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Gene co-expression networks contributing to reproductive development in Holstein-Friesian bull calves

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ABSTRACT

Enhanced early life nutrition stimulates the functionality of the hypothalamic-pituitary–testicular (**HPT**) biochemical signalling axis, resulting in precocious reproductive development in bull calves. Additionally, there is evidence that peptides and hormones produced within adipose tissue depots are also central in mediating the effect of metabolic status with reproductive development. The objective of this study was to undertake gene co-expression analyses on transcriptional data of the HPT and adipose tissues derived from bull calves fed contrasting planes of nutrition up to 18 weeks of life. The relationship between networks of co-expressed genes in each tissue dataset with calf phenotypic data was also assessed using a Pearson correlation analysis. Phenotypic data were related to metabolic status (systemic concentrations of insulin, leptin, adiponectin and IGF-1) reproductive development (systemic concentrations of testosterone, FSH and LH) and markers of testicular development (seminiferous tubule diameter, seminiferous tubule lumen score, spermatogenic cells and Sertoli cells). In the hypothalamus, gene co-expression networks involved in biochemical signalling processes related to gonadotropin-releasing hormone (**GnRH**) secretion were positively associated ($P < 0.05$) with systemic concentrations of IGF-1 and insulin. Similarly, a network of gene transcripts involved in GnRH signalling in the anterior pituitary was positively associated ($P < 0.05$) with systemic concentrations of LH. In the testes and adipose tissues, networks of co-expressed genes implicated in cholesterol and fatty acid biosynthesis were positively associated ($P < 0.05$) with lumen score, Sertoli cell number, and stage of spermatogenesis. Additionally, gene co-expression networks significantly associated ($P < 0.05$) with both metabolic and reproductive trait data were found to be enriched ($P < 0.05$) for biological pathways related to energy production, cellular growth and proliferation, GnRH signalling and cholesterol biosynthesis across multiple tissues examined. Results from this study highlight networks of co-expressed genes directly associated with markers of enhanced metabolic status and subsequent earlier reproductive development. Furthermore, genes involved in biological processes mentioned above may hold potential for informing genomic selection breeding programmes for the selection of calves capable of displaying earlier reproductive development as a consequence of enhanced dietary intake.

Implications

Results from this study provide knowledge of the biology underlying the interaction between metabolic status and reproductive development. This is of interest to animal producers, as early pubertal onset in bull calves may facilitate semen collection from genetically elite sires at an earlier age. Thus, a greater understanding of the underlying biology and identification of key hub genes contributing to earlier reproductive development as a consequence of enhanced nutrition could be harnessed within animal management practices as well as genomic selection breeding programmes to optimise feeding strategies and breed cattle better able to reproductively respond to enhanced early life nutrition.

Introduction

Plane of nutrition in early calf-hood plays a critical role in orchestrating the advancement of reproductive development and the timing of puberty onset in bulls (Brito et al., 2007; Dance et al., 2015; Byrne et al., 2018). Indeed, work from our own group has clearly demonstrated that offering bull calves a high plane of nutrition prior to 6 months of age hastens age at puberty attainment by approximately 1 month compared with contemporaries

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offered a moderate dietary allowance (Byrne et al., 2018). The positive effect of improved metabolic status is mediated through a cascade of endocrine and neuroendocrine events affecting hypothalamic gonadotropin-releasing hormone (**GnRH**) pulsatility, which subsequently lead to enhanced and earlier secretion of the gonadotropins, LH and FSH. The transient rise of the former is of particular importance and typically occurs between 8 and 20 weeks of age (Rawlings et al., 2008). Earlier LH secretion can elicit testosterone synthesis and release, which in turn leads to advanced testicular development and pubertal onset (Dance et al., 2015; Byrne et al., 2018). Indeed, enhanced nutrition for the first 18 weeks of life has been clearly shown to contribute to greater testicular development in bull calves when compared to contemporary controls that received a lower plane of nutrition during the same time (English et al., 2018a). This was manifested as statistically greater values for the following parameters in the calves which received a high energy diet for the first 18 weeks of life: testes size, testosterone concentrations, LH response, seminiferous tubule diameter, seminiferous tubule lumen score, mature spermatogenic cells and Sertoli cell number (English et al., 2018a).

The integrated effect of enhanced nutrition and reproductive development is coordinated by the hypothalamic-pituitary–testicular (**HPT**) biochemical signalling axis (Ramasawamy and Weinbauer, 2014), with the arcuate nucleus region of the hypothalamus, in particular, central to conveying metabolic status throughout the body with subsequent reproductive development. More recently, adipose tissue has also been implicated in both metabolic and reproductive processes and it has been postulated that there is crosstalk between peptides and hormones produced within adipose tissues and the HPT axis (Tsatsanis et al., 2015). However, although it is clear that a higher plane of nutrition in early calf-hood can lead to an advancement of puberty attainment in bulls (Kenny et al., 2018), the biochemical mechanisms mediating this effect are yet to be fully elucidated (English et al., 2018b). We have recently evaluated the transcriptional response of tissues of the HPT axis in addition to adipose tissue, to varying planes of nutrition in young bull calves (English et al., 2018a, 2018b and 2018c). However, although results from each of these studies have provided novel information on the specific molecular response to nutrition, in the respective tissues, they do not explain the interactions between genes in response to dietary management regimen. Studies have shown that individual genes do not work alone, instead, genes interact with each other to elicit a particular physiological phenotype (Miklos and Rubin, 1996; Arnone and Davidson, 1997; Li et al., 2018). Thus, the identification of networks of co-expressed genes that contribute to the expression of complex traits may reveal more information on the molecular control of a particular phenotype (Kogelman et al., 2014). Overall gene networks can provide the potential to uncover the interactions between genes governing a particular phenotypic outcome, with this information important for predicting the functions of new genes and finding genes that play key roles in complex phenotypes (Kogelman et al., 2014).

Although our aforementioned earlier studies yielded novel information on the effect of early life nutritional status on the biochemical regulation of testes and adipose tissue functionality, insights into the molecular response in key neuroendocrine tissues such as the arcuate nucleus region of the hypothalamus and the anterior pituitary gland were more nuanced. Thus, we hypothesise that co-expression analyses may yield interesting insights into the molecular control of these tissues, through the identification of gene co-expression networks directly associated with enhanced metabolic status and subsequent earlier reproductive development. Earlier reproductive development in bull calves is of interest as it may facilitate semen collection from genetically elite sires at an earlier age. Thus, a greater understanding of the underlying biology and identification of key hub genes contributing to earlier reproductive development as a consequence of enhanced nutrition could be harnessed within animal management practices as well as genomic selection breeding programmes to optimise feeding strategies and breed cattle better able to reproductively respond to enhanced early life nutrition. The objective of the current study, therefore, was to construct networks of co-expressed genes from global transcriptomic data pertaining to the HPT and adipose tissues collected from Holstein-Friesian bull calves offered contrasting planes of nutrition during the first 18 weeks of life (English et al., 2018a, 2018b and 2018c). Furthermore, in order to facilitate the identification of key hub genes contributing to earlier reproductive development in bull calves, networks of co-expressed genes were correlated with important variables related to metabolic status (systemic concentrations of insulin, leptin, adiponectin and IGF-1) as well as reproductive development (systemic concentrations of testosterone, FSH and LH) and markers of testicular development (seminiferous tubule diameter, seminiferous tubule lumen score, spermatogenic cell and Sertoli cell number) pertaining to the bulls used in the same transcriptomics study.

Material and methods

Animal model

This study 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 have previously been described in detail by English et al. (2018a) and are only briefly outlined here. Following arrival at Grange Research Farm, 20 Holstein-Friesian bull calves were allowed a 5 day acclimatisation period, following which calves were blocked based on age, sire, live weight and farm of origin into one of two groups: a high or low plane of nutrition group, with 10 calves in each group. All calves were individually fed milk replacer and concentrates using an electronic feeding system (Forster-Tecknik Vario; Engen, Germany). Calves on the high plane of nutrition received 1 200 g of milk replacer in 8 l of water daily, together with concentrate ad libitum and 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. All calves were weaned at a mean age of 82 (±3.9) days, when consuming a minimum of 1 kg of concentrate for three consecutive days. Following weaning, the high plane of nutrition group was offered ad libitum concentrates, whilst 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. Blood samples were collected from all calves through jugular venepuncture for the determination of systemic concentrations of both metabolic (insulin, leptin, adiponectin and IGF-1) and reproductive hormones (testosterone, FSH, LH) (English et al., 2018a).

Tissue collection

All calves were euthanised at a mean age of 126 days (±1.1), through an intravenous overdose of sodium pentobarbitone. It was expected that all calves would have experienced an endogenous transient LH rise (Rawlings et al., 2008) by the time of slaughter. Death was confirmed by lack of ocular response and was followed by exsanguination and decapitation. The brain was removed from the skull and the tissue surrounding the arcuate nucleus region of the hypothalamus was dissected according to Komatsu et al. (2012). The anterior and posterior sections of the pituitary gland were separated, with the anterior portion retained. Parenchyma was dissected from the middle region of each testis

pair and subcutaneous adipose samples were obtained from the flank of the carcass following slaughter. All tissue samples were washed in sterile Dulbecco's phosphate-buffered saline and immediately snap-frozen in liquid nitrogen. All tissue samples were stored at −80 °C pending further processing.

Histological evaluation of testes

A portion of testes parenchyma was also sampled for histology. Testes parenchyma tissue samples were fixed in 10% neutral buffered formalin and then prepared for histological sectioning. Histology was employed on testes samples to assess outer seminiferous tubule diameter, seminiferous tubule lumen development, and both spermatogenic and Sertoli cell numbers. Full details of histological analyses are outlined in full in English et al. (2018a).

Statistical analysis of animal performance and phenotypic data

All animal performance data, hormone concentration and testes histology results were analysed using Statistical Analysis Software (SAS, version 9.3; described in full in English et al., 2018a). Data were analysed for the fixed effect of treatment within the statistical model. Differences between treatment groups were deemed statistically significant if $P < 0.05$.

RNA isolation and sequencing

Details related to RNA isolation from tissue samples and subsequent sequencing are outlined in full in English et al. (2018b and 2018c). Briefly, total RNA was extracted from arcuate nucleus, anterior pituitary and testes tissue samples using the RNeasy Universal plus Kit (Qiagen, Manchester, UK) by following the manufacturer's instructions. The RNeasy Lipid Tissue Mini Kit (Qiagen, Manchester, UK) was used for the isolation of total RNA from adipose tissue samples according to the manufacturer's instruction. The quality and quantity of resultant isolated total RNA from all tissue samples were verified on the Agilent Bioanalyzer 2100 (Agilent Technologies Ireland Ltd., Dublin, Ireland) and Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA), respectively. cDNA libraries were prepared from 1 μg of high-quality total RNA samples using an Illumina TruSeq RNA Sample Preparation kit v2 following the manufacturer's instructions (Illumina, San Diego, CA, USA). All libraries were validated on the Agilent Bioanalyzer 2100 using the DNA 1000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd, Dublin, Ireland). Individual RNA-seq 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. RNAseq count files used in the current study were downloaded from NCBI's Gene Expression Omnibus (**GEO**) through accession numbers GSE97673 and GSE97674 for HPT and adipose datasets, respectively. RNAsesq data downloaded from NCBI GEO were sequenced to an average depth of 20 M reads per sample and aligned to the UMD3.1 bovine reference genome.

Gene co-expression analysis

The weighted gene co-expression network analysis (**WGCNA**) software package (Langfelder and Horvath, 2008) was used to identify modules (or networks) of co-expressed genes. WGCNA was performed on each tissue dataset individually. Additionally, a separate WGCNA consensus analysis was also undertaken on the four tissue datasets together. In both instances, RNAseq read count data from each tissue were first filtered for lowly expressed genes, by only retaining genes that had reads in at least half the total n for each tissue. Filtered RNAseq count data were then normalised in EdgeR by read counts per million mapped reads, (adjusted for sample library size). Normalised count data were then $\text{Log}_2(x + 1)$ transformed in R. For the individual tissue analyses, following filtering for the removal of lowly expressed genes, RNAseq datasets contained 12 392; 14 294; 13 742 and 14 406 genes in adipose, arcuate nucleus, pituitary and testes datasets, respectively. Unsigned, weighted correlation network construction and module detection were performed using the automatic one-step function of WGCNA for each individual tissue dataset. An adjacency matrix was calculated for each tissue dataset by raising the co-expression matrix to a soft-threshold power, determined by scale-free topology of each network ($R^2 > 0.9$). For each tissue, a soft-thresholding power of 20 was required for scale-free topology $R^2 > 0.9$. Gene cluster dendrograms for each individual HPT and adipose datasets are presented in Fig. 1. For the combined tissue consensus analysis, only genes that were commonly expressed (10 881 genes) across each of the four tissues were included in the analysis. The consensus analysis was undertaken using the automatic one-step function, blockwise consensus analysis of WGCNA, incorporating unsigned, weighted correlation network construction and module detection. A soft-thresholding power of 20 was required for scale-free topology $R^2 > 0.9$ for each tissue in the consensus analysis. To determine co-expression between genes in both individual tissue analyses and the consensus analysis, pairwise weighted Pearson correlations were calculated between all pairs of genes in each tissue examined. A topology overlap matrix was derived for each tissue based on the transformed connection strengths between genes. Average linkage hierarchical clustering was then applied to the topology overlap matrix for each tissue resulting in the grouping of highly similarly expressed genes and modules of co-expressed genes for each tissue dataset. The resulting modules of co-expressed genes were assigned colour identifiers by the software for each separate analysis undertaken.

Modules of co-expressed genes for each analysis were then correlated with phenotypic trait parameters related to metabolic status (systemic concentrations of insulin, leptin, adiponectin and IGF-1) as well as reproductive development (systemic concentrations of testosterone, FSH and LH) and markers of testicular development (seminiferous tubule diameter, seminiferous tubule lumen score, number of spermatogenic cells and Sertoli cell number) pertaining to the bulls used in the same transcriptomics study. Module-trait correlations were calculated through Pearson correlations between the module eigengene of each module of co-expressed genes and trait data related to metabolic status and reproductive development as listed above. Modules with statistically significant ($P < 0.05$) correlations were then selected for further analysis as biologically interesting modules associated with reproductive development. Pathway analysis was subsequently performed through Ingenuity pathway analysis (Qiagen) (Krämer et al., 2014) on genes from each module identified as significantly correlated with trait data, in order to assign biological annotation and undertake biological pathway analysis.

Results

Animal performance

Results related to calf growth and performance following varying plane of nutrition during the first 18 weeks of life were outlined in full by English et al. (2018a). Briefly, results directly related to the analysis undertaken in the current study are as follows. Systemic concentrations of IGF-1 and insulin were greater in calves on the high plane of nutrition compared to those on the low plane of nutrition ($P < 0.05$). Early life dietary treatment did

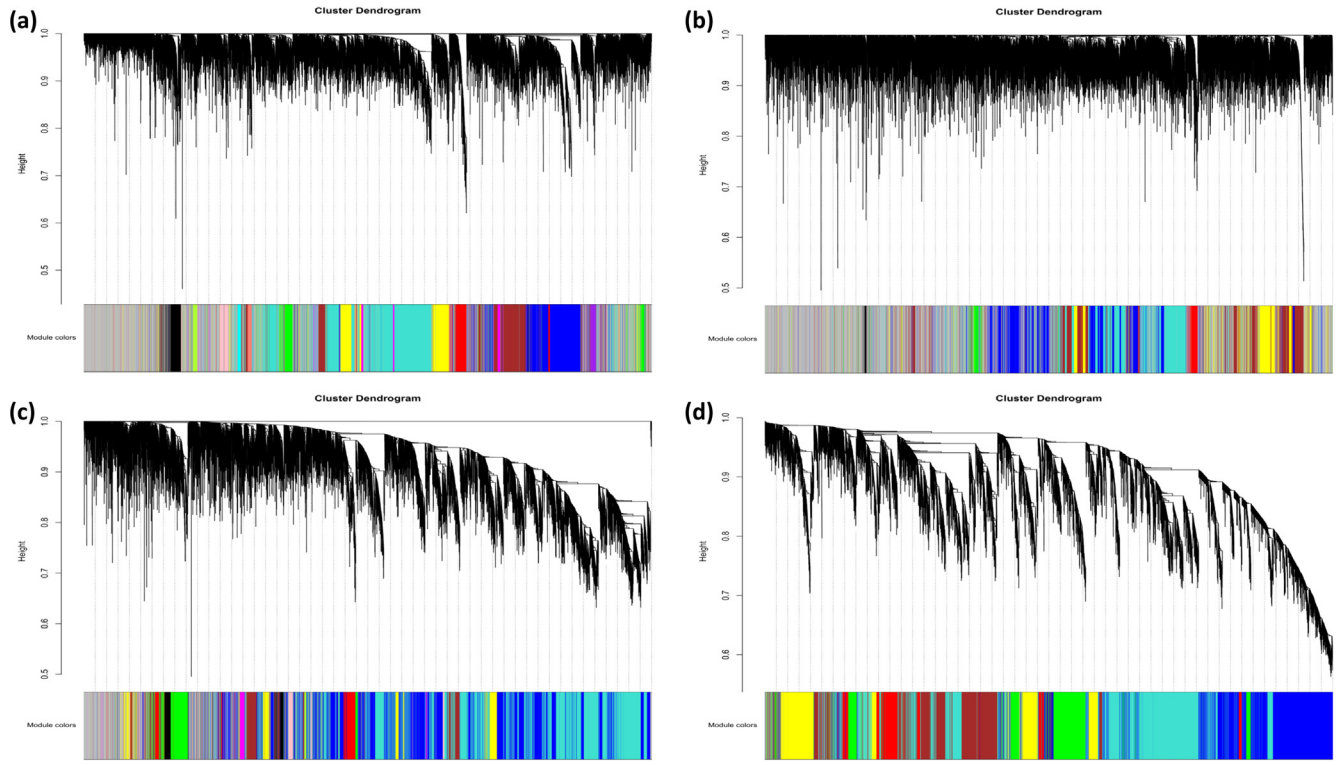

Fig 1. Gene cluster dendrograms for (a) arcuate nucleus; (b) anterior pituitary; (c) testes and; (d) adipose tissue datasets of Holstein-Friesian bull calves.

not impact circulating concentrations of either leptin or adiponectin ($P > 0.05$). Concentrations of both FSH and LH were unaffected by dietary treatment ($P > 0.05$); however, an evaluation of LH response through area under the curve analysis indicated a greater LH response ($P < 0.01$) in the calves on the high plane of nutrition compared to those on the lower plane of nutrition. Testosterone concentrations tended to be higher in the calves on the high diet ($P < 0.09$). Following 18 weeks of differential feeding, testicular seminiferous tubule diameter ($P < 0.001$), seminiferous tubule lumen score ($P < 0.001$), stage of spermatogenesis ($P < 0.001$) and number of Sertoli cells ($P < 0.05$) were all greater in the calves in the high plane of nutrition compared to their contemporaries on the lower plane of nutrition.

Individual tissue gene co-expression analysis

From the 14 294 genes retained for the arcuate nucleus co-expression analysis, a total of 17 networks of co-expressed genes, each denoted by a different colour identifier, were derived. Of these 17 networks, 4 were significantly associated with related traits examined. Sertoli cell number was negatively and positively correlated with the yellow network ($r = -0.53$, $P = 0.02$) and the midnightblue network ($r = 0.5$, $P = 0.04$), respectively. The latter of these two networks was also positively associated with testosterone concentrations ($r = 0.47$, $P = 0.05$). Two further networks were associated with systemic concentrations of IGF-1 (salmon network, $r = 0.61$, $P = 0.007$) and insulin (purple network, $r = 0.52$, $P = 0.03$). The full list of genes within each network is presented in Supplementary Table S1, and results of the module-trait association analysis for the arcuate nucleus are presented in Fig. 2a. Enriched pathways of biological relevance to the current study for each of the salmon, purple and midnightblue networks are presented in Table 1; no significantly enriched pathways were identified for the yellow network.

Ten separate networks of co-expressed genes were generated from the anterior pituitary transcriptome data. Genes included in each network within the anterior pituitary are outlined in full in Supplementary Table S1. Results of the module-trait association analysis for the anterior pituitary are presented in Fig. 2b. Of the ten networks, three in particular (black, brown and yellow networks) were associated with lumen score (black $r = -0.62$, $P = 0.02$; brown $r = -0.64$, $P = 0.01$; yellow $r = 0.56$, $P = 0.04$), Sertoli cell number (black $r = -0.66$, $P = 0.01$; brown $r = -0.68$, $P = 0.007$; yellow $r = 0.57$, $P = 0.03$) and stage of spermatogenesis (black $r = -0.67$, $P = 0.009$; brown $r = -0.78$, $P = 0.0009$; yellow $r = 0.87$, $P = 0.0006$). The brown network was also negatively associated with systemic concentrations of IGF-1 ($r = -0.67$, $P = 0.009$) and positively associated with LH concentrations ($r = -0.92$, $P = 0.0003$), whilst the yellow network was positively associated with concentrations of both testosterone ($r = 0.54$, $P = 0.04$) and LH ($r = 0.63$, $P = 0.02$). The turquoise network was positively associated with LH concentrations ($r = 0.55$, $P = 0.04$) and stage of spermatogenesis ($r = 0.58$, $P = 0.03$). Similarly, the magenta network was also positively correlated with LH concentrations ($r = 0.55$, $P = 0.04$), as well as with IGF-1 concentrations ($r = 0.63$, $P = 0.02$). Two final networks were positively and negatively associated with Sertoli cell number (red network, $r = 0.57$, $P = 0.03$) and tubule diameter (green network, $r = -0.61$, $P = 0.02$), respectively. Table 2 details biologically relevant enriched pathways pertaining to networks described above (black, brown, yellow, turquoise, green, red), and there were no significantly enriched pathways for the magenta network.

A total of 12 networks of co-expressed genes were derived from the testes dataset, of which 6 were significantly associated with a trait examined. The full list of genes within each testes derived network is presented in Supplementary Table S1. Module-trait associations for all comparisons within the testes are presented in Fig. 2c. Lumen score was associated with the following co-expressed networks: purple network ($r = -0.59$, $P = 0.03$), turquoise network ($r = -0.6$, $P = 0.02$), yellow network ($r = 0.67$, $P = -0.008$), red network ($r = 0.64$, $P = 0.01$) and blue network ($r = 0.62$, $P = 0.02$). Similar to lumen score, the purple network was also negatively

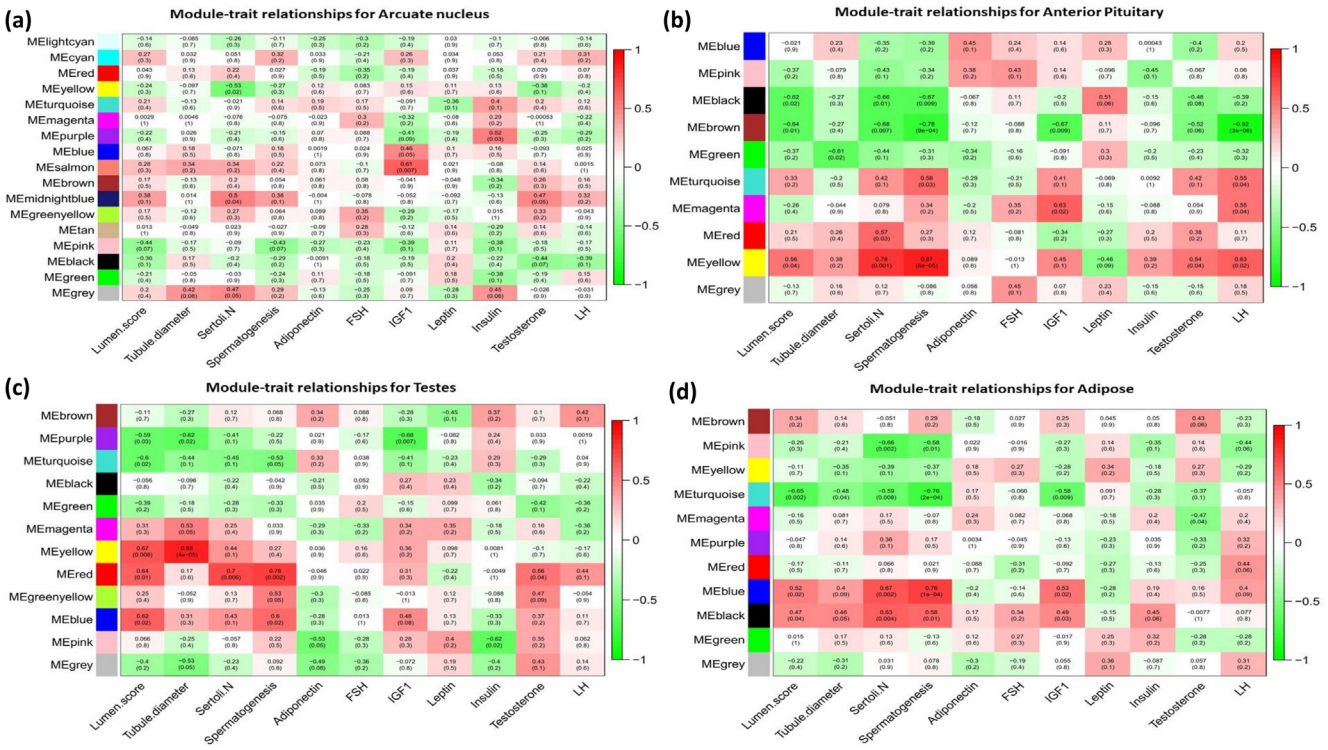

Fig 2. Module-trait relationships for detected modules (networks; y-axis) in (a) hypothalamic arcuate nucleus; (b) anterior pituitary; (c) testes parenchyma and; (d) subcutaneous adipose of Holstein-Friesian bull calves. The module-trait relationships were coloured based on the correlation between the module and traits (red = strong positive correlation; green = strong negative correlation). Correlation coefficient is presented for each module-trait association, followed by p-value in parentheses. X-axis legend: Lumen.score = seminiferous tubule lumen score; Tubule.diameter = seminiferous tubule diameter; Sertoli.N = Sertoli cell number; Spermatogenesis = stage of spermatogenesis; Adiponectin = adiponectin concentration; FSH = FSH concentration; IGF−1 = IGF-1concentration; Leptin = leptin concentration; Insulin = insulin concentration; Testosterone = testosterone concentration; LH = LH concentration.

Table 1
Top enriched biological pathways from networks of co-expressed genes significantly associated with metabolic and reproductive traits in the hypothalamic arcuate nucleus of Holstein-Friesian bull calves.

Network	Biological pathway	*P*-value
Midnightblue	PI3K/AKT Signalling	0.046
Salmon	PI3K/AKT Signalling	0.000
	G-Protein Coupled Receptor Signalling	0.000
	Opioid Signalling Pathway	0.001
	Actin Cytoskeleton Signalling	0.003
	Semaphorin Neuronal Repulsive Signalling Pathway	0.006
	cAMP-mediated signalling	0.030
	Synaptogenesis Signalling Pathway	0.032
Purple	Actin Cytoskeleton Signalling	0.000
	Epithelial Adherens Junction Signalling	0.000
	Synaptogenesis Signalling Pathway	0.000
	G-Protein Coupled Receptor Signalling	0.000
	GABA Receptor Signalling	0.004
	Tight Junction Signalling	0.005
	cAMP-mediated signalling	0.006
	Semaphorin Neuronal Repulsive Signalling Pathway	0.009

Table 2
Top enriched biological pathways from networks of co-expressed genes significantly associated with metabolic and reproductive traits in the anterior pituitary gland of Holstein-Friesian bull calves.

Network	Biological pathway	*P*-value
Black	AMPK Signalling	0.01072
	GNRH Signalling	0.0182
	Sirtuin Signalling Pathway	0.03388
	Growth Hormone Signalling	0.03631
	Oxidative Phosphorylation	0.04266
Brown		0.01479
	Sirtuin Signalling Pathway	0.0166
	AMPK Signalling	0.02291
Yellow	Kinetochore Metaphase Signalling Pathway	3.2E-09
	Cyclins and Cell Cycle Regulation	1.7E-06
	Cell Cycle: G1/S Checkpoint Regulation	0.00032
	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.00224
	Cell Cycle Control of Chromosomal Replication	0.00457
Turquoise	ERK/MAPK Signalling	3E-06
	GNRH Signalling	0.00012
Green	Sirtuin Signalling Pathway	0.00794
	AMPK Signalling	0.02884
Red	mTOR Signalling	2.5E-16
	Oxidative Phosphorylation	0.00575
	Sirtuin Signalling Pathway	0.00646

correlated with tubule diameter (r = −0.62, P = 0.02), and IGF-1 concentrations (r = −0.68, P = 0.007), whilst the red network was positively associated with Sertoli cell number (r = 0.7, P = 0.006), spermatogenesis stage (0.76, P = 0.002) and testosterone concentrations (r = 0.56, P = 0.04). The yellow and blue networks were also positively associated with tubule diameter (r = 0.86, 4e-05) and stage of spermatogenesis (0.6, P = 0.02), respectively, whilst the pink network was negatively associated with insulin concentrations (r = −0.62, P = 0.02). Enriched relevant biological pathways pertaining to each significantly associated network are described in Table 3; no significantly enriched pathways were identified for the pink network.

Of the 12 392 genes within the adipose tissue, 11 networks of co-expressed genes were formed. The full list of genes within each

network is presented in Supplementary Table S1, and Fig. 2d displays the module-trait relationships for all networks identified within the adipose tissue. Four networks in particular showed a number of associations with traits related to testicular development. These included the pink network, negatively associated with Sertoli cell number (r = −0.66, 0.002) and stage of spermatogenesis (r = −0.58, P = 0.01); the turquoise network negatively associated with lumen score (r = −0.65, P = 0.002), tubule diameter (r = −0.48, P = 0.04), Sertoli cell number (r = −0.59, P = 0.008) and spermatogenesis stage (r = −0.76, P = 0.0002); the blue network positively associated with lumen score (r = 0.52, P = 0.02), Sertoli cell number (r = 0.67, P = 0.002) and spermatogenesis stage (r = 0.76, P = 0.0001); and finally, the black network associated with lumen score (r = 0.47, P = 0.04), Sertoli cell number (r = 0.63, P = 0.004) and spermatogenesis stage (r = 0.58, P = 0.01). Systemic concentrations of IGF-1 were also significantly associated with the turquoise network (r = −0.58, P = 0.009), as well as the blue (r = 0.53, P = 0.02) and black (r = 0.49, P = 0.03) networks. Biologically relevant enriched pathways for each of these networks are presented in Table 4.

Consensus analysis

The WGCNA consensus analysis conducted across the four tissues resulted in 16 separate networks of co-expressed genes. Five of these networks were not significantly associated (P > 0.05) with any of the traits tested (brown, greenyellow, midnight blue, cyan and grey). Sertoli cell number was negatively associated with the tan network in both adipose and testes datasets (r = −0.58, P = 0.02); the yellow network across anterior pituitary and testes datasets (r = −0.6, P = 0.02); and the salmon network in the adipose and anterior pituitary datasets (r = −0.6, P = 0.01). The salmon network was also negatively associated with stage of spermatogenesis (r = −0.68, P = 0.006) in adipose and pituitary tissues. The green network was positively associated with Sertoli cell number (r = 0.6, P = 0.02) and stage of spermatogenesis (r = 0.74, P = 0.002) across both adipose and anterior pituitary datasets. Similarly, the blue network was positively associated with Sertoli cell number and stage of spermatogenesis across adipose and testes datasets. The pink network was positively associated with Sertoli cell number (r = 0.55, P = 0.04) in both pituitary and testes datasets.

Table 3
Top enriched biological pathways from networks of co-expressed genes significantly associated with metabolic and reproductive traits in the testes tissue of Holstein-Friesian bull calves.

Network	Biological pathways	*P*-value
Yellow	Sperm Motility	1.2E-05
	Sertoli Cell-Sertoli Cell Junction Signalling	2.4E-05
	Epithelial Adherens Junction Signalling	0.00056
	Tight Junction Signalling	0.00479
Red	Superpathway of Cholesterol Biosynthesis	6.3E-27
	Cholesterol Biosynthesis I	7.9E-15
	Androgen Biosynthesis	3.9E-05
Purple	mTOR Signalling	2.5E-12
	ERK/MAPK Signalling	0.02884
Blue	Oxidative Phosphorylation	2.3E-05
	Cyclins and Cell Cycle Regulation	0.00011
	Cell Cycle: G1/S Checkpoint Regulation	0.00049
	Insulin Receptor Signalling	0.00324
	TCA Cycle II (Eukaryotic)	0.01738
	Sertoli Cell-Sertoli Cell Junction Signalling	0.02344
	Androgen Signalling	0.03162
Turquoise	Cell Cycle Control of Chromosomal Replication	1.1E-06
	Androgen Signalling	1.9E-06
	Germ Cell-Sertoli Cell Junction Signalling	0.00089
	Insulin Receptor Signalling	0.00295
	mTOR Signalling	0.0263

Table 4
Top enriched biological pathways from networks of co-expressed genes significantly associated with metabolic and reproductive traits in subcutaneous adipose tissue of Holstein-Friesian bull calves.

Network	Biological pathway	*P*-value
Black	Integrin Signalling	0.00562
	Tight Junction Signalling	0.03467
	Actin Cytoskeleton Signalling	0.03548
Blue	Oxidative Phosphorylation	2.5E-39
	Superpathway of Cholesterol Biosynthesis	3.7E-09
	AMPK Signalling	1.6E-07
	Cholesterol Biosynthesis I	7.4E-07
	Fatty Acid Biosynthesis Initiation II	0.01096
Pink	Interferon Signalling	1.3E-15
	Activation of Cytosolic Pattern Recognition Receptors	3.5E-07
	Antigen Presentation Pathway	1.8E-05
Turquoise	Androgen Signalling	0.00018
	AMPK Signalling	0.00912

Stage of spermatogenesis was positively and negatively associated with the red network (r = 0.56, P = 0.04) and the turquoise network (r = −0.62, P = 0.02) in adipose and pituitary tissues. The purple network was positively associated with Sertoli cell number in arcuate nucleus and anterior pituitary datasets (r = 0.5, P = 0.03), arcuate nucleus and testes (r = 0.5, P = 0.03), and anterior pituitary and testes datasets (r = 0.6, P = 0.04). The anterior pituitary and testes datasets were also positively associated with stage of spermatogenesis (r = 0.59, P = 0.01) and IGF-1 concentrations (r = 0.52, P = 0.04) for the purple network. Sertoli cell number and stage of spermatogenesis were positively associated with the magenta network across adipose and pituitary datasets (Sertoli cell number: r = 0.74, P = 0.0002; spermatogenesis: r = 0.79, P = 0.00002); adipose and testes datasets (Sertoli cell number: r = 0.74, P = 0.0002; spermatogenesis: r = 0.79, P = 0.0001); and anterior pituitary and testes tissues (Sertoli cell number: r = 0.77, P = 0.0002; spermatogenesis: r = 0.79, P = 0.0001). The magenta network was also positively associated with lumen score across adipose and pituitary datasets (r = 0.54, P = 0.02). Across adipose and pituitary datasets, the black network was positively associated with lumen score (r = 0.5, P = 0.03); Sertoli cell number (r = 0.73, P = 0.001); stage of spermatogenesis (r = 0.68, P = 0.006); and LH concentration (r = 0.49, P = 0.03). The black network was also positively associated with traits examined across adipose and testes datasets including Sertoli cell number (r = 0.69, P = 0.002); stage of spermatogenesis (r = 0.73, P = 0.0008); and concentrations of IGF-1 (r = 0.52, P = 0.04). Within the pituitary and testes datasets, the black network was also positively associated with Sertoli cell number (r = 0.69, P = 0.002) and stage of spermatogenesis (r = 0.68, P = 0.006). The full list of genes included within each network is presented in Supplementary Table S2 with an overview of the significant module-trait associations presented in Fig. 3. Biologically relevant enriched pathways for each of the consensus analysis networks are presented in Table 5.

Integration of results across co-expression networks

In order to determine the interaction of genes between tissues, gene lists for all networks from the individual tissue analyses were compared with one another. Large numbers of genes were identified as commonly associated with the phenotypic traits examined across the four tissues examined. The full list of genes common across networks for each individual tissue analysis is presented in full in Supplementary Table S3. Additionally, a comparison between the networks of co-expressed genes derived from the current study and the differentially expressed genes reported from our

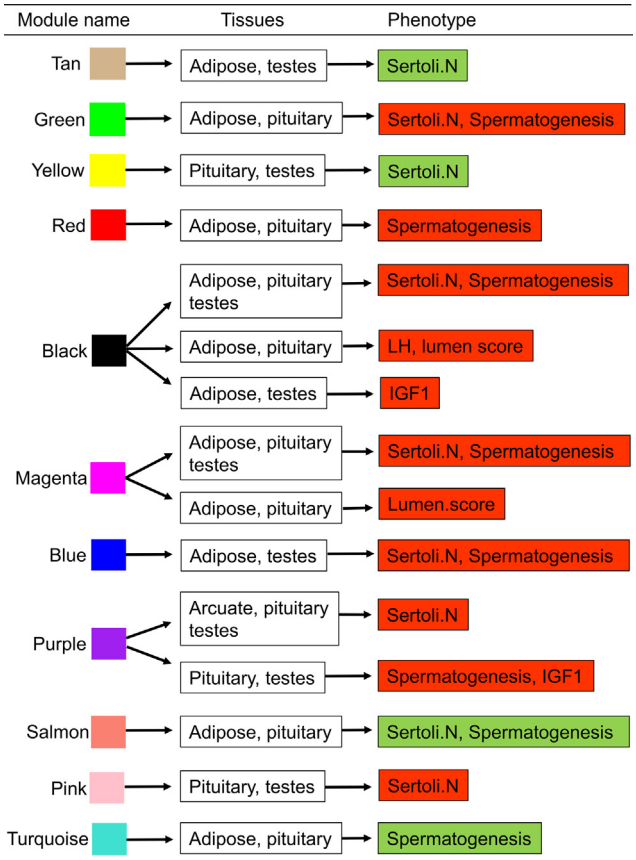

Fig 3. Overview of significant associations related to consensus analysis of Holstein-Friesian bull calves. Phenotype colour denotes correlation type between co-expressed network and trait examined (red = strong positive correlation; green = strong negative correlation). Phenotype legend Lumen.score = seminiferous tubule lumen score; Sertoli.N = Sertoli cell number; Spermatogenesis = stage of spermatogenesis; IGF−1 = IGF-1concentration; LH = LH concentration.

Table 5
Top enriched biological pathways from consensus analysis networks of co-expressed genes significantly associated with metabolic and reproductive traits in Holstein-Friesian bull calves.

Network	Biological pathway	*P*-value
Green	Oxidative Phosphorylation	7.24436E-08
	Sirtuin Signalling Pathway	2.45471E-06
	TCA Cycle II (Eukaryotic)	0.003388442
	Opioid Signalling Pathway	0.011220185
Blue	Triacylglycerol Biosynthesis	8.91251E-07
	Oxidative Phosphorylation	0.019952623
	Fatty Acid Activation	0.023988329
	Growth Hormone Signalling	0.030902954
Pink	Sertoli Cell-Sertoli Cell Junction Signalling	0.016596
Red	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.00631
	GNRH Signalling	0.007586
	IGF-1 Signalling	0.019498
Black	Oxidative Phosphorylation	3.0903E-10
	TCA Cycle II (Eukaryotic)	1.1749E-05
	Sirtuin Signalling Pathway	0.000645654
	Cyclins and Cell Cycle Regulation	0.005495409
	Fatty Acid Biosynthesis Initiation II	0.026915348
Magenta	Cholesterol Biosynthesis I	8.31764E-09
	Cholesterol Biosynthesis II	8.31764E-09
	Cholesterol Biosynthesis III	8.31764E-09
	Superpathway of Cholesterol Biosynthesis	1.62181E-08
Purple	Senescence Pathway	0.001202
	CD40 Signalling	0.002399
	IL-10 Signalling	0.033113
	IL-17 Signalling	0.038019
	Toll-like Receptor Signalling	0.038019

earlier study conducted on the same tissue samples was also undertaken. This was undertaken for both individual and consensus co-expression analyses, with the results presented in Supplementary Tables S4 and S5, respectively.

Discussion

Given the central contribution of the HPT axis and adipose tissue to regulating the influence of nutritional status on the ontogeny of sexual development in calves, studies have sought to uncover the molecular mechanisms contributing to this outcome (English et al., 2018a, 2018b and 2018c; Kenny et al., 2018, Johnson et al., 2019). Although molecular evaluations of the effect of early life nutrition on subsequent puberty attainment through the identification of differentially expressed genes can yield interesting effects of the particular phenotype, studies have shown that individual genes do not work alone, instead genes interact with each other to elicit a cascade of biochemical processes which result in a subsequent physiological phenotypic outcome (Miklos and Rubin, 1996; Arnone and Davidson, 1997; Li et al., 2018). Gene co-expression networks can provide the potential to uncover the interactions between genes governing a particular phenotypic outcome, with this information important for predicting the functions of genes that play key roles in complex phenotypes (Kogelman et al., 2014). Gene co-expression analyses may also provide a more holistic evaluation examining all expressed genes as opposed to those detected as differentially expressed, allowing for a more equitable comparison across diverse study designs (Keogh et al., 2019). Moreover, the identification of hub genes, which are important in regulating the expression of several other genes within a network of co-expressed genes, may hold potential as biomarkers for the selection of a trait of interest. However, a disadvantage of gene co-expression analyses is the reliance on correlation as opposed to causation which may be more apparent through differential gene expression analysis. Despite this limitation, gene co-expression analyses are a useful tool in molecular omics evaluations. Consequently, using previously published RNAseq datasets (English et al., 2018b and 2018c) we performed gene co-expression analyses to reveal further insights into the biological processes affected by dietary manipulation and which are contributing to the resultant phenotypic outcome of reproductive development in bull calves. Indeed, the utility of co-expression analyses compared to differential expression analysis was most apparent in the current study in both arcuate nucleus and anterior pituitary datasets where few genes were identified as differentially expressed in our previous evaluation (English et al., 2018b). Results from the current study build on our previous differential expression analysis evaluation whilst also identifying additional genes not identified in our earlier study as important to mediating enhanced metabolic status with subsequent reproductive development. An evaluation of biological processes affected across HPT and adipose tissues (Fig. 4) revealed commonality for pathways related to energy production, cellular growth and proliferation, GnRH signalling and cholesterol biosynthesis; thus, the remainder of this discussion will focus on these processes.

Energy production

Following 18 weeks of differential feeding, genes involved in cellular processes related to metabolic or energy sensing as well as ATP production were co-expressed in networks across both individual and consensus analyses for the anterior pituitary, testes and adipose tissues. Cellular processes affected across these networks and tissues included those related to sirtuin signalling, tricarboxylic acid (**TCA**) cycle and oxidative phosphorylation. Sirtuins

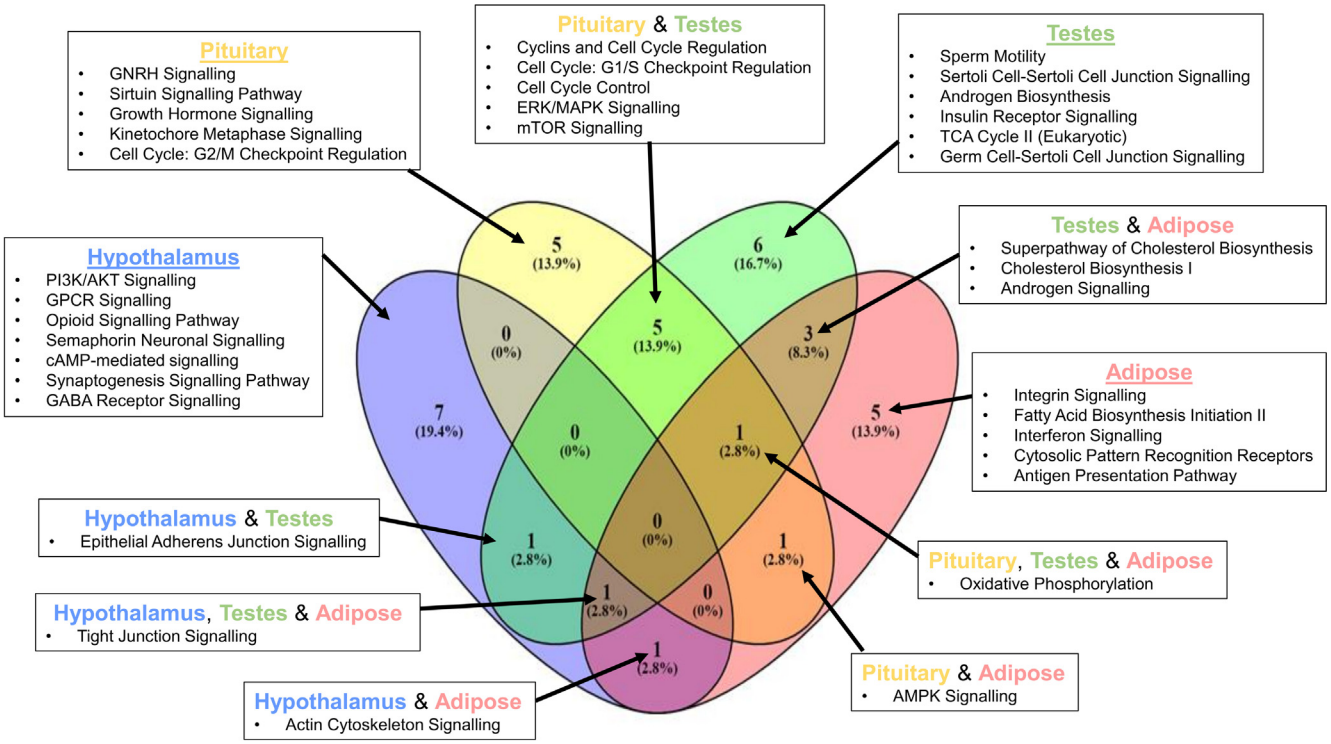

Fig 4. Venn diagram showing biological processes enriched in significant networks across hypothalamic-pituitary-testes and adipose tissues as a consequence of enhanced earlier life metabolic status in Holstein-Friesian bull calves.

represent a family of highly conserved signalling proteins involved in metabolic regulation, functioning as nutrient sensors responding directly to cellular nutrient availability by regulating cellular energy homeostasis. Whilst both the TCA cycle and oxidative phosphorylation are involved in cellular ATP production. Thus, given the planned differential feeding regime utilised in the current study, enrichment of such processes may not be unexpected. However, although it might be expected for these networks to be positively correlated with the traits examined (greater expression in calves on the high plane of nutrition), not all of these networks were positively correlated with some networks displaying negative associations with both metabolic and reproductive traits. This was most apparent within the anterior pituitary dataset where three separate networks of genes (black, brown and green) all displayed negative associations with both metabolic and reproductive traits examined (lumen score, Sertoli cell number, stage of spermatogenesis, systemic concentrations of IGF-1 and LH). However, the negative associations within the pituitary tissue may be due to the co-expressed genes within these networks representing negative regulators or inhibitory genes within the pathway. This may also explain the positive correlations between other pituitary networks involved in energy production processes and the traits examined.

Despite the aforementioned negative associations between pituitary networks and energy production processes, an additional network within the pituitary (red network) which included genes enriched for both oxidative phosphorylation and sirtuin signalling was positively associated with Sertoli cell number. Moreover, networks within both the testes and adipose datasets also displayed positive associations as well as being enriched for oxidative phosphorylation, with the testes network also enriched for TCA cycle. These associations included the blue network in the testes which was positively associated with lumen score and stage of spermatogenesis and the blue network in the adipose dataset which was positively associated with lumen score, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrations. Although adipose tissue is not directly linked within the HPT signalling axis, peptides produced by the adipose tissue depots throughout the body can elicit direct effects on the hypothalamus and thus can influence the resultant signalling between the tissues of the HPT (Tsatsanis et al., 2015). Overall though the function of adipose tissue in metabolic and reproductive processes is complex, it has been hypothesised that there is crosstalk between adipose tissue and the HPT axis (Kershaw and Flier, 2004). Indeed, this was evident through the results of the current study where networks of co-expressed genes including the blue network of the adipose dataset were significantly correlated with traits related to testicular development. Furthermore, the contribution of adipose tissue towards reproductive development is also established through the consensus analysis results, where networks enriched for oxidative phosphorylation, TCA cycle and sirtuin signalling were common between the adipose, pituitary and testes datasets. These included the green, black and blue networks from the consensus analysis which displayed positive associations with Sertoli cell number, stage of spermatogenesis, lumen score and systemic concentrations of both IGF-1 and LH. Indeed, differential expression analysis of the adipose tissue used in the current study also showed greater expression of genes involved in cellular energy production (English et al., 2018c). Taken together results from both the individual tissue and consensus analyses indicate towards a role for biological processes related to energy homeostasis and production towards enhanced metabolic processing and reproductive development across pituitary, testes and adipose tissues. Results suggest that as a consequence of enhanced dietary intake, there was a requirement for greater cellular energy production to contribute to growth processes within these tissues.

Cellular growth and proliferation

As per the experimental design, the high calves were consuming more feed compared to the low dietary intake group, thus, it is not

unexpected that cellular processes related to cellular division and growth were enriched in networks significantly associated with the phenotypic traits examined. Indeed, differential expression analysis of anterior pituitary tissue used in the current study showed greater expression of genes involved in cell cycle in the calves on the high plane of nutrition (English et al., 2018b). Similarly, networks including genes involved in cell cycle and cell cycle regulation were positively associated with reproductive traits tested; suggesting greater cellular growth is associated with earlier reproductive development. Networks positively associated with cell cycle pathways included the yellow network in the anterior pituitary and the blue network in the testes. Additional networks from the consensus analysis (red and black networks) were also positively associated with trait data in adipose, pituitary and testes datasets. Interestingly, the red consensus network also contained genes enriched for pathways related to reproductive development including both GnRH and androgen signalling. Indeed, the *GNAI1* gene which encodes a guanine nucleotide-binding protein involved in GnRH secretion was included within the red network as a hub gene, whilst also identified as differentially expressed within the subcutaneous adipose tissue used in this study (English et al., 2018c). The yellow network of the pituitary dataset was of particular interest due to its inclusion of genes that have previously been implicated towards reproductive development and puberty attainment. This network was positively correlated with lumen score, Sertoli cell number, stage of spermatogenesis, and systemic concentrations of testosterone and LH within the anterior pituitary. Genes contained within this network and identified as hub genes included the neuroendocrine secretory genes; *SCG2* and *SCG3,* which were found to contain single nucleotide polymorphisms (**SNPs**) associated with puberty at the transcript level (Dias et al., 2017). Indeed, positive associations with lumen score, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrations were also apparent for the blue network within the adipose dataset which also included the *SCG2* gene. Conversely though, *SCG3* was negatively associated with lumen score, tubule diameter, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrations in the adipose turquoise network, whilst both *SCG2* and *SCG3* were negatively associated with lumen score in the testes turquoise network. Despite the conflicting results from the current study, the implication of these genes to reproductive function is further established through the reduction in GnRH-induced FSH expression as a consequence of cellular knockout of the *SCG2* gene (Choi et al., 2016). Moreover, Johnson et al. (2019) reported differential expression of the *SCG2* gene in testes parenchyma between calves fed either a high or low diet during the first 16 weeks of life. Additionally, the *PROP1* gene was also contained within the yellow pituitary network as a hub gene. This gene encodes a protein involved in the development of the pituitary gland as well as the production of hormones including LH, FSH and GH (D'Elia et al., 2001; Raetzman et al., 2002; Scully and Rosenfeld, 2002; Kenny et al., 2018). Moreover, Cánovas et al. (2014) concluded that *PROP1* was a key transcription factor involved in the regulation of puberty in heifers, thus highlighting a role for this gene in reproductive development.

In addition to networks involving genes related to cell cycle processes, the consensus analysis revealed an enrichment for the growth hormone signalling pathway across the blue network in adipose and testes datasets. The blue consensus network was significantly positively associated with Sertoli cell number and stage of spermatogenesis, indicating a potential role for growth hormone signalling towards these traits. Moreover, the blue network within the individual adipose analysis (positively associated with lumen score, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrators) also contained genes of the somatotropic axis, including *GHR* and *IGF1*, both of which were identified as hub genes. *GHR* was also expressed within the yellow network of the anterior pituitary, displaying positive associations with lumen score, Sertoli cell number, stage of spermatogenesis and concentrations of LH, whilst the yellow network of the testes dataset contained *IGF1*, which was positively associated with lumen score and tubule diameter, suggesting a role for the somatotropic axis towards reproductive development across adipose, testes and pituitary tissues. Indeed, the high plane of nutrition calves used in the current study also displayed greater concentrations of systemic IGF-1 during the early life dietary trial (English et al., 2018a), which combined with the co-expression network results suggest a role for the somatotropic axis towards earlier reproductive development as a consequence of enhanced dietary intake in early life.

Gonadotropin-releasing hormone signalling

The positive effect of improved metabolic status and subsequent earlier reproductive development is mediated through a cascade of endocrine and neuroendocrine events affecting hypothalamic GnRH pulsatility, which subsequently leads to enhanced and earlier secretion of LH and FSH. Indeed, GnRH signalling was identified as an enriched pathway within the individual pituitary dataset (turquoise network) as well as across the adipose and pituitary datasets following the consensus analysis (red network). The turquoise network of the pituitary dataset was positively associated with both stage of spermatogenesis and systemic concentrations of LH, and additional pathways of interest also enriched within this network included insulin and IGF-1 receptor signalling as well as ERK/MAPK signalling. The enrichment of ERK/MAPK pathway is of particular interest due to the relationship between GnRH and MAPK, whereby GnRH may use alternate signalling cascades to regulate selective gonadotropin subunit genes, specifically LHβ (Harris et al., 2002). Thus, due to the role of ERK/MAPK in proliferation and differentiation, the expression of ERK/MAPK genes and subsequent biochemical signalling may be directly augmented by prevailing dietary management with the subsequent effect of LH synthesis within the anterior pituitary.

In addition to the positive associations for the pituitary gland, negative associations for networks involved in GnRH signalling were also identified for this tissue. These included the black and brown networks, which were negatively associated with lumen score, Sertoli cell number and stage of spermatogenesis, whilst the brown network was also negatively associated with systemic concentrations of both IGF-1 and LH. The timing of the early transient rise in LH pulsatility is a critical factor in determining the age at which puberty is reached (Rawlings et al., 2008). For bulls, this early rise has been reported to occur between 10 and 20 weeks of age and is thought to induce responsiveness of testicular Leydig cells to LH, leading to an increase in testosterone production. However, results from the current study as well as English et al. (2018a) suggest that following enhanced differential feeding up to 18 weeks of age, the age at which the early LH rise occurs may be reduced. Indeed, this may explain the negative correlations evident between networks of genes involved in energy sensing, as discussed above and GnRH signalling with determinants of reproductive development. Moreover, the negative correlations between the brown pituitary network are of particular interest as this network also included genes involved in the RNA-induced silencing complex, which ultimately functions in transcriptional repression (Kenny et al., 2018). Negative correlation between these genes and lumen score, Sertoli cell number and stage of spermatogenesis suggests a continuation of cellular transcription and prevention of transcriptional repression within the anterior pituitary which may be contributing to the documented outcome traits in the calves on the high plane of nutrition (Kenny et al., 2018). Moreover, specific

genes included within the brown network as hub genes have previously been implicated in reproductive development or pubertal attainment. For example in their evaluation of genes contributing to puberty in heifers, Cánovas et al. (2014) attributed the transcription factor *PITX2* to be critical to puberty in heifers. Moreover, Dias et al. (2017) identified transcriptional variants within this gene as associated with puberty in cattle. However, the negative association between the network in which this gene is involved and markers of testicular development established through our own analyses suggests that this gene is not contributing to earlier puberty attainment in animals fed a higher plane of nutrition. This is further apparent through the identification of the *PITX2* gene as negatively associated with lumen score within the turquoise network of the testes. Similarly, other genes with functions in the negative regulation of cellular processes including *E2F3*, *INHBE* and *PLAG1* were also found to be negatively associated with lumen score, Sertoli cell number and stage of spermatogenesis in the pituitary dataset. The *E2F3* gene, involved in cell cycle control, has previously been implicated in puberty in heifers across two separate studies (Cánovas et al., 2014; Fortes et al., 2016). This gene was also included in networks positively associated with lumen score and stage of spermatogenesis in testes (blue network), and negatively associated with lumen score, tubule diameter, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrations in adipose (turquoise network). Moreover, *INHBE* inhibits FSH secretion, whereas expression of the transcription factor *PLAG1* can lead to uncontrollable cell proliferation. However, although our results suggest a negative role of *PLAG1* for puberty attainment in bulls, SNPs in the *PLAG1* gene have been found to be associated with puberty onset in bulls determined through scrotal circumference or age at a scrotal circumference of 26 cm (Hawken et al., 2012; Fortes et al., 2013; Bolormaa et al., 2015). Additionally, a number of genome-wide association studies (**GWAS**) have shown that SNPs in the *PLAG1* gene in cattle are also associated with circulating IGF-1 concentrations (Hawken et al., 2012; Fortes et al., 2013). Moreover, *INSL3*, a marker of testicular Leydig cell functional capacity (Anand-Ivell et al., 2019), was also included as a hub gene within the brown pituitary network, despite this gene displaying greater expression in the testes tissue of the high calves compared to the low calves (English et al., 2018b). However, although our results seem contradictory to those mentioned above, the use of an early life nutritional calf model such as that used in this study must be acknowledged as different to the aforementioned studies which were based on non-interventional studies of cattle undergoing puberty. Additionally, as per the experimental design, the high calves were consuming more feed compared to the low group, with potential for various tissues and organs within the body to have developed earlier in the high group. Thus, the negative correlations may represent a divergence of energy to other parts of the body as a consequence of sustained higher nutrient intake, with the development of the anterior pituitary gland still occurring within the low dietary intake calves at the time of tissue sampling.

Although GnRH signalling was not identified as an enriched process within the arcuate nucleus datasets across either individual or consensus analyses, biological processes related to GnRH were enriched within the arcuate nucleus. These included the purple and salmon networks of the arcuate dataset, which were positively associated with systemic concentrations of insulin and IGF-1 respectively. Both of these networks contained genes involved in GnRH migration and regulation, for example the semaphorin neuronal repulsive signalling pathway. Semaphorins and their receptors, neurophilins and plexins, represent a large family of molecules implicated in neuronal development and plasticity and are known as key regulators of GnRH neuron biology and deficiency (Oleari et al., 2019). Specifically, these signalling molecules have been shown to play diverse roles in GnRH neuron biology by regulating migration and survival during embryonic development as well as secretion in adulthood (Oleari et al., 2019). Moreover, additional pathways positively correlated with insulin and IGF-1 concentrations in these networks included those involved in further signalling processes related to GnRH regulation. For example, cAMP-mediated signalling, involved in GnRH secretion (Vitalis et al., 2000); G-Protein coupled receptor signalling, critical for normal reproductive development and function (Noel and Kaiser, 2011), as well as GABA receptor signalling, which although only associated with insulin concentrations, has long been implicated as one of the major players in the regulation of GnRH neurons (Watanabe et al., 2014). Furthermore, the salmon network associated with IGF-1 concentrations also contained genes involved in the opioid signalling pathway. The opioid signalling pathway is responsible for producing neuroendocrine products that are involved in the release of GnRH and consequently FSH and LH from the anterior pituitary (Subiran et al., 2011). Interestingly, a gene of the opioid signalling pathway, *PENK* (pro-enkephalin), has previously been implicated in puberty in cattle, through the identification of SNPs within this gene associated with puberty attainment (Fortes et al., 2016; Dias et al., 2017). Moreover, opioid signalling was also an enriched process within the green network across both adipose and pituitary datasets pertaining to the consensus analysis. Additionally, the salmon network also included the *C3* gene as a hub gene, which has recently been implicated as a potential mediator between enhanced metabolic status and subsequent reproductive development in the arcuate nucleus of 12-week-old bull calves (Keogh et al., 2021). The *C3* gene was positively associated with IGF-1 concentrations in the salmon arcuate nucleus network and also displayed positive associations for lumen score and stage of spermatogenesis in the blue networks of both adipose and testes datasets. Additionally, the adipose blue network which included *C3* was also positively associated with Sertoli cell number and IGF-1 concentrations, indicating a potential positive role for this gene towards early life reproductive development across the tissues examined. Thus, although our differential expression analysis of the same arcuate nucleus transcriptomic data used in this current study yielded no genes of interest (English et al., 2018b), the current co-expression analysis clearly shows a direct association between higher dietary intake resulting in greater concentrations of insulin and IGF-1 and subsequent enrichment of biochemical pathways related to GnRH release and signalling.

Cholesterol biosynthesis and Sertoli cell development

The final endpoint of the HPT signalling axis is the production and secretion of testosterone within the testes, with androgen biosynthesis characterised through cholesterol biosynthesis (Miller, 2002). Indeed, genes involved in cholesterol biosynthesis were up-regulated in the calves on the high plane of nutrition used in this study (English et al., 2018b). A similar outcome was also evident from the results of the current gene co-expression analyses, where gene networks from both the testes and adipose datasets displayed enrichment for biological pathways related to cholesterol biosynthesis. The blue network within the adipose was positively associated with lumen score, Sertoli cell number, IGF-1 concentration and stage of spermatogenesis. Whilst the red network within the testes was positively associated with lumen score, Sertoli cell number, stage of spermatogenesis and systemic concentrations of testosterone. Moreover, the magenta network of the consensus analysis was positively associated with Sertoli cell number, lumen score and stage of spermatogenesis across adipose, pituitary and testes datasets. Indeed, the previous differential expression analysis of the datasets used for this current study identified the *EBP* gene, which was identified as a hub gene within the red network of the testes dataset as well as in the magenta dataset

of the consensus analysis, as up-regulated in calves on the high diet in both adipose and testes tissues (English et al., 2018b and 2018c). Moreover, *EBP* expression, involved in cholesterol biosynthesis, was also greater in bull calves that were offered a high plane of nutrition compared to a moderate plane of nutrition for the first 12 weeks of life in Coen et al. (2021a).

In addition to enrichment of processes related to cholesterol biosynthesis, enrichment of Sertoli cell-Sertoli cell junction signalling was also apparent both within the individual testes analyses and the consensus analysis (pituitary and testes datasets). Specifically, within the consensus analysis, the pink network was positively associated with Sertoli cell number, whilst in the individual testes dataset, the yellow network was positively associated with lumen score and tubule diameter, with the blue testes network positively associated with lumen score and stage of spermatogenesis. Indeed, the blue co-expression network of the testes, previously discussed within the cellular growth section, was also enriched for additional biological processes including insulin receptor signalling and androgen signalling, which may suggest a role for insulin receptor signalling towards testes development as a consequence of prevailing dietary management. Moreover, the yellow network of the testes dataset also contained the *CLDN11* gene as a hub gene. This gene encodes a protein involved in the blood-testis-barrier which also contributes to spermatogenesis. *CLDN11* was also included within the turquoise network in the adipose tissue; however, in the adipose, this gene was negatively associated with tubule diameter, lumen score, Sertoli cell number, stage of spermatogenesis and IGF-1 concentrations. Similarly, *CLDN11* was down-regulated in the subcutaneous adipose tissue between high and low fed calves (English et al., 2018c), indicating differential regulation dependent on tissue type. However, although the aforementioned results related to the testes co-expression analysis clearly show positive associations between specific traits examined, we also identified negative correlations between testes gene co-expression networks and lumen score in the turquoise network. Indeed, the turquoise network, negatively associated with lumen score, included the *CDH13* cadherin gene, which was recently reported as down-regulated in the testes tissue of bull calves fed an enhanced diet up to 12 weeks of age compared to a contemporary group offered a moderate diet (Coen et al., 2021b). Overall results indicate that the greater dietary intake in the calves receiving the high plane of nutrition is associated with increased cholesterol biosynthesis and ultimately with Sertoli cell development and androgen biosynthesis.

Conclusions

Results from this study provide further novel biological information on the cellular response of the HPT and adipose tissues to prevailing early life nutrition. This study builds on our existing differential expression analysis and through gene co-expression analyses provides further insight into the molecular control of reproductive development as a consequence of enhanced early life nutrition. In particular, gene co-expression analysis of arcuate nucleus and anterior pituitary tissues yielded novel results that were not apparent through differential expression analyses alone. Results from this study highlight networks of co-expressed genes enriched for biological processes related to energy production, cellular growth, GnRH signalling and cholesterol biosynthesis as directly associated with markers of enhanced metabolic status and subsequent earlier reproductive development across the HPT and adipose tissues. Furthermore, genes within networks positively associated with markers of enhanced metabolic status and reproductive development may hold potential for informing genomic selection breeding programmes for the selection of calves capable of displaying earlier reproductive development as a consequence of enhanced dietary intake. However, despite the interesting results presented, further functional analyses are warranted on biological pathways/processes discussed in order to fully elucidate the complex molecular processes regulating the underlying biology. Furthermore, specific key hub genes highlighted throughout the preceding sections require further evaluation in terms of their use as potential candidates within marker-assisted genomic selection programmes to support earlier onset of puberty in bull calves.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100527.

Ethics approval

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

Data and model availability statement

RNAseq data used in this study are publically available from NCBI's Gene Expression Omnibus (GEO) through accession numbers GSE97673 and GSE97674 for HPT and adipose datasets, respectively.

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Author contributions

KK and DAK conceived the study design. KK undertook data analysis, interpretation of results and drafted the manuscript. DAK edited the manuscript.

Declaration of interest

None.

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