of leptin on GnRH release have been shown to be mediated by kisspeptin in some species (Smith et al., 2006). AdipoQ, another adipokine hormone, has been shown to have its receptors, ADIPOR1 and ADIPOR2, expressed in the anterior pituitary and the ARC in the hypothalamus of cows (Tabandeh et al., 2011); therefore, AdipoQ employs two potential pathways to signal to the HPT axis.

It has been reported in children that consistent excessive energy intake during early life development can contribute to precocious puberty (Aksglaede et al., 2009). In bull calves of dairy breeds early pubertal onset is advantageous to facilitate early semen collection. The exact metabolic mechanisms are unknown; hence this study aimed to examine the effect of early life plane of nutrition on global gene expression profiles of adipose tissue in Holstein-Friesian bulls within the particular context of potential latent implications for HPT function. Much of the published work to-date on this topic has focused on the effect of body condition score (BCS) on adipose tissue hormone signalling in post pubertal animals and there is a dearth of information on the role of adipose tissue in stimulating the HPT axis and its potential as a key regulator of the timing of puberty onset in cattle.

4.3 Materials and methods

All procedures involving animals were approved by the Teagasc Animal Ethics Committee and were licensed by the Health Products Regulatory Authority, Ireland in accordance with the European Union Directive 2010/36/EU.
4.3.1 Animal model

This experiment was conducted as part of a larger study designed to examine the effect of early calf-hood nutrition on the physiological control of the HPT axis. Holstein-Friesian bull calves (n=20) with a mean (±S.D.) age and bodyweight of 19 (±8.2) days and 47.5 (±5.3) kg, were purchased from commercial dairy farms and blocked based on sire, initial weight, farm of origin and age and assigned to either a high (n=10) or low (n=10) plane of nutrition. Calves were individually fed milk replacer and concentrates using an electronic feeding system (Forster-Tecknik Vario, Engen, Germany). After five days of acclimatisation, the high treatment were offered 1200 g of milk replacer in 8 L of water daily, together with concentrate ad libitum. Animals in the low treatment group were allocated 500 g of milk replacer in 4 L of water and a further allocation of a maximum of 1 kg of concentrate daily. All calves were weaned when consuming a minimum of 1 kg of concentrate for 3 consecutive days, at a mean age (±S.D.) of 82 (±3.9) days. Following weaning, the high treatment group was offered ad libitum concentrates, while the low treatment group received 1 kg of concentrate daily. All calves had individual daily access to approximately 0.5 kg of straw as well as ad libitum access to fresh water.

4.3.2 Tissue collection

The calves were euthanized at a mean age (±S.D.) of 126 (±1.1) days, using an intravenous overdose of sodium pentobarbitone. Death was confirmed by lack of ocular response. Subcutaneous adipose samples were obtained from the flank of the carcass following slaughter and subsamples were either (i) snap-frozen in liquid nitrogen and subsequently stored at -80°C for long term storage pending further analysis or (ii) fixed in 10% neutral buffered formalin (Sigma-Aldrich, Wicklow, Ireland) and embedded in paraffin in accordance to the standard procedures. Sections (5 µm thick) were dehydrated in ascending
concentrations grades of alcohol, followed by clearing with xylene and were stained for gross anatomy using H&E stain.

4.3.3 Adipocyte cell number and diameter

Adipocyte cell number and cell size were analysed using Aperio ImageScope (v12; Leica Biosystems, Wetzlar, Germany). Five square sections (0.25 mm2) were electronically generated and randomly assigned on each slide and every adipocyte within these square sections was counted manually. The diameter of 200 adipocytes per animal, selected at random, within these square sections was measured at the widest point for each cell, using the ruler tool from the ImageScope software.

4.3.4 RNA isolation and purification

Total RNA was extracted using RNeasy Lipid Tissue Mini kit (Qiagen, Manchester, UK). RNA was purified using the RNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA). The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA quality was assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies Ireland Ltd, Dublin, Ireland) using the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd, Dublin, Ireland). Samples had an RNA Integrity Number (RIN) of mean (±SD) of 7.6 (±0.77).

4.3.5 RNASeq library preparation and sequencing

Twenty cDNA libraries were prepared from high quality RNA using an Illumina TruSeq RNA Sample Preparation kit v2 following the manufacturer’s instructions (Illumina, San Diego, CA, USA). For each sample, 1μg of total RNA was used for cDNA preparation. All libraries were validated on the Agilent Bioanalyzer 2100 using the DNA 1000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd, Dublin, Ireland). Individual RNASeq libraries
were pooled (10 libraries per lane) based on their respective sample-specific-6 bp adaptors and sequenced at 100 bp/sequence single-end reads using an Illumina HiSeq 2500 sequencer.

### 4.3.6 RNASeq data analyses

Raw sequence reads were checked for quality using FASTQC software (version 0.10.0) ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Input reads were then aligned to the bovine reference genome (UMD3.1) using Spliced Transcripts Alignment to a Reference (STAR) aligner. Reads were only aligned if they contained fewer than two mismatches with the reference genome and uniquely mapped to the reference genome. The software package HTSeq (v0.5.4p5) was used to calculate the number of sequence reads overlapping all protein coding genes from the ENSEMBLv74 annotation of the bovine genome. The number of read counts mapping to each annotated gene from HTSeq was then collated into a single file and used for calculation of subsequent differential gene expression. Only uniquely mapped reads were used for subsequent differential gene expression analysis. Genes with low read counts across all libraries were excluded from subsequent analysis. The R (v 3.01) Bioconductor package EdgeR (v3.2.4), was applied to identify statistically significant differentially expressed genes (DEG).

### 4.3.7 Pathway analysis

Biological pathways were identified using GOSeq software (v1.14.0; Young et al., 2010) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway annotations (Kanehisa and Goto, 2000) were used to identify biological pathways that were over-expressed among DEG. GOSeq is an application for executing gene ontology analysis on RNASeq data while accounting for biases (Young et al., 2010). The online tool BioMart (www.ensembl.org/biomart/martview) was used to convert the bovine gene IDs, extracted from GOSeq, into human orthologs. These human orthologs were inputted into KEGG
To examine the molecular functions and biological pathways, the RNASeq data was also analysed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com).

4.3.8 Statistical analysis

Data for adipocyte cell size and number were analysed using Statistical Analysis Software (SAS v9.3) and were tested for normality of distribution (UNIVARIATE procedure). Data were found to be normally distributed and were statistically analysed using ANOVA (MIXED procedure). Block was included as a variable. Animal was the experimental unit in all analysis and was included as a random effect. All results are presented as mean ± s.e.m.

4.4 Results

4.4.1 Animal performance and adipose tissue histology

Average daily bodyweight gain from the start to the end of the study was 1.08 (± 0.03) and 0.57 (± 0.03) for high and low plane of nutrition groups, respectively and this translated into a bodyweight difference of 53.8 kg at slaughter between the bulls of the high compared to the low treatment group (160.9 ± 3.98 kg versus 107.1 ± 3.19 kg, respectively; P<0.001). Adipocyte cell number (291 ± 47.2 cells/1.25 mm2 versus 7.5 ± 4.0 cells/1.25 mm2, P<0.001; Figure 4.4) and cell diameter (36.9 ± 2.89 µm versus 3.7 ± 1.94 µm, P<0.001) were both greater in the high compared with the low plane of nutrition animals, respectively.

4.4.2 Differential gene expression

The mean ± SD number of raw reads per sample was; 18,670,029 ± 4,093,759. Approximately 89 % of the reads were uniquely mapped to the reference bovine genome after alignment. A multi-dimensional scaling (MDS) plot was created in Edge-R which estimated the degree of similarity between samples from the high compared to the low nutritional treatments. The MDS plot showed a separation between the two treatments (Figure 4.1).
There were 745 differentially expressed genes (DEG) between the two nutritional treatments (P<0.05; False Discovery Rate<0.05; fold change >2.0). One hundred and sixty four genes had increased expression, while 581 had decreased expression. The RNASEq data have been deposited in NCBI’s Gene Expression Omnibus and area accessible via GEO series accession number GSE97674.

4.4.3 Pathway analysis

There were 678 DEG from the original 745 that mapped successfully to a molecular/biological pathway using IPA and these DEG were analysed and allocated to a biological function within IPA. Information on the effect of nutritional treatment on the molecular and cellular functions and on the biochemical pathways affected are presented in Figures 4.2 and 4.3, respectively. Results of KEGG pathway analysis using GOSeq (FDR <0.05) indicated that 23 KEGG pathways were over-represented among DEG in the low treatment compared to the high treatment at 18 weeks of age (Table 4.1). These enriched pathways included metabolic pathways, valine, leucine and isoleucine degradation and citrate cycle. After analysis using IPA, the following pathways and processes were identified as the most influenced by the differential nutritional regimen at the level of subcutaneous adipose tissue: energy production via mitochondrial dysfunction (P<0.0001), oxidative phosphorylation (P<0.0001), and tricarboxylic acid (TCA) cycle II (P<0.0001), amino acid metabolism via valine and isoleucine metabolism (P<0.0001). The directionality of the DEG relating to mitochondrial dysfunction, oxidative phosphorylation, TCA, valine and isoleucine degradation suggest upregulation of these pathways in the high treatment in comparison to the low treatment.

A total of 25 networks, regarded as having biological significance were identified (Table 4.2). Particular networks of interest including energy production, lipid metabolism and small
molecule biochemistry (Network 4) were identified as being enriched in the high compared to the low treatment. This network highlighted 28 associated molecules, 24 of which were upregulated, and 4 of which were down regulated in the high compared to the low treatment. Of particular interest, ADIPOQ (-1.62 log fold change) and its receptor ADIPOR2 (-1.772 log fold change) and LEP (leptin; -4.541 log fold change) were upregulated in the high compared to the low treatment. Using the Molecule Activity Predictor (MAP) function in IPA insulin has been predicted to be inhibited in the low compared to the high treatment.

4.4.4 Lipogenesis and adipogenesis

Animals on the low plane of nutrition had a lower of expression of MLXIPL (MLX interacting protein-like, also known as ChREBP; -1.375 log fold change), a transcription factor that regulates lipogenesis and adipogenesis, in comparison to the high plane of nutrition. Target genes linked with adipocyte differentiation were down regulated in the low treatment in comparison to the high treatment, such as DGAT2 (diacylglycerol acyltransferase 2; -5.215 log fold change), FASN (fatty acid synthetase; -5.184 log fold change), ACACA (acetyl-CoA carboxylase-α; -3.432 log fold change), LPL (lipoprotein lipase; -3.241 log fold change), PLIN1 (Perilipin 1; -2.279 log fold change) and FABP4 (fatty acid-binding protein 4; -2.273 log fold change), INSIG-1 (insulin-induced gene 1; -3.216 log fold change) and GDF-10 (growth differentiation factor 10; -2.014). Genes that act as inhibitors for adipogenesis such as GATA2 (GATA binding protein 2; 1.151 log fold change) and WNT2B (Wnt family member 2B; 1.672 log fold change) were up regulated in the low plane of nutrition compared to the high.

4.5 Discussion

This is the first study to apply next generation sequencing technology to examine the effect of early calf-hood nutrition on the transcriptomic profile of subcutaneous adipose in calves. The
histological and mRNA expression data indicate much lower adiposity (number and size of adipocytes) and greater number of preadipocytes in the calves subjected to the low plane of nutrition. This was consistent with evidence for the high plane of nutrition inducing an increase in both energy production and amino acid metabolic pathways in comparison to the low treatment. The ‘energy production, lipid metabolism and small molecule biochemistry network’ was also found to be enriched in the high compared to the low group with certain genes of particular interest within this network, including ADIPOQ, ADIPOR2 and LEP all consistent with increased adipogenesis and all upregulated in the high treatment group.

4.5.1 Lipogenesis and adipogenesis

It has been postulated that rapid growth during the post weaning period in humans can cause preadipocytes to enter terminal differentiation (Hausman et al., 2009). Some transcription factors have been highlighted as regulators of lipogenesis and adipogenesis at a transcriptional level, including sterol regulatory element binding factor 1 (SREBF1) and MLXIPL (Loor, 2010). The calves on the low plane of nutrition in our study had lower expression of MLXIPL in comparison to their contemporaries on the high plane of nutrition, which was supported by the complementary histology data. It has been reported that MLXIPL expression levels increase during preadipocyte differentiation in humans, mice and rats; however, there was a dramatic decrease in MLXIPL expression when cell differentiation took place in the absence of insulin (Hurtado del Pozo et al., 2011, He et al., 2004). It is well accepted that insulin is a metabolically active hormone responsible for anabolic processes promoting growth, development, and nutrient homeostasis (Taniguchi et al., 2006, Cheng et al., 2010). Indeed we recorded greater plasma concentrations of insulin in the high compared with the low treatment groups at the time of slaughter (data not presented), although no differences were observed at the mRNA level. Insulin-induced gene-1 (INSIG-1) has been reported to mediate sterol regulatory element binding protein-1c (SREBP-c) which is a
transcription factor involved in the regulation of the effect of insulin on lipid metabolism and adipogenesis (Dong and Tang, 2010). Studies have indicated that INSIG-1 increases in the fat tissue of mice developing diet induced obesity and also in differentiating 3T3-L1 preadipocytes (Li et al., 2003). This highlights the importance of insulin in lipogenesis.

In a recent study (Ambele et al., 2016) the authors reported a transcriptomic analysis of adipogenesis in human adipocyte tissue and identified a variety of genes as general adipocyte markers such as FABP4, PLIN1, LPL and ADIPOQ. These genes had increased expression during adipogenic differentiation consistent with the findings of others (Ullah et al., 2013). Upregulation of FABP4, FASN, ACACA and DGAT2 mRNA, which are target genes of PPARγ, are required for fatty acid biosynthesis and adipocyte differentiation (Obregon, 2008, Hausman et al., 2009). FASN is critical in the process of synthesising long chain saturated fatty acids and its function is inhibited by fasting (Wakil, 1989). Down regulation of ACACA has been reported in calorie restriction in adipose and liver tissue in pigs (Lkhagvadorj et al., 2010) and muscle in cattle (Keogh et al., 2016). It has been hypothesised that leptin controls adipocyte size via influencing DGAT expression (Suzuki et al., 2005). Even though, no difference in PPARγ expression was determined in our study, downstream genes affected by PPARγ were downregulated in tissue from the animals on the low plane of nutrition. There was no evidence of BMP (bone morphogenic protein) expression being affected by plane of nutrition. However, GDF10 which is linked to BMP3 was found to downregulated on the low plane of nutrition. As well as a down regulation in genes that stimulate adipogenesis, in our study we found an upregulation in a key gene found to inhibit adipogenesis in the low plane of nutrition compared to the high plane of nutrition. It has been reported that GATA2 is expressed in preadipocytes and is down-regulated during terminal differentiation (Tong et al., 2000). Studies in mouse preadipocytes show that GATA2 inhibits adipogenesis and traps cells at preadipocyte stage by direct suppression of PPARγ (Tong et al., 2000). In addition,
the histological data showing much lower adiposity (number and size of adipocytes) and greater number of preadipocytes in the animals on the low plane of nutrition corroborates the transcriptomic data aforementioned.

4.5.2 Endocrine regulatory networks

Insulin plays a role in regulating the synthesis and secretion of leptin, as adipocytes cultured in the presence of insulin increased the synthesis and secretion of leptin (Kim et al., 1998, Barr et al., 1997). It is therefore unsurprising that IPA predicted, when applied to our data, the stimulation of insulin as well as the known upregulation of LGALS12, BCAT2 and ADIPOQ, which are all located upstream of LEP and likely resulted in LEP to be upregulated in the high treatment. Plasma leptin is positively correlated with body fat in growing cattle (Ehrhardt et al., 2000) where it has been shown to signal energy abundance by informing the various bodily systems, including the HPT axis, of the prevailing metabolic status and energy reserves via circulating leptin protein concentrations (Ahima et al., 2000). For example, in adult animals, studies which examined the adipose tissue transcriptome of pre-partum dairy cows have shown that cows with greater BCS had greater expression of LEP (Vailati-Riboni et al., 2016). Consistent with this we found that calves on the high plane of nutrition in our study had >4.5 log fold higher LEP expression compared with animals the low animals. Despite this, when plasma concentrations were assayed, we failed to find any difference in leptin protein concentrations between the two groups (Chapter 2). This is in agreement with studies in young dairy and beef bulls (Brito et al., 2007b, Byrne et al., 2017c, Dance et al., 2015). The lack of differences in plasma leptin concentrations may be due to the minute quantity of adipose tissue laid down in calves at this stage of development and/or the relative insensitivity of protein immunoassays compared with transcript based molecular approaches.
Leptin does not directly affect GnRH neurons of the hypothalamus and knocking out of leptin receptors on the GnRH neurons does not apparently delay the onset of puberty nor indeed affect subsequent fertility (Quennell et al., 2009). However, leptin has been found to indirectly influence the timing of puberty via the regulation of the hypothalamic KISS1 neurons’ stimulation of GnRH (Sanchez-Garrido and Tena-Sempere, 2013). Leptin can also bypass the hypothalamus and act on the pituitary gland and the testes; with leptin modestly stimulating gonadotropin secretion at a pituitary level and both direct stimulatory and inhibitory actions of leptin been reported in the gonads (Tena-Sempere, 2007). At high leptin concentrations, it has been reported to inhibit testosterone secretion in vitro in adult rats but to have no effect in pubertal rats (Tena-Sempere et al., 1999). This is possibly due to the fact that pubertal testes produce more 5α-reduced androgens than testosterone (Corpéchot et al., 1981). In research carried out by Byrne et al. (2017b) bulls which had experienced dietary restriction prior to six months of age and an increase in plane of nutrition post six months of age leading to an increase in leptin concentration, were older at puberty than their contemporaries offered a high plane of nutrition; highlighting the importance of early life nutrition on age at puberty.

AdipoQ functions in the regulation of lipid and glucose metabolism, insulin sensitivity as well as inflammation (Kadowaki et al., 2006, Ahima and Lazar, 2008, Galic et al., 2010). Its concentration in blood has been shown to be negatively correlated to adipocyte size (Hammes et al., 2012). Food intake inhibits ADIPOQ mRNA expression in WAT and therefore, inhibits AdipoQ concentration in serum (Steinberg and Kemp, 2007). Globular AdipoQ protein has been found to inhibit the secretion of GnRH in GT1-7 cells derived from mouse hypothalamic GnRH neurons via the mediation of adenosine monophosphate-activated protein kinase (AMPK; Wen et al., 2008). However, high doses of adiponectin in MA-10 mouse Leydig cells has been reported to advance progesterone production through an
increase in StAR and the CYP11A1 steroidogenesis enzyme, suggesting that AdipoQ could stimulate testosterone production from the Leydig cells (Landry et al., 2015). It has also been shown that adiponectin reduces LH secretion directly from the gonadotropes in the anterior pituitary via AMPK (Lu et al., 2008). Pre-pubertal children with increased body mass index (BMI), though not classified as obese, had lower AdipoQ concentration in subcutaneous adipose (Sabin et al., 2006). Similarly, postpartum cows, in negative energy balance had decreased ADIPOR1 and ADIPOR2 abundance in subcutaneous adipose compared to antepartum cows (Lemor et al., 2009) which is in agreement with our findings with calves where we found AdipoQ and its receptor ADIPOR2 were downregulated in the low compared to the high treatment groups. It has been reported that ADIPOR2 null mice demonstrated seminiferous tubules with aspermia but had normal testosterone concentration (Bjursell et al., 2007). It has also been found that the abundance of AdipoQ, ADIPOR1 and ADIPOR2 is greater in high fertility compared to that of medium and low fertility Holstein bulls (Kasimanickam et al., 2013). There was no difference in serum concentrations of AdipoQ between the two groups in the current study (Chapter 2), which like leptin may be due to the overall low adipose deposition in young bull calves. Using a very similar animal model and the same assays as those employed here, Byrne et al. (2017b) similarly observed in leptin and AdipoQ concentrations during the same development as reported here. However, from eight months on age onwards, bull calves offered a high plane of nutrition had greater leptin compared to their contemporaries on a moderate plane of nutrition (ADG c.0.65 kg/day)

4.5.3 Branched chain amino acid metabolism

Branched chain amino acids (BCAAs), leucine, isoleucine and valine are essential amino acids and, unlike other amino acids which are catabolized by the liver, BCAAs are catabolized mainly in the muscle, adipose, kidney and the brain; as the liver does not contain
the branched amino acid transferase enzyme (BCAT2), necessary for BCAA catabolism (Herman et al., 2010). The isoleucine/valine degradation pathway enters the TCA cycle either directly or via an acetyl derived intermediate and in this study we found that genes involved in the valine degradation pathway encoding BCAT2, 2-oxoisovalerate dehydrogenase (BCKDHB and DLD), 2-methylacyl-CoA dehydrogenase (ACADSB), enoyl-CoA hydratase (ECHS1, EHHADH, HADHA and HADHB), 3-hydroxyisobutyryl-CoA hydrolase (HIBCH), methylmalonate-semialdehyde dehydrogenase (ALDH6A1) and (S)-3-amino-2-methylpropionate transaminase (ABAT) were all down regulated in the low in contrast to the high treatment. Similarly, within the isoleucine degradation pathway, genes encoding for branched-chain-amino-acid transaminase (BCAT2), branched-chain α-keto acid dehydrogenase complex (DLD), 2-methylacyl-CoA dehydrogenase (ACADSB), enoyl-CoA hydratase (ECHS1, EHHADH, HADHA and HADHB), 3-hydroxy-2-methylbutyryl-CoA dehydrogenase (HSD17B10) and acetyl-CoA C-acetyltransferase (ACAT1) were down regulated in the low compared to the high treatment. Expression of genes involved in branched chain amino acids metabolism were upregulated during 3T3-L1 adipocyte differentiation (Frerman et al., 1983, Kedishvili et al., 1994) suggesting that BCAA are involved in fatty acid synthesis (Crown et al., 2016). It has also been shown that BCCA, especially leucine, systemic concentrations increase leptin secretion in vitro in murine (Lynch et al., 2006) and decreases food intake in vitro in rats (Cota et al., 2006). Systemic concentrations of branched chain amino acid are elevated in response to over nutrition in children and adolescents (McCormack et al., 2013). Therefore, it is reasonable to suggest that the evidence for a decline in BCAA degradation may be a result of the moderate restriction in dietary allowance experienced by our low plane of nutrition calves. The BCAA catabolic by-products mentioned in this study are intended for integration into the TCA cycle and
therefore, any down regulation of the BCAA degradation pathway would cause a decrease in mitochondrial respiration in existing adipocytes (Pietiläinen et al., 2008).

### 4.5.4 Energy production and mitochondrial dysfunction

Mitochondria are key organelles in cellular energetics as they catabolise carbohydrates, lipids and proteins to synthesise ATP and metabolites for not only growth but also adipocyte differentiation and maturation (De Pauw et al., 2009). There are four stages involved in aerobic respiration. Glycolysis occurs in the cytoplasm and the remaining steps, pyruvate oxidation, the TCA cycle and electron transport chain and chemiosmosis (as employed in oxidative phosphorylation) take place within the inner membrane of the mitochondria (Goldenthal and Marin-Garcia, 2004). The two pyruvate molecules, the products of glycolysis, enter the mitochondrial and are converted to acetyl CoA. Acetyl CoA enters the TCA cycle which drives the cycle to produce nicotinamide-adenine dinucleotide (NADH), ATP and reduced flavin adenine dinucleotide (FADH2). Genes encoding for citrate synthase (CS) namely, aconitase (ACO1 and ACO2), isocitrate dehydrogenase (IDH3B), α-ketoglutarate dehydrogenase (OGDH and DLD), succinyl-CoA synthetase (SUCLG1), fumarase (FH) and malate dehydrogenase (MDH1), were all found to be down regulated in the low compared to the high treatment. NADH and FADH2 donate electrons to the electron transport chain, which powers ATP synthesis via oxidative phosphorylation. Genes associated with NADH dehydrogenase Complex I, succinate dehydrogenase Complex II, ubiquinol-cyt c oxidoreductase Complex III, cytochrome c oxidase Complex IV and ATP synthase Complex V were down regulated in the low plane of nutrition compared to the high plane of nutrition (Table 4.3). It has been reported that there is an increase in mRNA expression of nucleus-encoded mitochondrial genes for enzymes such as pyruvate carboxylase and pyruvate dehydrogenase complex during differentiating 3T3-L1 adipocytes (Wilson-Fritch et al., 2003). Pyruvate carboxylase has been found to be overexpressed upon
conversion of preadipocytes to adipocytes (Owen et al., 2002). This is consistent with the results of our study where the high plane of nutrition calves were at a more advanced stage of adipocyte differentiation, at tissue recovery, as indicated by the significant number of adipocytes following histological investigation when compared with their lower nutrition counterparts.

4.6 Conclusion

This study clearly demonstrates that the prevailing plane of nutrition of bull calves during the first 18 weeks of life can have a significant effect on the transcriptional and morphological functionality of subcutaneous adipose tissue. A high plane of nutrition during early calf hood has been shown to positively affect adipokines, leptin and AdipoQ whose functions include signalling metabolic status to the reproductive system. The data generated in this study broaden our knowledge base around the potential molecular processes underlying the biological cross-talk between peripheral tissues that may be metabolic mediators of important events such as timing of puberty onset etc. Furthermore, global differential gene expression patterns provide data which may have implications for the selection of robust biomarkers to identify animals with superior genetic potential for early pubertal onset, greater carcass adiposity and other economically important traits, shown to be latently affected by early life plane of nutrition.
Figure 4.1: Multidimensional scaling plot which shows the measured similarity of the samples in 2-dimensions. The samples labelled in yellow are the subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a low plane of nutrition and slaughtered at 18 weeks of age and those labelled in red are the subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a high plane of nutrition and slaughtered at 18 weeks of age. BCV = Biological Coefficient of Variation.
Figure 4.2: Top molecular and cellular function of differentially expressed genes of the subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a low versus high plane of nutrition and slaughtered at 18 weeks of age. The bars indicate the likelihood [-log (P-value)] that the specific molecular and cellular function was affected by a high plane of nutrition. The threshold line in the bar chart represents a p-value of 0.05.
Figure 4.3: Top biochemical pathway significantly enriched in the subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a low plane of nutrition in comparison to a high plane of nutrition, slaughtered at 18 weeks of age. Greens bars represent genes down regulated and red bars up regulated genes as percentages of the overall number of genes in each pathway. The significance of each pathway is represented by the orange line describing $-\log(p$-value). The numbers along the right hand side indicate the number of genes in total involved in each pathway. The P-value is calculated by the number of genes from our dataset of differentially expressed genes that participate in a particular pathway and dividing it by the total number of genes in the Canonical Pathway in Ingenuity Pathways Analysis (IPA) analysis.
Figure 4.4: Light micrograph of subcutaneous adipose tissue stained with haematoxylin and eosin. A; High plane of nutrition had large areas of adipocytes (400x), B; Low plane of nutrition had small pockets of adipocytes (400x).
Table 4.1: Genes differentially expressed in the subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a low plane of nutrition in comparison to a high plane of nutrition and slaughtered at 18 weeks of age returned by GOSeq (P<0.05; False Discovery Rate<0.05; fold change>2.0).

<table>
<thead>
<tr>
<th>Rank</th>
<th>KO Pathway</th>
<th>Pathway Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>01100</td>
<td>Metabolic pathways</td>
</tr>
<tr>
<td>2</td>
<td>00280</td>
<td>Valine, leucine and isoleucine degradation</td>
</tr>
<tr>
<td>3</td>
<td>00020</td>
<td>Citrate Cycle -carbohydrate metabolism</td>
</tr>
<tr>
<td>4</td>
<td>05012</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>5</td>
<td>00190</td>
<td>NADH:ubiquinone oxidoreductase</td>
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<tr>
<td>6</td>
<td>00640</td>
<td>Malonate semialdehyde pathway</td>
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<tr>
<td>7</td>
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<td>Butanoate metabolism</td>
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<tr>
<td>8</td>
<td>03010</td>
<td>Ribosome</td>
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<td>9</td>
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<td>Glyoxylate and dicarboxylate metabolism</td>
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<td>10</td>
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<td>00071</td>
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<td>00330</td>
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<tr>
<td>23</td>
<td>00010</td>
<td>Glycolysis / Gluconeogenesis</td>
</tr>
</tbody>
</table>

Rank = significance relative position (1 is the most significant, 23 is the least significant)  
KO = KEGG Orthology
**Table 4.2:** Network of biological interest generated through network analysis using Ingenuity Pathway Analysis (IPA) of subcutaneous adipose tissue of Holstein-Friesian dairy bulls fed on a low plane of nutrition in comparison to a high plane of nutrition, slaughtered at 18 weeks of age.

<table>
<thead>
<tr>
<th>Network ID</th>
<th>Top Functions</th>
<th>Affected Molecules in Network</th>
<th>Score</th>
<th>Focus Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Energy Production, Lipid Metabolism, Small Molecule Biochemistry</td>
<td><em>ABLIM2, ACAT1, ADIPOQ, ADIPOR2, ALDOA, BCAT2, CISD1, CKB, FABP4, FITM2, GPD1, HOMER2, LEP, LGALS12, MT-ND1, MT-ND6, NDUFB5, NDUFB8, NDUFS2, NDUFV1, NNAT, NR4A1, SNCA, SORBS1, SYNE2, TUSC5</em></td>
<td>39</td>
<td>28</td>
</tr>
</tbody>
</table>

Molecules highlighted in bold are downregulated in the low plane of nutrition in comparison to the high plane of nutrition.
Table 4.3: Oxidative Phosphorylation genes differentially expressed in the subcutaneous adipose tissue of the Holstein-Friesian dairy bulls feed on a low plane of nutrition in comparison to a high plane of nutrition and slaughtered at 18 weeks of age.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Log Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complex I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDUFA4</td>
<td>NDUFA4, mitochondrial complex associated</td>
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<tr>
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<td>NADH:ubiquinone oxidoreductase subunit A5</td>
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<td>NADH:ubiquinone oxidoreductase subunit A8</td>
<td>-1.134</td>
</tr>
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<td>NADH:ubiquinone oxidoreductase subunit A9</td>
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<tr>
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<td>NADH:ubiquinone oxidoreductase subunit A12</td>
<td>-1.221</td>
</tr>
<tr>
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CHAPTER 5: General Discussion
The experiments described in this thesis involved a multi-disciplinary approach to examine the effects of early life nutritional management of bull calves on the molecular control of the HPT axis (Chapters two to four). This necessitated the establishment of an experimental animal model where Holstein Friesian bull calves were managed in order to create either a high or low growth trajectory from two to eighteen weeks of life, as the nutritional regimen employed resulted in considerable divergence in calf growth rate which was mirrored in metabolic hormone and metabolite status. High throughput mRNA transcriptional profiling technology (RNASeq) was employed to assess the effect of prevailing nutritional status during the early calf hood period (pre and early post-weaning) on the biochemical response of key tissues regulating reproductive function in the bull. Specifically we studied the arcuate nucleus region of the hypothalamus, the anterior pituitary, parenchymal tissue of the testes as well as subcutaneous adipose tissue. To verify these results histological and immunohistological analysis of these tissues was also conducted. The main results from the work undertaken demonstrate that a high plane of nutrition has a positive effect on gametogenesis and testicular development at both a structural and transcriptomic and proteomic level.

Recently work by our research group found bulls offered a high plane of nutrition pre-six months were younger at puberty (approx. 1 month) and at sexual maturation (Byrne et al., 2017b). This work also demonstrated that the effects of dietary restriction in early life cannot be mitigated by enhancing nutrient intake after six-months of age nor will post six-month dietary restriction reduce the advantages obtained from a high plane of nutrition pre six-months (Byrne et al., 2017b). It has been shown that the high plane of nutrition in the early calf-hood period advances the transient rise of LH (Evans et al., 1996) and the sensitivity of LH release to GnRH (Byrne et al., 2017a) which occurs between 8 and 20 weeks of age.
Therefore in this study we sacrificed bull calves’ on two divergent planes of nutrition at 18 weeks of age, which is still within the (arbitrary) 8-20 week window of influence and allowed the animals’ sufficient time to express their respective growth performance potential. This allowed us the opportunity to characterise the response of the HPT at a point in time within this relevant window. Therefore our hypothesis was that early calf nutrition would impact the transcriptome of the HPT.

Ideally an intensive bleed would have been carried out to get more specific data on the reproductive and metabolic hormone pulsatility; however, as these calves were very young and only on trial for a short period of time it was decided to take weekly blood samples to avoid any major stress that could have a long term effect on the calves.

**Early Life Nutritional effects on Arcuate Nucleus and Anterior Pituitary**

This is the first published study to investigate the effect of nutrition on the transcriptome of the HPT axis in bull calves using any technology. The effect of plane of nutrition during the first 18 weeks of life on the transcriptomic and immunohistochemical profile of the ARC and anterior pituitary of Holstein Friesian bull calves was characterised in chapters two and three of this thesis. The neurons in the central nervous system have the capability to alter gene expression in response to changes in the genetics and/or physiological environment such as stress, malnutrition, sleep, hormones and drugs (Sodhi and Sanders-Bush, 2004). There are two critical or vulnerable periods in brain development (Calikoglu et al., 2001); the initial development of the foetus and early in postnatal life, with the duration of these critical periods being species dependent. The ARC was chosen for study in the experiments in this thesis as its main function is in the regulation of both metabolic and reproductive processes and functions by communicating peripheral metabolic signals including those relating to
bodyweight and appetite to other regions of the brain such as the anterior pituitary gland (Filby et al., 2008, Allen et al., 2012, Schwartz et al., 2000). Previous studies have reported evidence for differences in metabolic function in the ARC of heifer calves fed a high concentrate diet and achieving a high ADG from three-seven months of age, when compared with contemporaries offered a predominantly forage based diet and growing at a lower rate (Allen et al., 2012). A recent study carried out by the same research group reported that increased rates of growth during the period of 4.5 to 8.5 months of age in heifers altered the DNA methylation pattern in the ARC and these alterations may be linked to more precocious puberty (Alves et al., 2017). In contrast to the aforementioned studies there were no significant differences in gene expression found in this study in ARC tissue of calves offered divergent planes of nutrition. However, the studies reported above involved animals of a different sex, age, nutritional treatment and analytical technologies compared to our work. As well as all the above differences, these studies nutritional treatments commenced much later than our study and therefore, may not be fair comparison. Despite calves on the low plane of nutrition in our study only achieving a growth rate of approximately 50% of that of their contemporaries on the high plane of nutrition, leading to these calves being only 66% of the calves on the high plane of nutrition treatment at 18 weeks of age. The transcriptomic signature of the ARC nucleus and anterior pituitary tissues found in this study did not reflect the major differences in phenotype for the two dietary groups employed, in that the DEG observed within the RNASeq analysis within anterior pituitary tissue only related to cell cycle and not specifically to metabolism or reproductive processes. However, the rate of cell cycle activity was related to the prevailing metabolic status. The lack of DEG in either the ARC region of the hypothalamus or anterior pituitary could be the result of a number of possibilities. First, the unavoidable differences in age of calves at the start. Calves had to be purchased from outside farms as there were no animals in calf to dairy calves on farm. Also it
was important that the calves received adequate colostrum. This avoided any major health problems with the calves during the trial. Secondly, the transient nature of gene transcription, particularly in brain tissue may have contributed to the difficulty in observing DEG between the divergent nutritional treatments employed (Bondy and Lee, 1993). Thirdly, the timing of 18 weeks of age for tissue recovery was chosen as it was within the developmental window during which bull calves, on average, experience a gonadotrophin rise weeks (Evans et al., 1996). This would allow an examination of the relative physiological maturity of the HPT between the two treatments, at least at a transcriptional level. However, as described in Chapter two, no statistically significant difference in systemic concentrations of LH was evident between the two nutritionally divergent treatment groups. This is in contrast to the findings of Byrne et al. (2017b) who reported greater concentrations of LH and testosterone between Holstein Friesian and Jersey calves on a high lane of nutrition subjected to an exogenous GnRH challenge at 16 weeks of age. However, had tissues been harvested at an earlier age it is possible that greater difference in DEG for both ARC and adenohypophyseal tissue between our treatment groups may have been observed. For example, while LH concentrations were not different at the time of tissue harvest, we did observe higher concentrations of LH in the systemic concentration of calves offered the high plane of nutrition at 12 weeks of age. Notwithstanding this, the large number of DEG observed in testicular tissue at 18 weeks of age is testament, no doubt, to major biochemical differences between the treatments in hypothalamic and hypophyseal activity at an earlier stage of development. Additionally, while numerous studies have investigated the effect of intravenous GnRH agonists on systemic gonadotrophin response, this is the first study to the author’s knowledge of the effect of nutrition on the global transcriptome of the anterior pituitary in calves.
The anatomical location of the ARC region lends itself to more easy identification and dissection than other nuclear regions within the hypothalamus. The method used to dissect out the ARC from the rest of the hypothalamus in the studies described in this thesis was based on the report of Komatsu et al. (2012). While this method is relatively precise and enables the collection of tissue with high quality RNA, the method of sectioning the tissue using a cryostat and selecting the exact location of the ARC using cresyl violet staining as per Allen et al. (2012) or using laser capture microdissection as per Lee et al. (2012) would have been more specific. However, these methods have technical difficulties in obtaining enough high quality and quantity of mRNA for transcriptomic analysis, due to the high level of processing increasing the likelihood mRNA degradation, thus compromising the primary outcome of interest. Although, our study did not employ these methods, immunohistochemistry was carried out to corroborate that the ARC region was captured from each animal. Two proteins, NPY and KISS1 were selected as NPY has been found to be particularly abundant in the ARC of the bovine brain (Bahar and Sweeney, 2008). KISS1 neurons are found in the ARC and the preoptic nucleus of cattle (Hassaneen et al., 2016) and as these nuclei are not close in proximity, it was deemed a good biomarker for the ARC. While both proteins were found to be abundant within the tissue which we harvested, surprisingly though there were no differences between protein expression and intensity for calves on either plane of nutrition.

KISS1 neurons relay metabolic status to the GnRH neurons (Castellano et al., 2010, Kalamatianos et al., 2008, Quennell et al., 2011, Wahab et al., 2011). Approximately 40% of KISS1 neurons present in the ARC region of the mouse brain were shown to express the active leptin receptor (Ob-Rb) mRNA, demonstrating that, at least in mice, KISS1 neurons are direct targets for regulation by leptin (Smith et al., 2006). This study carried out in situ
hybridisation on cryostat cut tissue only from the diagonal band of Broca to the mammillary bodies which contained the ARC. IGF-I is widely associated as a mediator of the effect of nutrition on reproduction via the HPT axis (Brito et al., 2007b) and its binding proteins regulate IGF-I activity by controlling the availability of IGF-I to their receptors. Administration of IGF-I activates the KISS1 gene in the anteroventral periventricular and ARC of the hypothalamus in prepubertal female rats (Hiney et al., 2009). Therefore, it was surprising that there was not differences in expression at either the transcriptomic or proteomic level between either plane of nutrition given the significantly higher systemic concentrations of IGF-I in calves on the high plane of nutrition. As mentioned above, leptin targets both KISS1 and NPY neurons. Leptin receptor mRNA and NPY neurons are co-localised in the ARC of ewes (Williams et al., 1999). Low leptin concentrations stimulate NPY and AgRP release, feed intake and inhibit POMC and CART activity as well as energy expenditure (Schwartz et al., 2000). However, other studies (Brito et al., 2007a, Dance et al., 2015, Byrne et al., 2017b) have reported no difference between treatments with respect to leptin which is in agreement with our study.

In Chapter two, a qPCR study was carried out on the ARC and anterior pituitary focusing on genes involved in metabolic and reproductive signalling. Unlike any of the previous reported studies (Allen et al., 2012, Cardoso et al., 2015, Alves et al., 2017), no difference was found in the expression of any of the candidate genes examined in ARC tissue between either plane of nutrition with the exception of GHSR. This is despite the fact that in the aforementioned studies, nutritional treatments commenced much later in the life of the calves employed than in our study. Ghrelin concentrations in blood plasma are regulated by food intake (Stanley et al., 2005) and therefore, it was not unexpected that overall GHSR expression was higher for calves on the low compared to the high plane of nutrition in the current study. Gene
expression levels are commonly analysed as relative expression values; however, this doesn’t allow for determination of actual expression levels. Also the SEM values reported in the ARC are large and this could have been improved by using laser capture microdissection technology.

**Early Life Nutritional effects on the Testes**

Restricted feed during calf hood has been shown to impede the hypothalamic GnRH pulse generator and its interaction with the anterior pituitary gland can impair steroidogenesis in the testes, delay puberty and decrease testicular weight at 70 weeks of age (Brito et al., 2007a). In the study described in Chapter two, a high plane of nutrition resulted in heavier paired testicular weights, greater number of more mature spermatogenic cells and Sertoli cells, greater seminiferous tubule diameter and a more mature lumen at slaughter than for those calves offered the low plane of nutrition. In the early postnatal life of the bull calf, undifferentiated Sertoli cells form and by approximately 30-40 weeks of age the adult Sertoli cells, which each support a set number of germ cells, are established (Rawlings et al., 2008). Sertoli cell number is fixed at puberty and cannot change post puberty (Sharpe et al., 2003, Buzzard et al., 2003). Therefore, larger testes would suggest more Sertoli cells and more advanced spermatogenesis and, therefore, the capacity to produce more sperm (O'Donnell et al., 2000). These results indicate greater nutrient intake positively influence the onset of puberty via advancing germ cell development (Chandolia et al., 1997). Additionally as referred to earlier, a number of studies have shown that a high plane of nutrition before 31 weeks of age leads to an earlier onset of puberty in Holstein-Friesian bulls (Dance et al., 2015, Byrne et al., 2017b). Although rapid growth in sheep due to over nutrition can lead to health problems in later life and early culling (Johnsen et al., 2013) however; stud bulls have
a very short working life as each breeding season new genetic superior bulls enter the stud farm and therefore, are culled long before joint problems arise.

In Chapter two of this thesis we focused on the genes that are reported as markers for Sertoli cell development, namely, transcription factor *GATA4* (Jimenez-Severiano et al., 2005) and *AMH* (Vigier et al., 1984). Our rationale for including the adult testes dimension into our study was to investigate if the calves benefiting from the high plane of nutrition had any similarities in gene expression to the adult bulls in comparison to the calves on the low plane of nutrition. It has been reported that Sertoli cells proliferate until 6 months of age (Ortavant et al., 1977) and data from our study reflects this; with the significantly higher *GATA4* and *AMH* expression levels in the prepubertal calves in comparison to the adult bulls. For spermatogenesis to function properly, tight junctions need to form between Sertoli cells; two genes that regulate this are *CLDN11* and *ZO1*. There was a greater expression of *CLDN11* in both the adult and the calves on the high nutrition treatment when compared with those on the low treatment. This suggests that calves on the high treatment were at a more mature physiological stage of development than those on the low treatment. Similarly in the rabbit, *CLDN11* mRNA and protein content was lower in immature testes (postnatal Day 10) in comparison to those of adult animals (postnatal Day 180; Park, et al. 2011). It has been reported that an increase in Sertoli cell number is linked to an increase in endogenous FSH (Bagu et al., 2004). In agreement Harstine et al. (2017b) demonstrated that Angus calves treated with FSH from 1-3 months of age had a greater number of Sertoli cells when castrated at 3 months. In another study published by that group on the effect of FSH treatment on calves from 2-6 months of age, they reported an increase in activin A in the blood which has a beneficial role in spermatogenesis (Harstine et al., 2017a). However, in the study reported in Chapter 2 we failed to observe any difference in systemic concentrations of FSH between
the calves on the divergent planes of nutrition employed which corresponds with other studies with animals at a similar stage of development (Brito, 2014, Byrne et al., 2017c). Expression of FSHR has been found to decrease with testes development in bulls (Dias and Reeves, 1982). The numbers of receptors in the testes have been shown to decrease quickly from birth to 2 months of age and then decrease at a slower rate from 2 months to 2-5 years of age in bulls (Dias and Reeves, 1982). This agrees with the significantly higher expression for calves on both treatment groups compared to that of the adult bulls. No difference was seen in FSHR abundance between the nutrition treatments; however, the RNASeq data in Chapter 3 showed that FSHR was down regulated in the high compared to the low plane of nutrition in the testes. This discrepancy between techniques is likely due to the slight decrease in the number of animals used in the RNASeq study due to a low number of reads compared to that examined for PCR. Studies have shown that there is a decline in FSHR number in rats and mice which is linked to a decrease in Sertoli cell interaction with FSH; due to having reached maturity (Ketelslegers et al., 1978).

It has been reported that an increase in systemic FSH, testosterone and especially LH in early life is positively related to larger and more mature testes (Brito et al., 2007b). Our study found that there was a peak in systemic concentrations of LH at 12 weeks, however, unexpectedly; there was not a statistically significant difference in LH concentration between the treatment groups. This was further corroborated with LHR expression while no difference was evident between the calves on the high and low planes of nutrition; it was significantly greater than the adult bulls. This early rise is reported to induce responsiveness of testicular Leydig cells to LH, leading to an increase in testosterone production and sexual maturation (Schanbacher, 1979, Amann and Walker, 1983). However, even though there was no difference in LH concentration between the treatment groups, there was a greater rise in
testosterone in the calves on the high compared to those on the low plane of nutrition. This indicates that the divergent planes of nutrition were effective and there is a high probability that there were differences in both the frequency and amplitude of LH pulsatility earlier but the high animal to animal variation combined with the pulsatile nature of LH makes it difficult to characterise differences between treatments with only one blood sample per time point. The lower concentration of IGF-I in calves on the low plane of nutrition may also have played a role in negatively affecting the peri-pubertal testosterone concentrations and therefore, negatively influencing Leydig cells (Brito et al., 2007c).

The high plane of nutrition induced an increase in DEGs in the testes affecting hormone production including androgen and cholesterol biosynthesis in comparison to the low plane of nutrition. Genes encoding for the enzymes catalysing the biosynthesis of cholesterol were found to be down regulated in calves on the low compared to the high plane of nutrition in the testes. This is of interest as under normal physiological conditions, de novo cholesterol synthesis replenishes cholesterol stores in Leydig cells (Hu et al., 2010). The low plane of nutrition had a negative effect on the expression of genes that mediate the transport of cholesterol. Interestingly, the uptake of HDL cholesteryl esters via binding HDL-ApoE (Fofana et al., 2000), the selective uptake pathway of HDL cholesterol to the testes via the SCARB1 (Acton et al., 1996) and the LDLR pathway (Miller, 2007) are negatively affected by the low plane of nutrition. In the Δ5 pathway, pregnenolone is converted to 17α-hydroxyprogrenolone then to dehydroepiandrosterone and then to testosterone through either androstenediol or androstenedione. Our data shows that this pathway was negatively affected by the low plane of nutrition which corroborates the significant lower testosterone concentrations of these calves.
It has been reported that StAR is the primary determinant in the process of steroid synthesis (Christenson and Strauss, 2001). The expression of StAR has previously been shown to be expressed in the testes of the bull (Pilon et al., 1997) and has been reported to regulate the transport of cholesterol from the outer into the inner mitochondrial membrane (Aspden et al., 1998). Calves offered the low plane of nutrition had a lower expression of StAR compared to the high plane of nutrition. The expression of StAR has been reported to be stimulated by IGF-I in fetal and adult mice Leydig cells (Manna et al., 2006). This is consistent with previous reports from our group showing greater plasma IGF-I concentrations when the same animals in this study were offered a high versus a low plane of nutrition (Byrne et al., 2017b). Surprisingly, genes purported to be involved in gametogenesis were upregulated in testis tissue of calves on the low in comparison to that of those calves on the high plane of nutrition, which may be due to the low plane of nutrition calves experiencing a late spurt of gametogenesis. Despite this the testes from the calves on the high plane of nutrition showed a more mature spermatogenic profile with regard to testes weight, seminiferous tubule diameter, stage of spermatogenesis, Sertoli cell number and lumen development at slaughter.

**Early Life Nutritional effects on the Subcutaneous Adipose**

Adipocytes are acutely sensitive to changes in nutritional status (Bjorndal et al., 2011). Hormones such as leptin and adiponectin, which are secreted from adipocytes are understood to play a part in the regulation of reproductive processes. Leptin increases proportionally in line with an increase in body fat and signals energy abundance (Landry et al., 2013) and high systemic concentrations of adiponectin have been found to negatively affect the reproductive axis (Campos et al., 2008). Much of the published work to-date on this topic has focused on the effect of body lipid status on adipose tissue hormone signalling in post pubertal heifers and cows and there is a dearth of information on the role of adipose tissue status in
stimulating the HPT axis and its potential as a key regulator of the timing of puberty onset in male cattle.

The histological and mRNA expression data in this thesis indicate much lower adiposity (number and size of adipocytes) and greater number of preadipocytes in the calves subjected to the low plane of nutrition. It has been reported that MLXIPL expression levels increase during preadipocyte differentiation in humans, mice and rats (Hurtado del Pozo et al., 2011, He et al., 2004). The calves on the low plane of nutrition had lower expression of MLXIPL in comparison to their contemporaries on the high plane of nutrition. Genes required for adipogenic differentiation such as FABP4, FASN, ACACA and DGAT2 were upregulated on the high plane of nutrition compared to the low. As well as an upregulation of genes that stimulate adipogenesis in the high plane of nutrition, in our study we found an upregulation in a key gene, GATA2, found to inhibit adipogenesis in the calves on the low compared to the high plane of nutrition. It has been reported that GATA2 is expressed in preadipocytes and is down-regulated during terminal differentiation (Tong et al., 2000). It has been reported that there is an increase in mRNA expression of nucleus-encoded mitochondrial genes for enzymes such as pyruvate carboxylase and pyruvate dehydrogenase complex during differentiating 3T3-L1 adipocytes (Wilson-Fritch et al., 2003). Pyruvate carboxylase has been found to be overexpressed upon conversion of preadipocytes to adipocytes (Owen et al., 2002). All data in our study are consistent with evidence that offering calves a high starch diet during early calf hood induces precocious adipogenesis and lipid filling (Graugnard et al., 2010) and therefore, theoretically advances puberty.

Leptin has been found to indirectly influence the timing of puberty via the regulation of the hypothalamic KISS1 mediated neuronal stimulation of GnRH release (Sanchez-Garrido and
Tena-Sempere, 2013). Leptin can also bypass the hypothalamus and act directly on the pituitary gland and the testes; with leptin modestly stimulating gonadotropin secretion at a pituitary level and both direct stimulatory and inhibitory actions of leptin been reported in the gonads (Tena-Sempere, 2007). The high plane of nutrition calves had a heavier slaughter weight than those on the low plane of nutrition and the RNASeq data found that calves on the high plane of nutrition in our study had >4.5 log fold higher LEP expression compared with calves on the low plane of nutrition. Despite this, when plasma concentrations were assessed, we failed to find any difference in leptin concentrations between the two groups. This is in agreement with studies in both young dairy and beef bulls (Dance et al., 2015, Brito et al., 2007a, Byrne et al., 2017b). The lack of differences in plasma leptin concentrations may be due to the minute quantity of subcutaneous adipose tissue laid down in calves at this stage of development, the relative insensitivity of protein immunoassays compared with transcript based molecular approaches or most likely because leptin is produced primarily by mature adipocytes (Diakonova, 2015).

Postprandial activity reduces ADIPOQ mRNA expression in WAT and has knock on effects on adiponectin concentration in serum (Steinberg and Kemp, 2007). It has been shown that adiponectin reduces LH secretion directly from the gonadotropes in the anterior pituitary via AMPK (Lu et al., 2008). Similarly, postpartum cows, in negative energy balance had decreased ADIPOR1 and ADIPOR2 abundance in subcutaneous adipose compared to cows just prior to calving (Lemor et al., 2009) which is in agreement with our findings with calves where we found ADIPOQ and its receptor ADIPOR2 were down regulated in the low compared to the high treatment groups. However, there was no difference in serum concentrations of adiponectin between the two groups in the current study, which like leptin may be due to the overall low adipose deposition in young bull calves. It has been reported
that leptin concentration was greater in the high plane of nutrition in Holstein Friesians post six months of age (Byrne et al., 2017b).

The high plane of nutrition induced an increase in both energy production and amino acid metabolic pathways in adipose tissue in the high in comparison to the low nutrition treatment. Branched chain amino acid concentrations are elevated in response to over nutrition in children and adolescents (McCormack et al., 2013). Therefore, it is reasonable to suggest that the evidence for a decline in BCAA degradation may be a result of the moderate restriction in dietary allowance experienced by our low plane of nutrition calves.

The main findings of this thesis were:

(i) 18 week old bull calves offered a high plane of nutrition from two weeks of age were heavier at slaughter, had larger testes, larger seminiferous tubule diameter, more mature spermatogenic cells, more Sertoli cells and a greater Sertoli cell volume density as well as more advanced seminiferous tubule lumen development.

(ii) From a selected panel of genes involved in both metabolic and reproductive signalling in the ARC region of the hypothalamus and the anterior pituitary, GHSR was the only gene affected by plane of nutrition, while when a RNASeq approach was taken plane of nutrition had no significant effect on gene expression in the ARC or anterior pituitary tissue

(iii) Offering bull calves a low plane of nutrition down regulated the expression of genes involved in cholesterol and androgen biosynthesis in the testes,

(iv) Histological and mRNA expression data from subcutaneous adipose tissue indicated much lower adiposity (number and size of adipocytes) and a greater
number of pre-adipocytes in subcutaneous adipose tissue of calves offered a low compared with a high plane of nutrition. This was consistent with evidence for altered expression of genes involved in cellular energy production and branched chain amino acid degradation in subcutaneous adipose tissue in these calves. Calves offered a high plane of nutrition had greater abundance of transcripts from adipokines such as leptin (LEP) and adiponectin (ADIPOQ) in subcutaneous adipose tissue, both of which are known to play direct roles in regulating aspects of reproductive function.

Suggestions for Future Direction of Research in this Area

- It would be informative to replicate the experiment with a larger cohort of calves and sample the HPT at various time points between 4-20 weeks of age and at puberty (39-41 weeks of age); to characterise the transcriptomic and proteomic response of the effect of nutrition at key time points.
- Further work should be carried out to examine the effect of early life metabolic status on the microRNA transcriptome, given the importance of the latter in regulating spermatogenesis.
- Additionally, comprehensive proteomic characterisation of subcutaneous adipose would yield valuable insights into the effects of early life nutrition on the hyperplasia and heterotrophy of adipose tissue and its downstream effects on the HPT axis.
CHAPTER 6: Publication List
6.1 Published papers:

I was involved in the performing of experiments, analysing the data and editing and revising of each manuscript.


6.2 Submitted papers:


Byrne, C.J., Fair, S., English, A.M., Lonergan, P., Kenny D.A. Plane of nutrition during pre and post-six months of age in Holstein-Friesian bulls: I. Effect on performance, body composition, age at puberty and post-pubertal semen production (Submitted)

Byrne, C.J., Fair, S., English, A.M., Urh, C., Sauerwein, H., Crowe, M.A., Lonergan, P., Kenny D.A. Plane of nutrition during pre- and post-six months of age in Holstein-Friesian bulls: II. Effect on metabolic and reproductive endocrinology and identification of physiological markers of puberty and sexual maturation (Submitted)

6.2 Conference publications

bull calves. International Society of Animal Genetics (ISAG), Dublin, Ireland, 18th June 2017. (Poster)


Byrne, C.J., English, A.M., Fair, S., Lonergan, P., Kenny, D.A. 2016. Effect of plane of nutrition during the first and second six months of life on age at puberty and subsequent semen production in Holstein Friesian bulls. 18th International Congress on Animal Reproduction, Tours, France. 27th June 2016 (Poster)

Byrne, C.J., English, A.M., Fair, S., Lonergan, P., Kenny, D.A. 2016 Effect of plane of nutrition during the first and second six months of life on age at puberty and blood metabolites in Holstein Friesian bulls. 67th Annual Meeting of European Association of Animal Production. 29th August 2016. (Poster)
Chapter 7: Bibliography


ARITA, Y., KIHARA, S., OUCHI, N., TAKAHASHI, M., MAEDA, K., MIYAGAWA, J., HOTTA, K.,
SHIMOMURA, I., NAKAMURA, T., MIYAOKA, K., KURIYAMA, H., NISHIDA, M., YAMASHITA, S.,
OKUBO, K., MATSUBARA, K., MURAGUCHI, M., OHMOTO, Y., FUNAHASHI, T. & MATSUZAWA,

Neuropeptides, 40, 375-401.

ASPDEN, W. J., RODGERS, R. J., STOCCO, D. M., SCOTT, P. T., WREFORD, N. G., TRIGG, T. E., WALSH, J.
& D’OCCHIO, M. J. 1998. Changes in testicular steroidogenic acute regulatory (STAR) protein,
steroidogenic enzymes and testicular morphology associated with increased testosterone
secretion in bulls receiving the luteinizing hormone releasing hormone agonist deslorelin.
Domest Anim Endocrinol, 15, 227-38.

AZHAR, S. & REAVEN, E. 2002. Scavenger receptor class B1 and selective cholesteryl ester uptake:

BADO, A., LEVASSEUR, S., ATTOUNB, S., KERMORGANT, S., LAIGNEAU, J. P., BORTOLUZZI, M. N.,

gonadotropin receptors, serum gonadotropin, and testosterone concentrations and

BAGU, E. T., MADGWICK, S., DUGGAVATHI, R., BARTLEWSKI, P. M., BARRETT, D. M., HUCKKOWSKY,
S., COOK, S. J. & RAWLINGS, N. C. 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, 861-73.

BAHAR, B. & SWEENEY, T. 2008. Mapping of the transcription start site (TSS) and identification of
SNPs in the bovine neuropeptide Y (NPY) gene. BMC Genetics, 9, 91.


Publications.

& STEINER, R. A. 1996. Leptin is a metabolic signal to the reproductive system. Endocrinology, 137, 3144-7.

BARB, C. R., BARRETT, J. B. & KRAELING, R. R. 2004. Role of leptin in modulating the hypothalamic-
pituitary axis and luteinizing hormone secretion in the prepuberal gilt. Domestic Animal
Endocrinology, 26, 201-214.


both leptin secretion and production by rat white adipose tissue. Endocrinology, 138, 4463-
72.

body composition of dairy calves fed milk replacers containing different amounts of protein


Plays an Essential Role with DNA Damage Response Proteins in Meiotic Recombination and
Gene Silencing. PLOS Genetics, 9, e1003435.


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HAFez, B. & HAFez, E. S. E. 2000. Reproduction in Farm Animals, Lippincott Williams & Wilkins.


Immunohistochemical characterization of the arcuate kisspeptin/neurokinin B/dynorphin (KNDy) and preoptic kisspeptin neuronal populations in the hypothalamus during the estrous cycle in heifers. The Journal of Reproduction and Development, 62, 471-477.


KALAMATIANOS, T., GRIMSHAW, S. E., POORUN, R., HAHN, J. D. & COEN, C. W. 2008. Fasting reduces KISS-1 expression in the anteroventral periventricular nucleus (AVPV): effects of fasting on
the expression of KiSS-1 and neuropeptide Y in the AVPV or arcuate nucleus of female rats. Journal of Neuroendocrinology, 20, 1089-97.


OBREGON, M.-J. 2008. Thyroid hormone and adipocyte differentiation. Thyroid, 18, 185-195.


