Milestone report

Increased pasture intake and reduced supplement requirements of sheep and cattle

Project code: B.NBP.0813

Prepared by: Simon Quigley
The University of Queensland

Date published: 31-March-2021

PUBLISHED BY
Meat and Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Feed intake of ruminants in northern Australia is reduced by 20 to 60% when consuming diets deficient in protein (dry season) and phosphorus (wet season), resulting in decreased productivity (i.e. liveweight gain, reproduction rates). Nutritional models of intake suppression in response to deficiencies in dietary protein and phosphorus content, and total energy intake were successfully established. Whole transcriptome sequencing and bioinformatics analysis of gene expression profiles of three regions in the hypothalamus, liver, and rumen and duodenum walls were conducted to understand the mechanisms involved in the regulation of feed intake in cattle (Experiment 1) and sheep (Experiment 2).

Differential expression of a greater number of genes were evident in the peripheral (liver, rumen) tissues in response to nutrient deficiencies than in the hypothalamus. The anticipated first-order gene (neuropeptide) pathways are involved in the central regulation of intake in ruminants in response to nutrient deficiencies, although minor differences may exist in the profile of differentially expressed genes depending on the type of nutrient deficiency. However, it appears that dysregulation of the normal appetite regulatory system occurs in ruminants consuming nutrient deficient diets. Nutrient deficient animals appear to be in a state of hunger (as indicated by anticipated signalling of key appetite regulatory neurotransmitters) but suppress intake through other currently undetermined control mechanisms within the central nervous system in response to signals from the peripheral tissues.

This project was terminated by MLA. The report for Milestone 3 represents a final deliverable for the work completed.

Table of contents

1. Milestone

Milestone 3.1 Completion of Experiments 1, 2 and 3

- RNAseq analysis completed and analysed (Experiments 1 and 2)
- Multi-tissue gene expression networks developed (Experiment 3)
- Gene expression data linked with intake data (Experiments 1 and 2)
- Potential methods to manipulate intake reviewed

Milestone 3.2 Design of Experiments (Activities) 4 and 5 proposed

A pathway to continue research to further advance our understanding of intake regulation of ruminants fed nutrient deficient diets was proposed including,

Activity 4. Interrogation of existing data-sets and stored samples

- Interrogate gene expression data in 'targeted' metabolic pathways / tissues (Experiment 2)
- Interrogate gene expression data (original Quigley/Poppi ovine model)
- Analysis of stored plasma samples (amino acid profile, biomarkers linked to higher-order brain activity) (Experiment 2)
- Testing pharmaceuticals to increase intake in an ovine deficient model

Activity 5. Testing pharmaceuticals (and nutrients) to increase intake

- Establish two groups of weaned sheep (CP and P deficient and CP and P adequate models)
  - Test a number of long-acting pharmaceuticals on intake in both models
  - Examine the acute intake response to dietary P and N in both models
  - Examine the effect of orexigenic neuropeptides on intake in both models

Milestone 3.3 Workshop

- Workshop with project team and MLA to discuss results and proposed manipulation methods
- Determine feasibility of research to manipulate intake in ruminants through targeted compound delivery
- Design subsequent experiments to identify and test delivery of compounds to manipulate intake in ruminants consuming nutrient deficient diets

2. Project objectives

The objectives of this project are to,

1. Identify and integrate the gene and hormone pathways in the hypothalamus and other tissues which control intake of nutrient deficient diets in ruminants (through multi-tissue network analysis of sequence data from tissues involved in intake regulation), and
2. Identify (and test) non-nutritional delivery methods to increase intake of nutrient deficient (crude protein, phosphorus) pastures or crop residues by ruminants.

3. Methodology

Experiment 1

Experiment 1 involved the analysis of tissues collected from *Bos indicus* crossbred weaner steers in an experiment previously described by Kidd et al. (2018) and Antari (2018). The overall experiment included a placebo and an exogenous hormone (bovine somatotropin) treatment of steers allocated to three different nutritional treatments (i.e. a hormone x diet design). Only those tissues collected from placebo steers are included in the analysis undertaken within the current project.

Briefly, *Bos indicus* crossbred steers [n=15; 185.2 ± 2.4 kg liveweight (mean ± standard deviation)] were allocated to one of three nutritional treatments,

- High crude-protein (CP) and high metabolisable energy (ME) intake (*HCP-HMEI*; HCP-HDMD-U)
    - *ad libitum* access to lucerne (*Medicago sativa*) chaff (900 g OM, 200 g CP, 420 g NDF, 12.7 g Ca and 3.3 g P/kg DM),
- Low CP and low ME intake (*LCP-LMEI*; LCP-LDMD-U)
    - *ad libitum* access to Mitchell grass (*Astrebla* spp) hay (910 g OM, 40 g CP, 678 g NDF, 5.2 g Ca and 1.5 g P/kg DM)
    - 50 g cottonseed meal/kg Mitchell grass (924 g OM, 485 g CP, 290 g NDF, 2.8 g CP and 14.2 g P/kg DM)
- High CP and low ME intake (*HCP-LMEI*; HCP-LDMD-R); restricted access to lucerne chaff to provide an equivalent ME intake as that measured in steers offered the LCP-LME intake treatment.

Steers were maintained on the above treatments for 98 days with liveweight and feed intake measured each week. Dry matter (DM) and ME intake, digestibility of dry matter in the diet (DMD) and the concentration of glucose, urea-N, inorganic-P, total Ca, total protein, and non-esterified fatty acids (NEFA) in the plasma were determined at the end of the experiment (key phenotypic data are presented in Appendix 1). Steers were euthansed and the hypothalamus was dissected into arcuate (ARC), lateral (LHA) and ventromedial (VMH) regions, and samples of liver (right frontal lobe), and rumen (ventral sac), abomasum and duodenum wall were dissected. All tissues (7 tissues x 3 nutritional treatments x 5 biological replicates/treatment) were frozen in liquid N and stored at -80^{o}C until subsequent RNA extraction and RNAseq analysis. RNA was extracted using tissue specific variations of a TRizol based protocol followed by RNeasy cleanup with on-column DNAase treatment, prior to cDNA library preparation using the Illumina TruSeq stranded mRNA preparation kit and sequencing on the Illumina NovaSeq 6000 platform generating 100 base-pair single-end reads.

Experiment 2

The experimental design and methods were previously described in more detail by Innes (2021) and Quigley et al. (2019, Milestone Report 2). Briefly, Merino wethers [n=40; 23.7 ± 1.4 kg liveweight (mean ± standard deviation)] were allocated to one of five nutritional treatments,

- *ad libitum* access to a high CP, high P diet (*HCP-HP-UMEI*; 114 g CP, 330 g NDF, 2.2 g P, 3.7 g Ca/kg DM)
    - representative of a wet season pasture grown on adequate soil P
- *ad libitum* access to a high CP, low P diet (*HCP-LP-UMEI*; 115 g CP, 370 g NDF, 0.7 g P, 3.8 g Ca/kg DM)
    - representative of a wet season pasture grown on acutely deficient soil P
- *ad libitum* access to a low CP, high P diet (*LCP-HP-UMEI*; 56 g CP, 360 g NDF, 2.4 g P, 3.6 g Ca/kg DM)
    - representative of a dry season pasture grown on adequate soil P
- *ad libitum* access to a low CP, low P diet (*LCP-LP-UMEI*; 52 g CP, 370 g NDF, 0.7 g P, 7.5 g Ca/kg DM)

    - representative of a dry season pasture grown on acutely deficient soil P
- restricted access to the HCP-HP treatment diet above (*HCP-HP-RMEI*)
    - this was an artificial treatment designed to establish a 'hunger' model

The nutritional treatments were formulated to provide a ME content representative of the early wet season (9 MJ/kg DM) and were generated using an 8 mm diameter pellet (JNJ, Willowbank, QLD) based on barley straw (~58%; 39 g CP, 610 g NDF, 0.5 g P, 2.1 g Ca/kg DM), raw sugar (~22%) with the content of gluten, starch, urea, limestone, gypsum, mono-di-calcium-phosphate, MgO and KCl included in varying portions to achieve the desired CP and P content of the four treatment diets; each ration contained 1 kg/T of a lamb TMV (Advanced Feeds, WA). Each wether was offered pellets *ad libitum* with 2.5 g barley straw DM/kg liveweight offered to maintain rumen function, with the exception of wethers allocated to the HCP-HP(R) treatment. These wethers were offered a restricted allocation of pellets each day at an equivalent ME intake of wethers allocated to the LCP-LP treatment.

Wethers were maintained on the above treatments for 56 days with liveweight and feed intake measured each week. Dry matter and ME intake, DMD and the concentration of glucose, urea-N, inorganic-P, total Ca, total protein, full biochemical profile, ghrelin, insulin, leptin, and insulin-like growth factor-1 (IGF1) in the plasma, rumen pH and digesta load, the concentration of ammonia-N and the molar proportions of volatile fatty acids in rumen fluid were determined at the end of the experiment. Wethers were euthansed and the hypothalamus was dissected into ARC, LHA and VMH, and samples of liver (right frontal lobe), rumen wall (ventral sac) and dudenum wall were collected. All tissues (6 tissues x 5 nutritional treatments x 8 biological replicates/treatment) were frozen in liquid N and stored at -80^{o}C until subsequent RNA extraction and RNAseq analysis. RNA extraction and cDNA library synthesis was as described above for Experiment 1.

Bioinformatics

A similar bioinformatics pipeline was used to analyse and interpret RNAseq data generated in Experiments 1 and 2 and used to develop the multi-tissue gene expression networks (Experiment 3) (Figure 1). In brief, integrity and confidence of FASTQ files were assessed and trimmed, with surviving reads aligned against the *Bos taurus* release 9 (ARS.UCD1.2, 2018) and *Ovis aries* release 3 (Oar_V3.1) reference genomes Ensemble (releases 99 and 100) for Experiments 1 and 2 respectively. Gene reads were filtered, counted and normalised and pair-wise nutritional treatment comparisons of gene counts were conducted within each tissue to identify genes differentially expressed (DE) between treatments. Gene pathways enriched for DE genes were identified and visualised in Cytoscape. Genes with a significant Phenotypic Impact Factor (PIF) and Regulatory Impact Factor (RIF; transcription factors) were identified within specific tissues (TS).

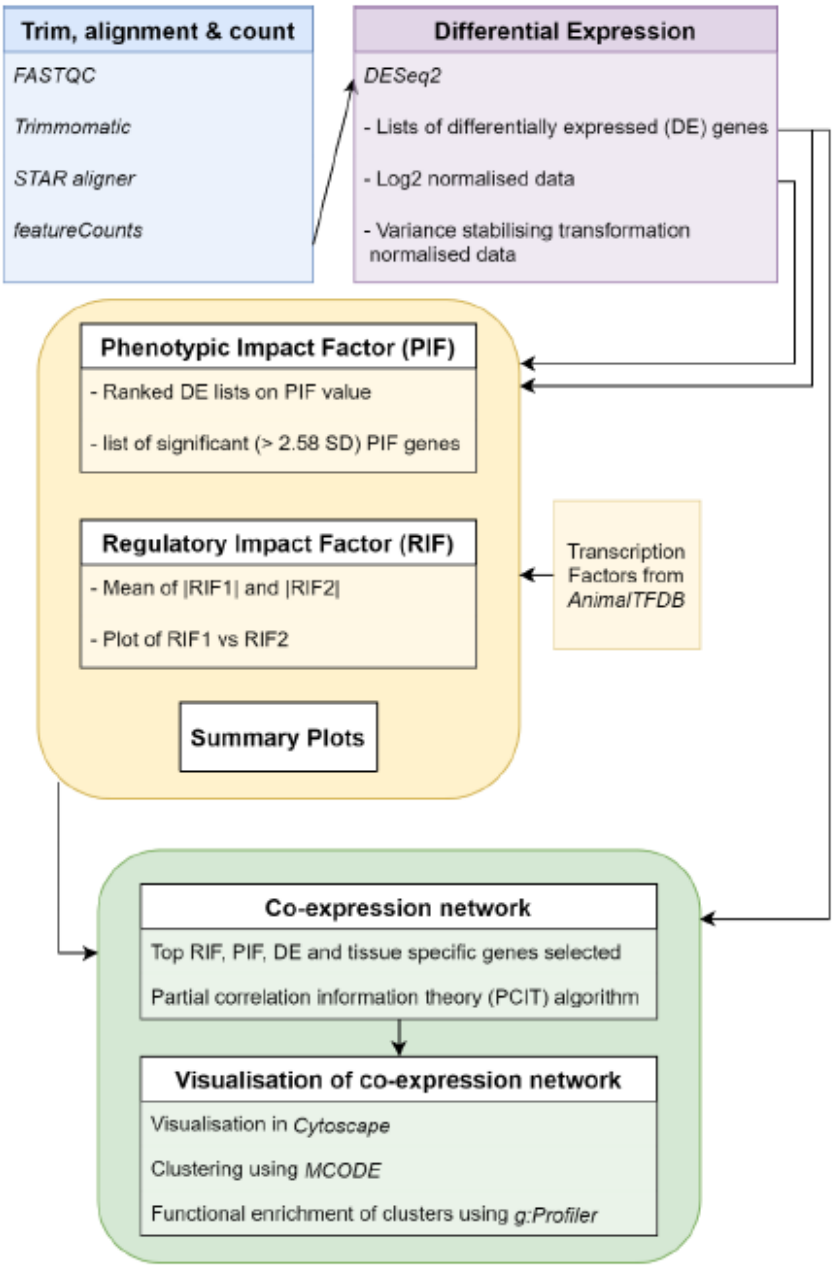


Figure 1. Bioinformatics analysis pipeline used in the analysis of the RNAseq data generated for tissue samples collected from three regions of the hypothalamus, liver, and the rumen and duodenum wall of steers (Experiment 1) and Merino wethers (Experiment 2).

Multi-tissue functional co-expression network

A selection of genes from the four main datasets (DE, PIF, RIF and TS) were included as nodes in a multi-tissue co-expression analysis with significant edges determined using the partial correlation information theory (PCIT) algorithm. The output of PCIT was filtered to include only significant correlations > |0.9| for visualisation using *Cytoscape*.

In Experiment 1, the genes included in the PCIT were selected on the following criteria,

- Top 100 genes that were DE (adjusted $P < 0.05$) for each pairwise comparison and ranked by lowest adjusted *P* value
- Top 40 positive and negative PIF per treatment comparison (n = 80) including only significant PIF values (> 2.58 SD mean)
- Top 40 positive and negative average RIF (average of RIF1 and RIF2) per treatment comparison (n = 80) including only significant RIF values (> 1.96 SD mean)
- All TS genes

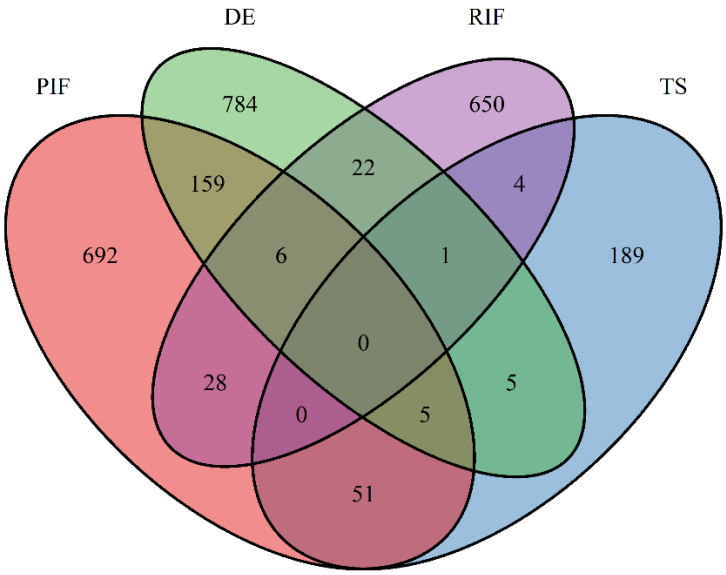
Resulting in 982 DE, 940 PIF, 711 RIF and 254 TS genes selected for inclusion in the co-expression network, however 2596 unique genes were included as some genes were selected based on multiple criteria.

In Experiment 2, the genes included in the PCIT were selected on the following criteria,

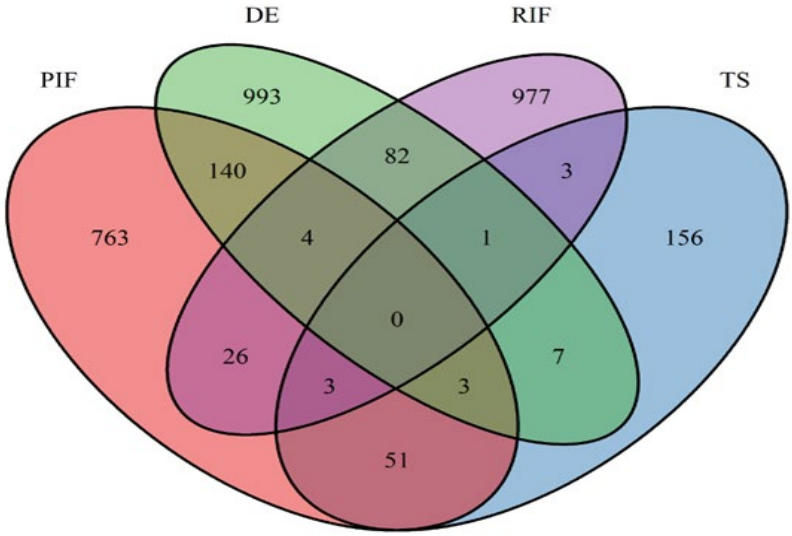
- Top 50 genes that were DE (adjusted $P < 0.05$) for each pairwise comparison and ranked by lowest adjusted *P* value
- Top 25 positive and negative PIF per treatment comparison (n = 50) including only significant PIF values (> 2.58 SD mean)
- Top 40 positive and negative average RIF (average of RIF1 and RIF2) per treatment comparison (n = 80) including only significant RIF values (> 2.58 SD mean)
- All TS genes

Resulting in 1230 DE, 990 PIF, 1096 RIF and 224 TS genes selected for inclusion in the co-expression network, however 2333 unique genes were selected as some genes were selected based on multiple criteria.

The normalised expression data selected for PCIT analysis in both experiments (Experiment 1, 105 samples by 2596 genes; Experiment 2, 236 samples by 2333 genes) was deemed to represent the most relevant differences between regions and treatment comparisons within each Experiment (Figure 2).



a.



b.

Figure 2. Venn plot showing the number of genes selected for each criteria as input for the multi-tissue partial correlation information theory (PCIT) analysis for Experiments 1 (a.) and 2 (b.). Criteria included Phenotypic Impact factor (PIF), Differential Expression (DE), Regulatory Impact Factor (RIF) and Tissue Specific (TS).

Review of literature on non-nutritional methods of intake regulation

A brief review was undertaken of the recent literature regarding

1. non-nutritional / pharmacological methods to stimulate intake, and
2. recent advances in methods to deliver therapeutic agents into the central nervous system (CNS)

The information was largely derived from advances in the human biomedical sciences in the treatment of degenerative brain diseases.

4. Results (to-date)

The results presented here are a summary from Experiments 1 and 2 that describe the animal models, gene expression analysis in the hypothalamus and the multi-tissue co-expression network analysis undertaken with data generated in Experiments 1 and 2. Additional results from Experiment 1 are included in the Appendix. The results are adapted from the PhD thesis of Mr David Innes (submitted for examination in February-2021).

Experiment 1

Intake, liveweight gain and plasma metabolites

Steers allocated to the HCP-HME treatment had higher DM and estimated ME intake and liveweight gain than steers allocated to the LCP-LME and HCP-LME treatments (Table 1), with no difference in liveweight gain measured between steers allocated to the latter two treatments with an equivalent estimated ME intake. The concentration of urea-N in the plasma of steers allocated to the LCP-LME treatment was lower than in the plasma of steers allocated to the HCP treatments. However, steers with a LME intake had a lower concentration of IGF-1 and glucose in the plasma than steers with a HME intake. The concentration of inorganic P in the plasma was similar for all steers and was within a range which would indicate that dietary P was not limiting in these treatments.

Table 1. Phenotypic description of steers offered nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI), low CP and low ME intake (LCP-LMEI) and high CP and low ME intake (HCP-LMEI).

Parameter	HCP-HMEI[1]	LCP-LMEI	HCP-LMEI	SEM[1]	P-value
DM[2] intake, g/kg LW.day	28.4^{c}	17.4^{b}	9.9^{a}	0.7	<0.0001
DM digestibility of diet, %	57.3^{b}	37.4^{a}	59.1^{b}	1.2	<0.0001
ME[2] intake, KJ/kg LW.day	231.0^{b}	82.8^{a}	83.6^{a}	5.8	<0.0001
Average daily gain, kg	1.16^{b}	-0.02^{a}	-0.03^{a}	0.04	<0.0001
Plasma urea-N, mmol/L	9.0^{b}	2.2^{a}	8.6^{b}	0.4	<0.0001
Plasma total protein, g/L	64.6	60.4	66.4	1.7	0.075
Plasma glucose, mmol/L	5.3^{b}	3.7^{a}	3.7^{a}	0.2	<0.0001
Plasma inorganic P, mmol/L	2.5	2.3	2.3	0.1	0.33
Plasma NEFA[2], meq/L	0.28	0.24	0.36	0.11	0.74
Plasma insulin, uUI/mL	8.7	5.6	5.3	1.3	0.17
Plasma IGF-1[2], ng/mL	283.4^{b}	41.8^{a}	59.8^{a}	37.2	0.001

[1]Values are treatment mean with an overall standard error of the mean (SEM); different alphabetical superscripts indicate a significant difference (P<0.05) between treatment means of each parameter

[2]Dry matter, DM; metabolisable energy, ME; non-esterified fatty acid, NEFA; insulin-like growth factor-1, IGF-1

[3]Plasma samples collected on day 98 of the experiment

Gene expression

The number of DE genes in each of the tissues provides a broad indication of transcriptome responses to nutritional treatments across the tissues and regions studied (Table 2) albeit in no way informative of the specific biological processes responding. The liver, rumen and duodenum had more differentially expressed genes in response to nutritional treatments than the hypothalamic regions and the abomasal wall. The transcriptome of the ARC of the steers appeared more sensitive than the VMH and LHA to the nutritional treatments imposed in the current experiment. More DE genes were observed in response to differences in ME intake (HCP-HMEI v LCP-LMEI and HCP-HMEI v HCP-LMEI) rather than the CP content of the diet (HCP-LMEI v LCP-LMEI). A similar trend was observed for the number of DE genes in the liver, with the opposite observed in both the rumen and duodenum where the number of DE genes was higher in response to diet CP content rather than ME intake.

Table 2. Number of differentially expressed (DE) genes in response to nutritional treatments in three regions of the hypothalamus, liver, and rumen and duodenum wall in steers.

Tissue[1]	HCP-HMEI vs LCP-LMEI[2]			HCP-HMEI vs HCP-LMEI			HCP-LMEI vs LCP-LMEI		
	Total	Up	Down[3]	Total	Up	Down	Total	Up	Down
ARC	188	132	56	129	85	44	10	2	8
VMH	5	4	1	11	7	4	1	0	1
LHA	1	0	1	0	0	0	2	1	1
LIV	720	477	243	275	181	94	69	30	39
RUM	348	193	155	61	27	34	478	207	271
ABO	22	7	15	28	14	14	2	2	0
DUO	201	136	65	58	34	24	811	484	327

[1]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall

[2]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI), low CP and low ME intake (LCP-LMEI) and high CP and low ME intake (HCP-LMEI)

[3]Total number of DE genes within a tissue type of steers fed the two nutritional treatments indicated; Up = the number of DE genes that were up-regulated (more abundant) in the first of the two nutritional treatments within each pair-wise comparisons; Down = the number of DE genes that were down-regulated (less abundant) in the first of the two nutritional treatments within each pair-wise comparison.

Hypothalamus

Gene sets

Individual genes with the highest significant DE and PIF in the ARC of steers were CYP1A1, NPY, AGRP and POMC. The latter three genes encode neuropeptides with known potent effects on appetite, with NPY, AGRP and POMC all downregulated in the ARC of steers allocated to the HCP-HMEI treatment (see example MA plot, Figure 3). This result is consistent with other experimental models and provides confidence in both the soundness of the animal models, experimental techniques and bioinformatics pipeline.

Genes within a tissue region with a significant ($P \leq 0.05$) pairwise difference (higher or lower) and a fold change > 2 between the treatment of interest and both other treatments, and subsequently no difference between these other two treatments, were deemed 'uniquely different genes' (Table 3). Therefore, uniquely different genes in the steers fed the HCP-HMEI treatment represent individual genes responding to a higher DM and ME intake, but not the CP content of the diet. The AgRP and NPY genes were down-regulated in steers with *ad libitum* access to a high quality (HCP-HDMD) diet in agreement with previous studies in sheep indicative of their role in regulating intake when nutrient adequate diets are fed. Uniquely different genes in the steers fed the LCP-LMEI treatment represent individual genes responding to a voluntary reduction in feed intake ('satiety' model) due to the lower CP content of the diet, the lower DMD of the diet or the different forage type, but not ME intake. The CYP1A1 gene was consistently and uniquely up-regulated in all three regions of the hypothalamus of steers allocated to the LCP-LMEI treatment. This gene encodes an enzyme that is part of the cytochrome P450 family which is important in xenobiotic and drug metabolism with a particular affinity for aromatic hydrocarbons. Uniquely different genes in the steers fed the HCP-LMEI treatment represent individual genes uniquely responding to an enforced feed restriction ('hunger' model) but not to differences in the CP content of the diet, DMD of the diet or ME intake. These genes included the ENSBTAG00000025258 (an uncharacterised gene known to be responsive to nutritional treatments in hand-reared calves) and GBP2 (activated by cytokines and recently associated with growth and efficiency traits in cattle).

Regulatory impact factor analysis

The genes that were determined to be significant RIFs were further ranked on their highest average of |RIF1| and |RIF2| within each treatment comparison with the top five significant transcription factors for each nutritional comparison within each tissue included in Table 4.

The listed transcription factors include those genes which are known to control physiological processes by regulating the transcription of mRNA, as listed by the AnimalTFDB (transcription factor database) and are therefore more likely to be key regulators of the physiological differences between the steers in each treatment. Transcription factors in the ARC are associated with neurogenesis, brain disorders and inflammatory responses; DMBX1 is associated with the AgRP regulation of feeding behaviour.

Gene pathways

The DE genes in the ARC enriched for gene pathways related to the extracellular matrix, tissue growth and development, immune function and neuropeptide signalling (see example enrichment, Figure 4).

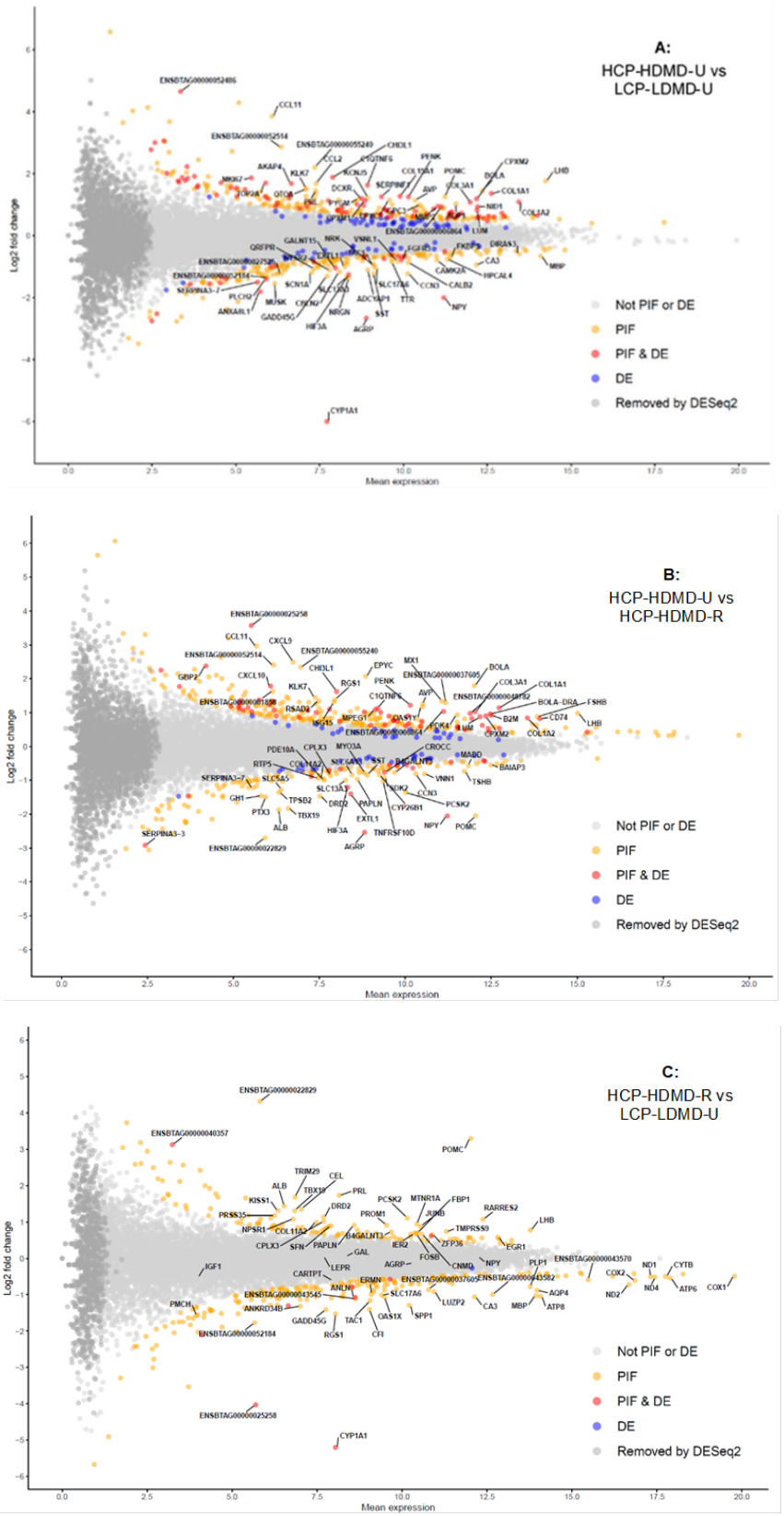


Figure 3. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the arcuate hypothalamus of steers allocated to the HCP-HMEI (HCP-HDMD-U), LCP-LMEI (LCP-LDMD-U) and HCP-HDMD-R) treatments. Each dot is an individual gene, see legend for colour coding. Nutritional treatments included *ad libitum* (U) or restricted (R) amounts of high (H) or low (L) crude protein (CP) resulting in H or L metabolisable energy intake (MEI)

Table 3. List of genes that are significantly higher or lower (adjusted *P* ≤ 0.05 and fold-change > 2) in one nutritional treatment compared to the other two nutritional treatments[1] for each region of the hypothalamus[2] in steers.

Treatment	Region	Relative expression	No. of genes	Genes[3]
HCP-HMEI	ARC	higher	14	*COL1A1, CHI3L1, CPXM2, COL3A1, PENK, C1QTNF6, COL15A1, SERPINF1, TOP2A, MKI67, THRSP, RRM2, TTK, CENPF*
		lower	4	*AGRP, NPY, HIF3A, bta-mir-2350*
LCP-LMEI	ARC	higher	1	*CYP1A1*
	LHA	higher	1	*CYP1A1*
	VMH	higher	1	*CYP1A1*
HCP-LMEI	ARC	lower	2	*ENSBTAG00000025258, GBP2*

[1]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI), low CP and low ME intake (LCP-LMEI) and high CP and low ME intake (HCP-LMEI)
[2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA)
[3]Genes listed in order of highest absolute phenotypic impact factor (PIF)

Table 4. Top five transcription factors (TF) or co-factors ranked on absolute RIF1 and RIF2[1] for nutritional treatment[1] comparisons for each region[2] of the hypothalamus of steers.

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
ARC	HCP-HMEI vs LCP-LMEI	EAF2	3.99	1.84	2.92	co-factor	ELL associated factor 2
		ARID5A	3.40	2.03	2.72	TF	AT-rich interaction domain 5A
		SNAI3	4.52	0.76	2.64	TF	snail family transcriptional repressor 3
		CSDE1	3.05	2.09	2.57	TF	cold shock domain containing E1
		ZNF391	4.28	0.67	2.47	TF	zinc finger protein 391
	HCP-HMEI vs HCP-LMEI	ALX4	3.87	-1.02	2.45	TF	ALX homeobox 4
		FERD3L	4.10	-0.63	2.37	TF	Fer3 like bHLH transcription factor
		DMBX1	2.46	-2.16	2.31	TF	diencephalon/mesencephalon homeobox 1
		CENPA	4.00	-0.62	2.31	TF	centromere protein A
		POU3F4	-1.81	-2.78	2.30	TF	POU class 3 homeobox 4
	HCP-LMEI vs LCP-LMEI	PBXIP1	-3.36	-1.90	2.63	co-factor	PBX homeobox interacting protein 1
		LRRFIP1	-3.35	-1.91	2.63	TF	LRR binding FLII interacting protein 1
		TSHZ1	-3.26	-1.87	2.57	TF	teashirt zinc finger homeobox 1
		ZBED5	-3.16	-1.78	2.47	TF	zinc finger BED-type containing 5
		HEY2	-2.71	-2.08	2.40	TF	hes related family bHLH transcription factor with YRPW motif 2
LHA	HCP-HMEI vs LCP-LMEI	SOX30	-3.82	-2.23	3.02	TF	SRY-box transcription factor 30
		ZNF572	-3.59	-1.92	2.76	TF	zinc finger protein 572
		FHL5	-4.04	-1.47	2.75	co-factor	four and a half LIM domains 5
		ZNF175	-4.05	-1.15	2.60	TF	zinc finger protein 175
		ASF1B	-4.06	-1.12	2.59	co-factor	anti-silencing function 1B histone chaperone
VMH	HCP-HMEI vs LCP-LMEI	ZNF16	-3.88	-0.87	2.38	TF	zinc finger protein 16
		PMF1	1.84	2.85	2.34	co-factor	polyamine modulated factor 1
		ZNF227	2.20	2.45	2.32	TF	zinc finger protein 227
		HMGXB3	-3.49	-1.09	2.29	TF	HMG-box containing 3
		ZBTB2	-3.59	-0.96	2.28	TF	zinc finger and BTB domain containing 2
	HCP-HMEI vs HCP-LMEI	TFAP4	-3.82	2.55	3.19	TF	transcription factor AP-4
		ZBTB33	-3.62	2.74	3.18	TF	zinc finger and BTB domain containing 33
		TBX18	-3.38	2.88	3.13	TF	T-box transcription factor 18
		HLF	-3.23	2.91	3.07	TF	HLF transcription factor, PAR bZIP family member
		TRRAP	-2.71	3.02	2.86	co-factor	transformation/transcription domain associated protein

[1]Regulatory impact factor (RIF1, RIF2). RIF analysis was not conducted for regions with less than two differentially expressed genes

[2]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI=HCP-HDMD-U), low CP and low ME intake (LCP-LMEI=LCP-LDMD-U) and high CP and low ME intake (HCP-LMEI=HCP-HDMD-R)

[3]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA)

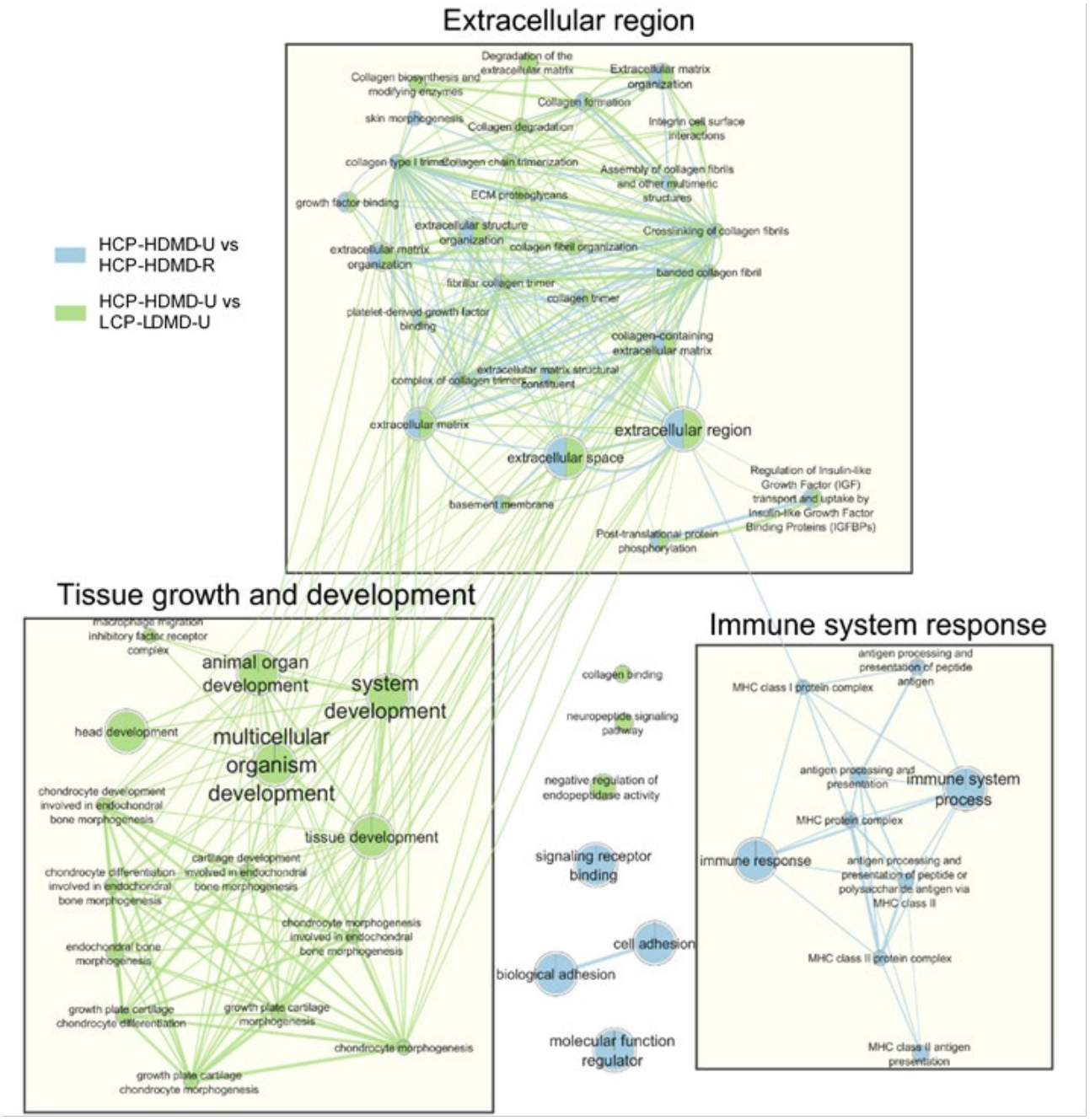


Figure 4. Enriched pathways of all differentially expressed genes in the arcuate hypothalamus of steers fed HCP-HMEI, HCP-LMEI and LCP-LMEI nutritional treatments. Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI; HCP-HDMD-U), low CP and low ME intake (LCP-LMEI; LCP-LDMD-U) and high CP and low ME intake (HCP-LMEI; HCP-HDMD-R)

Experiment 2

Intake, liveweight gain, rumen parameters and plasma hormones and metabolites

Dry matter intake of the HCP-HP treatment diet was almost 45 g DM/kg LW.day during the pre-experiment co-variate period demonstrating that this diet was likely to allow wethers to fully express their intake potential. Wethers allocated to the HCP-HP-UMEI treatment had higher DM and ME intake and higher liveweight gain than wethers allocated to all other diets that were deficient in either CP, P, or CP and P, or ME intake (Table 5). Dry matter digestibility of the diets was relatively consistent across diets, resulting in similar estimated ME content. Hence, differences in intake are attributed solely to the targeted nutrient deficiency model (CP or P), and restriction of the HCP-HP-RMEI treatment on a DM basis was appropriate given the similar ME content of the diets. Rumen fill was highest in wethers fed the HCP-HP treatment and was significantly lower in wethers fed the CP and P deficient diets, demonstrating that the observed suppression of intake was unlikely to be due to physical limitations. The higher rumen fill in wethers fed the HCP-HP(R) treatment was likely due to the more rapid consumption of their total allocation of feed in the first 2 hours after feeding compared to the more consistent consumption pattern observed for wethers fed the other treatments (data not included here). The concentration of urea-N and inorganic-P in the plasma at the end of the experiment and throughout the experiment further support the successful establishment of the targeted models of nutritional deficiency.

Table 5. Dry matter (DM) and metabolisable energy (ME) intake, digestibility of DM (DMD), liveweight (LW) gain, concentration of inorganic-P (PiP), urea-N (PUN) and total-Ca (PCa) in plasma, and rumen parameters of wethers with restricted or *ad libitum* access to diets that were high (H) or low (L) in crude protein (CP) and/or phosphorus (P)[1].

Parameter	HCP-HP-RMEI[2]	HCP-HP-UMEI	HCP-LP-UMEI	LCP-HP-UMEI	LCP-LP-UMEI	SEM[2]	*P*
Straw DM intake, g DM/kg LW.day	2.8^{b}	2.5^{a}	2.7^{ab}	2.7^{ab}	2.8^{b}	0.07	0.014
Pellet DM intake, g DM/kg LW.day	16.9^{a}	34.7^{c}	22.6^{b}	19.1^{ab}	16.3^{a}	1.00	<0.001
Total DM intake, g DM/kg LW.day	19.7^{a}	37.2^{c}	25.3^{b}	21.8^{ab}	19.1^{a}	0.97	<0.001
ME content, MJ ME/kg DM	9.8	9.2	8.8	8.9	8.6	0.29	0.063
ME intake, MJ/kg LW.day	0.19^{ab}	0.34^{c}	0.22^{b}	0.19^{ab}	0.16^{a}	0.01	<0.001
LW gain, g/day	-6^{b}	141^{c}	-1^{b}	-47^{a}	-60^{a}	0.01	<0.001
PiP, mmol/L[3]	2.4^{d}	2.0^{c}	1.1^{a}	2.4^{d}	1.6^{b}	0.09	<0.001
PUN, mmol/L[3]	4.1^{b}	4.1^{b}	4.4^{b}	1.1^{a}	1.6^{a}	0.22	<0.001
PCa, mmol/L[3]	2.3^{a}	2.5^{b}	2.7^{c}	2.2^{a}	2.4^{b}	0.03	<0.001
Insulin, uIU/mL[4]	10.8^{bc}	13.4^{c}	7.7^{ab}	5.5^{a}	9.6^{abc}	0.9	<0.001
Leptin, ng/mL[4]	7.0	6.1	6.0	6.3	7.1	0.5	NS
Ghrelin, pg/mL[4]	613	477	524	538	532	48	0.09
Insulin-like growth factor-1, ng/mL[4]	59.9^{b}	116.3^{c}	42.5^{ab}	25.6^{a}	21.6^{a}	6.1	<0.001
Rumen pH[5]	5.46^{a}	6.04^{b}	6.47^{c}	6.39^{bc}	6.75^{c}	0.10	<0.001
Abomasum pH[5]	2.88	2.57	2.60	2.54	2.66	0.13	0.33
Rumen NH_3N, mg/L[5]	180^{c}	143^{bc}	79^{ab}	42^{a}	34^{a}	19.3	<0.001
Rumen acetate, %[5]	58.0	60.5	60.2	63.2	57.1	2.09	0.29
Rumen propionate, %[5]	28.5	29.0	27.7	28.3	28.3	2.62	0.99
Rumen butyrate, %[5]	12.0	9.2	10.5	7.7	13.4	1.51	0.09
Rumen digesta weight, g DM[5]	442^{c}	499^{c}	322^{b}	300^{ab}	199^{a}	26.7	<0.001
Rumen digesta weight, g DM/kg LW[5]	19^{c}	16^{bc}	14^{ab}	14^{ab}	10^{a}	1.00	<0.001
Apparent retention time, h[5]	27^{b}	10^{a}	14^{a}	14^{a}	14^{a}	1.12	<0.001

[1]Nutritional treatments were: high protein-high phosphorus diet offered *ad libitum* (HCP-HP-UMEI), high protein-low phosphorus diet offered *ad libitum* (HCP-LP-UMEI), low protein-high phosphorus diet offered *ad libitum* (LCP-HP-UMEI), low protein-low phosphorus diet offered *ad libitum* (LCP-LP-UMEI) and high protein-high phosphorus diet offered at a restricted ME intake equivalent to LCP-LP-UMEI (HCP-HP-RMEI)

[2]Values are least-square means with standard error of the mean (SEM); treatment means were not significantly different at the significance level of $P \leq 0.05$)

[3]Mean concentration across experiment

[4]Samples collected prior to feeding on day 53 of the experiment

[5]Samples collected from sheep at euthanasia 2 hours after feeding

Gene expression

Consistent with Experiment 1, the liver had more DE genes in all pairwise comparisons than the other tissues analysed, with the largest responses in the non-deficient (HCP-HP-UMEI) vs deficient (LCP-HP-UMEI, HCP-LP-UMEI, LCP-LP-UMEI) and non-deficient vs restricted ME intake (HCP-HP-RMEI) treatment comparisons (Table 6). A similar trend was observed for the rumen and duodenum, albeit with fewer DE genes in response to the nutritional treatment in each tissue. Overall the results would suggest that the transcriptome in these three tissues primarily responds to total nutrient flux, although nutrient specific differences were observed and these may be additive.

Hypothalamus

Gene sets

The ARC was the most responsive region within the hypothalamus to the nutritional treatments with 195 DE genes in the HCP-HP-UMEI vs LCP-LP-UMEI treatment comparison (Figure 5). Nutritional treatments had very little effect on differential gene expression in the LHA and VMH with the exception of a relatively large number (123) of DE genes in the VMH of wethers offered the P deficient (HCP-LP-UMEI) and restricted ME intake (HCP-HP-RMEI) treatments. Expression of established first-order neurotransmitters (AGRP, NPY, POMC) were down-regulated in the ARC and VMH of wethers with *ad libitum* consumption of the nutrient adequate nutritional treatment (associated with a high total ME intake). In contrast, expression of the CART neurotransmitter was upregulated in the ARC and VMH (CARTP) of wethers with *ad libitum* access to the non-deficient (HCP-HP-UMEI) nutritional treatment. These trends in neuropeptide expression are consistent with responses reported in fasted sheep. A cluster of solute transporters were also significantly down-regulated in wethers allocated to the HCP-MP-UMEI nutritional treatment.

Genes that are uniquely DE in one of the nutritional treatments are informative of the sensitivity of key genes to specific nutrients (CP, P, ME) in individual tissues/regions however there were no uniquely DE genes in any of the three regions of the hypothalamus.

Gene pathways

Genes that were up-regulated in the ARC of wethers fed the HCP-HP-UMEI relative to the LCP-LP-UMEI treatment were enriched for terms including 'enzyme binding', 'ATP synthesis coupled proton transport', 'oxidative phosphorylation', and 'nucleotide metabolic process' (GO-BP:0009117; adjusted P = 1.11E-02). Genes that were down-regulated in the ARC of wethers fed the HCP-HP-UMEI treatment relative to the LCP-LP-UMEI treatment were enriched for terms including 'cellular macromolecule metabolic process', 'cellular response to hormone stimulus' and 'methylation'. The enriched terms for the DE genes in the VMH that were upregulated in the wethers fed the HCP-LP-UMEI relative to the HCP-HP-RMEI treatment included 'synaptic vesicle cycle', 'anion transmembrane transporter activity', 'transmembrane transport', and 'neuron projection morphogenesis'.

Regulatory impactor factor analysis

Notable transcription factors in the hypothalamus include TIRAP (in the ARC) which is involved with the resistin/toll like receptor 4 signalling pathway that causes neuronal inflammation in the hypothalamus; FOS (in the LHA) which is part of the activator protein 1 transcription factor and a marker for neuronal activity; ARNT2 and BCL11B (in the VMH) which are important for the development of the hypothalamus and neuronal differentiation.

Table 6. Number of differentially expressed (DE)[1] genes in three regions of the hypothalamus[2], liver, and rumen and duodenum wall in wethers in response to nutritional treatments[3], presented by a) nutrient deficient, and b) restricted metabolisable energy intake comparisons.

a) Tissue region	HCP-HP-UMEI vs LCP-LP-UMEI	HCP-HP-UMEI vs LCP-HP-UMEI	HCP-HP-UMEI vs HCP-LP-UMEI	HCP-LP-UMEI vs LCP-HP-UMEI	HCP-LP-UMEI vs LCP-LP-UMEI	LCP-HP-UMEI vs LCP-LP-UMEI
Liver	3756	2486	2290	328	208	380
Rumen	1041	226	383	6	1	8
Duodenum	170	21	36	6	1	3
ARC	195	10	16	6	2	0
LHA	3	1	1	0	1	1
VMH	5	1	12	0	2	0

b) Tissue region	HCP-HP-UMEI vs HCP-HP-RMEI	LCP-HP-UMEI vs HCP-HP-RMEI	HCP-LP-UMEI vs HCP-HP-RMEI	LCP-LP-UMEI vs HCP-HP-RMEI
Liver	3666	571	615	1169
Rumen	1398	155	179	245
Duodenum	173	3	149	69
ARC	24	0	38	10
LHA	0	0	0	1
VMH	4	0	123	1

[1]Genes considered differentially expressed when adjusted $P \leq 0.05$ as determined by the Wald test using *DESeq2* package (version 1.28.1) in R (version 4.0).

[2]Arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and lateral hypothalamus (LHA).

[3]Nutritional treatments were: high protein-high phosphorus diet offered *ad libitum* (HCP-HP-UMEI), high protein-low phosphorus diet offered *ad libitum* (HCP-LP-UMEI), low protein-high phosphorus diet offered *ad libitum* (LCP-HP-UMEI), low protein-low phosphorus diet offered *ad libitum* (LCP-LP-UMEI) and high protein-high phosphorus diet offered at a restricted ME intake equivalent to LCP-LP-UMEI (HCP-HP-RMEI).

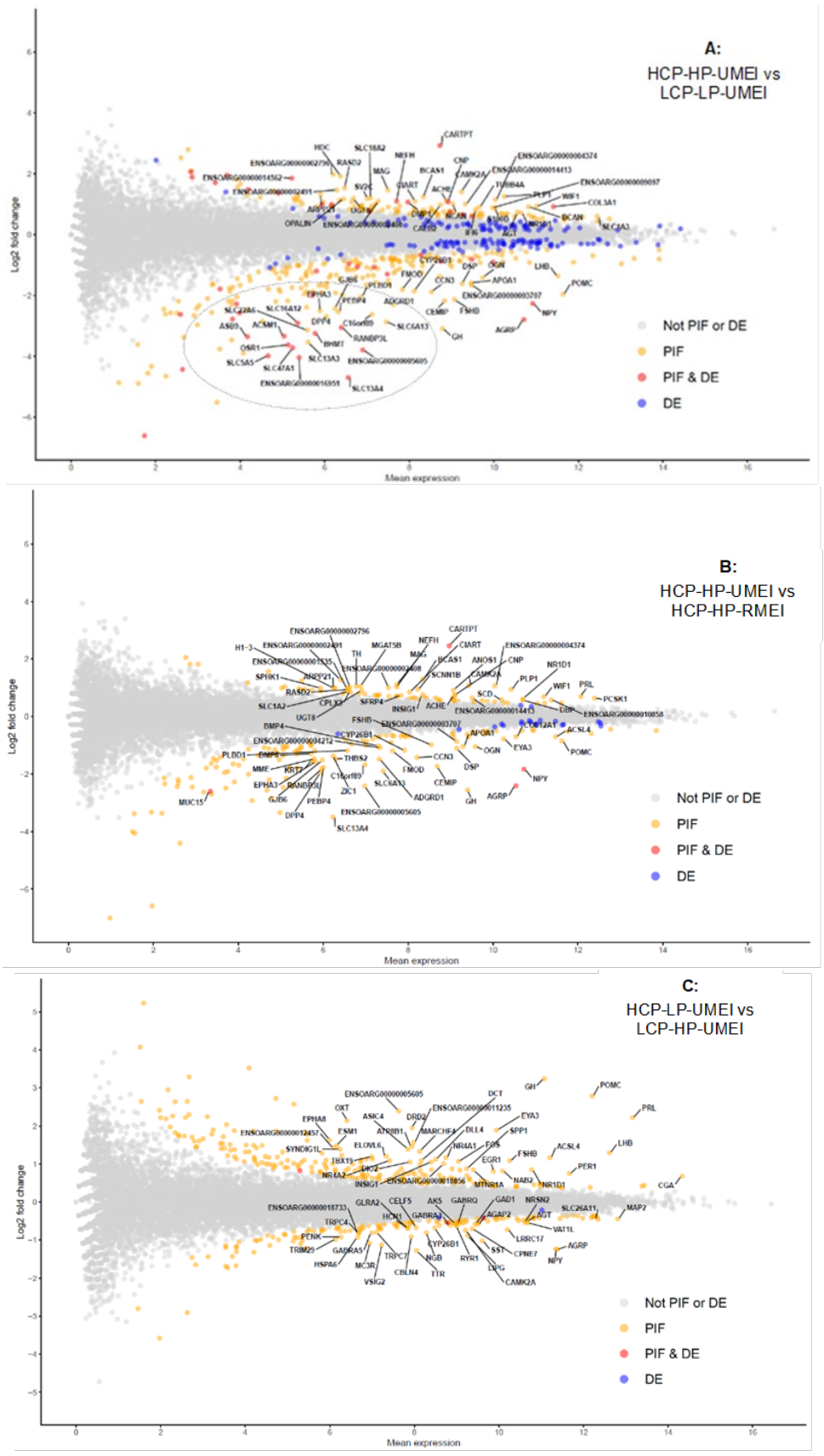


Figure 5. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the arcuate hypothalamus of Merino wethers allocated to the HCP-HP-UMEI and LCP-LP-UMEI treatments. Each dot is an individual gene, see legend for colour coding.

Table 7. Top five transcription factors (TF) or cofactors ranked on absolute RIF1 and RIF2[1] for nutritional treatment[2] comparisons for each region[3] of the hypothalamus of Merino wethers.

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
ARC	HCP-HP-UMEI vs HCP-HP-RMEI	HMGXB4	7.18	0.75	3.97	TF	HMG-box containing 4
		ENSOARG00000013117	6.90	0.53	3.71	TF	forkhead box D4
		JDP2	4.60	1.85	3.22	TF	Jun dimerization protein 2
		ALX1	5.73	0.44	3.08	TF	ALX homeobox 1
		ENSOARG00000009419	5.67	-0.19	2.93	cofactor	zinc finger MYND-type containing 8
	HCP-HP-UMEI vs HCP-LP-UMEI	TFAP2E	-3.88	-2.80	3.34	TF	transcription factor AP-2 epsilon
		ENSOARG00000000468	-4.00	-2.52	3.26	TF	nuclear transcription factor Y subunit beta
		ZBTB6	-4.92	-1.51	3.22	TF	zinc finger and BTB domain containing 6
		SUZ12	-4.01	-2.10	3.06	cofactor	SUZ12 polycomb repressive complex 2 subunit
		ETV3	-3.62	-2.10	2.86	TF	ETS translocation variant 3
	HCP-HP-UMEI vs LCP-HP-UMEI	MED8	-6.13	-2.06	4.10	cofactor	mediator complex subunit 8
		CHMP1A	-5.55	-1.82	3.69	cofactor	charged multivesicular body protein 1A
		PICALM	-5.85	-1.45	3.65	cofactor	phosphatidylinositol binding clathrin assembly protein
		ZNF461	-4.59	2.50	3.54	TF	zinc finger protein 461
		JUNB	-5.10	-1.81	3.46	TF	JunB proto-oncogene, AP-1 transcription factor subunit
	HCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000001987	4.79	0.93	2.86	cofactor	None
		HNF1B	-4.57	-1.04	2.81	TF	HNF1 homeobox B
		ENSOARG00000015037	2.85	2.72	2.78	TF	None
		TBX18	-2.39	-3.16	2.78	TF	T-box transcription factor 18
		TIRAP	2.28	3.24	2.76	cofactor	TIR domain containing adaptor protein
	HCP-LP-UMEI vs HCP-HP-RMEI	ENSOARG00000004242	-4.90	-1.80	3.35	cofactor	transcription factor B2, mitochondrial
		SP4	-3.78	-1.32	2.55	TF	Sp4 transcription factor
		ZNF133	-1.09	-3.77	2.43	TF	zinc finger protein 133
		ZBTB41	-3.00	-1.80	2.40	TF	zinc finger and BTB domain containing 41
		PCGF1	-2.51	-2.13	2.32	cofactor	polycomb group ring finger 1
	HCP-LP-UMEI vs LCP-HP-UMEI	ENSOARG00000007917	-5.78	1.32	3.55	TF	None
		SHOX2	-3.50	3.15	3.33	TF	short stature homeobox 2
		ENSOARG00000017535	-4.38	1.89	3.13	TF	None
		ENSOARG00000011916	-3.31	-2.72	3.02	TF	None
		OVOL3	-2.85	2.82	2.84	TF	ovo like zinc finger 3
	HCP-LP-UMEI vs LCP-LP-UMEI	RBPMS2	-4.59	0.36	2.47	cofactor	RNA binding protein, mRNA processing factor 2
		LHX2	-3.51	1.41	2.46	TF	LIM/homeobox protein Lhx2
		PSIP1	-1.44	3.15	2.29	cofactor	PC4 and SFRS1 interacting protein 1
		ZMAT3	-2.76	1.79	2.27	TF	zinc finger matrin-type 3
		ENSOARG00000013839	-3.54	-0.93	2.23	cofactor	transcription initiation factor TFIID subunit 11
	LCP-LP-UMEI vs	FOXJ3	-4.50	-2.92	3.71	TF	forkhead box J3

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
LHA	HCP-HP-RMEI	SERTAD3	-4.71	-2.44	3.57	cofactor	SERTA domain containing 3
		KLF10	-3.74	-3.15	3.44	TF	Kruppel like factor 10
		ENSOARG00000004242	-3.71	-3.14	3.42	cofactor	transcription factor B2, mitochondrial
		GZF1	-4.67	-1.75	3.21	TF	GDNF inducible zinc finger protein 1
	HCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000002014	4.88	1.33	3.10	TF	GC-rich promoter binding protein 1 like 1
		HOXA1	4.59	1.54	3.06	TF	homeobox A1
		FOS	2.30	3.54	2.92	TF	Fos proto-oncogene, AP-1 transcription factor subunit
		ZNF235	4.25	1.59	2.92	TF	zinc finger protein 235
		TTF2	3.01	2.53	2.77	cofactor	transcription termination factor 2
VMH	HCP-HP-UMEI vs HCP-HP-RMEI	SETD7	-5.59	-1.42	3.51	cofactor	SET domain containing 7, histone lysine methyltransferase
		PHF1	-6.11	-0.57	3.34	cofactor	PHD finger protein 1
		DAXX	-5.15	-1.38	3.27	cofactor	death domain associated protein
		PCGF5	-4.57	-1.93	3.25	cofactor	polycomb group ring finger 5
	HCP-HP-UMEI vs HCP-LP-UMEI	NFKBIZ	-4.77	1.41	3.09	cofactor	NFKB inhibitor zeta
		RAF1	4.33	1.92	3.12	cofactor	Raf-1 proto-oncogene, serine/threonine kinase
		SUPT16H	3.54	2.35	2.94	cofactor	SPT16 homolog, facilitates chromatin remodeling subunit
		MLXIPL	3.03	2.74	2.89	TF	MLX interacting protein like
		KLF1	3.27	2.46	2.86	TF	Kruppel like factor 1
		ZNFX1	-3.18	-2.54	2.86	TF	zinc finger NFX1-type containing 1
	HCP-HP-UMEI vs LCP-LP-UMEI	BCL11B	-5.60	-1.44	3.52	TF	BAF chromatin remodeling complex subunit BCL11B
		KDM8	-4.16	-2.39	3.27	cofactor	lysine demethylase 8
		C6orf89	-6.18	0.10	3.14	cofactor	chromosome 20 C6orf89 homolog
		HES1	-5.05	-1.18	3.12	TF	hes family bHLH transcription factor 1
	HCP-LP-UMEI vs HCP-HP-RMEI	ARNT2	-3.60	-2.53	3.07	TF	Ovis aries aryl hydrocarbon receptor nuclear translocator 2 (ARNT2), mRNA.
		OVOL2	-5.82	-0.87	3.34	TF	ovo like zinc finger 2
		CHCHD3	-6.13	-0.49	3.31	cofactor	coiled-coil-helix-coiled-coil-helix domain containing 3
		MRTFA	-5.04	-1.06	3.05	cofactor	myocardin related transcription factor A
		ENSOARG00000016973	-4.18	-1.76	2.97	TF	None
		ETV1	-4.43	-1.51	2.97	TF	ETS variant transcription factor 1
	HCP-LP-UMEI vs LCP-LP-UMEI	TFAP2E	5.33	2.47	3.90	TF	transcription factor AP-2 epsilon
		SUPT5H	6.00	1.21	3.60	cofactor	SPT5 homolog, DSIF elongation factor subunit
		ZNF574	4.46	2.05	3.26	TF	zinc finger protein 574
		TTF1	5.21	-0.87	3.04	TF	transcription termination factor 1
		ERCC2	4.37	1.60	2.99	cofactor	ERCC excision repair 2, TFIIH core complex helicase subunit

[1]Regulatory impact factor (RIF1, RIF2). RIF analysis was not conducted for regions with less than two differentially expressed genes.

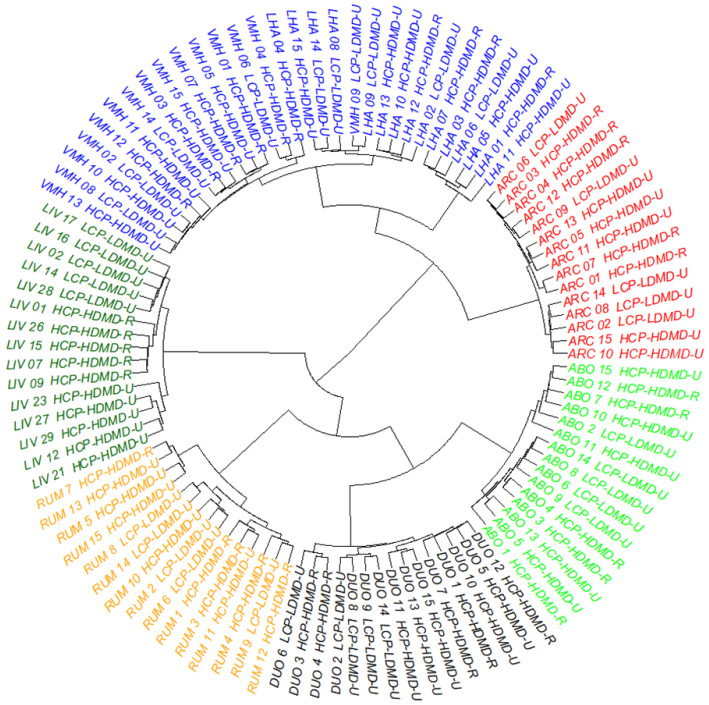
[2]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

[3]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall.

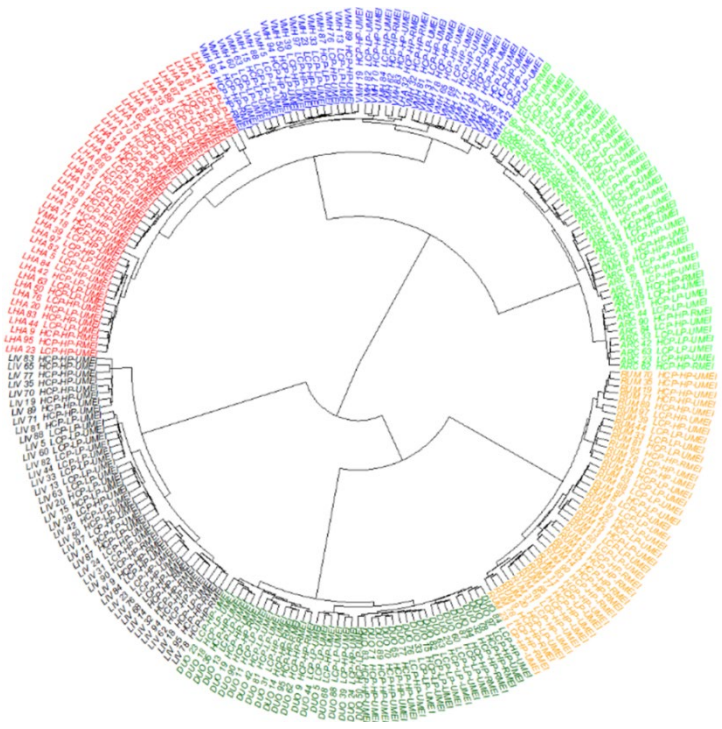
Experiment 3

Develop multi-tissue gene expression networks

In both Experiments 1 and 2, genes selected for inclusion in the PCIT analysis were clustered based on tissue/tissue region (Figure 6) with genes clustering on the basis of nutritional treatment only in the liver of steers (Experiment 1).



a.



b.

Figure 6. Individual samples of steers (a. Experiment 1) and wethers (b. Experiment 2), fed various nutritional treatments, hierarchically clustered based on the genes selected for co-expression network analysis.

Gene lists (combined phenotypic impact factor)

Genes that are consistently ranked highly by the PIF metric across different tissues represent a system-wide co-ordinated response to dietary treatment in the steers. The value of the sum of all |PIF| values, albeit abstract, was calculated to help identify such genes; however, it may also rank genes with a very strong |PIF| score in one tissue. This variation needs to be explored on a case by case basis.

In Experiment 1, the CYP1A1 gene, which ranked as DE in at least one pairwise comparison in all regions of the hypothalamus and a significant PIF gene in all tissues except the rumen, was ranked first with a total |PIF| nearly twice as high as the next gene in the list (Table 8). For example, CYP1A1 was expressed 64-fold higher in the ARC of steers fed the HCP-HMEI treatment compared to steers offered the LCP-LMEI treatment. Moreover, boxplots of the CYP1A1 gene indicate it was consistently numerically higher in the LCP-LMEI treatment for all tissues and had the highest mean expression in the liver. The coordinated upregulation of CYP1A1 in the steers fed the LCP-LMEI treatment is noteworthy because the biological function of the represented tissues are so diverse, representing various aspects of brain function, digestion and central regulation of metabolism. The three collagen related genes (COL1A1, COL1A2 and COL3A1) were consistently significantly higher in the HCP-HMEI treatment compared to the LCP-LDMD-U for the ARC, duodenum, liver and rumen tissues. The THRSP gene was also consistently numerically higher in the steers fed the HCP-HMEI treatment than in steers fed the LCP-LMEI treatment across all tissues, and was significantly higher in the steers fed the HCP-HMEI treatment compared to the other treatments in the ARC and liver. Notably, the expression of THRSP was 32-fold higher in the liver of steers fed the HCP-HMEI treatment compared to steers offered the other treatments. The top 20 genes, ranked on the sum of |PIF| across all tissues and treatments, were hierarchically clustered (on Euclidean distance of treatment group mean normalised expression) into their respective tissue groups, except for in the LHA and VMH which clustered by treatment before tissue group (Figure 7). The genes also clustered into four groups representing positive co-expression and likely similar function between the genes clustered closest together, including a cluster representing signalling in the hypothalamus (NPY, AGRP and CGA), extracellular matrix (COL1A1, COL1A2, COL3A1, THBS1, PI3 and PI16) and cell growth and differentiation (IGFBP1, IGFBP2 and GPC3). These modules included genes with the highest expression in the liver, rumen and ARC, and in each of these tissues the LCP-LMEI and (intake restricted) HCP-MEI treatment were most closely correlated based on Euclidean distance.

In Experiment 2, most genes included in the top 20 list (Table 9) were genes that responded most in the liver or rumen of the wethers. The ENSOARG00000003744 (haptoglobin), ENSOARG00000003426 (uncharacterised) and PAQR7 (progestin and adipoQ receptor family member 7) genes were ranked distinctly higher than the rest which can be attributed to their significant responses in the liver. However, CARTPT was also included and can be attributed to its significant response in both the ARC and VMH. The top 20 genes, ranked on the sum of |PIF| across all tissues and treatments, were hierarchically clustered (on Euclidean distance of treatment group mean normalised expression) into their respective tissue groups, except for some crossover in the LHA and VMH and for the liver of wethers fed the HCP-HP-UMEI treatment which was separated from the rest of the liver samples (Figure 8).

Table 8. Top 20 significantly different genes ranked on total absolute phenotypic impact factor (PIF), summed across all tissues and treatment comparisons, in steers fed various nutritional treatments[1] in Experiment 1

Gene name	Total |PIF| (all comparisons)	Gene description
CYP1A1	86.67	cytochrome P450, subfamily I, polypeptide 1
COL1A1	43.53	collagen type I alpha 1 chain
COL1A2	36.32	collagen type I alpha 2 chain
THRSP	36.30	thyroid hormone responsive
COL3A1	35.26	collagen type III alpha 1 chain
THBS1	31.37	thrombospondin 1
GPC3	27.53	glypican 3
ENSBTAG00000025258	26.85	keratin-associated protein 5-1
CGA	25.14	glycoprotein hormones, alpha polypeptide
ENSBTAG00000004565	25.06	None
SPARC	25.04	secreted protein acidic and cysteine rich
IGFBP1	24.22	insulin like growth factor binding protein 1
SERPINF1	23.29	serpin family F member 1
IGFBP2	22.74	insulin like growth factor binding protein 2
AGRP	22.11	agouti related neuropeptide
NPY	21.88	neuropeptide Y
PI3	20.64	peptidase inhibitor 3, skin-derived (SKALP)
PI16	20.45	peptidase inhibitor 16
GSTM1	20.39	*Bos taurus* glutathione S-transferase M1 (GSTM1), mRNA.
SLC1A1	20.21	solute carrier family 1 member 1

[1]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI), low CP and low ME intake (LCP-LMEI) and high CP and low ME intake (HCP-LMEI)

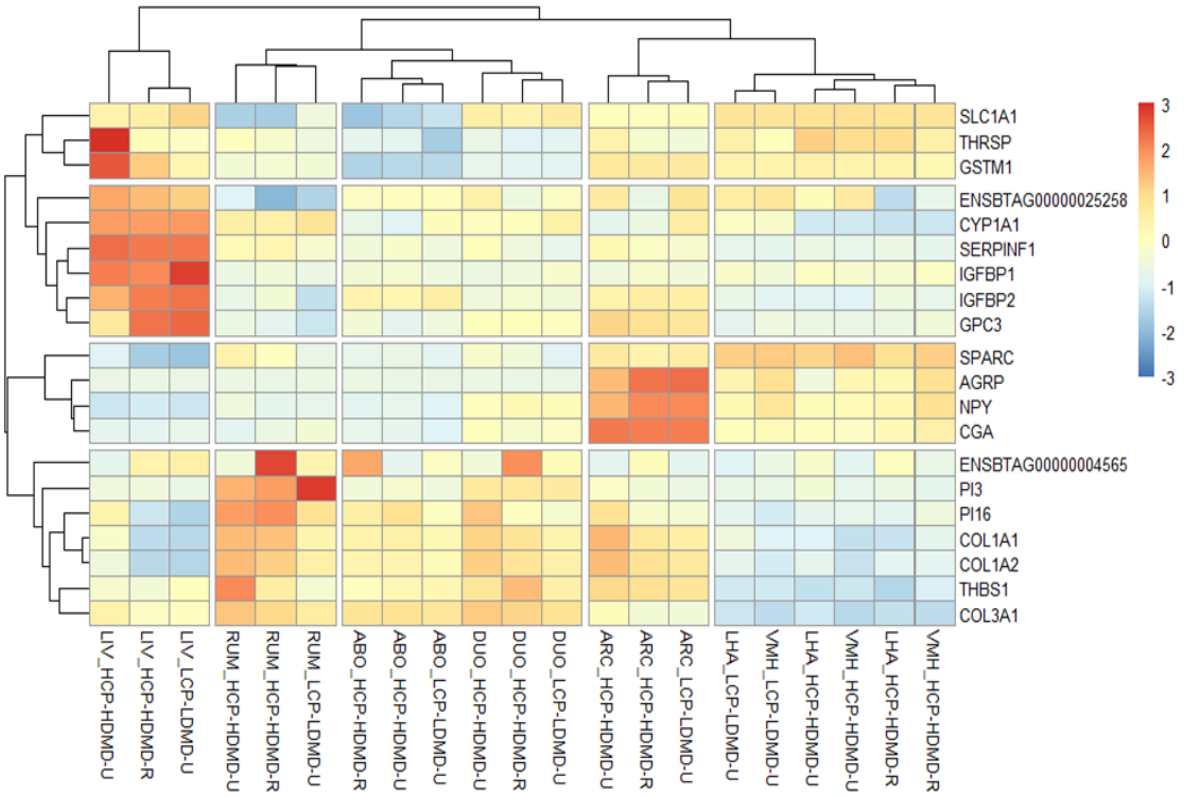


Figure 7. Heat-map of scaled expression of top 20 significantly different genes in steers fed different nutritional treatments[1], averaged by tissue and treatment group and ranked on total absolute phenotypic impact factor as summed across all tissues[2] and treatment comparisons.

[1]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI=HCP-HDMD-U), low CP and low ME intake (LCP-LMEI=LCP-LDMD-U) and high CP and low ME intake (HCP-LMEI=HCP-HDMD-R). [2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM), abomasum (ABO) and duodenum (DUO) wall

Table 9. Top 20 significantly different genes ranked on total absolute phenotypic impact factor (PIF), summed across all tissues and treatment comparisons, in Merino wethers fed all nutritional treatments[1].

Gene name	Total PIF (all comparisons)	Gene description
ENSOARG00000003744	144.36	haptoglobin
ENSOARG00000003426	127.49	None
PAQR7	124.63	progestin and adipoQ receptor family member 7
ENSOARG00000009159	79.62	amine sulfotransferase-like
ENSOARG00000009308	79.19	None
FGF21	79.11	fibroblast growth factor 21
ENSOARG00000008800	78.08	None
PLA2G2F	78.07	phospholipase A2 group IIF
ENSOARG00000017398	77.44	None
LGALS1	76.49	galectin 1
COL3A1	76.03	collagen type III alpha 1 chain
HSPA1A	75.84	heat shock 70kDa protein 1A
PIGR	75.61	polymeric immunoglobulin receptor
ENSOARG00000012585	73.76	None
CARTPT	70.32	CART prepropeptide
ATF3	69.13	activating transcription factor 3
ENSOARG00000019297	67.79	glutathione S-transferase Mu 1 (GSTM1)
ENSOARG00000006050	67.72	alpha-1-acid glycoprotein
GPX3	66.01	glutathione peroxidase 3
ENSOARG00000009865	64.82	serum amyloid A-4 protein

[1]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

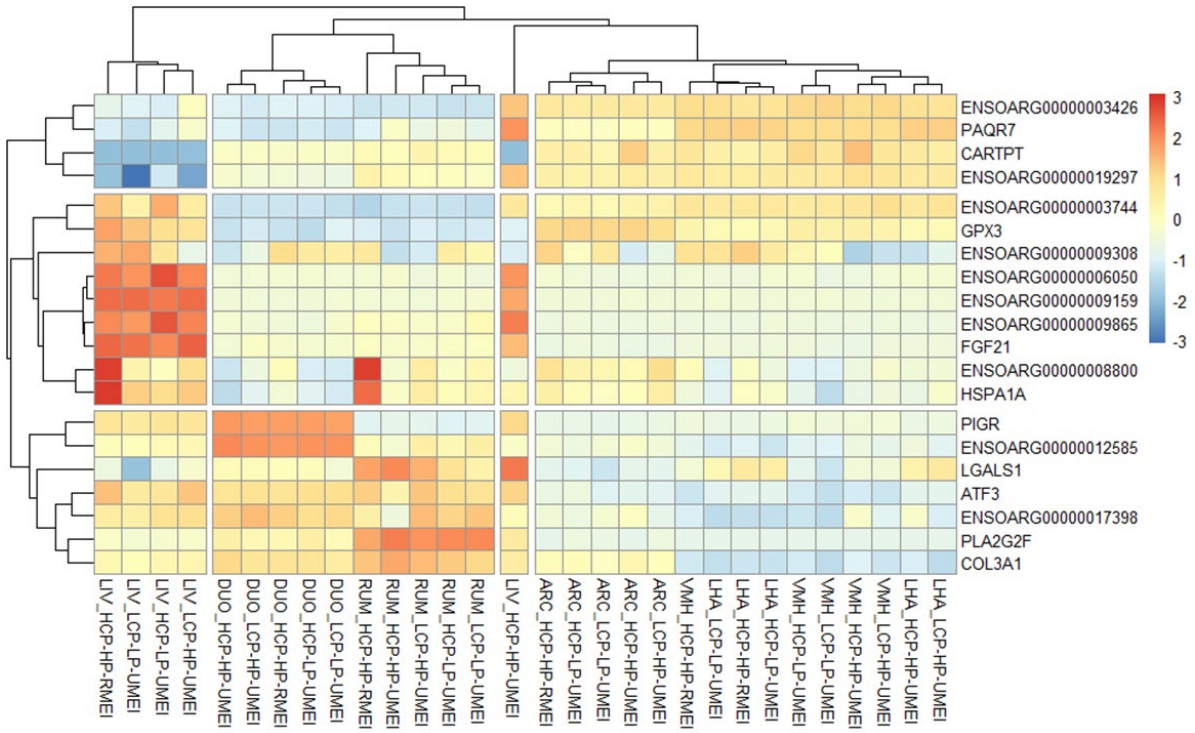


Figure 8. Heat-map of scaled expression of top 20 significantly different genes in steers fed different nutritional treatments[1], averaged by tissue and treatment group and ranked on total absolute phenotypic impact factor as summed across all tissues[2] and treatment comparisons.

[1]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

[2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall

Gene pathways

In Experiment 1, genes within the co-expression network clustered mainly by tissue of highest expression, regardless of the criteria by which it was selected for inclusion in the PCIT analysis (Figure 9). Some clusters also include genes that are functionally important in various tissues and therefore the 'tissue of highest expression' metric may not truly represent the biological role of the cluster. Therefore, the MCODE clustering algorithm was used with default settings within Cytoscape v3.8.0 to automatically identify dense clusters of genes. A functional enrichment of each cluster identified the main pathways that the clusters represented and were labelled onto the network (Figure 10). The co-expression network includes clusters for important cellular functions regardless of tissue type, such as ribosomal proteins (cluster 9) and oxidative phosphorylation (cluster 11), as well as tissue specific functions. The largest clusters represent the significant metabolic differences in the liver (cluster 1) and pathways involved in neurological signalling that were most highly expressed in the hypothalamus (cluster 2), with a small cluster related specifically to the glycoprotein hormones in the ARC (cluster 10) which is situated next to the NPY and AGRP genes.

In Experiment 2, genes also clustered mainly by the tissue of highest expression (Figure 11) which again highlights the small cluster of *AGRP, NPY, MC3R* and *GHRH* genes related to feed intake regulation in the arcuate nucleus. A more refined network was selected using a threshold of >|0.95| for further interpretation (Figure 12). The largest clusters included oxidation-reduction process and regulation of coagulation (Cluster 1), neurogenesis (Cluster 2), steroid hormone synthesis (Cluster 7) and extracellular region and keratinocyte differentiation (Cluster 3).

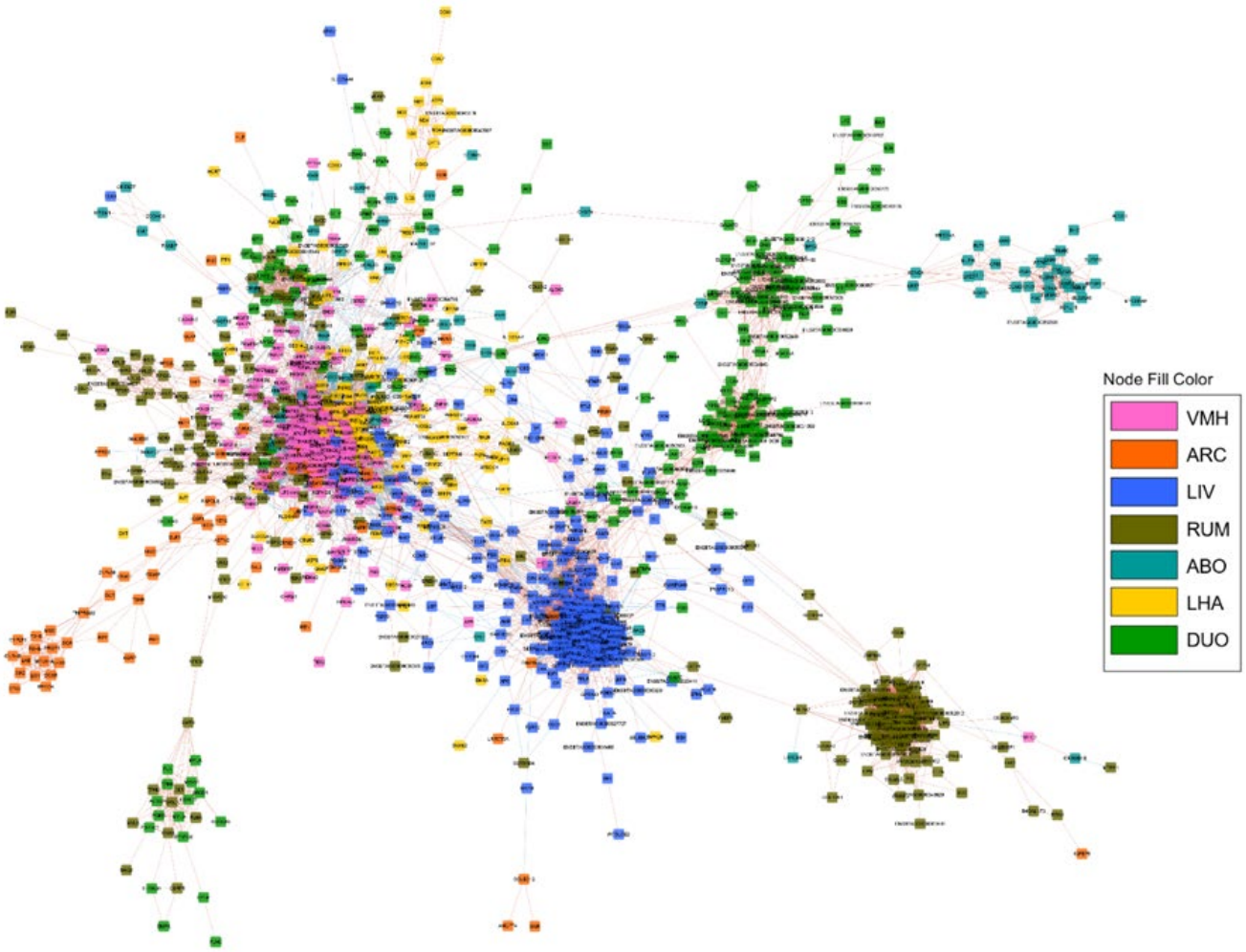


Figure 9. Co-expression network of significant correlations between selected genes (using PCIT) in steers fed different nutritional treatments[1] (Experiment 1) with node colour indicating the tissue[2] with the highest expression of each gene.

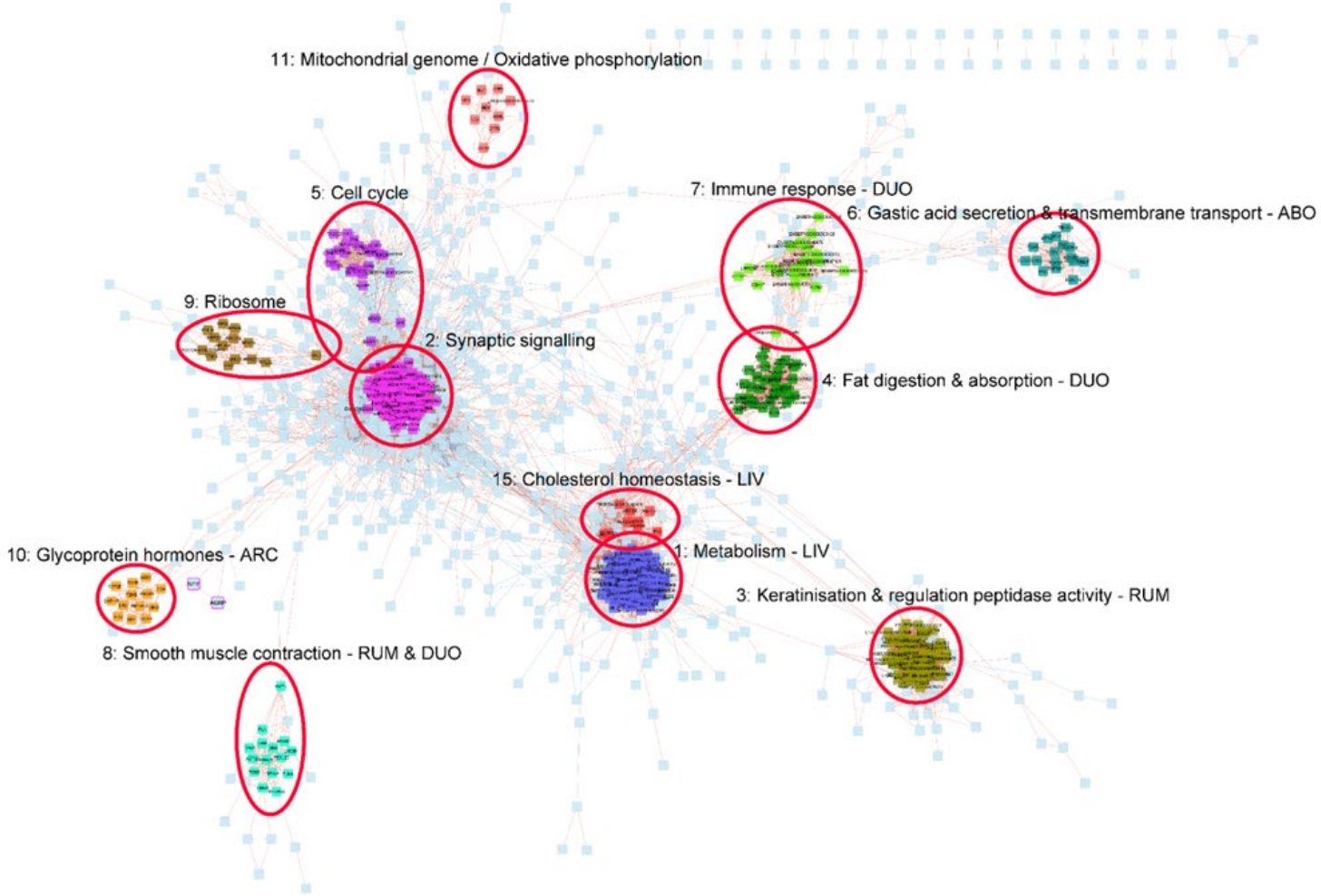


Figure 10. Co-expression network of significant correlations between selected genes (using PCIT) in steers fed different nutritional treatments[1] (Experiment 1) with node colour indicating functional clusters of interconnected genes.

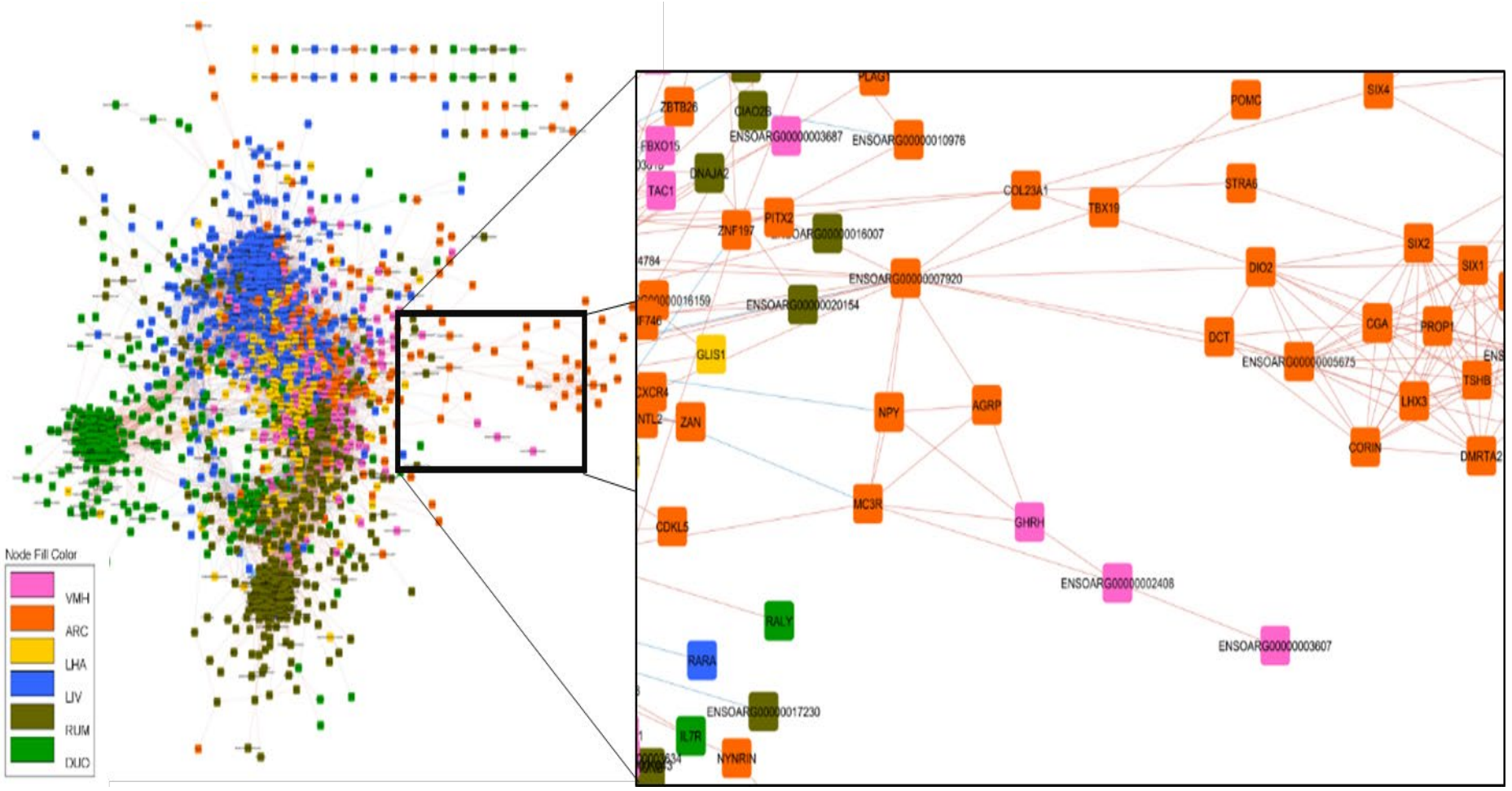


Figure 11. Co-expression network of significant correlations between selected genes (using PCIT) in Merino wethers fed different nutritional treatments[1] (Experiment 2) with node colour indicating the tissue[2] with the highest expression of each gene.

[1]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

[2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall.

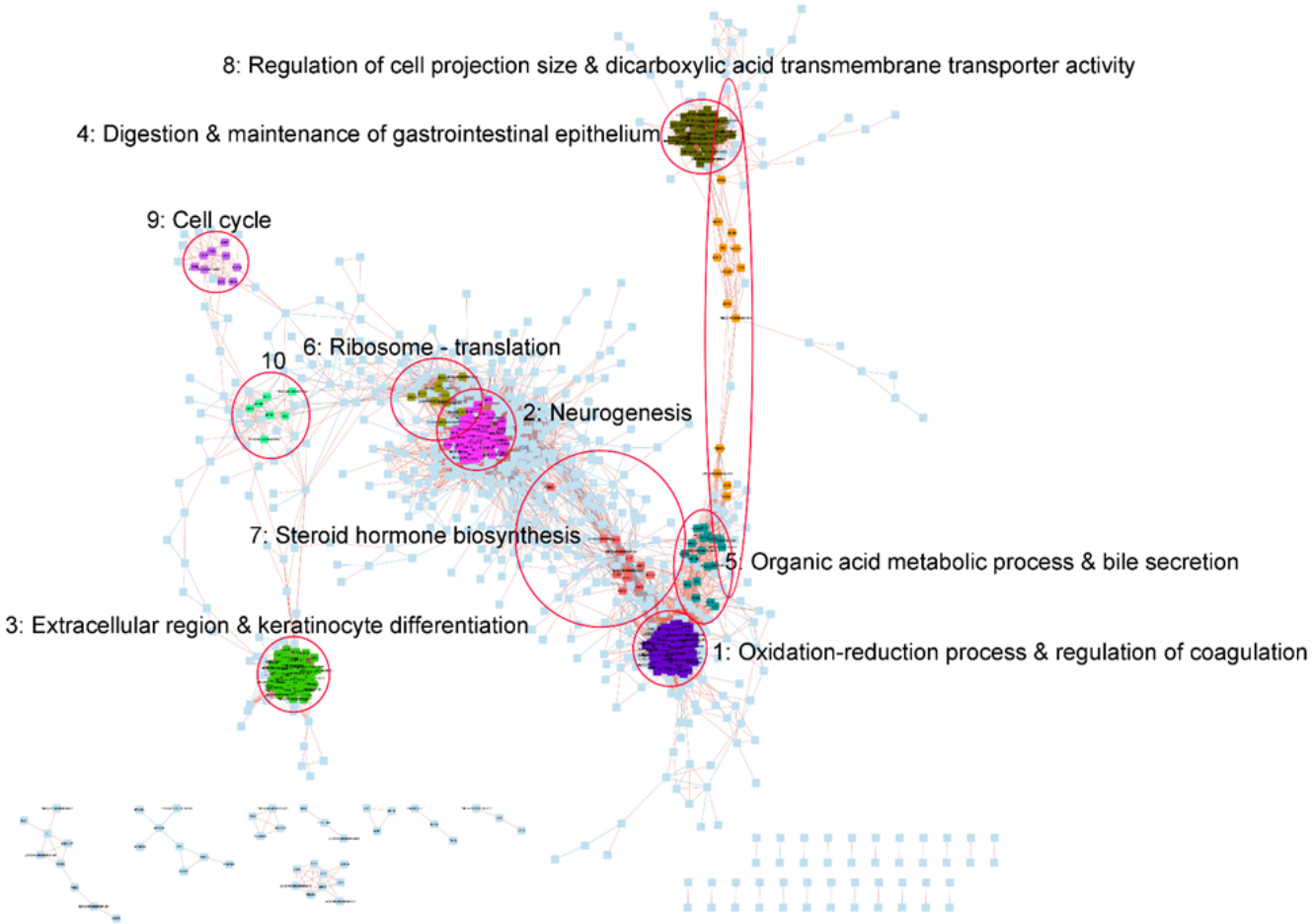


Figure 12. Co-expression network of significant correlations between selected genes (using PCIT) in Merino wethers fed different nutritional treatments[1] (Experiment 2) with node colour indicating functional clusters of interconnected genes.

[1]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

[2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall.

Discussion

Industry relevant animal models for investigating gene-pathway regulation of intake suppression were established in Experiments 1 and 2. The model established in Experiment 2 was designed for this specific purpose, whereas the model established in Experiment 1 was to investigate the effect of nutritional treatments (diet CP:ME intake) on skeletal growth in cattle. As such, less detailed measurements related to the physical and endocrine control of intake were applied in Experiment 1. Nevertheless, across both experiments nutrient deficiency (CP and/or P) resulted in a 40 to 60% reduction in ME intake at which point it was assumed animals fed *ad libitum* had reached satiety when consuming nutrient deficient diets and this reduced ME intake accounted for the low liveweight gain or liveweight losses measured.

It is noted here that a large number of DE, PIF and RIF genes were identified in the liver, rumen and duodenum. Some of these results are included in the Appendix of this Milestone Report and are discussed in detail in Innes (2021). The brief discussion below focuses on responses observed in the hypothalamus only.

The most immediate observation on gene expression in the hypothalamus, consistent across both experiments, was the relatively higher responsiveness of the transcriptome in the ARC to the nutrient treatments compared to the LHA and VMH (as indicated by the number of DE genes in each region). Whilst the response of the ARC to the nutritional treatments is unsurprising given its role in integrating signals (and the source of potent first-order neurons), it is the lack of responsiveness in the LHA and VMH which is perhaps more surprising as it is the second order neurons located at these sites that are believed to ultimately dictate feeding behaviour. In Experiments 1 (steers) and 2 (wethers), genes encoding peptides that are potent regulators of feeding behaviour were DE (NPY, AgRP, POMC, CARTPT) in the ARC in response to nutritional treatment; these were typically down-regulated (AgRP and NPY) or up-regulated (POMC, CARTPT) in the ARC of animals with *ad libitum* access to the nutritionally adequate treatment diet, suggestive of a cessation in feeding (indicative of satiety). These findings provide a form of biological confirmation that the nutritional models, sampling techniques and bioinformatics analysis were appropriate.

Aside from similar trends in expression profiles of known potent regulators of feeding behaviour (NPY and AgRP and, to a lesser extent, POMC and CARTPT) there were very few common gene expression patterns in the hypothalamus between Experiments 1 and 2. While it is noted that functional gene clusters were observed for synaptic signalling and neurogenesis in both experiments, these were largely a function of the tissue-specific inputted genes. Responses in immune pathways were also evident in various tissues across both experiments. The lack of consistent findings between experiments may suggest species specific responses (sheep v cattle), form of nutrient specific responses (i.e. unprocessed roughages v formulated pellets), variations in methodology (i.e. in relation to time of sample collection after feeding) and different background information (extent of annotation) and methods for the bioinformatics approaches employed. For example, the CYP1A1 gene was consistently and uniquely up-regulated across most tissues in steers with *ad libitum* access to Mitchell grass hay (LCP-LMEI) in Experiment 1 but was not observed to be DE or PIF in Experiment 2 in any tissue in response to any nutritional treatment which might suggest a nutrient form (Mitchell grass) rather than nutrient content (LCP-LMEI) effect on expression. Similarly, AFF1 was consistently down-regulated in the ARC of wethers with *ad libitum* intake of a non-deficient diet compared to wethers with reduced intake, regardless if the suppression was due to low CP and/or low P, or restricted ME allowances, but was not differentially expressed in any region of the hypothalamus of steers in response to nutritional treatments.

In Experiment 2, there were far more DE genes (195) in the ARC of wethers with *ad libitum* access to a non-deficient diet relative to wethers fed a dual-deficient diet relative to all other nutrient/intake comparisons, including the comparison between the *ad libitum* and restricted intake of the non-deficient diet. This would be indicative of a nutrient deficient effect on the transcriptome in the ARC

of sheep. Interestingly, pair-wise comparisons of the different nutritional models demonstrated consistent down-regulation of NPY and AgRP and up-regulation of CARTPT genes in the ARC of wethers fed the non-deficient diet compared to wethers consuming CP deficient, CP and P deficient and ME restricted nutritional treatments, whereas wethers consuming a P deficient (CP adequate) nutritional treatment had DE genes that encoded solute carriers but not the neuropeptides described above. The AFF1 gene was consistently down-regulated in the ARC of wethers with *ad libitum* intake of the non-deficient diet, compared to all other nutritional treatments where intake suppression resulted from either a CP, P, CP and P deficiency or a restriction in ME intake of the non-deficient diet. The gene is a member of the AF/FMR2 family of transcriptional activators which encourage RNA elongation. In the LHA, the POMC gene was up-regulated in wethers fed the non-deficient diet *ad libitum* compared to all other nutritional treatments. In the VMH, CARTPT gene was up-regulated in the wethers fed the non-deficient diet *ad libitum* compared to the CP and restricted ME nutritional treatments but not the P deficient treatment. There were very few DE genes in the LHA and VMH suggesting dysregulation of the traditionally viewed, first-order / second-order hierarchy of intake control in this model.

Neither Experiment 1 nor 2 investigated the effects of nutritional treatments on functional anatomy of the central nervous system (CNS; e.g. vagus nerve activation, brain volume, cerebral blood flow, neuronal activity, nutrient transport across the blood-brain barrier) or gene expression in other regions of the CNS (e.g. nucleus tractus solitarius [NTS], known to be a satiety centre; ventral tegmental area [VTA], origin of neurons involved in the mesolimbic dopamine pathway). Such methods may have been more informative of the responses of the steers and wethers to the nutritional treatments.

Whilst animals fed CP deficient nutritional treatments had no further appetite and were assumed to be satiated (i.e. suppressed intake) they were, in all likelihood, still hungry. This is evidenced from the up-regulated expression of NPY and AgRP genes (orexigenic), and down-regulated expression of POMC and CARTPT (anorexigenic) genes in the ARC and the similar gene profiles between satiated and hungry wethers, and the anecdotal observation that intake would increase rapidly when a nutrient deficiency was rectified. Such a response is evident in other models such as immune response in diseased animals, where animals may be hungry but have no appetite (don't want to eat) so intake is suppressed, or in anorexia nervosa in humans, where physiological hunger signals (NPY, ghrelin) are evident but appetite is supressed due to psycho-biological mechanisms, with the biological component potentially related to dysregulation of dopamine-serotonin signalling and opioid-cannabinoid receptors.

Path forward

The results here again demonstrate our ability to establish nutrient deficient models in ruminants. A large data set has been generated from which further, more specific hypothesis driven research questions might be asked in future. The animal models generated provide an opportunity to further test responses to nutrients and pharmaceuticals. A range of research questions have been developed from the completed experiments above, and these form the basis for the proposed pathways forward for the remainder of the project.

- What (specific) metabolic pathways have been perturbed by the nutritional treatments imposed?
- What affect does an immune response have on intake in ruminants fed nutrient deficient diets?
- What higher-order factors in different regions of the CNS are involved in intake suppression in ruminants fed nutrient deficient diets?
- What changes in functional anatomical and metabolism (e.g. nutrient transporters) in the brain have occurred in ruminants fed nutrient deficient diets?

- What hedonic and homeostatic signal cascades influence intake in ruminants are fed nutrient deficient diets?
- Do the accepted theories of orexigenic neuropeptide stimulation of intake hold in ruminants fed nutrient deficient diets? Why is there a lack of DE in second-order neuropeptides and known receptors? Do changes mRNA result in parallel changes in proteins?
- What role does hepatic oxidation theory play in intake suppression in ruminants fed nutrient deficient diets?

Three broad points are considered below in how to address the above questions and provide a suggested path forward for the remainder of the project.

1. *Genetic variation in intake experiment*

We consider implementation of the originally proposed selection and ranking experiment based on divergence in intake as unlikely to yield any additional information to that already generated in the nutrient specific experiments completed above. Further, the idea of ranking animals on high and low quality diet has been done to some extent in other recent MLA funded initiatives (Silva et al. N-recycling; and Bond et al. Better-Doers) albeit not with the extreme nutrient deficient models we typically work with. Whilst still an interesting experimental concept, we recommend the originally proposed experiment does not proceed within this project allowing resources to be focussed on strategies to manipulate intake based on existing data generated within the project.

2. *Continued use of existing data-sets and sample resources*

The above Experiments 1 and 2 have generated large data-sets from which additional hypothesis driven research questions might be answered. For example, analysis could focus on specific genes or gene pathways of interest (e.g. second order neurons, neuropeptide receptors, key metabolic pathways – amino acid catabolism, glycolysis), or on specific tissue connectivity (e.g. connectivity between the ARC-Liver only) rather than the data driven approach used and presented above (both approaches are valid). This could be expanded to include the original RNAseq dataset developed by Quigley, Nattrass and Poppi on the ARC of sheep fed diets that varied in ME:CP; the raw data could be retrieved from collaborators (Nattrass) and re-analysed using updated ovine genome annotation information. This activity would answer the first question above, at little cost.

The development of biomarkers (presumably in the circulation) that reflect activity of different regions of the brain is an exciting development in the field. In addition, a more detailed understanding of the profile of amino acids in the circulation may be informative of the availability of amino acid precursors for specific neurotransmitters in the brain (e.g. serotonin, dopamine, norepinephrine, glutamate, GABA). This activity would involve the analysis of existing stored plasma samples for amino acids, biomarkers for localised brain activity and metabolites (or precursors) of the neurotransmitters.

The histomorphometric analysis of bone samples from the wethers in Experiment 2 will be completed (Dr Lisa Kidd and Honours student) and this will provide more information on the nutritional regulation of P resorption from bone. The interactions between CP and P intake on bone histomorphometry and P reserves will have implications beyond the current project.

3. *Additional animal experiments (Experiments 4 and 5)*

It is proposed to establish two groups of growing wethers using our nutritional models above for use in Experiments 4 and 5. The two groups would be,

1. Nutrient deficient (fed diet deficient in CP and P)
2. Nutrient adequate (fed diet adequate in CP and P)

From these two groups a range of short experiments would be conducted examining the intake response of the wethers to a number of pharmaceutical, nutrient and neuropeptide treatments. The

experiments would require testing of dose rates and responses in both the deficient and adequate state (ideally an antagonist treatment would be included to demonstrate a targeted effect). A deficient animal model is required to reduce the effects of short-term compensatory metabolic mechanisms (e.g. P resorption from bone, N-recycling) which are likely to alleviate intake suppression in the short-term. A brief description of the proposed experiments and treatments, and justification is provided below.

Experiment 4. Effect of systemic (intravenous or intramuscular) administration of pharmaceutical agents and consumption (oral) of nutrient supplements on intake of nutrient deficient diets

Pharmaceuticals to be tested should focus on longer-acting or sustained release compounds and would be selected from the following broad classes of compounds.

Glucocorticosteroids (dexamethasone trimethylacetate, methyl prednisolone acetate) are selected based on differential expression of gene pathways related to immune responses in Experiments 1 and 2. Greer et al. used immune-suppression (prednisolone) to maintain intake of parasitised lambs, while Adams et al. used immune-suppression (dexamethasone) to increase intake of lambs during adaptation to diets and group pens prior to live export.

Benzodiazapines (Diazepam (i.e. Valium), Brotizolam, Midazolam; these are not anti-psychotics) enhance the inhibitory effect of GABA neurotransmitters which are secreted from neurons that have the co-localisation of the orexigenic neuropeptides (NPY and AgRP). In rodents, the partial GABA (receptor) agonist (Bretazenil, short-acting) stimulated feed intake in NPY/AgRP knockout models; antagonists suppressed intake. Dynes et al. (1992) increased intake of parasitised sheep over the short-term using Brotizolam (a short-acting BZD). Investigating the ability of a longer-acting BZD (e.g. Diazepam) pharmaceutical to stimulate feed intake independent of NPY/AgRP is warranted given the general lack of response to increased NPY and AgRP in Experiments 1 and 2 above.

Anti-psychotics (e.g. Olanzapine) are considered extreme appetite stimulants. Olanzapine is an atypical (second generation) anti-psychotic inhibiting serotonergic (5-HT2) and dopaminergic (D2) system, and has been utilized in the treatment of major depression and certain mood disorders including schizophrenia and bipolar disorder. Olanzapine has the greatest effect on appetite and weight gain of all the atypical anti-psychotic compounds. Many trials of olanzapine in patients with anorexia nervosa in combination with psychotherapy and nutritional support have been performed with promising outcomes. Accumulated data suggest that increasing appetite and food intake, as well as delayed satiety signalling, are key behavioural changes related to weight gain/obesity induced by antipsychotics and these may be related to stimulation of neurotransmitters and hormones involved in appetite control. Given the hypothesis that higher-order signalling (potentially involving serotonin-dopamine neurotransmitters) has a role in intake in Experiment 1 and 2, pharmaceuticals that target these pathways may be worthy of investigation in ruminants.

Cannabinoids (e.g. Tetrahydrocannabinol (THC), Dronabinol) are present in cannabis, but are also endogenous, and act via the CB1 receptor which is localised in the basal ganglia (enhance eating pleasure), forebrain (enhance food palatability), hypothalamus (increase food intake) and stomach and small intestine (ghrelin secretion) in rodent studies. In addition to direct action via the CB1 receptor, cannabinoids are reported to stimulate a ghrelin surge in rodents, which typically initiates a meal (when food is available). Dronabinol is currently used to stimulate appetite and weight gain in the treatment of cachexia in HIV patients, and has had minor testing in individuals with anorexia nervosa where it induced a modest increase in body weight over a 4-week period. By activating the CB1 receptor, endocannabinoids decrease PYY, activate mTOR and increase ghrelin, and can increase central appetite neurons in the hypothalamus. Endocannabinoid antagonists (block CB1 receptors) suppress intake and result in low of BW (but with severe side effects). While results from Experiments

1 and 2 provide no evidence for a role of CB receptors and intake, there is very little research on the effect of cannabinoids on intake in ruminants and as such research, although highly speculative, may be warranted.

Opioids (endogenous opioids include endorphin, enkephalin, dynorphin) are implicated in appetite and intake regulation in range of species, including ruminants. A synthetic opiate receptor agonist (SD33) or an opioid analogue (met-enkaphalinamide) increased intake of sheep without (Obese et al. 2007) or with (Dynes, PhD thesis 1993) parasite burdens while an opioid antagonist (Naloxone), suppressed intake in sheep in the same experiments (Obese et al. 2007 and Dynes, PhD thesis, 1993). Endorphin is derived from post-translational modifications of the POMC gene and enkephalin is derived from the PENK (pro-enkephalin) gene. In Experiments 1 and 2 above, the POMC gene was implicated in suppression of intake and signalling indicative of satiety in animals with ad libitum access to a non-deficient diet, while the PENK gene was up-regulated and the OPRD1 gene was down-regulated in the arcuate nucleus of steers fed the suggesting roles of endogenous pro-opioid proteins and their receptors in mediating intake. The PENK gene was also associated with higher DMI and RFI in steers (Alexandre et al. 2019) and in the cerebral-spinal fluid of dairy cows (Kuhla et al. 2015).

Psychedelics (e.g. psilocybin) have received increased interest in the treatment of eating disorders in humans (Foldi et al. 2020), again due to their interaction with the serotonin system, reduced binding and reduced concentration of primary metabolite in the cerebral-spinal fluid. Psychedelics are identified as a possible pharmaceutical to stimulate intake for their potential role in higher-order reward signalling.

The above list provides a range of pharmaceutical classes that may be of particular interest to investigate their effects on intake in ruminants. Some have been evaluated under models of nutrient balanced diets, diet transitions or parasite burdens but none have been evaluated in ruminants fed nutrient deficient diets, others have never been tested in ruminants. It would not be possible to screen all of the above classes, however a priority list will be developed, with an initial focus on the glucocorticoids, BZDs, opioids and cannabinoids. For each pharmaceutical two types of experiment are proposed.

Experiment 4 a. Determine the effect of the pharmaceutical on intake

For each pharmaceutical the following experimental treatments are proposed (Table 10) with a single high level of pharmaceutical administered in the first instance (based on dose rates used elsewhere in the literature).

Table 10. Proposed experimental design to test the effect of pharmaceuticals on intake of growing wethers

Wether group status	Diet	Pharmaceutical[1]
Nutrient deficient	Nutrient deficient	Saline
Nutrient deficient	Nutrient deficient	Pharmaceutical
Nutrient deficient	Nutrient adequate[2]	Saline
Nutrient adequate	Nutrient deficient	Saline
Nutrient adequate	Nutrient adequate	Pharmaceutical
Nutrient adequate	Nutrient adequate[2]	Saline

[1]Pharmaceuticals to be tested include – dexamethasone, diazepam, THC, SD33 (administered i.m. or i.v.)

[2]Nutrient adequate diet could be either a complete nutrient adequate diet used to generate the two groups, or the nutrient deficient diet with a CP and P supplement (monosodium phosphate and urea plus ammonium-sulphate) offered orally.

Experiment 4 b. Dose-response to determine the optimum pharmaceutical dosage rate

For the above experiments where an increase in intake in response to a pharmaceutical is evident, a dose-response experiment will be conducted. Deficient wethers would be fed the deficient diet *ad libitum*, with each target pharmaceutical administered in

i. increasing doses to determine the optimum efficacious dose rate, or
ii. increasing number of days between doses to determine the optimum dose frequency.

All experiments would be conducted over 14-days (after adaptation to pens and feeding) and the variables measured could include,

- Intake – rate of intake, number of meals, total intake
- Plasma – metabolites, hormones, biomarkers of brain function, concentration of administered pharmaceutical
- Brain activity – to be determined if method possible in conscious animal (e.g. fMRI, PET, ECG)
- Behaviour – activity, depression
- Other potential samples (depending on experiment) – rumen fluid (VFA, ammonia-N, microbiome), cerebrospinal fluid, tissue biopsies

Experiment 5. The effect of the intracerebroventricular administration of orexigenic neuropeptides on intake of nutrient deficient diets

Experiments 1 and 2 demonstrated up-regulated expression of mRNA of some orexigenic neuropeptides in the arcuate nucleus of nutrient deficient ruminants. However, unlike in ruminants fed nutrient adequate diets, this upregulation did not stimulate intake of the nutrient deficient diets. It is not clear if this was due to a lack of translation of neuropeptide proteins from these mRNA, limited availability of receptors, or due to higher-order regulation from other regions of the CNS. Regardless, it is important to test if the orexigenic neuropeptides that stimulate intake of nutrient adequate diets have the same effect in nutrient deficient diets. The neuropeptides to be infused include, NPY, AgRP and MCH or Orexin. Each of the compounds would be infused at a biologically high level (determined from the literature) and would be infused into wethers consuming both nutrient deficient and nutrient adequate diets to determine if these neuropeptides stimulate intake of nutrient deficient diets.

Variables outlined above will be monitored to determine the effect of inclusion of deficient nutrients on acute intake over a 14-day period. Euthanasia will be an end-point for these studies, so full tissue collections for mRNA analysis (and proteomics) will be possible to link back to Experiments 1 and 2 reported above.

4. Success in meeting the milestone

Milestone 3.1

Bioinformatics analysis of RNAseq data generated in Experiments 1 (steers) and 2 (Merino wethers) is complete. Data generated was used in the development of multi-tissue functional co-expression networks for both Experiments (Experiment 3).

Milestone 3.2

A pathway to continue research to further advance our understanding of intake regulation of ruminants fed nutrient deficient diets was proposed. The proposed approach involves additional analysis of existing experimental resources (data and plasma samples) and additional experimentation testing interventions based on data generated in Experiments 1 and 2. The proposed including,

Activity 4. Interrogation of existing data-sets and stored samples

- Interrogate gene expression data in 'targeted' metabolic pathways / tissues (Experiment 2)
- Interrogate gene expression data (original Quigley/Poppi ovine model)
- Analysis of stored plasma samples (amino acid profile, biomarkers linked to higher-order brain activity) (Experiment 2)
- Analysis of bone samples (Experiment 2)

Activity 5. Testing pharmaceuticals (and nutrients) to increase intake (Experiments 4 and 5)

- Establish two groups of weaned sheep (CP and P deficient and CP and P adequate models)
    - Test a number of long-acting pharmaceuticals on intake in both models
    - Examine the acute intake response to dietary P and N in both models
    - Examine the effect of orexigenic neuropeptides on intake in both models

Milestone 3.3

A workshop was held at The University of Queensland, St Lucia on 29-March-2021 with the project team (Quigley, Innes, Hudson, Anderson, Tillbrook, Poppi) and MLA project manager (Tim Huggins) to overview results and progress to date, and to discuss and propose future research activities (summarised above). Opportunities to value-add existing data and samples were discussed including specific hypothesis driven interrogation of existing RNAseq data, analysis of existing plasma samples for various brain biomarkers and completion of bone analysis all from Experiment 2. Potential linkages and synergies with other MLA funded projects were identified,

- Better-Doers project (NSWAG, UNE UQ)
- N-recycling and efficiency (UQ)
- Breeder performance in NB2 (UQ, QDAF, NTDPIR)
- Vitamin D (Heggarty and previous work by Tomkins & Elliot)

5. Overall progress of the project

The project is approximately 6 months behind the original schedule but is progressing in line with the revised timeline in the variation. The project has completed Experiments 1, 2 and 3 as planned and has developed a more detailed understanding of intake regulation of ruminants fed nutrient deficient diets. The project has reviewed a range of strategies to deliver compounds into the central nervous system of ruminants. The project has developed a proposed pathway for remaining research activities to test strategies to manipulate intake of ruminants fed nutrient deficient diets.

David Innes (UQ and MLA top-up postgraduate scholarships) has submitted his PhD thesis using data generated in this project, and plans to prepare four scientific papers from this work in 2021. Abstracts and poster/short-oral presentations from this work were presented at the International Symposium of Ruminant Physiology (2019) and Australian Association for Animal Sciences (2021) conferences. A number of UQ undergraduate and honours students have gained valuable research experience from their participation in the project.

Whilst there is continued uncertainty regarding COVID19 impacts on research in the short-term, it is expected that the proposed activities utilising generated data and samples, and the conduct of short-term animal experiments means impacts will be minimal over the remainder of the project. There would be an opportunity for a HDR student to undertake research within the proposed remaining activities within the project, and this will be explored if the project is approved to continue.

6. Conclusions/recommendations

Models for nutrient-deficient suppression of intake in ruminants and appropriate bioinformatics pathways were successfully established. Animals few nutrient deficient diets display signals at the mRNA level (in the hypothalamus) that are indicative of hunger, and yet intake is suppressed presumably due to some negative metabolic consequence of consuming an excess of a nutrient deficient diet. While no consistent over-arching regulator pathway was identified, neuropeptides implicated in feeding behaviour were differentially expressed in response to treatment diets, as were gene pathways implicated in metabolism, neuropeptide signalling and immune function. It is hypothesised that both up-stream (satiety signals of the nucleus tractus solitarius) and higher-order reward signals (dopamine-serotonin) may have a role in integrating and relaying signals related to these metabolic consequences to the feeding centre in the hypothalamus. The results present priorities for the proposed remaining research which will focus on treatments (pharmaceuticals, nutrients, neuropeptides) which target the above genes and gene pathways to increase intake of nutrient deficient diets with a number of potential experiments and treatments proposed. It is recommended that

- The project proceed in line with the broadly described activities above
- Quigley and Tilbrook to confirm feasibility of plasma analysis of brain biomarkers
- Quigley to source raw RNAseq data from ex-SARDI colleagues for re-analysis after alignment against updated ovine genome
- Submit four manuscripts for publication from Experiments 1 and 2
- Project team to meet to confirm design of proposed Experiments 4 and 5
- Recruit PhD student (and Honours students) to participate in Experiments 4 and 5

7. References

8. Appendix

Experiment 1

Gene expression

Liver

Individual genes with the highest significant DE and PIF in the liver of steers were genes related to growth (GPC3, FGF21, IGFBP1) and glutathione metabolism (GPX3, GSTM1) (see example MA plot, Figure 13). There were over 100 significantly enriched pathways for DE genes in the liver, with blood vessel development, extracellular organisation and growth factor binding prominent (see example enrichment, Figure 14).

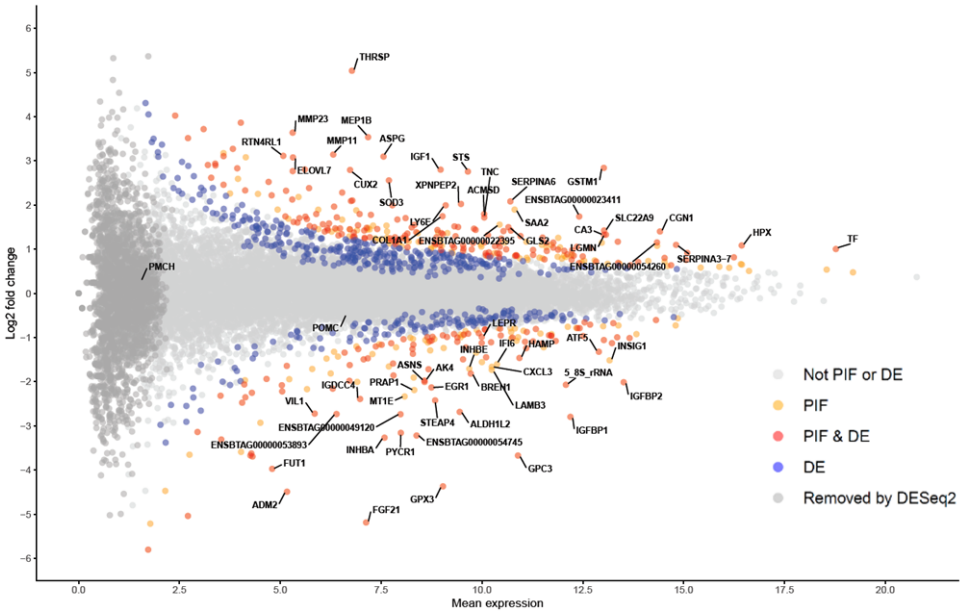


Figure 13. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the liver of steers allocated to the HCP-HMEI and LCP-LMEI treatments. Each dot is an individual gene, see legend for colour coding.

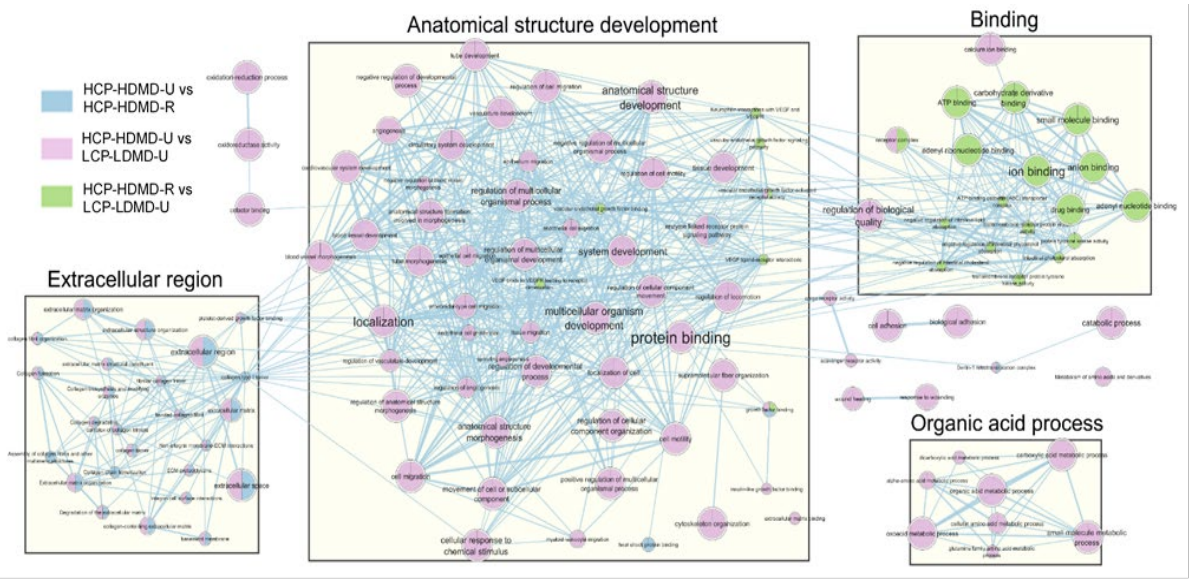


Figure 14. Enriched pathways of all differentially expressed genes in the liver of steers fed HCP-HMEI, HCP-LMEI and LCP-LMEI nutritional treatments.

Rumen

Individual genes with the highest significant DE and PIF in the rumen of steers were THBS1, CCN2, S100A9, PI3 and CHI3L2 (see example MA plot, Figure 15). Prominent enriched pathways for DE genes in the rumen included extracellular region, vascular development, inflammatory response and signalling receptor binding (see example enrichment, Figure 16).

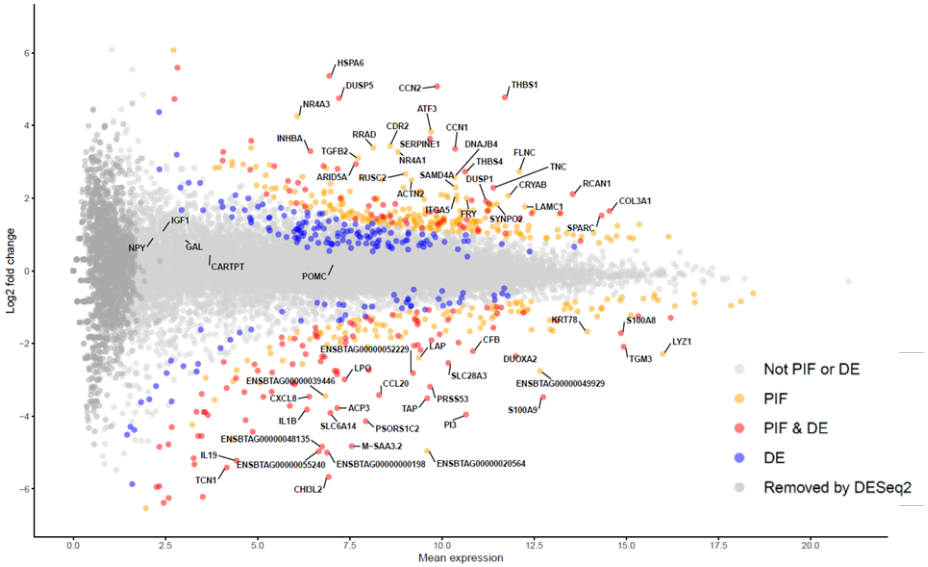


Figure 15. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the rumen wall of steers allocated to the HCP-HMEI and LCP-LMEI treatments. Each dot is an individual gene, see legend for colour coding.

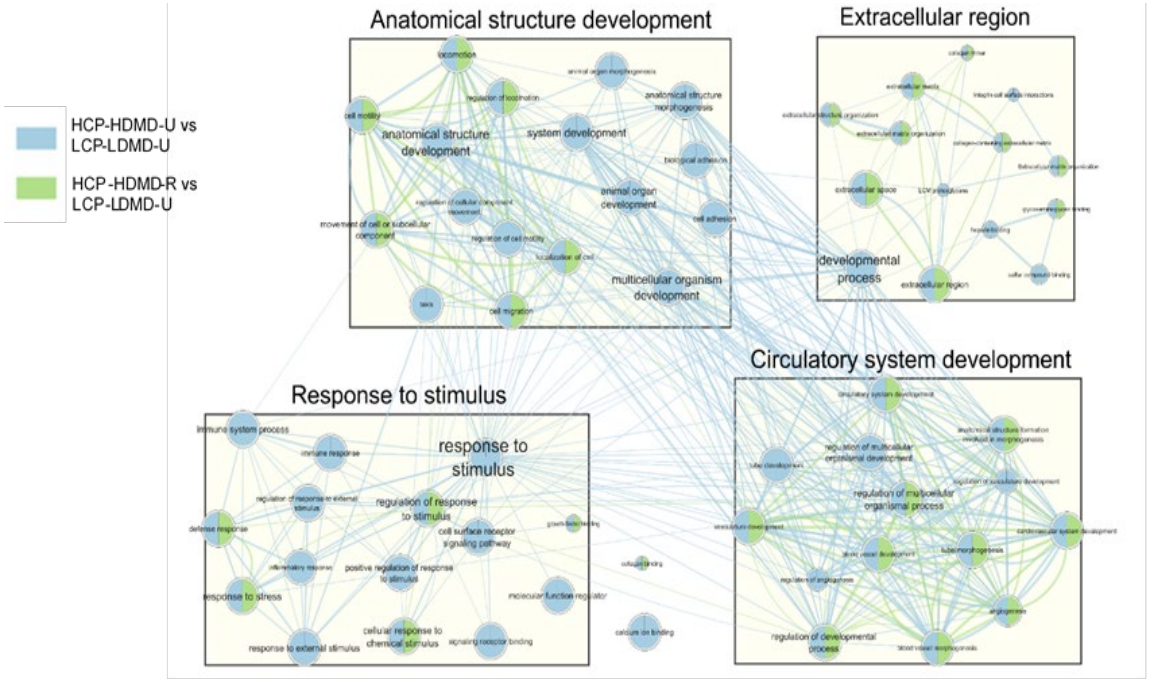


Figure 16. Enriched pathways of all differentially expressed genes in the rumen wall of steers fed HCP-HMEI, HCP-LMEI and LCP-LMEI nutritional treatments.

Duodenum

Individual genes with the highest significant DE and PIF in the rumen of steers were a non-annotaded gene (ENSBTAG0000052233), REG4, CL46, MS4A1 and GP2 (see example MA plot, Figure 17). Prominent enriched pathways for DE genes in the rumen included extracellular region, vascular development, inflammatory response and signalling receptor binding (see example enrichment, Figure 18).

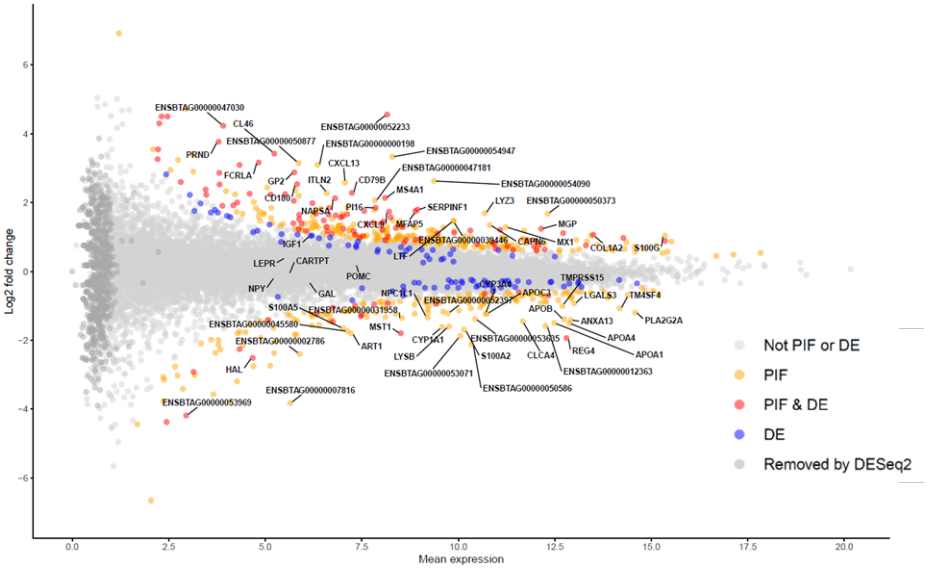


Figure 17. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the duodenum wall of steers allocated to the HCP-HMEI and LCP-LMEI treatments. Each dot is an individual gene, see legend for colour coding.

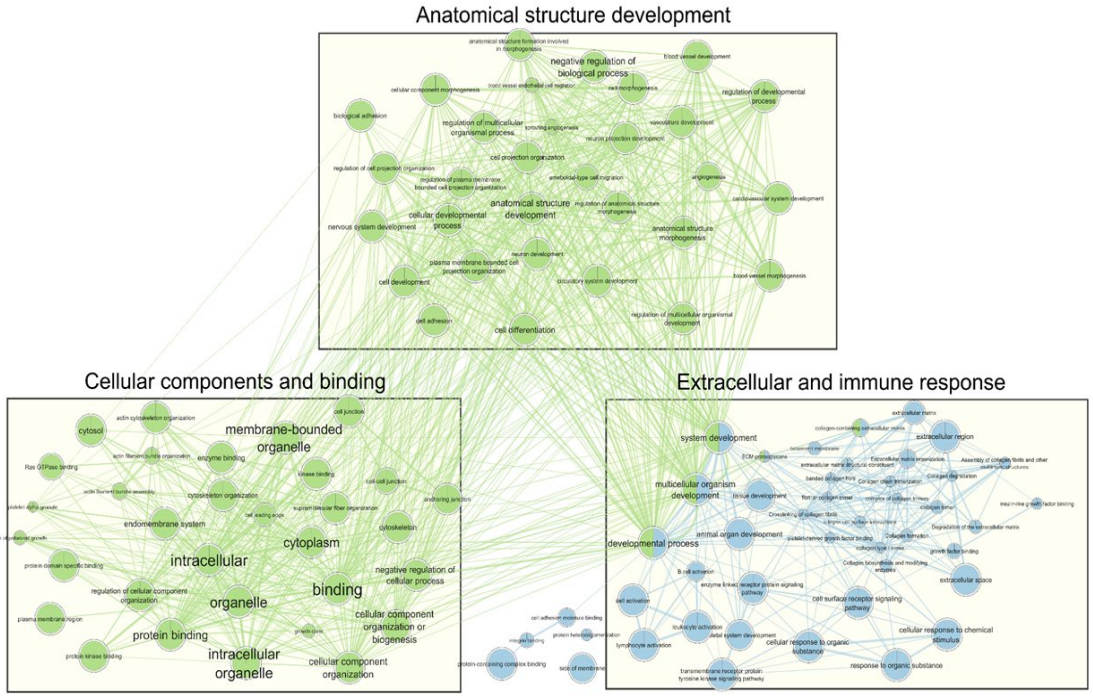


Figure 18. Enriched pathways of all differentially expressed genes in the duodenum wall of steers fed HCP-HMEI, HCP-LMEI and LCP-LMEI nutritional treatments.

Uniquely different genes in response to nutritional treatments

Genes within a tissue region with a significant ($P \leq 0.05$) pairwise difference (higher or lower) and a fold change > 2 between the treatment of interest and both other treatments, and subsequently no difference between these other two treatments, were deemed 'uniquely different genes' (Table 11). Therefore, uniquely different genes in the steers fed the HCP-HMEI treatment represent pathways responding to a higher DM and ME intake, but not the CP content of the diet. Likewise, uniquely different genes in the steers fed the LCP-LMEI treatment represent pathways responding to a voluntary reduction in feed intake ('satiety' model) due to the lower CP content of the diet, the lower DMD of the diet or the different forage type, but not ME intake. Uniquely different genes in the steers fed the HCP-LMEI treatment represent pathways uniquely responding to an enforced feed restriction ('hunger' model), but not to differences in the CP content of the diet, DMD of the diet or ME intake.

Table 11. List of genes that are significantly higher or lower (adjusted *P* ≤ 0.05 and foldchange > 2) in one nutritional treatment compared to the other two nutritional treatments[1] for each tissue region[2] in steers.

Treatment	Region	Relative expression	No. of genes	Genes[3]
HCP-HMEI	ABO	lower	3	*ADAMDEC1, BMP3, MME*
	ARC	higher	14	*COL1A1, CHI3L1, CPXM2, COL3A1, PENK, C1QTNF6, COL15A1, SERPINF1, TOP2A, MKI67, THRSP, RRM2, TTK, CENPF*
		lower	4	*AGRP, NPY, HIF3A, bta-mir-2350*
	DUO	higher	17	*FCRLA, MS4A1, CD79B, FCRL4, NAPSA, SERPINF1, ELL3, CXCR5, PI16, PRND, GAPT, IFITsp13, FCRL1, ALOX12E, ENSBTAG00000053318, ESM1, ENSBTAG00000054738*
		lower	2	*BTNL9, FFAR4*
	LIV	higher	71	*THRSP, GSTM1, IGF1, ENSBTAG00000023411, HPX, SERPINA6, CUX2, SOD3, MMP11, SERPINA3-7, SLC22A9, LGMN, COL1A1, ELOVL7, XPNPEP2, C18H16orf70, ACMSD, RETSAT, TEX12, PRODH, COL1A2, MAMDC2, MN1, PIGR, ENSBTAG00000022715, ADRB2, CD5L, SPARC, SIPA1L1, QSOX1, CNN1, COL3A1, CYP4B1, ENSBTAG00000052912, UPP2, NDNF, CCL26, TAFA4, PI16, ACTG2, TMED6, HIST1H2BD, TRIM31, PTPRB, SIX4, ENSBTAG00000038233, ELN, TP53INP2, HTR2B, TENM3, CHRNE, ENSBTAG00000046193, PLCD4, RILPL2, SLC26A7, CCN3, PAMR1, ENSBTAG00000053379, CKAP2, ADAMTS2, GPBAR1, ARMH1, SESN3, CSF2RB, ITIH5, CCDC80, ENSBTAG00000045854, KCNK3, TM4SF18, MPP3, PBX3*
		lower	30	*GPC3, IGFBP2, PYCR1, ALDH1L2, HSPA1A, ADM2, PTGR1, ASNS, HYOU1, IGDCC4, ENSBTAG00000049291, PSPH, C18H19orf12, SLC16A5, CRELD2, ENSBTAG00000050173, PDLIM1, SLC7A11, CITED1, SRXN1, MT1E, MAMSTR, ENSBTAG00000050563, ENSBTAG00000052965, ENSBTAG00000025383, NCMAP, MANEAL, PDE6C, SCARNA2, SRSF12*
	RUM	higher	5	*THBS1, HSPA6, PRND, FAIM2, BACH2*
		lower	6	*ENSBTAG00000055240, ENSBTAG00000050723, ENSBTAG00000047121, NTRK2, ENSBTAG00000050586, ENSBTAG00000024492*
LCP-LMEI	ARC	higher	1	*CYP1A1*
	DUO	higher	2	*ENSBTAG00000053969, TFCP2L1*
		lower	8	*ENSBTAG00000047621, CD248, POPDC2, ENSBTAG00000053073, RIMS3, ENSBTAG00000018009, PRICKLE2, CNMD*
	LHA	higher	1	*CYP1A1*
	LIV	higher	13	*IGFBP1, BREH1, AK4, FUT1, XDH, ENSBTAG00000051408, SLC1A1, ENC1, PRLR, KLF10, ENSBTAG00000003236, PDLIM2, MAFF*
		lower	15	*CA3, ARG1, STAB2, RASSF6, MYLK4, ENSBTAG00000049436, CYP4F2, ENSBTAG00000036099, DNASE1L3, LRIG3, GPR182, PCDH12, ENSBTAG00000053608, TNFSF10, THSD1*
	RUM	higher	55	*PI3, S100A9, CHI3L2, ENSBTAG00000000198, PRSS53, M-SAA3.2, PSORS1C2, TGM3, ACP3, PLA2G4F, SLC6A14, IL19, PLA2G4D, LPO, LBP, TCN1, CFB, SLC1A1, CXCL8, SAA3, GJB6, ABO, SLC5A8, ENSBTAG00000032873, CHST4, ERO1A, GABRR3, IL17A, CARD14, BNIP3L, ENSBTAG00000053991, WFDC5, APOBEC3Z1, ENSBTAG00000046375, IL22RA1, IL17F, MAB21L3, EPS8L1, IL22, SMPDL3B, TMPRSS11A, AZGP1, NOD2, ACSL6, RASAL1, MACC1, CASP7, NUCB2, ENSBTAG00000054953, CTLA4, HRH3, LTB4R2, SLC24A1, C5AR2, ENSBTAG00000054540*
		lower	39	*COL3A1, COL1A1, GPNMB, COL1A2, MGP, MMP2, TNXB, GPX1, MFAP5, FBLN1, CD248, ELN, MRC2, COL5A1, THBS2, THY1, AEBP1, ADAMTS2, PDGFRL, PCOLCE, SPON2, HTRA3, TCF21, ISLR, ANG2, NES, LRRN2, CPXM1, FST, GRIA3, KAZALD1, CTHRC1, MMP23, MFAP2, FAR2, MCHR1, ENSBTAG00000053911, DTX1, STC2*
	VMH	higher	1	*CYP1A1*

Treatment	Region	Relative expression	No. of genes	Genes[3]
HCP-LMEI	ARC	lower	2	*ENSBTAG00000025258, GBP2*
	DUO	higher	5	*ENSBTAG00000052264, PHOX2B, SGCA, ENSBTAG00000048509, RFX2*
		lower	2	*SLC13A2, ENSBTAG00000007280*
	LIV	lower	1	*ENSBTAG00000009760*
	RUM	higher	5	*ENSBTAG00000004565, C1QTNF12, NUMBL, GPRC5A, PTHLH*
		lower	5	*MGC157368, BAMBI, NCOA7, ENSBTAG00000051836, GBP4*

[1]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI), low CP and low ME intake (LCP-LMEI) and high CP and low ME intake (HCP-LMEI)
[2]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM), abomasum (ABO) and duodenum (DUO) wall
[3]Genes listed in order of highest absolute phenotypic impact factor (PIF).

Regulatory impact factor analysis

The genes that were determined to be significant RIFs were further ranked on their highest average of |RIF1| and |RIF2| within each treatment comparison with the top five significant transcription factors for each nutritional comparison within each tissue included in Table 12.

The listed transcription factors include those genes which are known to control physiological processes by regulating the transcription of mRNA, as listed by the AnimalTFDB (transcription factor database) and are therefore more likely to be key regulators of the physiological differences between the steers in each treatment. Notable transcription factors include *ARID5A* in ARC, linked with regulation of adipogenesis; *POU3F4* in ARC, required for maintenance of hypothalamus function and linked with proglucagon transcription in the pancreas; *ESR1*, *ARNT* and *AHR* in duodenum, part of the aryl hydrocarbon receptor complex involved in intestinal homeostasis and regulation of the transcription of *CYP1A1*; *PPARA*, *NR1H2* and *PPARD* in liver, nuclear hormone receptors with central metabolic roles related to lipid sensing, and in the case of the PPARs cellular organelle (peroxisome and mitochondria) content; and *GRHPR* in rumen involved in glyoxylate and hydroxy pyruvate metabolism. Two nuclear factor transcription factors, *NFKB1* and *NFE2L1*, related to a broad range of cellular functions including inflammation and oxidative stress, respectively, were ranked in the top five RIF in the rumen of steers fed HCP-HDMD-U vs LCP-LDMD-U. The *NFE2L1* transcription factor also down-regulates *GPX1* which is an antioxidant enzyme that reduces oxidative stress and was uniquely lower in the rumen of steers fed the LCP-LDMD-U treatment, indicating a potential oxidative stress response in these animals.

Table 12. Top five transcription factors (TF) or co-factors ranked on absolute RIF1 and RIF2[1] for nutritional treatment[1] comparisons for each tissue region[2] of the steers.

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
ABO	HCP-HMEI vs LCP-LMEI	CD3D	-1.96	-2.06	2.01	co-factor	CD3d molecule
		TAF3	-1.33	2.67	2.00	co-factor	TATA-box binding protein associated factor 3
		HTATIP2	-2.65	1.34	2.00	co-factor	HIV-1 Tat interactive protein 2
		SPIB	-1.46	-2.52	1.99	TF	Spi-B transcription factor
		TRERF1	-2.67	1.24	1.95	TF	transcriptional regulating factor 1
	HCP-HMEI vs HCP-LMEI	ZNF329	-3.15	-2.10	2.63	TF	zinc finger protein 329
		PHIP	-3.58	-1.61	2.60	co-factor	pleckstrin homology domain interacting protein
		NFAT5	-3.90	-0.96	2.43	TF	nuclear factor of activated T cells 5
		EAPP	-3.56	-1.23	2.39	co-factor	E2F associated phosphoprotein
		MYSM1	-3.93	-0.78	2.35	TF	Myb like, SWIRM and MPN domains 1
	HCP-LMEI vs LCP-LMEI	ZNF536	2.76	2.83	2.80	TF	zinc finger protein 536
		SUPT4H1	2.76	2.27	2.52	co-factor	SPT4 homolog, DSIF elongation factor subunit
		PIH1D1	2.90	2.10	2.50	co-factor	PIH1 domain containing 1
		NOTCH2	3.44	1.37	2.40	co-factor	notch receptor 2
		CALR	2.09	2.67	2.38	co-factor	calreticulin
ARC	HCP-HMEI vs LCP-LMEI	EAF2	3.99	1.84	2.92	co-factor	ELL associated factor 2
		ARID5A	3.40	2.03	2.72	TF	AT-rich interaction domain 5A
		SNAI3	4.52	0.76	2.64	TF	snail family transcriptional repressor 3
		CSDE1	3.05	2.09	2.57	TF	cold shock domain containing E1
		ZNF391	4.28	0.67	2.47	TF	zinc finger protein 391
	HCP-HMEI vs HCP-LMEI	ALX4	3.87	-1.02	2.45	TF	ALX homeobox 4
		FERD3L	4.10	-0.63	2.37	TF	Fer3 like bHLH transcription factor
		DMBX1	2.46	-2.16	2.31	TF	diencephalon/mesencephalon homeobox 1
		CENPA	4.00	-0.62	2.31	TF	centromere protein A
		POU3F4	-1.81	-2.78	2.30	TF	POU class 3 homeobox 4
	HCP-LMEI vs LCP-LMEI	PBXIP1	-3.36	-1.90	2.63	co-factor	PBX homeobox interacting protein 1
		LRRFIP1	-3.35	-1.91	2.63	TF	LRR binding FLII interacting protein 1
		TSHZ1	-3.26	-1.87	2.57	TF	teashirt zinc finger homeobox 1
		ZBED5	-3.16	-1.78	2.47	TF	zinc finger BED-type containing 5
		HEY2	-2.71	-2.08	2.40	TF	hes related family bHLH transcription factor with YRPW motif 2
	HCP-HMEI vs LCP-LMEI	ESR1	4.47	-0.68	2.58	TF	estrogen receptor 1
		ARNT	3.83	-1.24	2.53	TF	aryl hydrocarbon receptor nuclear translocator
		TFAM	3.80	-1.14	2.47	TF	transcription factor A, mitochondrial
		RERE	3.56	-1.19	2.38	TF	arginine-glutamic acid dipeptide repeats
		HDAC4	3.35	-1.35	2.35	co-factor	histone deacetylase 4

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
DUO	HCP-HMEI vs HCP-LMEI	BHLHE40	2.38	2.90	2.64	TF	basic helix-loop-helix family member e40
		AHR	2.21	2.76	2.48	TF	aryl hydrocarbon receptor
		CDC5L	2.71	2.19	2.45	TF	cell division cycle 5 like
		CDK12	1.97	2.86	2.42	co-factor	cyclin dependent kinase 12
		TFAM	2.82	1.90	2.36	TF	transcription factor A, mitochondrial
	HCP-LMEI vs LCP-LMEI	CEBPZ	3.83	1.36	2.60	TF	CCAAT enhancer binding protein zeta
		DEK	3.40	1.77	2.59	co-factor	DEK proto-oncogene
		PAXBP1	3.51	1.66	2.58	TF	PAX3 and PAX7 binding protein 1
		SS18	3.63	1.50	2.57	co-factor	SS18 subunit of BAF chromatin remodeling complex
		RPRD1A	3.61	1.44	2.52	co-factor	regulation of nuclear pre-mRNA domain containing 1A
LHA	HCP-HMEI vs LCP-LMEI	SOX30	-3.82	-2.23	3.02	TF	SRY-box transcription factor 30
		ZNF572	-3.59	-1.92	2.76	TF	zinc finger protein 572
		FHL5	-4.04	-1.47	2.75	co-factor	four and a half LIM domains 5
		ZNF175	-4.05	-1.15	2.60	TF	zinc finger protein 175
		ASF1B	-4.06	-1.12	2.59	co-factor	anti-silencing function 1B histone chaperone
LIV	HCP-HMEI vs LCP-LMEI	ZNF358	3.71	0.63	2.17	TF	zinc finger protein 358
		TEAD4	3.56	-0.74	2.15	TF	TEA domain transcription factor 4
		PPARA	2.91	1.27	2.09	TF	peroxisome proliferator activated receptor alpha
		ZNF70	-0.93	-3.21	2.07	TF	zinc finger protein 70
		ZNF180	-1.17	-2.95	2.06	TF	zinc finger protein 180
	HCP-HMEI vs HCP-LMEI	TCEA2	3.35	1.52	2.44	co-factor	transcription elongation factor A2
		CREB1	2.33	2.04	2.19	TF	cAMP responsive element binding protein 1
		ELF3	3.04	1.27	2.16	TF	E74 like ETS transcription factor 3
		ASXL1	2.74	1.56	2.15	co-factor	ASXL transcriptional regulator 1
		SNAPC4	2.84	1.38	2.11	TF	small nuclear RNA activating complex polypeptide 4
	HCP-LMEI vs LCP-LMEI	NFE2L3	2.98	-2.85	2.92	TF	nuclear factor, erythroid 2 like 3
		MIER1	3.38	-2.44	2.91	TF	MIER1 transcriptional regulator
		NR1H2	3.35	-2.15	2.75	TF	nuclear receptor subfamily 1 group H member 2
		PPARD	-3.18	-1.86	2.52	TF	peroxisome proliferator activated receptor delta
		TCF7L2	2.50	-2.34	2.42	TF	transcription factor 7 like 2
RUM	HCP-HMEI vs LCP-LMEI	ZCCHC12	-5.15	-2.65	3.90	co-factor	zinc finger CCHC-type containing 12
		ZNF469	-4.95	-1.78	3.36	TF	zinc finger protein 469
		NFKB1	-4.53	-2.15	3.34	TF	nuclear factor kappa B subunit 1
		NFE2L1	-4.30	-1.97	3.14	TF	nuclear factor, erythroid 2 like 1
		PYCARD	-4.18	-1.83	3.00	co-factor	PYD and CARD domain containing
	HCP-HMEI vs HCP-LMEI	DBP	-3.55	-2.59	3.07	TF	D-box binding PAR bZIP transcription factor
		GTF2A2	-3.34	-2.46	2.90	co-factor	general transcription factor IIA subunit 2
		CBX6	-2.37	-3.25	2.81	co-factor	chromobox 6
		TCF4	-3.24	-2.06	2.65	TF	transcription factor 4

Region	Treatment comparison	Gene name	RIF1	RIF2	Average |RIF|	TF type	Gene description
VMH	HCP-LMEI vs LCP-LMEI	ARID3C	-2.94	-2.25	2.60	TF	AT-rich interaction domain 3C
		ZNF7	-4.05	-1.24	2.64	TF	zinc finger protein 7
		ATF6	2.35	2.28	2.32	TF	activating transcription factor 6
		HIF1AN	1.93	2.62	2.27	co-factor	hypoxia inducible factor 1 subunit alpha inhibitor
		GRHPR	-3.41	-1.13	2.27	co-factor	glyoxylate and hydroxypyruvate reductase
		RBM15	1.97	2.47	2.22	co-factor	RNA binding motif protein 15
	HCP-HMEI vs LCP-LMEI	ZNF16	-3.88	-0.87	2.38	TF	zinc finger protein 16
		PMF1	1.84	2.85	2.34	co-factor	polyamine modulated factor 1
		ZNF227	2.20	2.45	2.32	TF	zinc finger protein 227
		HMGXB3	-3.49	-1.09	2.29	TF	HMG-box containing 3
		ZBTB2	-3.59	-0.96	2.28	TF	zinc finger and BTB domain containing 2
	HCP-HMEI vs HCP-LMEI	TFAP4	-3.82	2.55	3.19	TF	transcription factor AP-4
		ZBTB33	-3.62	2.74	3.18	TF	zinc finger and BTB domain containing 33
		TBX18	-3.38	2.88	3.13	TF	T-box transcription factor 18
		HLF	-3.23	2.91	3.07	TF	HLF transcription factor, PAR bZIP family member
		TRRAP	-2.71	3.02	2.86	co-factor	transformation/transcription domain associated protein

[1]Regulatory impact factor (RIF1, RIF2). RIF analysis was not conducted for regions with less than two differentially expressed genes.

[2]Nutritional treatments with high crude-protein (CP) and high metabolisable energy (ME) intake (HCP-HMEI=HCP-HDMD-U), low CP and low ME intake (LCP-LMEI=LCP-LDMD-U) and high CP and low ME intake (HCP-LMEI=HCP-HDMD-R)

[3]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM), abomasum (ABO) and duodenum (DUO) wall

Experiment 2

Gene expression

Liver

The majority of the DE genes in the liver were between the wethers fed the HCP-HP-UMEI vs LCP-LP-UMEI and HCP-HP-UMEI vs HCP-HP-RMEI treatment comparisons, and these two treatment comparisons were similar (Figure 19). From the list of the top 20 DE genes (ranked by PIF) 10 genes were common in these two treatment comparisons, including genes related to growth and development (*IGFBP1, IGFBP2, FGF21*), cell signalling (*GPC3),* glutathione processes (*GPX3, ENSOARG00000019297, ENSOARG00000019285),* progesterone signalling *(PAQR7),* amine sulfotransferase-like gene (*ENSOARG00000009159)* and an uncharacterised gene *(ENSOARG00000003426).* Whilst there were relatively less DE genes amongst the nutrient deficient treatment comparisons, a common interesting output was the significant up-regulation of a cluster of genes related to the acute-phase immune response in wethers allocated to the HCP-LP-UMEI treatment.

Differentially expressed genes that were up-regulated in the liver of wethers fed the HCP-HP-UMEI treatment compared to the LCP-LP-UMEI treatment were enriched for 81 terms including 'steroid biosynthesis', 'organic substance transport', 'glutathione transferase activity', 'regulation of response to stress', 'fatty acid metabolic process' and 'PPAR signaling pathway'; whereas down-regulated DE genes were enriched for 39 terms including 'structural constituent of ribosome', 'regulation of gene expression', 'negative regulation of cellular metabolic process' and 'regulation of gluconeogenesis'.

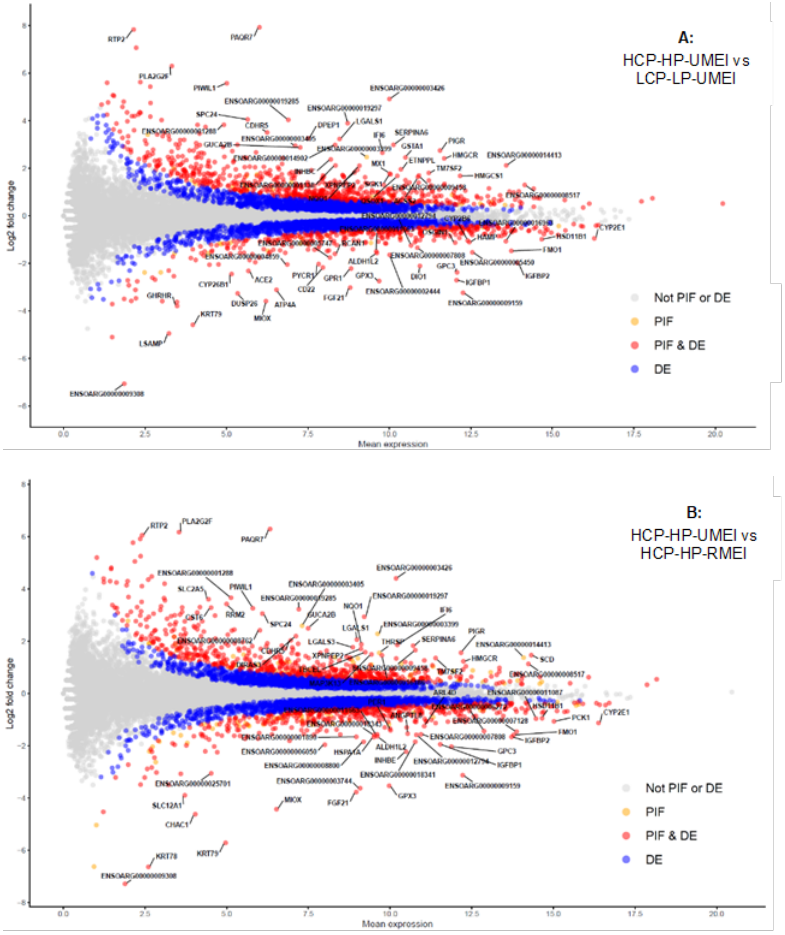


Figure 19. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the liver of Merino wethers allocated to the HCP-HP-UMEI, LCP-LP-UMEI and HCP-HP-RMEI treatments. Each dot is an individual gene, see legend for colour coding.

Rumen

The majority of the DE genes in the rumen were between the wethers fed the HCP-HP-UMEI vs LCP-LP-UMEI (Figure 20) and HCP-HP-UMEI vs HCP-HP-RMEI treatment comparisons. From the top 20 DE genes ranked by PIF, genes related to an immune response had a significantly lower expression in the rumen of wethers fed the HCP-HP-UMEI treatment when compared to the LCP-LP-UMEI treatment (*IGHM, CD177*, *ENSOARG00000014486*, *PGLYRP2*), whereas genes related to the extracellular matrix were more highly expressed in HCP-HP-UMEI relative to LCP-LP-UMEI (*COL1A1, COL3A1,* and *COL1A2*). In the rumen of wethers fed the HCP-HP-UMEI vs HCP-HP-RMEI treatments, 8 genes had a similar response to the HCP-HP-UMEI vs LCP-LP-UMEI treatment comparison (*ENSOARG00000021083, ENSOARG00000014486, ENSOARG00000002492, SLC26A9, CRNN, ENSOARG00000017398, CD177* and *PGLYRP2*).

Genes that were differentially upregulated in the rumen of wethers fed the HCP-HP-UMEI treatment compared to the LCP-LP-UMEI treatment were enriched for 39 terms including 'collagen binding', 'anatomical structure morphogenesis', 'PI3K-Akt signaling pathway', 'oxidoreductase activity' and 'positive regulation of Wnt signaling pathway'; whereas down-regulated DE genes were enriched for 24 terms including 'peptidoglycan binding', 'NF-kappa B signaling pathway', 'response to bacterium' and 'B cell receptor signaling pathway'. Genes that were differentially upregulated in the rumen of wethers fed the HCP-HP-UMEI treatment compared to the ME restricted (HCP-HP-RMEI) treatment were enriched for 26 terms including 'regulation of cellular metabolic process', 'cell cycle', 'regulation of cellular response to stress' and 'regulation of primary metabolic process'; whereas down-regulated DE genes were enriched for 35 terms including 'regulation of mRNA splicing, via spliceosome', 'regulation of cellular protein metabolic process', 'cellular response to lipid' and 'enzyme binding'.

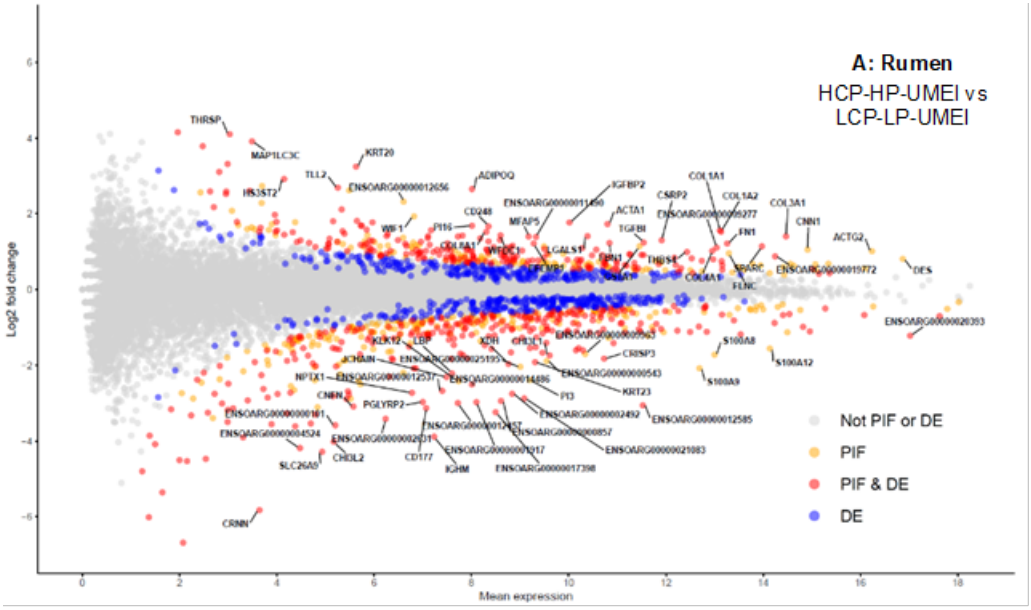


Figure 20. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the rumen wall of Merino wethers allocated to the HCP-HP-UMEI and LCP-LP-UMEI treatments. Each dot is an individual gene, see legend for colour coding.

Duodenum

There were less DE genes in the duodenum wall compared with the rumen wall. Consistent with the rumen, the majority of the DE genes in the duodenum wall were between the wethers fed the HCP-HP-UMEI vs LCP-LP-UMEI (Figure 21) and HCP-HP-UMEI vs HCP-HP-RMEI treatment comparisons.

Genes that were differentially upregulated in the duodenum wall of wethers fed the HCP-HP-UMEI treatment compared to the LCP-LP-UMEI treatment were enriched for 26 terms including 'extracellular matrix structural constituent', 'platelet-derived growth factor binding', 'cellular response to amino acid stimulus' and 'fatty acid metabolism'. Genes that were differentially upregulated in the rumen of wethers fed the HCP-HP-UMEI treatment compared to the ME restricted (HCP-HP-RMEI) treatment were enriched for eight terms including 'fatty acid metabolism' and 'extracellular space'; whereas down-regulated DE genes were enriched for 16 terms including 'structural constituent of ribosome', 'formation of cytoplasmic translation initiation complex' and 'regulation of primary metabolic process'.

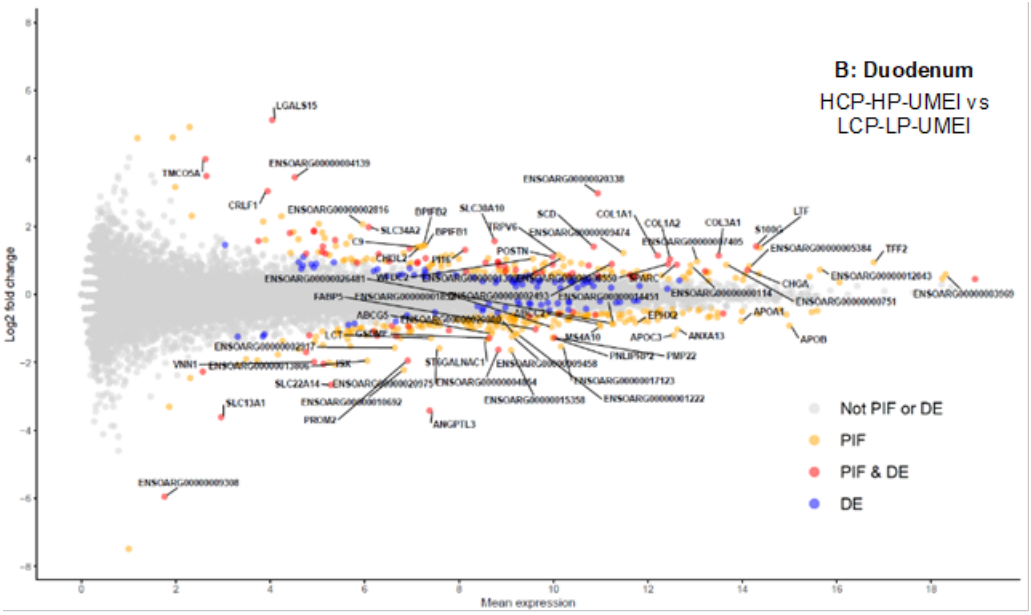


Figure 21. Effect of nutritional treatment on differential expression (DE) and phenotypic impact factor (PIF) of individual genes in the duodenum wall of Merino wethers allocated to the HCP-HP-UMEI and LCP-LP-UMEI treatments. Each dot is an individual gene, see legend for colour coding.

Uniquely different genes

Genes that are uniquely DE in one of the nutritional treatments are informative of the sensitivity of key genes to specific nutrients (CP, P, ME) in individual tissues/regions. The approach allows the answering of the following questions (Table 13),

1. What genes were responding to a lower DM intake, regardless of how the lower DM intake was derived (nutrient deficient diet or artificial restriction of available feed, and hence, intake)? (i.e. genes that were DE in HCP-HP-UMEI compared to all other treatments, but not DE between the other treatments)
2. What genes were responding uniquely to dietary restriction, but not nutrient content of the diet fed? (i.e. genes that were DE in HCP-HP-RMEI compared to all other treatments, but not DE between the other treatments)

There were no uniquely DE genes in any of the three regions of the hypothalamus.

The liver had the highest number of unique DE genes in wethers allocated to the HCP-HP-UMEI treatment. These 'nutritional treatment' unique genes are likely responding to the increased supply of nutrients compared to the other nutritional treatments. No pathways were enriched for these unique gene lists in liver, but a series of metabolically important genes were ranked highly by PIF. The genes that were uniquely lower in the liver of wethers fed the HCP-HP-UMEI treatment included those related to growth and glucose utilisation (FGF21, GHRHR), IGF binding proteins (IGFBP1, IGFBP2, and IGFBP5), response to oxidative stress (GPX3, PYCR1, OSGIN1), cell signalling (GPC3, DUSP26 and CD22), xenobiotic metabolism (FMO1), fatty acid metabolism (CYP2E1) and solute transport. Unique up-regulated DE genes in the liver included those related to signalling receptor (PAQR7), glutathione transferase (ENSOARG00000019297 and ENSOARG00000019285), inactivation of circulating cortisol via corticosteroid-binding globulin (SERPINA6), regulation of apoptosis (IFI6, LGALS1, NDRG1 and CKAP2), activation of the NF-kappa-B transcription factor (SGK1, TRIP6 and MAP3K13), urea cycling (ARG2), cell cycle (POC1A, KIF24 and CDK1), serine protease inhibition (SERPINI1 and SERPINE1), glycogen synthesis (GYG2), immune system suppression (BTLA), inhibition of glycolysis (CBFA2T3) and translation of mRNA via eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2). Genes that were uniquely lower in the rumen of wethers fed the HCP-HP-UMEI treatment included those related to epidermal development, tissue reorganisation and immune response via immunoglobin complex. Genes uniquely higher in the rumen of wethers fed the HCP-HP-UMEI treatment include those related to lipogenesis (THRSP), natriuretic peptide receptor (NPR3), inhibition of transforming growth factor beta (TGF-β) signalling (BAMBI) and immune regulation (GCA, PIANP and TRIM36).

Two genes encoding for heat shock proteins (HSPA1A and HSPA6) were uniquely higher in the rumen of restricted fed wethers (HCP-HP-RMEI), one of which (HSPA1A) was also uniquely higher in the liver. Upregulation of heat shock proteins indicate a response to cellular stress to protect the integrity of the proteins within the cell. The most highly ranked uniquely different gene in the rumen and liver of wethers fed the HCP-HP-RMEI treatment was ENSOARG00000008800 in both the rumen and liver. This gene is uncharacterised but is located within close proximity to HSPA1A on chromosome 20, and the protein from these two genes are both included in the Ensembl protein family PTHR19375_SF155.

Additional comparisons have been completed but are omitted from this Milestone report.

Table 13. List of genes that are significantly higher or lower (adjusted *P* ≤ 0.05 and fold-change > 2) in one nutritional treatment compared to the other four nutritional treatments for each tissue region in steers.

Treatment[1]	Region[2]	Relative expression	No. of genes	Genes[3]
HCP-HP-UMEI	DUO	Lower	2	*ENSOARG00000002582, CA4*
	LIV	Lower	63	*ENSOARG00000009159, FGF21, IGFBP1, GPX3, MIOX, IGFBP2, GPC3, FMO1, DIO1, CYP2E1, ENSOARG00000005450, ALDH1L2, ENSOARG00000007808, PYCR1, ENSOARG00000011663, CD22, ENSOARG00000002444, OSGIN1, ENSOARG00000025701, ARL4D, SLC12A1, GHRHR, ENSOARG00000005747, DUSP26, ENSOARG00000001890, ENSOARG00000004859, LSAMP, SLC13A2, ENSOARG00000014841, RCAN1, DENND2A, COL27A1, ATP10A, CYP26B1, DLEC1, SLC26A8, TTC9, SLC16A4, ACE2, EIF4EBP1, ENSOARG00000004703, PALB2, ROS1, SLC13A3, GRIN3A, LRATD1, NLN, NAALAD2, FKBP5, IGFBP5, PCSK4, ENSOARG00000016183, FST, SNED1, CSF3R, NRL, GPC1, SNX22, ENSOARG00000004875, CARMIL3, ENSOARG00000015875, ENSOARG00000004132, ENSOARG00000008320*
		Higher	133	*ENSOARG00000003426, PAQR7, ENSOARG00000019297, IFI6, ENSOARG00000019285, PIGR, SERPINA6, PIWIL1, LGALS1, PLA2G2F, ENSOARG00000001288, SPC24, ENSOARG00000009458, GUCA2B, ENSOARG00000008517, CST6, ETNPPL, XPNPEP2, NMRK2, RETREG1, INHBC, SGK1, TBCEL, TM4SF5, QSOX1, PAPLN, ENSOARG00000020076, MAP3K13, ENSOARG00000003412, ENSOARG00000016186, TRIP6, ENSOARG00000004828, TTC8, CRYAB, ENSOARG00000018199, ABHD3, PCDH12, MX2, NYNRIN, DEGS2, ENSOARG00000013973, ENSOARG00000014816, MEST, NDRG1, TRANK1, TNFRSF12A, ZNF185, TMED3, CKAP2, ENSOARG00000017022, ENSOARG00000001131, GPLD1, GASK1B, DCDC2, COL1A1, ADAM12, SLC28A1, CAPN13, HJURP, FKBP1B, FLRT3, CDK1, AVIL, ABCA4, PTK2B, FRMD7, ENSOARG00000005759, UBE2C, DUSP14, FAM71F1, CLDN6, ENSOARG00000000935, EIF2AK2, LAMC2, CCL25, CAPSL, PREX2, CBFA2T3, ENSOARG00000002839, CA7, MOB3B, CA9, BTLA, SLC51A, SIDT1, ENSOARG00000002874, SPNS3, GYG2, ENSOARG00000001004, SERPINE1, PLSCR1, C1orf194, TOMM40L, ENSOARG00000016499, INPP5J, ENSOARG00000019455, LYNX1, CDKL4, DYNC2LI1, FLNC, SERPINI1, HSPA12A, MAP2K6, PROX2, IL17RB, COL26A1, ENSOARG00000012752, SPTSSB, ENSOARG00000009176, ENSOARG00000018998, ENSOARG00000000115, ENSOARG00000008548, BST-2B, PCP4L1, PROCR, E2F3, ENSOARG00000017596, POC1A, KIF24, ELAPOR2, ARG2, DNAH1, DNER, ZYG11A, CCNB2, CCNF, ENSOARG00000012827, CENPL, MROH2B, NIPA1, MAPK8IP2, ELAPOR1, CPXM2*
	RUM	Lower	11	*ENSOARG00000017398, ENSOARG00000021083, ENSOARG00000002492, IGHM, CRNN, ENSOARG00000008862, KRT4, PLAUR, PSORS1C2, ACP7, CD79A*
		Higher	10	*THRSP, NPR3, FAM184B, BAMBI, GABRR3, RGL3, SLC5A5, TRIM36, GCA, PIANP*
HCP-HP-RMEI	LIV	Lower	1	*SLITRK5*
		Higher	6	*ENSOARG00000008800, HSPA1A, RASD1, ENSOARG00000004314, ENSOARG00000025856, ENSOARG00000016874*
	RUM	Lower	1	*RBBP8NL*
		Higher	4	*ENSOARG00000008800, HSPA1A, HSPA6, H1-6*

[1]Nutritional treatments with high crude-protein (CP), high phosphorus (HP) and high metabolisable energy (ME) intake (HCP-HP-UMEI), high CP, high P and restricted ME intake (LCP-LP-RMEI)
[2]Liver (LIV), and rumen (RUM) and duodenum (DUO) wall
[3]Genes listed in order of highest absolute phenotypic impact factor.

Combined phenotypic impact factor

Genes that are consistently ranked highly by the PIF metric across different tissues represent a system-wide co-ordinated response to dietary treatment in the steers. The value of the sum of all |PIF| values, albeit abstract, was calculated to help identify such genes; however, it may also rank genes with a very strong |PIF| score in one tissue. This variation needs to be explored on a case by case basis.

Most genes included in the top 20 list (Table 14) were genes that responded most in the liver or rumen of the wethers. The ENSOARG00000003744 (haptoglobin), ENSOARG00000003426 (uncharacterised) and PAQR7 (progestin and adipoQ receptor family member 7) genes were ranked distinctly higher than the rest which can be attributed to their significant responses in the liver. However, CARTPT was also included and can be attributed to its significant response in both the ARC and VMH.

Table 14. Top 20 significantly different genes ranked on total absolute phenotypic impact factor (PIF), summed across all tissues and treatment comparisons, in Merino wethers fed all nutritional treatments[1].

Gene name	Total PIF (all comparisons)	Gene description
ENSOARG00000003744	144.36	haptoglobin
ENSOARG00000003426	127.49	None
PAQR7	124.63	progestin and adipoQ receptor family member 7
ENSOARG00000009159	79.62	amine sulfotransferase-like
ENSOARG00000009308	79.19	None
FGF21	79.11	fibroblast growth factor 21
ENSOARG00000008800	78.08	None
PLA2G2F	78.07	phospholipase A2 group IIF
ENSOARG00000017398	77.44	None
LGALS1	76.49	galectin 1
COL3A1	76.03	collagen type III alpha 1 chain
HSPA1A	75.84	heat shock 70kDa protein 1A
PIGR	75.61	polymeric immunoglobulin receptor
ENSOARG00000012585	73.76	None
CARTPT	70.32	CART prepropeptide
ATF3	69.13	activating transcription factor 3
ENSOARG00000019297	67.79	glutathione S-transferase Mu 1 (GSTM1)
ENSOARG00000006050	67.72	alpha-1-acid glycoprotein
GPX3	66.01	glutathione peroxidase 3
ENSOARG00000009865	64.82	serum amyloid A-4 protein

[1]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

Regulatory impactor factor analysis

The genes that were determined to be significant RIFs were further ranked on their highest average of |RIF1| and |RIF2| within each treatment comparison with the top five significant transcription factors for each nutritional comparison within each tissue included in Table 15. The listed transcription factors include those genes which are known to control physiological processes by regulating the transcription of mRNA, as listed by the AnimalTFDB (transcription factor database) and are therefore more likely to be key regulators of the physiological differences between the steers in each treatment.

Table 15. Top five transcription factors (TF) or co-factors ranked on absolute RIF1 and RIF2[1] for nutritional treatment[2] comparisons for each tissue region[3] of the Merino wethers.

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
ARC	HCP-HP-UMEI vs HCP-HP-RMEI	HMGXB4	ENSOARG00000018373	7.18	0.75	3.97	TF	HMG-box containing 4
		ENSOARG00000013117	ENSOARG00000013117	6.90	0.53	3.71	TF	forkhead box D4
		JDP2	ENSOARG00000001796	4.60	1.85	3.22	TF	Jun dimerization protein 2
		ALX1	ENSOARG00000015379	5.73	0.44	3.08	TF	ALX homeobox 1
		ENSOARG00000009419	ENSOARG00000009419	5.67	-0.19	2.93	cofactor	zinc finger MYND-type containing 8
	HCP-HP-UMEI vs HCP-LP-UMEI	TFAP2E	ENSOARG00000019518	-3.88	-2.80	3.34	TF	transcription factor AP-2 epsilon
		ENSOARG00000000468	ENSOARG00000000468	-4.00	-2.52	3.26	TF	nuclear transcription factor Y subunit beta
		ZBTB6	ENSOARG00000019166	-4.92	-1.51	3.22	TF	zinc finger and BTB domain containing 6
		SUZ12	ENSOARG00000013017	-4.01	-2.10	3.06	cofactor	SUZ12 polycomb repressive complex 2 subunit
		ETV3	ENSOARG00000006704	-3.62	-2.10	2.86	TF	ETS translocation variant 3
	HCP-HP-UMEI vs LCP-HP-UMEI	MED8	ENSOARG00000000100	-6.13	-2.06	4.10	cofactor	mediator complex subunit 8
		CHMP1A	ENSOARG00000014268	-5.55	-1.82	3.69	cofactor	charged multivesicular body protein 1A
		PICALM	ENSOARG00000004654	-5.85	-1.45	3.65	cofactor	phosphatidylinositol binding clathrin assembly protein
		ZNF461	ENSOARG00000005312	-4.59	2.50	3.54	TF	zinc finger protein 461
		JUNB	ENSOARG00000010854	-5.10	-1.81	3.46	TF	JunB proto-oncogene, AP-1 transcription factor subunit
	HCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000001987	ENSOARG00000001987	4.79	0.93	2.86	cofactor	None
		HNF1B	ENSOARG00000003338	-4.57	-1.04	2.81	TF	HNF1 homeobox B
		ENSOARG00000015037	ENSOARG00000015037	2.85	2.72	2.78	TF	None
		TBX18	ENSOARG00000013317	-2.39	-3.16	2.78	TF	T-box transcription factor 18
		TIRAP	ENSOARG00000012557	2.28	3.24	2.76	cofactor	TIR domain containing adaptor protein
	HCP-LP-UMEI vs HCP-HP-RMEI	ENSOARG00000004242	ENSOARG00000004242	-4.90	-1.80	3.35	cofactor	transcription factor B2, mitochondrial
		SP4	ENSOARG00000010410	-3.78	-1.32	2.55	TF	Sp4 transcription factor
		ZNF133	ENSOARG00000018436	-1.09	-3.77	2.43	TF	zinc finger protein 133
		ZBTB41	ENSOARG00000015070	-3.00	-1.80	2.40	TF	zinc finger and BTB domain containing 41
		PCGF1	ENSOARG00000012749	-2.51	-2.13	2.32	cofactor	polycomb group ring finger 1
	HCP-LP-UMEI vs LCP-HP-UMEI	ENSOARG00000007917	ENSOARG00000007917	-5.78	1.32	3.55	TF	None
		SHOX2	ENSOARG00000002613	-3.50	3.15	3.33	TF	short stature homeobox 2
		ENSOARG00000017535	ENSOARG00000017535	-4.38	1.89	3.13	TF	None
		ENSOARG00000011916	ENSOARG00000011916	-3.31	-2.72	3.02	TF	None
		OVOL3	ENSOARG00000005260	-2.85	2.82	2.84	TF	ovo like zinc finger 3
	HCP-LP-UMEI vs LCP-LP-UMEI	RBPMS2	ENSOARG00000020727	-4.59	0.36	2.47	cofactor	RNA binding protein, mRNA processing factor 2

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
DUO	LCP-LP-UMEI vs HCP-HP-RMEI	LHX2	ENSOARG00000013742	-3.51	1.41	2.46	TF	LIM/homeobox protein Lhx2
		PSIP1	ENSOARG00000014002	-1.44	3.15	2.29	cofactor	PC4 and SFRS1 interacting protein 1
		ZMAT3	ENSOARG00000020713	-2.76	1.79	2.27	TF	zinc finger matrin-type 3
		ENSOARG00000013839	ENSOARG00000013839	-3.54	-0.93	2.23	cofactor	transcription initiation factor TFIID subunit 11
		FOXJ3	ENSOARG00000020401	-4.50	-2.92	3.71	TF	forkhead box J3
		SERTAD3	ENSOARG00000011370	-4.71	-2.44	3.57	cofactor	SERTA domain containing 3
		KLF10	ENSOARG00000017131	-3.74	-3.15	3.44	TF	Kruppel like factor 10
		ENSOARG00000004242	ENSOARG00000004242	-3.71	-3.14	3.42	cofactor	transcription factor B2, mitochondrial
		GZF1	ENSOARG00000005257	-4.67	-1.75	3.21	TF	GDNF inducible zinc finger protein 1
	HCP-HP-UMEI vs HCP-HP-RMEI	ENSOARG00000018472	ENSOARG00000018472	2.12	2.76	2.44	TF	None
		TAF9B	ENSOARG00000018853	2.90	1.92	2.41	cofactor	TATA-box binding protein associated factor 9b
		MECOM	ENSOARG00000000899	-3.23	-1.24	2.24	TF	MDS1 and EVI1 complex locus
		TASOR	ENSOARG00000015004	-1.92	2.52	2.22	cofactor	transcription activation suppressor
		SMAD1	ENSOARG00000009089	-3.43	-1.00	2.22	TF	SMAD family member 1
	HCP-HP-UMEI vs HCP-LP-UMEI	TFAP4	ENSOARG00000003120	3.81	1.51	2.66	TF	transcription factor AP-4
		TAB1	ENSOARG00000016603	3.24	2.00	2.62	cofactor	TGF-beta activated kinase 1 (MAP3K7) binding protein 1
		ASH2L	ENSOARG00000001208	3.08	2.15	2.62	cofactor	ASH2 like, histone lysine methyltransferase complex subunit
		CSRNP2	ENSOARG00000017411	2.60	2.57	2.58	TF	cysteine and serine rich nuclear protein 2
		SIRT1	ENSOARG00000004826	3.53	1.62	2.57	cofactor	sirtuin 1
	HCP-HP-UMEI vs LCP-HP-UMEI	SETD7	ENSOARG00000013062	4.91	3.14	4.03	cofactor	SET domain containing 7, histone lysine methyltransferase
		ZFPM2	ENSOARG00000015495	3.35	3.16	3.26	TF	zinc finger protein, FOG family member 2
		HDAC9	ENSOARG00000009641	3.71	2.79	3.25	cofactor	histone deacetylase 9
		LMX1A	ENSOARG00000011324	4.81	1.41	3.11	TF	LIM homeobox transcription factor 1 alpha
		ENSOARG00000013344	ENSOARG00000013344	4.87	1.29	3.08	TF	None
	HCP-HP-UMEI vs LCP-LP-UMEI	ZNF397	ENSOARG00000005768	2.30	-2.81	2.56	TF	zinc finger protein 397
		SIX2	ENSOARG00000006034	-2.02	-2.83	2.42	TF	SIX homeobox 2
		PRPF6	ENSOARG00000008776	2.42	2.13	2.27	cofactor	pre-mRNA processing factor 6
		LYL1	ENSOARG00000009454	-1.76	-2.75	2.25	TF	LYL1 basic helix-loop-helix family member
		ENSOARG00000001296	ENSOARG00000001296	2.65	1.79	2.22	TF	zinc finger protein 696
	HCP-LP-UMEI vs HCP-HP-RMEI	TAF1D	ENSOARG00000002155	-4.08	1.48	2.78	cofactor	TATA-box binding protein associated factor, RNA polymerase I subunit D
		TNP1	ENSOARG00000019426	-4.78	-0.73	2.75	cofactor	transition protein 1
		ENSOARG00000009729	ENSOARG00000009729	-4.64	-0.83	2.73	cofactor	None
		MCIDAS	ENSOARG00000007921	-3.45	-1.40	2.42	cofactor	multiciliate differentiation and DNA synthesis associated cell cycle protein

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
		NR2E3	ENSOARG00000018808	-2.63	-2.12	2.38	TF	nuclear receptor subfamily 2 group E member 3
	HCP-LP-UMEI vs LCP-HP-UMEI	ENSOARG00000015955	ENSOARG00000015955	-6.68	0.41	3.55	TF	None
		ENSOARG00000012595	ENSOARG00000012595	-5.67	-0.84	3.25	cofactor	WW domain containing E3 ubiquitin protein ligase 1
		PAX8	ENSOARG00000020812	-5.13	-1.36	3.25	TF	paired box 8
		ANKRD1	ENSOARG00000015908	-5.01	1.28	3.15	cofactor	ankyrin repeat domain 1
		SLC25A15	ENSOARG00000009523	-4.57	1.40	2.98	cofactor	solute carrier family 25 member 15
	LCP-HP-UMEI vs HCP-HP-RMEI	ENSOARG00000015955	ENSOARG00000015955	-5.00	-1.74	3.37	TF	None
		MED8	ENSOARG00000000100	-6.02	0.20	3.11	cofactor	mediator complex subunit 8
		PADI4	ENSOARG00000011018	-3.67	-1.87	2.77	cofactor	peptidyl arginine deiminase 4
		NKX1-2	ENSOARG00000010209	-3.49	-2.00	2.75	TF	NK1 homeobox 2
		RORB	ENSOARG00000012659	-5.15	-0.31	2.73	TF	RAR related orphan receptor B
	LCP-HP-UMEI vs LCP-LP-UMEI	SP5	ENSOARG00000002449	4.62	-1.30	2.96	TF	Sp5 transcription factor
		RB1	ENSOARG00000008246	-2.95	-2.73	2.84	cofactor	RB transcriptional corepressor 1
		ENSOARG00000000974	ENSOARG00000000974	-2.08	-3.49	2.79	cofactor	histone H1.4-like
		HLF	ENSOARG00000005301	3.81	-1.71	2.76	TF	HLF transcription factor, PAR bZIP family member
		EBF3	ENSOARG00000014932	3.69	-1.82	2.76	TF	EBF transcription factor 3
	LCP-LP-UMEI vs HCP-HP-RMEI	BRCA2	ENSOARG00000011179	2.69	2.32	2.51	cofactor	BRCA2 DNA repair associated
		NRIP1	ENSOARG00000001166	2.00	2.47	2.24	cofactor	nuclear receptor interacting protein 1
		HINFP	ENSOARG00000013643	1.89	2.57	2.23	TF	histone H4 transcription factor
		SLC30A9	ENSOARG00000013974	2.84	1.50	2.17	cofactor	solute carrier family 30 member 9
		ENG	ENSOARG00000011387	2.36	1.89	2.13	cofactor	endoglin
LHA	HCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000002014	ENSOARG00000002014	4.88	1.33	3.10	TF	GC-rich promoter binding protein 1 like 1
		HOXA1	ENSOARG00000009850	4.59	1.54	3.06	TF	homeobox A1
		FOS	ENSOARG00000001783	2.30	3.54	2.92	TF	Fos proto-oncogene, AP-1 transcription factor subunit
		ZNF235	ENSOARG00000009044	4.25	1.59	2.92	TF	zinc finger protein 235
		TTF2	ENSOARG00000020266	3.01	2.53	2.77	cofactor	transcription termination factor 2
LIV	HCP-HP-UMEI vs HCP-HP-RMEI	ENSOARG00000000192	ENSOARG00000000192	2.23	-3.07	2.65	TF	zinc finger protein 347-like
		TLE2	ENSOARG00000013277	2.53	2.67	2.60	cofactor	TLE family member 2, transcriptional corepressor
		KAT2B	ENSOARG00000015796	-2.90	-2.20	2.55	cofactor	lysine acetyltransferase 2B
		LRRFIP1	ENSOARG00000019011	3.03	-1.88	2.46	TF	LRR binding FLII interacting protein 1
		WNT3A	ENSOARG00000006544	2.81	-2.07	2.44	cofactor	Wnt family member 3A
	HCP-HP-UMEI vs HCP-LP-UMEI	DPF2	ENSOARG00000014337	3.92	3.17	3.55	cofactor	double PHD fingers 2
		ELK4	ENSOARG00000004182	4.76	1.99	3.38	TF	ETS transcription factor ELK4

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
		TGFB1I1	ENSOARG00000009529	3.83	2.82	3.33	cofactor	transforming growth factor beta 1 induced transcript 1
		ARNTL2	ENSOARG00000019779	2.71	3.64	3.17	TF	aryl hydrocarbon receptor nuclear translocator like 2
		ALPK3	ENSOARG00000013306	3.72	2.50	3.11	cofactor	alpha kinase 3
	HCP-HP-UMEI vs LCP-HP-UMEI	TDP2	ENSOARG00000005997	4.04	2.32	3.18	cofactor	tyrosyl-DNA phosphodiesterase 2
		ZNF436	ENSOARG00000007586	4.92	0.78	2.85	TF	zinc finger protein 436
		SMAD4	ENSOARG00000004662	2.67	2.95	2.81	TF	SMAD family member 4
		DPF2	ENSOARG00000014337	2.44	2.93	2.69	cofactor	double PHD fingers 2
		ELK4	ENSOARG00000004182	3.14	2.23	2.68	TF	ETS transcription factor ELK4
	HCP-HP-UMEI vs LCP-LP-UMEI	FGFR2	ENSOARG00000005375	4.16	1.68	2.92	cofactor	fibroblast growth factor receptor 2
		ENSOARG00000012881	ENSOARG00000012881	-2.13	2.98	2.56	TF	TEA domain transcription factor 2
		ZNF366	ENSOARG00000005075	3.54	1.49	2.52	TF	zinc finger protein 366
		ARID5A	ENSOARG00000013883	4.25	0.62	2.44	TF	AT-rich interaction domain 5A
		ALPK3	ENSOARG00000013306	-1.57	3.23	2.40	cofactor	alpha kinase 3
	HCP-LP-UMEI vs HCP-HP-RMEI	CREM	ENSOARG00000016854	-2.56	3.03	2.79	TF	cAMP responsive element modulator
		ENSOARG00000000925	ENSOARG00000000925	-2.46	3.01	2.74	cofactor	EP300 interacting inhibitor of differentiation 1
		NFATC3	ENSOARG00000003342	4.03	-1.41	2.72	TF	nuclear factor of activated T cells 3
		TAF13	ENSOARG00000019038	2.23	-3.15	2.69	cofactor	TATA-box binding protein associated factor 13
		HIF1AN	ENSOARG00000015423	-3.26	1.99	2.62	cofactor	hypoxia inducible factor 1 subunit alpha inhibitor
	HCP-LP-UMEI vs LCP-HP-UMEI	TSC22D2	ENSOARG00000004604	2.82	-4.28	3.55	TF	TSC22 domain family member 2
		ENSOARG00000000925	ENSOARG00000000925	4.14	-2.77	3.46	cofactor	EP300 interacting inhibitor of differentiation 1
		HABP4	ENSOARG00000008461	3.71	-2.97	3.34	cofactor	hyaluronan binding protein 4
		LRRFIP2	ENSOARG00000016501	3.45	-3.18	3.31	TF	LRR binding FLII interacting protein 2
		CDX2	ENSOARG00000012435	2.93	-3.58	3.26	TF	caudal type homeobox 2
	HCP-LP-UMEI vs LCP-LP-UMEI	HAND2	ENSOARG00000015352	-3.92	1.57	2.75	TF	heart and neural crest derivatives expressed 2
		GTF2A1	ENSOARG00000002918	-3.62	1.78	2.70	cofactor	general transcription factor IIA subunit 1
		FOXP1	ENSOARG00000009693	-2.66	2.62	2.64	TF	forkhead box P1
		PBX4	ENSOARG00000007632	-2.37	2.80	2.58	TF	PBX homeobox 4
		RALGAPA1	ENSOARG00000007701	-3.00	2.12	2.56	cofactor	Ral GTPase activating protein catalytic subunit alpha 1
	LCP-HP-UMEI vs HCP-HP-RMEI	PSEN1	ENSOARG00000000358	3.81	-4.09	3.95	cofactor	presenilin 1
		NOD2	ENSOARG00000017441	3.60	-3.73	3.66	cofactor	nucleotide binding oligomerization domain containing 2

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
RUM	LCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000018104	ENSOARG00000018104	4.12	-3.07	3.59	TF	None
		ZBTB8A	ENSOARG00000020930	3.49	-3.33	3.41	TF	zinc finger and BTB domain containing 8A
		MIDEAS	ENSOARG00000000680	2.82	-3.59	3.20	TF	mitotic deacetylase associated SANT domain protein
		EOMES	ENSOARG00000014467	2.98	3.95	3.47	TF	eomesodermin
		COPS2	ENSOARG00000021006	5.46	1.31	3.38	cofactor	COP9 signalosome subunit 2
		ENSOARG00000015037	ENSOARG00000015037	3.64	2.85	3.25	TF	None
		MYRF	ENSOARG00000013482	5.56	-0.50	3.03	TF	myelin regulatory factor
		DDX5	ENSOARG00000015045	4.19	1.87	3.03	cofactor	DEAD-box helicase 5
	LCP-LP-UMEI vs HCP-HP-RMEI	MAF1	ENSOARG00000014895	-3.09	2.76	2.92	TF	MAF1 homolog, negative regulator of RNA polymerase III
		ERBB2	ENSOARG00000011865	-2.25	3.10	2.68	cofactor	erb-b2 receptor tyrosine kinase 2
		SOX8	ENSOARG00000014473	4.78	-0.37	2.57	TF	SRY-box transcription factor 8
		ENSOARG00000003745	ENSOARG00000003745	-1.83	2.89	2.36	cofactor	peptidylprolyl isomerase D
		NCOR2	ENSOARG00000000357	-2.05	2.66	2.36	TF	nuclear receptor corepressor 2
	HCP-HP-UMEI vs HCP-HP-RMEI	HINFP	ENSOARG00000013643	-4.08	-2.58	3.33	TF	histone H4 transcription factor
		FOXL1	ENSOARG00000011648	4.65	1.77	3.21	TF	forkhead box L1
		HIVEP1	ENSOARG00000014356	2.72	3.29	3.00	TF	HIVEP zinc finger 1
		MALT1	ENSOARG00000005525	3.92	1.92	2.92	cofactor	MALT1 paracaspase
		RUNX1	ENSOARG00000013599	-4.75	-0.97	2.86	TF	RUNX family transcription factor 1
	HCP-HP-UMEI vs HCP-LP-UMEI	LPIN2	ENSOARG00000010081	-6.44	-1.71	4.07	cofactor	lipin 2
		HOXC5	ENSOARG00000016284	-6.13	-1.52	3.82	TF	homeobox C5
		ZNF341	ENSOARG00000008687	-6.89	-0.58	3.73	TF	zinc finger protein 341
		ENSOARG00000016973	ENSOARG00000016973	-6.73	-0.33	3.53	TF	None
		E2F3	ENSOARG00000009655	-4.93	-2.11	3.52	TF	E2F transcription factor 3
	HCP-HP-UMEI vs LCP-HP-UMEI	ENSOARG00000001815	ENSOARG00000001815	2.18	2.92	2.55	TF	None
		CITED2	ENSOARG00000002608	-3.62	-1.40	2.50	cofactor	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2
		LBX2	ENSOARG00000012737	1.96	2.83	2.40	TF	ladybird homeobox 2
		ENSOARG00000001314	ENSOARG00000001314	-4.17	-0.61	2.39	TF	zinc finger protein 628
		RPRD1B	ENSOARG00000018435	-2.50	-2.18	2.34	cofactor	regulation of nuclear pre-mRNA domain containing 1B
	HCP-HP-UMEI vs LCP-LP-UMEI	ENSOARG00000020934	ENSOARG00000020934	-6.79	-0.35	3.57	TF	zinc finger and BTB domain containing 8B
		ENSOARG00000007662	ENSOARG00000007662	-4.76	-1.22	2.99	cofactor	scaffold attachment factor B
		TBX18	ENSOARG00000013317	-4.94	1.04	2.99	TF	T-box transcription factor 18
		AGO2	ENSOARG00000003763	-4.46	-1.46	2.96	cofactor	argonaute RISC catalytic component 2
		ENSOARG00000018861	ENSOARG00000018861	3.68	2.12	2.90	TF	None
	HCP-LP-UMEI vs HCP-HP-RMEI	PAK6	ENSOARG00000020143	2.19	2.76	2.48	cofactor	p21 (RAC1) activated kinase 6
		ENSOARG00000007837	ENSOARG00000007837	1.81	2.91	2.36	TF	progesterone receptor

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
	HCP-LP-UMEI vs LCP-HP-UMEI	MED24	ENSOARG00000012736	-3.06	1.61	2.34	cofactor	mediator complex subunit 24
		TBX5	ENSOARG00000006614	-2.01	2.36	2.19	TF	T-box transcription factor 5
		ENSOARG00000003162	ENSOARG00000003162	-2.30	-2.03	2.16	TF	None
		H2AZ1	ENSOARG00000013955	-4.63	-1.79	3.21	cofactor	histone H2A.Z
		ENSOARG00000005496	ENSOARG00000005496	-3.35	-2.92	3.14	cofactor	general transcription factor IIH subunit 2
		ZNF514	ENSOARG00000014179	-3.08	-2.98	3.03	TF	zinc finger protein 514
		GTF2H5	ENSOARG00000004345	-4.83	-1.19	3.01	cofactor	general transcription factor IIH subunit 5
		TDP2	ENSOARG00000005997	-5.09	-0.77	2.93	cofactor	tyrosyl-DNA phosphodiesterase 2
	LCP-HP-UMEI vs HCP-HP-RMEI	MED31	ENSOARG00000001213	-4.87	-2.20	3.54	cofactor	mediator complex subunit 31
		ARNTL2	ENSOARG00000019779	-3.00	-3.64	3.32	TF	aryl hydrocarbon receptor nuclear translocator like 2
		PRRX1	ENSOARG00000012029	-4.22	-2.31	3.27	TF	paired related homeobox 1
		CRK	ENSOARG00000012278	-5.47	1.00	3.23	cofactor	CRK proto-oncogene, adaptor protein
		DTX1	ENSOARG00000007699	-3.14	-3.18	3.16	cofactor	deltex E3 ubiquitin ligase 1
	LCP-HP-UMEI vs LCP-LP-UMEI	PBX4	ENSOARG00000007632	4.21	1.97	3.09	TF	PBX homeobox 4
		FOS	ENSOARG00000001783	3.83	2.20	3.01	TF	Fos proto-oncogene, AP-1 transcription factor subunit
		SS18L2	ENSOARG00000003486	3.29	2.48	2.88	cofactor	SS18 like 2
		BCOR	ENSOARG00000001020	3.27	2.44	2.85	cofactor	BCL6 corepressor
		ENSOARG00000001314	ENSOARG00000001314	4.12	1.24	2.68	TF	zinc finger protein 628
	LCP-LP-UMEI vs HCP-HP-RMEI	PURB	ENSOARG00000008527	3.59	1.77	2.68	TF	purine rich element binding protein B
		ZNF354A	ENSOARG00000006794	2.39	-2.73	2.56	TF	zinc finger protein 354A
		THAP2	ENSOARG00000014616	3.14	1.96	2.55	TF	THAP domain containing 2
		TAF5	ENSOARG00000003445	-3.33	-1.63	2.48	cofactor	TATA-box binding protein associated factor 5
		KLF3	ENSOARG00000010144	-3.60	1.27	2.43	TF	Kruppel like factor 3
	HCP-HP-UMEI vs HCP-HP-RMEI	SETD7	ENSOARG00000013062	-5.59	-1.42	3.51	cofactor	SET domain containing 7, histone lysine methyltransferase
		PHF1	ENSOARG00000009736	-6.11	-0.57	3.34	cofactor	PHD finger protein 1
		DAXX	ENSOARG00000009519	-5.15	-1.38	3.27	cofactor	death domain associated protein
		PCGF5	ENSOARG00000015934	-4.57	-1.93	3.25	cofactor	polycomb group ring finger 5
		NFKBIZ	ENSOARG00000018628	-4.77	1.41	3.09	cofactor	NFKB inhibitor zeta
		RAF1	ENSOARG00000015630	4.33	1.92	3.12	cofactor	Raf-1 proto-oncogene, serine/threonine kinase
	HCP-HP-UMEI vs HCP-LP-UMEI	SUPT16H	ENSOARG00000019652	3.54	2.35	2.94	cofactor	SPT16 homolog, facilitates chromatin remodeling subunit
		MLXIPL	ENSOARG00000012375	3.03	2.74	2.89	TF	MLX interacting protein like
		KLF1	ENSOARG00000010245	3.27	2.46	2.86	TF	Kruppel like factor 1
		ZNFX1	ENSOARG00000012500	-3.18	-2.54	2.86	TF	zinc finger NFX1-type containing 1

Region	Treatment comparison	Gene name	Ensembl ID	RIF1	RIF2	Average |RIF|	TF type	Gene description
VMH	HCP-HP-UMEI vs LCP-LP-UMEI	BCL11B	ENSOARG00000000266	-5.60	-1.44	3.52	TF	BAF chromatin remodeling complex subunit BCL11B
		KDM8	ENSOARG00000018751	-4.16	-2.39	3.27	cofactor	lysine demethylase 8
		C6orf89	ENSOARG00000014088	-6.18	0.10	3.14	cofactor	chromosome 20 C6orf89 homolog
		HES1	ENSOARG00000020422	-5.05	-1.18	3.12	TF	hes family bHLH transcription factor 1
		ARNT2	ENSOARG00000015391	-3.60	-2.53	3.07	TF	Ovis aries aryl hydrocarbon receptor nuclear translocator 2 (ARNT2), mRNA.
	HCP-LP-UMEI vs HCP-HP-RMEI	OVOL2	ENSOARG00000018244	-5.82	-0.87	3.34	TF	ovo like zinc finger 2
		CHCHD3	ENSOARG00000006757	-6.13	-0.49	3.31	cofactor	coiled-coil-helix-coiled-coil-helix domain containing 3
		MRTFA	ENSOARG00000017567	-5.04	-1.06	3.05	cofactor	myocardin related transcription factor A
		ENSOARG00000016973	ENSOARG00000016973	-4.18	-1.76	2.97	TF	None
		ETV1	ENSOARG00000007763	-4.43	-1.51	2.97	TF	ETS variant transcription factor 1
	HCP-LP-UMEI vs LCP-LP-UMEI	TFAP2E	ENSOARG00000019518	5.33	2.47	3.90	TF	transcription factor AP-2 epsilon
		SUPT5H	ENSOARG00000006174	6.00	1.21	3.60	cofactor	SPT5 homolog, DSIF elongation factor subunit
		ZNF574	ENSOARG00000008200	4.46	2.05	3.26	TF	zinc finger protein 574
		TTF1	ENSOARG00000006189	5.21	-0.87	3.04	TF	transcription termination factor 1
		ERCC2	ENSOARG00000009857	4.37	1.60	2.99	cofactor	ERCC excision repair 2, TFIIH core complex helicase subunit

[1]Regulatory impact factor (RIF1, RIF2). RIF analysis was not conducted for regions with less than two differentially expressed genes.

[2]Nutritional treatments with high (H) or low (L) crude-protein (CP), phosphorus (P) and ad libitum (U) or restricted (R) metabolisable energy (ME) intake (HCP-HP-RMEI, HCP-HP-UMEI, HCP-LP-UMEI, LCP-HP-UMEI, LCP-LP-UMEI).

[3]Arcuate hypothalamus (ARC), ventromedial hypothalamus (VMH), lateral hypothalamus (LHA), liver (LIV), and rumen (RUM) and duodenum (DUO) wall.

Review

Background context

Variability in feed intake by ruminants occurs in response to different nutrient content of the diet [e.g. 20 to 50% reduction in intake in response to protein and phosphorus deficiency in cattle (Panjaitan et al. 2010; Quigley et al 2016)], health status [e.g. 20 to 90% reduction in intake of growing lambs when immune function is compromised under parasite infections (Dynes, 1993)], genotype [e.g. differences in residual feed intake (RFI) between individual animals], diet transitions (feedlots, live export), heat stress and when animals undergo compensatory liveweight gain (DaSilva, 2017). Low liveweight gain, or liveweight loss, occurs in ruminants when grazing nutrient deficient diets and this is due to a reduction in feed intake. For example, steers fed a diet representative of wet season pasture low in phosphorus (P) consumed approximately 50% less metabolisable energy (ME) than steers fed the same diet with adequate P (Quigley et al. 2016) resulting in a 0.8 kg/day difference in liveweight gain. Similar reductions in intake are evident when dry season pastures or crop residues low in crude protein (CP) and/or ME and high in fibre are consumed by grazing ruminants. Forbes (2007) proposed that intake is regulated by the additive effects of discomforts arising from nutrient deficiencies or excesses (protein, energy, phosphorus, fibre) that deviate from the requirements of the animal. This manifests in animals adjusting intake to minimise this discomfort (Minimal Total Discomfort) typically resulting in a reduction in intake to avoid the discomfort associated with nutrient imbalances.

A metabolic regulation of intake of ruminants fed a low CP roughage diet (Egan, 1965) and low P diet (Milton and Ternouth, 1985) has long been established. Whilst physical factors will ultimately set the upper limit of intake of a low quality diet (Detmann et al. 2014) there is significant capacity to increase intake before these physical limits are reached in diets that are low in CP and P. Even when diets are balanced for nutrients some classes of cattle (i.e. dairy cows in early lactation) demonstrate a capacity to increase intake above theoretical predicted maximum intakes. Regardless of the mechanism, feed intake is ultimately controlled in the hypothalamus (located in the brain) in the central nervous system (CNS) which integrates metabolic (leptin, insulin, ghrelin), nutrient (glucose, amino acid, fatty acid) and physical signals from other tissues (gastro-intestinal, liver, adipose, muscle) to activate hunger or satiety neurons which motivate an animal to eat or not (Sartin et al. 2010). These dietary signals are not independent but are proposed to be additive and are largely integrated through the arcuate nuclei (ARC) of the hypothalamus resulting in an increase or decrease in feed intake mediated through nuclei in the lateral (LHA) and paraventricular and ventromedial (VMH) regions of the hypothalamus. Whilst the work of Egan (1965) and subsequent studies have demonstrated that both metabolic and physical mechanisms regulate intake, very few studies in the past 50 years in ruminants have attempted to understand and integrate the underlying control by genes/gene pathways across the peripheral and central tissues.

Neural transmitters ultimately involved in the regulation of intake and energy balance include neuropeptide-Y (NPY), agouti-related protein (AgRP), melanin concentrating hormone (MCH), orexin (ORX) (increase intake) and cocaine- and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC) (suppress intake). A range of short-term infusion studies have demonstrated direct effects of these neurotransmitters on feed intake in sheep (for examples, NPY infusion see Miner et al. 1989, AgRP infusion see Wagner et al. 2004, MCH infusion see Whitlock et al. 2005, ORX see Sartin et al. 2001) and rodents (for examples, CART infusion see Rohner-Jeanrenaud et al. 2002). However it is unknown how these factors are stimulated or inhibited in response to nutrient deficient diets in ruminants, or in fact they elicit the same response when animals are fed nutrient deficient diets. We have recently developed methods to dissect the regions of the hypothalamus that control food intake in sheep and cattle. We have conducted preliminary transcriptome wide gene expression analysis to identify differential gene expression in two regions of the hypothalamus in sheep (Quigley et al. 2016). Preliminary results identified 4000 to 6000 differentially expressed genes in different regions of the hypothalamus of sheep fed the same diet, but a much smaller number (~20) of differentially expressed genes within a region of the hypothalamus in response to diet quality and intake (Quigley et al. 2016). Some of these differentially expressed genes were also identified in the arcuate nuclei of Angus-sired steers divergent in residual feed intake (Perkins et al. 2014).

Previous studies have demonstrated therapeutic and nutritional treatments that can increase intake in ruminants. For example, post-rumen infusion of casein increased intake of a low quality (<4% CP) chaff by sheep (Egan, 1965), injection of Brotizolam (Dynes et al. 1992) or SD33 (Obese et al. 2007) in the circulation or Loxiglumide in the brain (Dynes et al. 1998) increased intake in sheep. However all these approaches are invasive and not possible to implement under commercial conditions. There is increasing research on the use of pharmaceutical agents to stimulate appetite and alternative therapies to transport agents (agonists/antagonists) across the blood brain barrier in a range of human disease models and neurodegenerative disorders. It is proposed that, once developed, such methods may be able to deliver compounds (agonists/antagonists) into the brain to target key genes/gene pathways to treat diseases, and these methods may be transferable to a range of scenarios in ruminants, including the stimulation of feed intake of nutrient deficient diets.

The review covers the following sections,

1. Anatomical regions of the central nervous system implicated in feed intake
2. Orexigenic neuroendocrine and hormonal factors
3. Antagonists of anorexigenic factors
4. Anorexia nervosa as a model for suppression of feeding
5. Appetite stimulating pharmaceutical agents
6. Approaches to target the central nervous systems

Comprehensive reviews of the neuroendocrine, hormonal and physiological regulation of intake in ruminants have been undertaken recently by Roche et al. (2008), Sartin et al. (2010), Sartin et al. (2011), Daniel et al. (2013), Innes (2021). As such only a summary of the key known factors and their reported roles in intake regulation in ruminants is presented below and more detailed mechanistic descriptions are found in the more comprehensive reviews and the source papers. Similarly, recent reviews by Dong (2018), Upadhyay (2014) and Lu et al. (2014) describe methods to target the CNS including approaches to cross or circumnavigate the blood-brain barrier.