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Differential voluntary feed intake and whole transcriptome profiling in the hypothalamus of young sheep offered CP and phosphorus-deficient diets

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ABSTRACT

A reduction in voluntary feed intake is observed in ruminants consuming nutrient-deficient diets, such as those with a low CP or P content, and has been attributed to active metabolic regulation, rather than a physical constraint. The hypothalamus is the key integrator of feed intake regulation in mammals. The objectives of this experiment were to (1) establish a model of metabolic feed intake regulation in ruminants consuming diets of variable CP and P content, and (2) determine key biochemical pathways and influential points of regulation within the hypothalamus. Merino wethers [n = 40; 23.7 ± 1.4 kg liveweight (mean ± SD)] were fed one of five dietary treatments (n = 8/treatment) for 63 days in individual pens. The treatments included targeted combinations of high (**H**) and low (**L**) CP (110 and 55 g/kg DM) and high and low P (2.5 and 0.7 g/kg DM) with 9 MJ metabolisable energy (**ME**) per kg DM which were fed *ad libitum* (**UMEI**; unrestricted ME intake) resulting in four experimental diets (HCP-HP-UMEI, LCP-HP-UMEI, HCP-LP-UMEI and LCP-LP-UMEI). An additional nutritional treatment (HCP-HP-RMEI) restricted intake of the HCP-HP diet to an equivalent ME intake of wethers consuming the LCP-LP-UMEI treatment. Wethers offered the LCP-HP-UMEI, HCP-LP-UMEI and LCP-LP-UMEI treatments consumed 42, 32 and 49% less total DM ($P \leq 0.05$), respectively than the HCP-HP-UMEI treatment, and this was not attributable to any physical limitation of the rumen. Plasma concentrations of urea nitrogen and inorganic phosphate indicated that these nutrient deficiencies were successfully established. To assess potential mechanisms, RNA-seq was conducted on samples from the arcuate nucleus (**ARC**), ventromedial hypothalamus and lateral hypothalamus of the wethers, yielding a total of 301, 8 and 148 differentially expressed genes across all pairwise comparisons, respectively. The expression of *NPY*, *AGRP* and *CARTPT*, known for their regulatory role in mammalian feed intake regulation, had a similar transcriptional response in the ARC of wethers consuming nutrient-deficient treatments and those consuming a ME-restricted treatment, despite these wethers expressing behaviours indicative of satiated and hungry states, respectively. In addition, genes involved with glycolysis (*TPI1*), the citric acid cycle (*CS, OGDH, GLUD1, GOT1*) and oxidative phosphorylation (*COX5A, ATP5MC1, ATP5F1B, ATP5MC3*) were downregulated in the ARC of wethers fed a nutrient deficient (LCP-LP-UMEI) relative to the non-deficient (HCP-HP-UMEI) treatment. In summary, a model for voluntary feed intake restriction was established to determine genome-wide molecular changes in the hypothalamus of young ruminants.

Implications

The role of the hypothalamus in the regulation of feed intake in ruminants consuming diets deficient in CP and/or P is poorly understood. Young wethers (castrated male sheep) offered diets deficient in CP, P or both, consumed 42, 32, and 49% less than wethers fed a non-deficient diet. The hypothalamic gene expression of these 'satiated' wethers were similar to 'hungry' wethers

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fed a restricted intake treatment. These results identify potential gene or gene pathway targets to manipulate feed intake in ruminants consuming nutrient-deficient diets.

Introduction

Voluntary feed intake in ruminants is regulated by an integration of numerous physical and metabolic signals within the central nervous system. These signals collectively represent the animal's nutrient status, ambient environment, social experiences and diet characteristics [reviewed in Baile and Forbes (1974) and Roche et al. (2008)]. Voluntary feed intake is also a critical determinant of liveweight (**LW**) gain in growing ruminants. For example, ruminants grazing nutrient-deficient diets in northern Australia often experience low LW gain or LW loss due to low DM intake.

Intake suppression is observed in ruminants consuming very low-quality diets, such as diets with an extremely low CP or P content. For example, in regions of northern Australia with low soil P, the reduction in CP content of pastures in the dry season is considered the first limiting nutrient, despite both a P and CP deficiency at this time, whereas in the wet season, P is considered the first limiting nutrient in these pastures. It is likely that the contribution of metabolic signals (Forbes, 2007) suppresses the intake of ruminants consuming these diets before any physical constraint (i.e. rumen fill) on intake occurs (Poppi et al., 1994; Panjaitan et al., 2010).

The capacity of a low CP content of a diet to reduce feed intake in ruminants is commonly associated with reduced microbial growth. In turn this leads to poorer digestion, a longer retention time of digesta in the rumen, and increased rumen fill that apparently results in satiety (Faverdin, 1999). However, previous studies demonstrate that when the CP content of the diet is low (<60 g CP/kg DM) local or circulating (e.g., hormones) metabolic factors may be responsible for reducing feed intake independently of physical effects in both sheep (Egan and Moir, 1965; Egan, 1977; Poppi et al., 1994; Quigley et al., 2012a) and cattle (Bandyk et al., 2001; Wickersham et al., 2004; Panjaitan et al., 2010). A similar phenomenon is also true for feed intake of ruminants in response to a P deficiency in the diet. Growing lambs had a 30–40% reduction in feed intake when consuming diets deficient in P, regardless of Ca intake (Field et al., 1975; Ternouth and Sevilla, 1990). Reductions in feed intake due to dietary P deficiency are also known to be independent of physical effects, such as rate of passage and rate of digestion (Sevilla, 1984; Milton and Ternouth, 1985). It is also generally understood that there is no additive effect of diet CP and P deficiencies on voluntary feed intake (Ternouth et al., 1993).

Whilst various models of nutrient-mediated reductions in feed intake have been reported, very few studies have attempted to identify the underlying molecular mechanisms responsible in the hypothalamus. Identification of these mechanisms will inform potential targets for further research on novel intervention strategies to minimise the requirement for supplementation of grazing animals in extensive production systems.

The hypothalamus in mammals is thought to be the most significant central regulator of intake as it is closely associated anatomically and physiologically with two circumventricular organs that have the capacity to allow the transport of hormones into and out of the brain and permit the entry of certain compounds from the blood circulation into the brain (Ganong, 2000; Miyata, 2015). The arcuate nucleus (**ARC**), lateral hypothalamus (**LHA**) and ventromedial hypothalamus (**VMH**) are important hypothalamic nuclei implicated in feed intake regulation in ruminants (Baile and McLaughlin, 1987; Sartin et al., 2010). The expression of messenger RNA (**mRNA**) for feed intake regulatory genes in the ARC, LHA and VMH of wethers (castrated male sheep) fed diets varying in CP content indicated significant regional specificity for some genes (Quigley et al., 2016). There was a tendency for upregulation of orexigenic and downregulation of anorexigenic genes in the LHA and vice-versa in the VMH, which indicates that a model of a hunger (LHA) and satiety (VMH) centre has relevance in ruminants.

The objectives of this experiment were to (1) establish a model of metabolic feed intake regulation in young wethers consuming diets of variable CP and P content, and (2) identify key regulatory gene pathways in the hypothalamus involved in this model of feed intake regulation. It was hypothesised that young wethers fed a diet deficient in CP and/or P would have lower feed intake than young wethers fed a diet with adequate CP and P, and this response would not be due to a physical limitation of the rumen (i.e., rumen fill). It was also expected that differentially abundant mRNA in three regions of the hypothalamus, measured by RNA-seq, would identify physiological pathways related to the metabolic regulation of feed intake in wethers fed diets deficient in CP and/or P.

Material and methods

The following experiment was conducted at the Queensland Animal Science Precinct, The University of Queensland, Gatton, Qld, Australia between May and August 2019. The mean temperature within the building was 15.3 °C with a minimum and maximum temperature of 3.6 and 25.8 °C, respectively.

Animal experiment design

The experiment consisted of a 24-day pre-experimental period, a 7-day pretreatment covariate period and a 63-day experimental period. Sixty Merino wethers of approximately 7 months of age and 21.4 ± 1.1 kg LW (mean ± SD) were sourced from a single commercial supplier (Armidale, NSW) that reared them together under the same conditions. All wethers were administered a 1 mL subcutaneous injection of Glanvac 6 vaccine (Zoetis, Australia) for protection against clostridial diseases, with a second dose administered 28 days later. Wethers were drenched with Startect (Zoetis, Australia; 5 mL/wether) for the control of internal parasites. A pretreatment and 21-day post-treatment faecal egg count on a representative sub-sample (n = 15) of wethers confirmed the drench successfully eliminated intestinal parasites prior to the commencement of the experimental period.

Wethers were fed barley straw *ad libitum* [39 g CP/kg DM, 7.1 MJ metabolisable energy (**ME**)/kg DM, 2.1 g Ca/kg DM, 0.5 g P/kg DM] and a restricted quantity of a commercial pellet (109 g CP/kg DM, 11.7 MJ ME/kg DM, 7.3 g Ca/kg DM, 2.8 g P/kg DM) in a single outdoor group pen for 7 days, with continuous access to water. The 60 wethers were then placed into individual indoor pens and fed barley straw *ad libitum* and a restricted quantity of the non-deficient (high CP-high P) experimental pellet. Over the next 17 days, the quantity of pellets offered was increased from approximately 0.7 g DM/kg LW per day to *ad libitum*.

Forty-five wethers were selected to participate in a covariate measurement during the final week of the adaptation period based on LW, feeding behaviour and temperament, and were offered *ad libitum* access to the non-deficient pellets and 5 g barley straw DM/kg LW per day for 7 days, with feed intake measured. Forty of these wethers were selected for inclusion in the experiment, weighed, ranked and blocked on LW (23.7 ± 1.42 kg) and randomly allocated to adjacent individual pens and one of five nutritional treatments within each block, resulting in eight replicates (i.e. individual wether) per treatment in a randomised block design.

Experimental treatment and diets

The experiment involved five nutritional treatments, four of which had different CP and P content and were considered experimental satiety models. These treatments targeted high (**H**) and low (**L**) CP and P content that broadly represented diets consumed by ruminants in northern Australia under the following conditions:

1. Wet season with adequate P – *ad libitum* intake of a high CP, high P diet (110 g CP/kg DM, 2.5 g P/kg DM; HCP-HP-**UMEI**; unrestricted ME intake)
2. Wet season with deficient P – *ad libitum* intake of a high CP, low P diet (110 g CP/kg DM, 0.5 g P/kg DM; HCP-LP-UMEI)
3. Dry season with adequate P – *ad libitum* intake of a low CP, high P diet (50 g CP/kg DM, 2.5 g P/kg DM; LCP-HP-UMEI)
4. Dry season with deficient P – *ad libitum* intake of a low CP, low P diet (50 g CP/kg DM, 0.5 g P/kg DM; LCP-LP-UMEI)

These diets were formulated to provide 9 MJ ME per kg DM and were fed *ad libitum*. The fifth nutritional treatment (HCP-HP-**RMEI**; restricted ME intake) was designed to represent an experimental hunger model, where wethers were fed the HCP-HP diet restricted to provide the equivalent ME intake of wethers allocated to the LCP-LP-UMEI treatment.

The experimental diets were pellets (8 mm diameter) containing varying proportions of barley straw (~56%), sugar (~24%), cassava starch (~11%), gluten, monocalcium phosphate (as Biofos; The Mosaic Company, Florida, USA), lime, urea, bicarbonate of soda, gypsum, potassium chloride, magnesium oxide and trace mineral mix to generate the intended nutrient contents. Actual nutrient content of pellets and barley straw are described in Table 1 and actual ingredients for each pellet are described in Supplementary Table S1 in Innes et al. (2023). All wethers were offered 5 g barley straw (35 mm chop length) DM/kg LW per day for the first 14 days of the experimental period to maintain rumination and to prevent acidosis. For the remainder of the experiment all wethers were offered 2.5 g barley straw (35 mm chop length) DM/kg LW per day to maintain rumen function and salivation.

Feeding

Wethers were offered barley straw and their allocated pellet in separate feeders at 0800 h each morning after feed residues from the previous day were collected. The allocations of barley straw and pellets for wethers allocated to the HCP-HP-RMEI treatment were calculated every 7 days as a proportion of LW and weighed into daily allocations for each wether. Each HCP-HP-RMEI wether was fed the HCP-HP pellet at an amount restricted to the same intake (g DM kg/LW) as the LCP-LP-UMEI wether within its experimental block. For all other treatments, approximately 10% daily pellet residue was targeted to ensure the allocation of pellets remained *ad libitum*.

Pellet, straw and total feed intake were calculated for each wether every 7 days by subtracting the cumulative weight of feed residue from the total weight of feed offered. Mean daily feed intake was calculated from the measured 7-day feed intake. Intake over the first 7 days of the experimental period is omitted from the analysis as this period was used to determine feeding allowances for wethers fed the restricted treatment (HCP-HP-RMEI). Representative sub-samples of the four pellet diets and the barley straw were collected every 7 days prior to feed preparation for analysis of DM, organic matter, CP, ash-free NDF, ash-free ADF and mineral content.

Table 1
Nutrient concentration of the pellets and barley straw fed to wethers.

Nutrient, g/kg DM	HCP-HP pellet	HCP-LP pellet	LCP-HP pellet	LCP-LP pellet	Barley straw
OM	932	929	928	921	919
CP	114	115	56	52	39
Ash-free NDF	332	372	364	369	610
Ash-free ADF	168	188	186	184	317
P	2.2	0.7	2.5	0.7	0.5
Ca	3.7	3.8	4.3	7.5	2.1
Mg	1.6	1.7	1.7	1.8	1.6
K	14.3	14	14.2	14.3	26.3
Na	3.4	3.6	3.7	4.0	4.5
S	1.6	1.5	1.5	1.4	1.0

Abbreviations: HCP-HP = high CP-high P; HCP-LP = high CP-low P; LCP-HP = low CP-high P; LCP-LP = low CP-low P; OM = Organic Matter.

Liveweight

The LW of the wethers was measured prior to feeding every 7 days throughout the experiment. Liveweight gain for each treatment group was calculated as the mean linear regression coefficient representing the change in LW over time.

Digestibility

Total faecal output was collected and weighed for all wethers over seven consecutive days commencing on day 42 of the experimental period. The cumulated faecal output of each wether was stored at 4 °C throughout the collection period and a final weight was recorded at the conclusion of the collections. A representative sub-sample of the faeces was dried at 60 °C in triplicate and stored for subsequent analysis of DM and organic matter.

Blood sampling

Blood samples were collected prior to feeding every 14 days to monitor the concentration of metabolites (urea-N, inorganic P and Ca) and prior to feeding on days 51 and 53 to measure the concentration of hormones (insulin, leptin, ghrelin and IGF-1) in the plasma of wethers in response to treatment. Samples were collected from the jugular vein of the wethers into lithium heparin-coated and dipotassium-ethylenediaminetetraacetic acid-coated vacutainers (Becton Dickinson, North Ryde, NSW, Australia) for metabolites and hormones, respectively. Vacutainers were gently inverted six to eight times and stored in ice prior to centrifugation at 1 200*g* at 4 °C for 15 min.

Euthanasia

Wethers were euthanased at the end of the experiment over four consecutive days, with two blocks euthanased each day in sequential pen order. All wethers were fed their respective nutritional treatments until euthanasia. Feeding time was staggered the day before euthanasia so that wethers were all fed exactly 2 h before they were euthanased. Wethers were euthanased by a sodium pentobarbitone overdose administered via the jugular vein, followed by exsanguination.

Hypothalamus dissection

The dissection of the hypothalamus was based on previous experience and publications identifying the anatomical structures of the sheep hypothalamus (Tillet and Thibault, 1989; Anderson et al., 1997; Colthorpe and Martin, 1998; Anderson et al., 2001) and is detailed in Supplementary Material S1 (Innes et al. 2023).

Rumen digesta weight and sampling

The rumen was isolated using clamps and all digesta was removed and weighed. The rumen digesta was then mixed thoroughly and sub-sampled for the determination of DM and organic matter content. Rumen fluid passed through a 1 mm screen and the pH was measured, with sub-samples of rumen fluid collected for analysis of volatile fatty acids and NH_3-N.

Rumen retention time

The apparent retention time (h) of digesta in the rumen was estimated as the digesta weight at slaughter (kg DM) divided by the mean DM intake rate preslaughter (kg DM/h). This is a modified version of the method described previously for sheep and cattle (Minson, 1966; Poppi et al., 1981). In this experiment, mean hourly feed intake was derived from the 24-h period for the day before but wethers were fed once in the morning with *ad libitum* access to feed (except for the HCP-HP-RMEI treatment) instead of fed hourly.

Analytical procedures

DM and organic matter

DM content was determined by drying duplicate sub-samples of feed offered, feed residues and faeces at 60 °C until a constant weight was achieved. The feed offered and feed residue samples were ground through a 4 mm screen and then a 2 mm screen using a Retsch SM 100 mill (Retsch GMBH, Haan, Germany). The faeces were ground through a 2 mm screen using a Foss Cyclotec mill (FOSS, Hillerød, Denmark). A sub-sample of the ground samples was dried at 105 °C for 24 h to determine residual DM content and then approximately 1 g was combusted in an electric muffle furnace (Modutemp Pty. Ltd.; Perth, WA, Australia) for 4.5 h at 550 °C to determine ash and organic matter content.

CP

The total N content of feeds offered were measured by the Kjeldahl method using an auto-digestor (Tecator 2520; FOSS, Hillerød, Denmark) and a N analyser (Kjeltec 8400; FOSS, Hillerød, Denmark) following the manufacturer's guidelines. CP content was calculated using a conversion factor of 6.25.

Fibre analysis

The Van Soest et al. (1991) method was used to determine the ash-free NDF and ADF content of feed samples using an ANKOM 220 fibre analyser (ANKOM Technology, Macedon, New York).

Minerals

The mineral content of feeds offered was determined using an inductively coupled plasma atomic emission spectrometer (Optima 7300 DV, Perkin Elmer, Waltham, MA, USA) after a nitric-perchloric acid digestion.

Rumen ammonia-N

Duplicate samples of rumen fluid (8 mL) were combined with 1 M sulphuric acid (2 mL) mixed and stored at −20 °C until analysis. The NH_3-N was distilled from the samples into boric acid using a semi-automatic distillation unit (UDK 139, Velp Scientifica, Ustmate, MB, Italy). The concentration of NH_3-N in the distillate was determined by titration with 0.0096 *M* hydrochloric acid using a TIM 840 auto-titrator (Radiometer Analytical SAS, Villeubanne Cedex, France).

Volatile fatty acids

Rumen fluid (4 mL) was combined with 20% metaphosphoric acid with an internal standard (4-methyl n-valeric acid; 0.48 g/L) (1 mL) and then dispensed into four approximately equal aliquots which were stored at −20 °C until analysis. The molar proportion of individual volatile fatty acids in the rumen fluid was analysed by gas-liquid chromatography (GC-2010, Shimadzu; Kyoto, Honshu, Japan) fitted with a polar capillary column (ZB-FFAP, Phenomenex; Lane Cove, NSW, Australia).

Metabolisable energy

The ME content of the feeds was estimated using the equation:

$M/D = 0.172 \times DMD - 0.707$ [equation 1.12A in Freer et al. (2007)],

where M/D is the ME content per kg DM and DMD is the DM digestibility estimated from total faecal collections and intake as described above.

Plasma biochemical and metabolite analysis

Blood plasma metabolites were measured on an OLYMPUS auto-analyser (AU480, Beckman Coulter Inc., Brea, California, USA) by a commercial laboratory.

Plasma hormone assays

Plasma insulin concentrations were determined on a sub-set of samples (n = 4/treatment; Blocks 5–8) using a commercial immunoradiometric assay kit (DIAsource INS-IRMA Kit, DIAsource; Louvain-la-Neuve, Belgium). The concentration of leptin in plasma was determined on a sub-set of samples (n = 4/treatment; Blocks 5–8) using a commercial multi-species radioimmunoassay kit (XL-85 K, Millipore Corporation; St Charles, MO, USA). The concentration of active ghrelin in plasma was determined on a sub-set of samples (n = 6/treatment; Blocks 3–8) using a commercial radioimmunoassay kit (GHRA-88HK, Millipore Corporation; St Charles, MO, USA). The concentration of total IGF-1 in plasma was determined on all wethers on a single prefeeding sample using a commercial immunoradiometric assay (A15729, Immunotech, Beckman Coulter; Prague, Czech Republic).

Radioimmunoassays were conducted using the manufacturer's protocols. Assay tubes containing ^{125}Iodine were counted in duplicate using a gamma counter (2470 Wizard2; Perkin Elmer, Waltham, MA, USA) for 1 min each. The output of counts per minute for each tube was then processed using the AssayZap software (Biosoft; Cambridge, United Kingdom). An appropriate curve fitting procedure (immunoradiometric or radioimmunoassay) was used for the standards which allowed for the calculation of unknown concentrations in both quality controls and samples.

The inter- and intra-assay CV were 4.8 and 4.0%, 0.1 and 13.5%, and 3.3 and 8.2%, respectively for insulin, leptin and ghrelin. The intra-assay CV of IGF-1 was 6.8% and no inter-assay CV was calculated as only one assay was required.

Statistical analysis for animal experiment

All data were statistically analysed using the R environment (R Core Team, 2020) within RStudio (RStudio Team, 2019). Variables with repeated measures over time, including all intakes (DM and ME) and the concentration of metabolites (urea-N, inorganic P and Ca) in plasma, were analysed using the linear mixed effects model function ('lme') within the package *nlme* (Pinheiro et al., 2019) with the following model:

$$Y_{ijklm} = \mu + T_i + D_j + (T \times D)_k + Block_l + ID_m + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, T_i is the i^{th} fixed effect of dietary treatment (n = 5), D_j is the j^{th} fixed effect of day of experiment (every 7 d for intakes and every 14 d for blood metabolites), $(T \times D)_k$ is the k^{th} fixed effect of the interaction between treatment and day, $Block_l$ is the l^{th} fixed effect of the experimental block (n = 5), ID_m is the m^{th} random intercept for each individual wether (n = 40), and ε_{ijklm} is the residual error. The 'autocorrelation structure of order 1' ('corAR1') with the form of day of

experiment grouped by individual wether was used as the covariance structure to account for the repeated measurements on the same experimental unit over time. For each variable, the effect of block or day was removed if not significantly contributing to the model.

Variables with non-repeated measures, including DM digestibility, ME concentration, LW gain, concentration of hormones (insulin, leptin, ghrelin and IGF-1) in plasma, the pH, concentration of NH_3-N, and molar proportion of volatile fatty acids in rumen fluid, and the weight and retention time of digesta, were analysed using the linear model function ('lm') within the *stats* package (R Core Team, 2020) with the following model:

$$Y_{ij} = \mu + T_i + Block_j + \varepsilon_{ij}$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, T_i is the i^{th} fixed effect of dietary treatment (n = 5), $Block_j$ is the j^{th} fixed effect of experimental block (n = 5), and ε_{ijklm} is the residual error. For each variable, the effect of block was removed if not significantly contributing to the model. For all models, treatment means were estimated using the 'lsmeans' function from the package *emmeans* (Lenth, 2020) and compact letter display (letters indicating significantly different treatment) were calculated with the 'cld' function from the package *multicomp* (Hothorn et al., 2008). The residuals from all models were checked for normality and homoscedasticity to ensure these assumptions were correct. Pearson correlations were calculated using the 'stat_cor' function from the *ggpubr* package (Kassambara, 2020). A reproducible example of the R code used for fitting the linear mixed effect models and linear models is provided in Supplementary Material S1 (Innes et al. 2023).

Hypothalamus RNA-seq analysis

RNA extraction

Total RNA was isolated from the ARC, LHA and VMH samples using a combined TRIzol® (Life Technologies; Carlsbad, CA, USA) and RNEasy Plus Mini Kit protocol (Qiagen, Hilden, Germany) detailed in Supplementary Material S1 (Innes et al. 2023). This protocol was required as only 30 mg of starting tissue was available. One ARC sample from the HCP-LP-UMEI treatment group was lost during preparation for shipping and was subsequently unable to be sequenced. The RNA integrity number was determined for the ARC (8.4 ± 0.2), LHA (7.3 ± 0.5) and VMH (7.6 ± 0.4) samples on an Agilent 2200 TapeStation.

Sample preparation and sequencing

The sample preparation and sequencing for the RNA were completed by the Ramaciotti Centre for Genomics, University of NSW, Sydney, NSW, Australia. The complementary DNA libraries were prepared using Illumina's TruSeq stranded mRNA sample preparation kit following the manufacturer's protocol and the libraries were sequenced using an Illumina NovaSeq 6000 producing 100 base pair single-end reads with a target sequencing depth of at least 20 million reads. Briefly, mRNA was purified and fragmented with divalent cations and heat followed by a first-strand complementary DNA synthesis with random primers and a second-strand complementary DNA synthesis step. The complementary DNA library preparation protocol includes the repair of DNA fragment ends, 3′ Adenylation of DNA fragments, sequencing adapter ligation, amplification of library via polymerase chain reaction, and library verification.

Trimming, alignment and counting

The 119 RNA-seq 100 bp FASTQ files were assessed by sequencing confidence and integrity using FASTQC (Andrews, n.d.), version 0.11.8. Reads were removed that failed to pass quality thresholds and trimmed of low-quality bases (Sliding Window of four bases at minimum quality Q15, minimum length 36 bases, trim leading three bases, trim trailing three bases) using the *trimmomatic* tool (Bolger et al., 2014), version 0.36. Surviving reads were mapped using *STAR* (Dobin et al., 2012), version 2.6.0b, against the Ovis aries (Oar_v3.1) reference genome from Ensembl (Yates et al., 2019) release 100 (Ovis_aries.Oar_v3.1.dna.toplevel.fa). Gene reads were counted using the *featureCounts* tool (Liao et al., 2013) referenced to the Ensembl (Yates et al., 2019) 100 annotation file (Oar_v3.1.100.gtf). This produced count tables for downstream normalisation and determination of differentially expressed (**DE**) genes. The entire analysis was completed on the Australian Galaxy web platform (Afgan et al., 2018), using wrappers for the above-mentioned tools. Approximately 86% of the average 22.3 million reads per sample in the hypothalamus (ARC, LHA and VMH) were aligned to the ovine reference genome. Approximately 43% of these reads were annotated to one gene, resulting in 20 904 of 27 054 genes (77%) in the ovine reference genome with more than 10 reads across all samples.

Differential expression

Pairwise comparisons of the raw count data were conducted using the *DESeq2* package version 1.28.1 (Love et al., 2014) in R environment version 4.0.0 (R Core Team, 2020) within RStudio version 1.2.5033 (RStudio Team, 2019). Briefly, models were calculated separately for each region of the hypothalamus, and each treatment pairwise comparison was produced using the 'results' function with the $\log_2$ fold change threshold set to 0 (Love et al., 2014). The *P*-values were corrected using the Benjamini-Hochberg method, with an adjusted *P*-value below 0.05 indicating a statistically significant difference in that gene between the two treatments and an adjusted *P*-value less than 0.1 indicating a tendency to be different. Function defaults were not always used so a detailed explanation is included in Supplementary Material S1 in Innes et al. (2023), along with the method used for the preparation of the plots.

Phenotypic impact factor

The phenotypic impactor factor (**PIF**) is a modified differential expression metric that is calculated as the mean expression of a gene for the two conditions being compared multiplied by the differential expression (fold change) between them (Hudson et al., 2009). This technique attempts to account for the biological relevance of genes by ranking genes that are both highly abundant and highly different between treatments as the most significant. It also de-emphasises noisy, lowly expressed genes. A gene was considered to have a significant PIF value if it was >2.58 SD from the mean (for a nominal $P < 0.01$).

Functional enrichment analysis

For each pairwise treatment comparison, genes identified as DE were used as input to a functional enrichment analysis to identify pathways from gene ontology and Reactome databases using *g:Profiler* (version e99_eg46_p14_f929183) via their R package *gprofiler2* with the 'gost' function (Kolberg et al., 2020). Pathways were deemed significant when $P < 0.05$ after adjustment using the inbuilt *g:SCS* multiple testing correction method (Raudvere et al., 2019). In addition, the genes identified as DE were differentiated into those which were up- and downregulated as indicated by the $\log_2$ fold change value and used as input to the same functional enrichment analysis. This produced a separate enrichment for pathways which were up- or downregulated in condition 1 vs condition 2 in each pairwise comparison, noting that this method does not account for the activatory or inhibitory roles of the encoded protein products. However, this method did produce similar terms

to the original lists with the added benefit of implying if the pathway was likely up- or downregulated. To reduce the number of redundant terms, the output lists from all enrichment analyses were further filtered to produce additional lists that included only the lowest 'child' terms by removing any terms which had a 'parent' term included.

Results

Nutrient deficiency models

DM intake, digestibility and liveweight gain

Total intake (43.6 ± 4.3 g DM/kg LW per day; $P = 0.86$) during the 7-day pretreatment covariate period was not different between wethers subsequently allocated to nutritional treatments. Pellet, total DM intake and ME intake of wethers fed the HCP-HP-UMEI treatment were higher than wethers allocated to the other treatments (Table 2). Wethers offered the HCP-LP-UMEI, LCP-HP-UMEI and LCP-LP-UMEI treatments consumed 32, 42 and 49% less total DM than the HCP-HP-UMEI treatment, respectively. Wethers offered the restricted (HCP-HP-RMEI) and the combined CP- and P-deficient (LCP-LP-UMEI) treatments consumed significantly more barley straw than wethers fed the non-deficient (HCP-HP-UMEI) treatment (Table 2).

The DM digestibility (and consequently the estimated ME content) of the consumed diets was high in all treatments, with only a tendency ($P = 0.063$) to be higher in the wethers fed the HCP-HP-RMEI treatment (Table 2). As expected, ME intake was significantly higher in the HCP-HP-UMEI wethers; however, there was a significant difference between the HCP-LP-UMEI and LCP-LP-UMEI wethers which reflected differences in total DM intake (Table 2). Total DM intake and ME intake of the wethers were highly correlated ($r = 0.99$, $P < 0.001$), which confirms that the dietary ME contents were similar between treatments and that the variation of ME intake was due to the differences in total DM intake.

The wethers fed the non-deficient treatment (HCP-HP-UMEI) had a significantly higher mean LW gain than wethers offered the other treatments (Table 2). The mean LW gain of the wethers was positively correlated with total DM intake (r = 0.89, $P < 0.001$) and ME intake ($r = 0.93$, $P < 0.001$).

Plasma and rumen parameters

There were no differences in the concentration of inorganic P (1.7 ± 0.34 mmol/L; $P = 0.47$), urea-N (3.9 ± 0.85 mmol/L; $P = 0.82$) and total Ca (2.5 ± 0.07 mmol/L; $P = 0.39$) in the plasma of wethers prior to introduction to nutritional treatments (day 0). However, the mean concentration of inorganic P, urea-N and Ca in the plasma of wethers on days 21, 35, 49 and 63 of the experiment were significantly influenced by the nutritional treatments (Table 3). Wethers fed treatments with adequate P (HP) had a higher concentration of inorganic P in the plasma than those fed deficient P (LP) diets, regardless of CP content and ME intake (Table 3). However, wethers consuming an adequate P diet with a reduced ME intake (HCP-HP-RMEI and LCP-HP-UMEI) had a higher concentration of plasma inorganic P. Wethers fed treatments with a high CP (HCP) content had a higher concentration of urea-N in plasma than wethers fed treatments with a low CP (LCP) content (Table 3). The treatment effects on the concentration of inorganic P and urea-N in plasma persisted throughout the experimental period (Fig. 1).

The concentration of insulin was highest in the plasma of wethers fed the HCP-HP-UMEI treatment prior to feeding on days 51 and 53 and lowest in wethers fed the LCP-HP-UMEI treatment, with no differences detected between other treatments (Table 3). There were no differences in the concentration of leptin and ghrelin in the plasma of wethers (Table 3). The concentration of total IGF-1 in the plasma was highest in wethers fed the HCP-HP-UMEI treatment and lowest in wethers fed the three nutrient-restricted diets, with no significant difference detected between the latter (Table 3).

The concentration of NH_3-N in the rumen fluid was highest in the wethers fed the HCP-HP-RMEI and HCP-HP-UMEI treatments and lowest in the wethers fed the LCP-HP-UMEI and LCP-LP-UMEI treatments (Table 4). The pH of rumen fluid collected after

Table 2
Mean DM intake, DM digestibility, estimated metabolisable energy (ME) intake and liveweight (LW) gain of wethers fed different nutritional treatments[1] for 63 days.

Item	HCP-HP-RMEI	HCP-HP-UMEI	HCP-LP-UMEI	LCP-HP-UMEI	LCP-LP-UMEI	SEM	*P*-value
Straw intake, g DM/kg LW per day	2.8[b]	2.5[a]	2.7[ab]	2.7[ab]	2.8[b]	0.07	<0.01
Pellet intake, g DM/kg LW per day	16.9[a]	34.7[c]	22.6[b]	19.1[ab]	16.2[a]	1.00	<0.001
Total intake, g DM/kg LW per day	19.7[a]	37.2[c]	25.3[b]	21.8[ab]	19.1[a]	0.97	<0.001
DM digestibility, %	66.9	63.6	61.0	61.7	60.0	1.67	0.063
ME concentration, MJ ME/kg DM	9.8	9.2	8.8	8.9	8.6	0.29	0.063
ME intake, MJ/kg LW per day	0.19[ab]	0.34[c]	0.22[b]	0.19[ab]	0.16[a]	0.01	<0.001
Liveweight gain, g/day	−6[b]	141[c]	−1[b]	−47[a]	−60[a]	9.23	<0.001

[1] See text for description of nutritional treatments. H (high) and L (low); CP, P (phosphorus), UMEI (unrestricted ME intake) and RMEI (restricted ME intake).
[a–c] Different superscripts across a row indicate significantly different treatment means ($P \leq 0.05$).

Table 3
Mean concentration of inorganic P, urea-N and Ca measured in the plasma prior to feeding every 14 days and mean concentration of insulin, leptin, ghrelin and insulin-like growth factor-1 (IGF-1) measured in the plasma prior to feeding on day 51 or 53 in wethers fed different nutritional treatments[1] for 63 days.

Item	HCP-HP-RMEI	HCP-HP-UMEI	HCP-LP-UMEI	LCP-HP-UMEI	LCP-LP-UMEI	SEM	*P*-value
Inorganic P, mmol/L	2.4[d]	2.0[c]	1.1[a]	2.4[d]	1.6[b]	0.09	<0.001
Urea-N, mmol/L	4.1[b]	4.1[b]	4.4[b]	1.1[a]	1.6[a]	0.22	<0.001
Ca, mmol/L	2.3[a]	2.5[b]	2.7[c]	2.2[a]	2.4[b]	0.03	<0.001
Insulin, uIU/mL	10.8[bc]	13.4[c]	7.7[ab]	5.5[a]	9.6[b]	0.76	<0.001
Leptin, ng/mL	7.0	6.1	6.0	6.3	7.1	1.1	0.93
Ghrelin, pg/mL	613	477	371	538	533	94.0	0.52
IGF-1, ng/mL	59.9[b]	116[c]	42.5[ab]	25.6[a]	21.6[a]	6.3	<0.001

[1] See text for description of nutritional treatments. H (high) and L (low); CP, P (phosphorus), N (Nitrogen), Ca (Calcium), ME (metabolisable energy); UMEI (unrestricted ME intake) and RMEI (restricted ME intake).
[a–d] Different superscripts across a row indicate significantly different treatment means ($P \leq 0.05$).

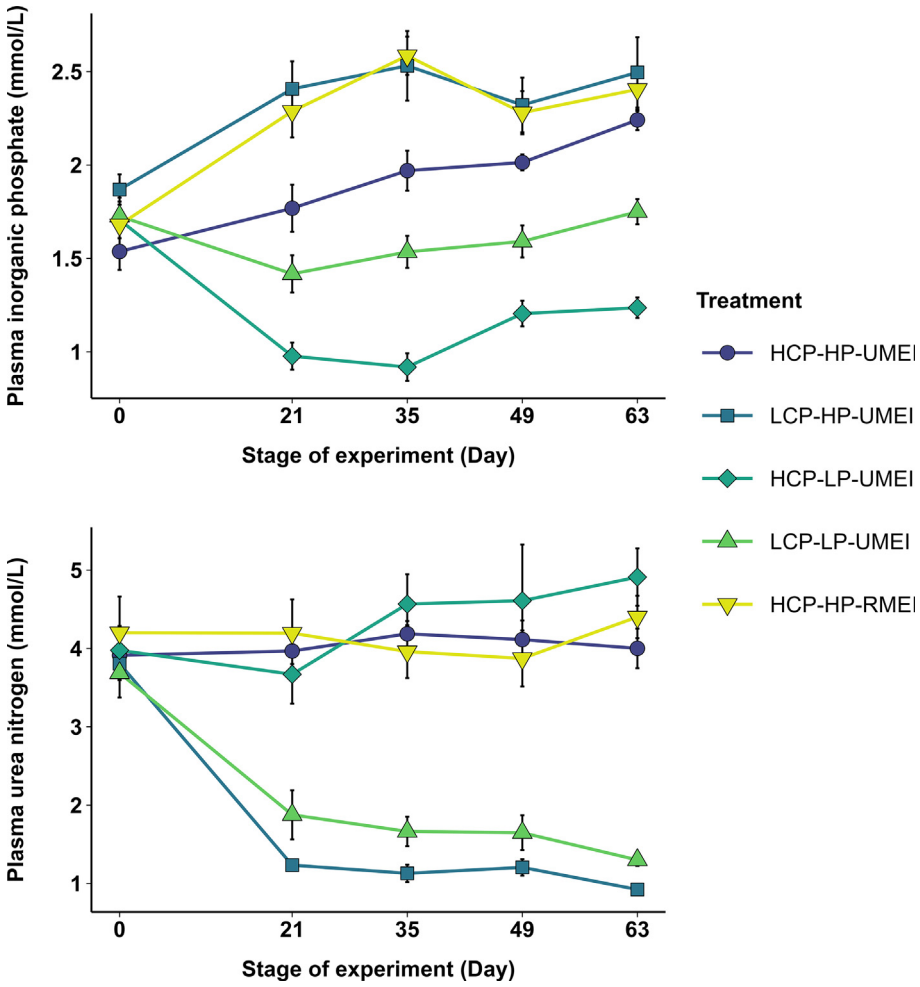

Fig. 1. Prefeeding concentration (mean ± SE) of inorganic phosphate and urea nitrogen in the plasma of wethers fed different nutritional treatments across the entire experimental period. [1]See text for description of nutritional treatments. H (high) and L (low); CP, phosphorus (P); UMEI (unrestricted metabolisable energy intake) and RMEI (restricted metabolisable energy intake).

Table 4
Mean pH, concentration of NH_3-N and molar proportions of volatile fatty acids in rumen fluid, rumen digesta DM load and apparent retention time of digesta in the rumen of wethers fed different nutritional treatments[1] for 63 days.

Item	HCP-HP-RMEI	HCP-HP-UMEI	HCP-LP-UMEI	LCP-HP-UMEI	LCP-LP-UMEI	SEM	*P*-value
Rumen pH	5.46[a]	6.04[b]	6.47[c]	6.39[bc]	6.75[c]	0.10	<0.001
Rumen NH_3-N, mg/L	180[c]	143[bc]	79[ab]	42[a]	34[a]	19.3	<0.001
Rumen acetic acid, %	58.0	60.5	60.2	63.2	57.1	2.09	0.29
Rumen propionic acid, %	28.5	29.0	27.7	28.3	28.3	2.62	0.99
Rumen butyric acid, %	12.0	9.2	10.5	7.7	13.4	1.51	0.09
Rumen valeric acid, %	1.2	1.2	1.3	0.7	1.1	0.37	0.81
Rumen digesta load, g DM	442[c]	499[c]	322[b]	300[ab]	199[a]	26.7	<0.001
Apparent retention time, h	27[b]	10[a]	14[a]	14[a]	14[a]	1.12	<0.001

[1] See text for description of nutritional treatments. H (high) and L (low); CP, P (phosphorus), N (Nitrogen), Ca (Calcium), ME (metabolisable energy); UMEI (unrestricted ME intake) and RMEI (restricted ME intake).
[a,b,c] Different superscripts across a row indicate significantly different treatment means ($P \leq 0.05$).

euthanasia was significantly lower in wethers fed the HCP-HP-RMEI treatment (Table 4). Wethers fed the LCP-LP-UMEI treatment had a higher rumen fluid pH than wethers fed the HCP-HP-UMEI treatment, with no other treatment effects observed. The wethers fed the HCP-HP-RMEI and HCP-HP-UMEI treatments had significantly more digesta within their rumens at euthanasia than wethers fed the deficient treatments (Table 4). The apparent retention time of the unrestricted ME intake treatments were all low and not different from each other (Table 4).

Gene expression

Differential expression

The differences between regions of the hypothalamus were represented by the number of DE genes for each treatment pairwise comparison (Table 5). The ARC was the region most responsive to the nutritional treatments with 195 DE genes in the HCP-HP-UMEI vs LCP-LP-UMEI treatment comparison, compared to only 24 in the HCP-HP-UMEI vs HCP-HP-RMEI treatment comparison,

Table 5
Number of differentially expressed genes[1] in three regions of the hypothalamus[2] of wethers between each nutritional treatment pairwise comparison[3], presented by (a) nutrient-deficient, and (b) restricted metabolisable energy intake comparisons.

(a) Hypothalamic 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
ARC	195	10	16	6	2	0
LHA	3	1	1	0	1	1
VMH	5	1	12	0	2	0

(b) Hypothalamic 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
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).
[2] Arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and lateral hypothalamus (LHA).
[3] See text for description of nutritional treatments. H (high) and L (low); CP, phosphorus (P); UMEI (unrestricted metabolisable energy intake) and RMEI (restricted metabolisable energy intake).

indicating that the ARC responded differently to a nutrient-deficient diet than a restriction of ME intake. The VMH had 123 DE genes between the P-deficient (HCP-LP-UMEI) and restricted ME intake (HCP-HP-RMEI) treatments, with only 12 DE genes in the next highest treatment comparison (HCP-HP-UMEI vs HCP-LP-UMEI). There were also 38 DE genes in the HCP-LP-UMEI vs HCP-HP-RMEI treatment comparison in the ARC. Therefore, despite relatively few DE genes between the two nutrient-deficient diets, there was some indication that the wethers responded to a P-deficient diet differently than to a restriction of ME intake. In addition to the results presented in the following sections, the full list of DE genes ranked on PIF for all pairwise comparisons are included in Supplementary Material S2, as well as all summary plots in Supplementary Material S3 (Innes et al. 2023).

The summary plots of three pairwise comparisons in the ARC (Fig. 2) represent (a) the genes responding differently when the wethers were fed diets with different nutrient contents, (b) the genes responding differently when the wethers were fed the same diet at either unrestricted ME intake (*ad libitum)* or restricted ME intake, and (c) the genes responding differently between wethers fed a low P or low CP diet. These plots incorporate the use of the PIF metric to highlight which DE genes possessed a combination of high differential expression and high overall abundance. In Fig. 2A, the *AGRP* (agouti-related protein), *NPY* (neuropeptide Y), *CARTPT* (cocaine- and amphetamine-regulated transcript pre-propeptide) and *POMC* (proopiomelanocortin) genes are placed on the extremities relative to the majority of genes which represents their high ranking by the PIF metric. There is also a cluster of DE genes ranked highly by the PIF metric on the lower half of Fig. 2A [dotted line circle; see Supplementary Material S3 in Innes et al. (2023) for a detailed version of this figure] which include genes encoding proteins related to the transport of sulphate (*SLC13A4*), toxin and drugs (*SLC47A1*), iodine (*SLC5A5*), monocarboxylates and creatinine (*SLC16A12*), as well as a transcription factor (*OSR1*) and the enzymes Betaine--Homocysteine S-Methyltransferase (*BHMT*) and Acyl-CoA Synthetase Medium-Chain Family Member 1 (*ACSM1*). A similar cluster is visually observable in the plots for the HCP-HP-UMEI vs HCP-LP-UMEI [Supplementary Material S3 in Innes et al. (2023)].

There were less DE genes in the ARC between wethers fed the non-deficient and the ME-restricted treatment comparison (HCP-HP-UMEI vs HCP-HP-RMEI; Fig. 2B) than the non-deficient and the combined CP and P-deficient (HCP-HP-UMEI vs LCP-LP-UMEI) treatment comparison (Fig. 2A). Only *AGRP*, *CARTPT*, *NPY* and *MUC15* (mucin 15) were ranked as significant by the PIF and DE analysis, although the location of the *AGRP*, *CARTPT*, *NPY* and *POMC* on the plot was very similar to Fig. 2A, indicating similar hunger and satiety responses in the hypothalamus between the nutrient-deficient and the ME-restricted wethers. The expression of *AGRP*, *CARTPT* and *NPY* were not identified as DE in the ARC between wethers fed either of the nutrient-deficient treatments (Fig. 2C). Likewise, of the DE genes that were detected, none had high PIF scores in this treatment comparison, indicating a potential similarity in the pathways responsible for the voluntary reduction in DM intake observed in wethers fed both the HCP-LP-UMEI and LCP-HP-UMEI treatments. Due to the high PIF ranking of *AGRP*, *CARTPT*, *NPY* and *POMC* genes in some pairwise comparisons and their established role in feed intake regulation literature, the distributions of their expression were compared between treatments within the ARC, LHA and VMH (Fig. 3). Overall, *CARTPT, AGRP* and *NPY* in the ARC and *POMC* in the VMH was DE between HCP-HP-UMEI treatment and treatments with low CP (LCP-HP-UMEI and LCP-LP-UMEI) or restricted ME intake (HCP-HP-RMEI), whereas *POMC* was DE between HCP-HP-UMEI and the three nutrient-deficient treatments in the LHA.

Functional enrichment

Genes that were upregulated in the ARC of wethers fed the HCP-HP-UMEI relative to the LCP-LP-UMEI treatment were enriched for terms including 'enzyme binding' (GO-MF:0019899; adjusted P = 1.35E−03), 'ATP synthesis coupled proton transport' (GO-BP:0015986; adjusted P = 6.17E−03), 'oxidative phosphorylation' (KEGG:00190; adjusted P = 7.05E−03) and 'nucleotide metabolic process' (GO-BP:0009117; adjusted P = 1.11E−02). Genes that were downregulated 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' (GO-BP:0044260; adjusted P = 2.06E−03), 'cellular response to hormone stimulus' (GO-BP:0032870; adjusted P = 3.89E−03) and 'methylation' (GO-BP:0032259; adjusted P = 8.56E−03). Genes that were downregulated in the ARC of wethers fed the HCP-HP-UMEI treatment relative to the HCP-HP-RMEI treatment were enriched for terms including 'RNA processing' (GO-BP:0006396; adjusted P = 2.28E−03) and 'nucleic acid metabolic process' (GO-BP:0090304; adjusted P = 3.64E−02).

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' (KEGG:04721; adjusted P = 4.74E−03), 'anion transmembrane transporter activity' (GO-MF:0008509; adjusted P = 8.02E−03), 'transmembrane transport' (GO-BP:0055085; adjusted P = 2.56E−02), and 'neuron projection morphogenesis' (GO-BP:0048812; adjusted P = 4.34E−02). No other meaningful enrichments were found in the other pairwise comparisons of all three

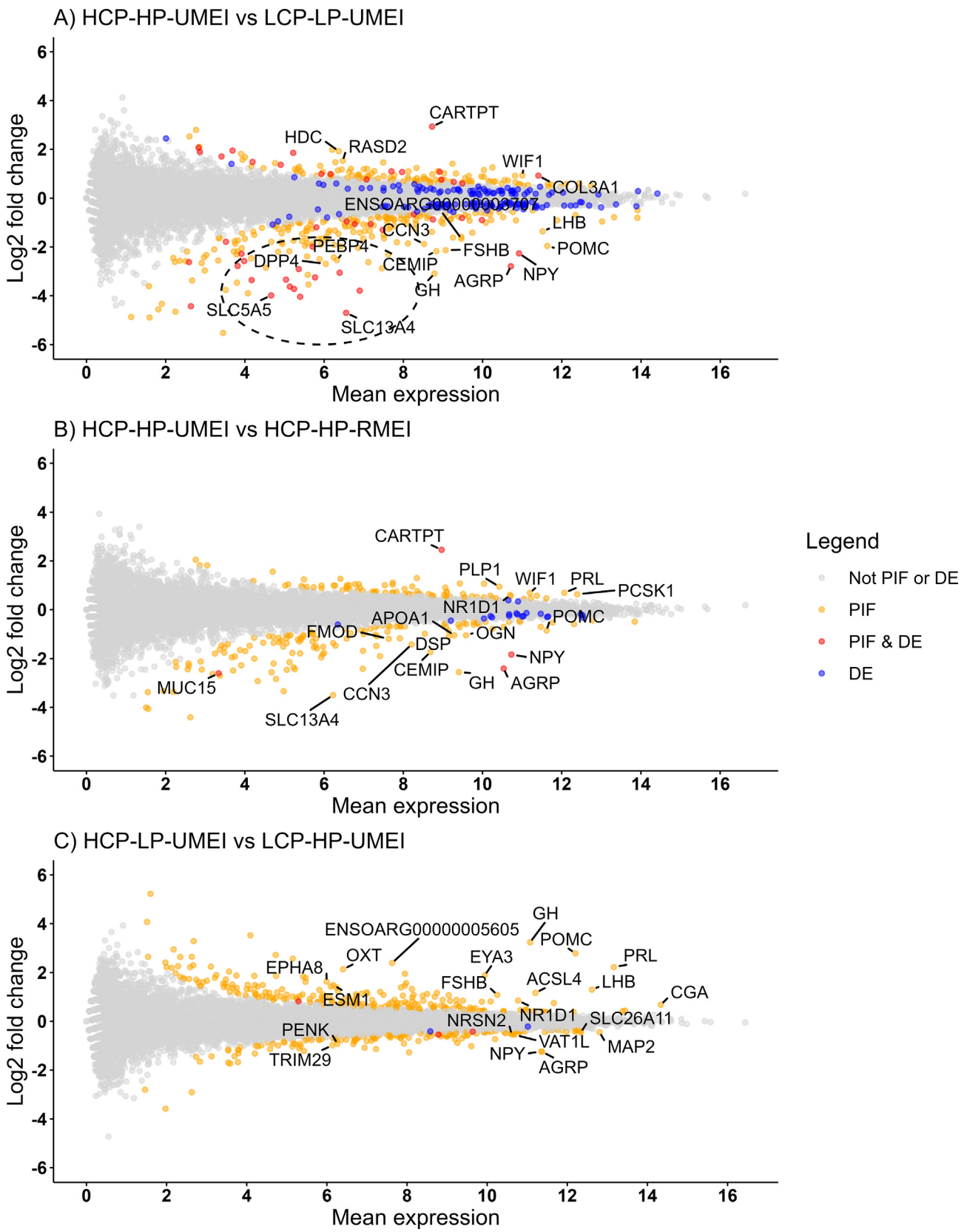

Fig. 2. Plots of mean $\log_2$ expression to $\log_2$ fold change[1] of genes between three selected nutritional treatment pairwise comparisons[2] (A), (B) and (C) within the arcuate nucleus of wethers. [1]PIF (phenotypic impact factor; >±2.58 SD), DE (differentially expressed genes; adjusted $P \leq 0.05$). [2]See text for description of nutritional treatments. Abbreviations: H = high; L = low; P = (crude protein)phosphorus; UMEI = unrestricted metabolisable energy intake; RMEI = restricted metabolisable energy intake. Dotted line identifies cluster referred to in the text.

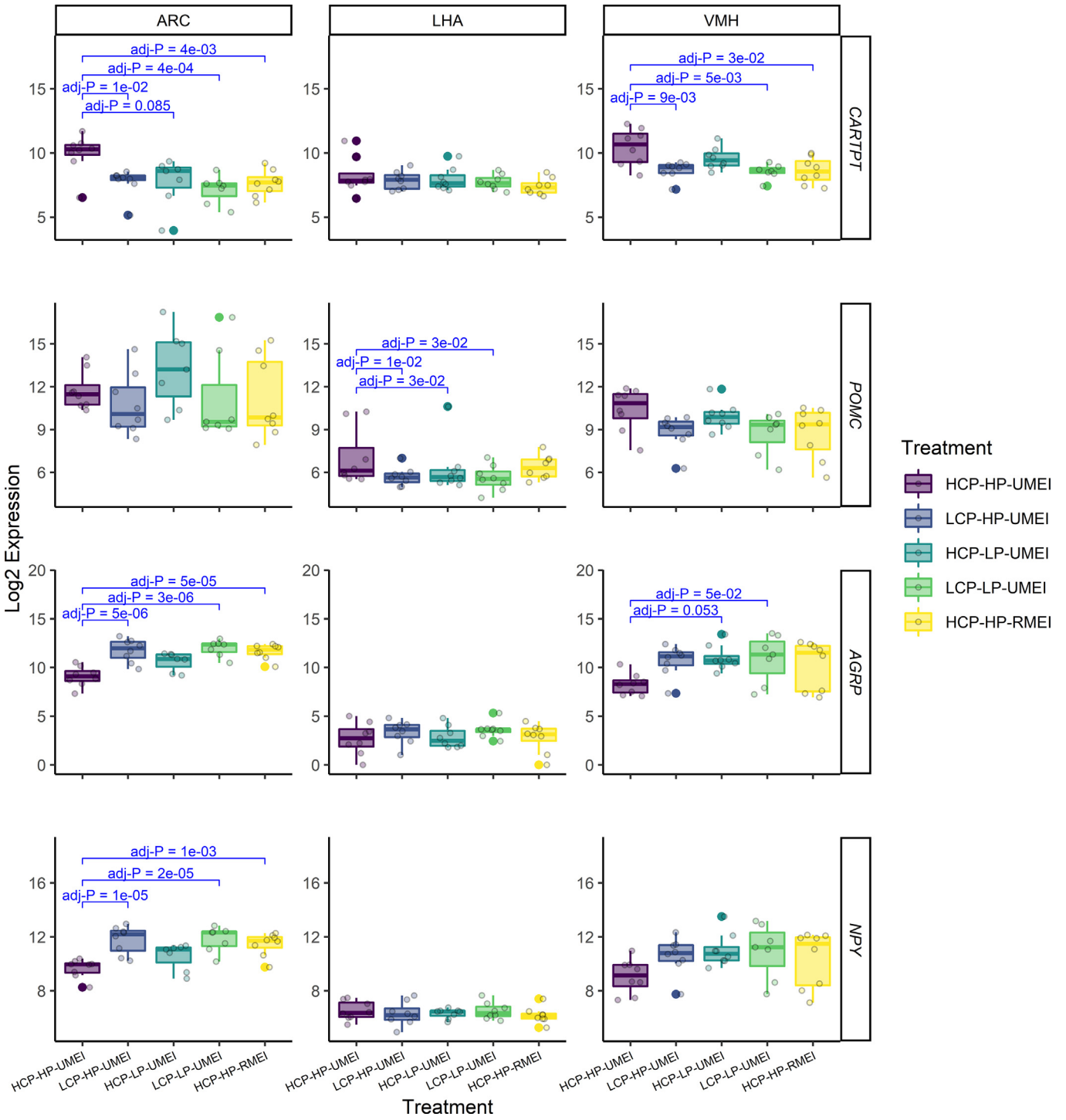

Fig. 3. Box plots of key genes related to feed intake, *CARTPT, POMC, AGRP* and *NPY,* in the arcuate nucleus (ARC), lateral hypothalamus (LHA) and ventromedial hypothalamus (VMH) of wethers fed different nutritional treatments[1]. Blue lines show adjusted *P*-values of less than 0.1 for treatment pairwise comparisons, as determined by *DESeq2* method, with adjusted $P < 0.05$ considered a statistically significant difference. Points represent individual wethers and are randomly spaced horizontally within treatment box plots to minimising overlaps. [1]See text for description of nutritional treatments. Abbreviations: H = high; L = low; P = (crude protein)phosphorus; UMEI = unrestricted metabolisable energy intake; RMEI = restricted metabolisable energy intake.

regions of the hypothalamus, but the lists of enriched terms, split by DE genes that were up- and downregulated, are included in Supplementary Material S4 (Innes et al., 2023). In addition, the lists of enriched terms for both up- and downregulated DE genes combined are included in Supplementary Material S5 (Innes et al., 2023).

Discussion

Nutrient deficiency models

Young wethers offered *ad libitum* diets deficient in CP, P, or combined CP and P voluntarily consumed less than wethers offered

a diet with adequate CP and P when ME intake was not limited. The lower DM intake in wethers fed the LCP-HP-UMEI, HCP-LP-UMEI and LCP-LP-UMEI treatments were also associated with a reduction in plasma urea-N, inorganic P and both urea-N and inorganic P, respectively, and all were associated with lower LW gain. Together this confirms the hypothesis that wethers fed *ad libitum* diets deficient in CP, P or both will voluntarily consume less than wethers fed a non-deficient diet.

Nitrogen deficiency

The DM intake of wethers fed the CP-deficient diet (LCP-HP-UMEI) was 42% lower than wethers fed the adequate CP diet (HCP-HP-UMEI). Ternouth et al. (1993) observed a 20% lower DM intake (28.3 g DM/kg LW per day) in wethers fed a diet with low CP (67.5 g/kg DM), high P (4.24 g/kg DM) and high ME (8.5 MJ/kg DM) content compared to wethers fed a diet with adequate CP and P (35 g DM/kg LW per day; 109 g CP/kg DM; 4.0 g P/kg DM) content. The more pronounced DM intake response to the low CP treatment in the current experiment was likely due to the lower dietary CP content (56 g/kg DM) than what was fed by Ternouth et al. (1993).

The consistent and significant decline of plasma urea-N to the low CP treatments (Table 3) indicates an appropriate CP deficiency was established. These values were also comparable to adult sheep that were fed a restricted amount of a wheaten chaff diet (22 g DM/kg LW per day; 56 g CP/kg DM) and had a plasma urea-N concentration of 2.1 mmol/L (McIntyre, 1970). In addition, the wethers fed the low CP treatments also had a NH_3-N (Table 4) concentration in the rumen fluid below the threshold of 50 mg/L required to meet the requirements for microbial protein production in ruminants fed high forage diets (Satter and Slyter, 1974).

When a rumen by-pass protein (fish meal) supplement was offered to Merino wether lambs fed a low-quality pasture hay (27 g CP/kg DM; 5.3 MJ ME/kg DM) they consumed 18.5 g DM/kg LW per day, which was significantly more than 15.3 g DM/kg LW per day consumed by wethers not offered the supplement (Quigley et al., 2012a; Quigley et al., 2012b). This suggests that metabolic mechanisms were likely involved in regulating DM intake due to dietary CP, independent of changes in rumen function. In addition, when a single dose of casein or urea was infused into the duodenum of sheep fed low CP diets (~40 g CP/kg DM), casein increased DM intake within 16 h but DM intake was not increased until the following day when urea was infused into the duodenum (Egan and Moir, 1965). The authors attributed this in part to acute metabolic mechanisms and not nitrogen recycling alone.

Therefore, feeding treatments with high ME content in this experiment was justified and was successfully used to develop a model of feed intake regulation in response to low dietary CP content that was independent of the mechanisms likely to be involved with rumen function.

Phosphorus deficiency

The DM intake for wethers fed the P-deficient diet (HCP-LP-UMEI) was 32% lower than the adequate P wethers (HCP-HP-UMEI). This is comparable to experiments by Ternouth and Sevilla (1990) who observed a 41 and 34% lower DM intake in young wethers fed deficient P diets (0.75 and 0.74 g P/kg DM, respectively) compared to adequate P diets (4.50 and 4.86 g P/kg DM, respectively).

The concentration of inorganic P in the plasma of wethers fed the HCP-LP-UMEI treatment (1.1 mmol/L; diet P 0.4 g/kg DM) were considered deficient, as previously determined in adult sheep fed a diet with 0.4 g P/kg DM (1.18 mmol/L; Ternouth et al. 1980), growing wethers fed a diet with 0.72 g P/kg DM (0.83 mmol/L; Schneider et al. 1985, and growing wethers fed a diet with 0.72 g P/kg DM (1.1 mmol/L; Ternouth & Sevilla 1990). It has also been suggested that a plasma inorganic P concentration of 1.3–1.9 mmol/L is 'marginal' in growing lambs (Suttle, 2010; Anderson et al., 2017), which is consistent with the wethers in the present experiment fed the LCP-LP-UMEI (plasma inorganic P 1.6 mmol/L; diet P 0.7 g/kg DM;) treatment.

Salivary P is able to buffer against the reduced dietary derived P in the rumen of wethers fed low P diets (Tomas et al., 1967), despite a strong positive relationship between P intake and salivary P production. The recycling of P to the rumen of the wethers in this experiment was unlikely to be limited as all wethers were offered 2.5 g DM/kg LW per day of chopped barley straw each day to help maintain salivary production, the pH of the rumens of wethers fed *ad libitum* were all within a normal range (6–7), and the total rumination time of wethers in all treatments was not different over a 24-h period (345 min ± 33.2; mean of all treatments ± SEM). Milton and Ternouth (1985) identified a clear increase in organic matter intake when sheep had higher blood, but not rumen, concentration of inorganic P. This confirms that it is plausible that wethers in the current experiment fed low P treatments were metabolically regulating their DM intake, independently of any effects on rumen function.

Hormones

Insulin has previously been implicated in feed intake regulation in sheep via signalling in the brain (Foster et al., 1991), and Ternouth (1990) suggested that glucose utilisation may be influenced by P deficiency in ruminants. Glucose and insulin have been correlated with digestible organic matter intake in an experiment where 27 different diets were fed to sheep (Bassett et al., 1971). The insulin concentration in that previous experiment ranged from 10 μU/mL in fasted sheep to greater than 25 μU/mL in sheep fed high-quality forages. Therefore, the plasma insulin concentration of wethers fed restricted and deficient treatments in our present study can be considered low, and differences observed are unlikely to be biologically important.

The concentration of IGF-1 was higher in the plasma of restricted-fed wethers compared to the LCP-LP-UMEI wethers, which may be in response to the higher CP intake of the restricted wethers, since they had the same ME intake. However, IGF-1 concentration was mostly correlated with ME intake (Pearson's $r = 0.77$, $P < 0.001$). A similar result has been previously described (Elsasser et al. 1989) where plasma IGF-1 of steers responded to CP intake independently of ME intake. Leptin and ghrelin both warranted inclusion due to their previously identified major roles in feed intake regulation, but they appear unlikely to be contributing to the nutrient deficiency-induced differences in DM intake observed in the current experiment.

Rumen retention time

Perhaps the most significant data to confirm the likelihood of a metabolic regulation of DM intake in the current experimental model is the rumen digesta weight and retention time. The higher digesta weight in the wethers fed the HCP-HP-RMEI treatment was observed because they had eaten 82 ± 16% of the DM intake of the previous day by the time the digesta weight was recorded at slaughter, 2 h after their last meal was offered. This is consistent with a higher rate of intake in the first h after feeding by wethers fed the HCP-HP-UMEI and HCP-HP-RMEI treatments (169 and 191 g DM/h, respectively; other data not shown). However, this indicates that the wethers fed the deficient treatment were not eating to their 'maximum' rumen capacity as the rumen digesta load of the wethers fed the deficient diets was 40–65% of the rumen fill of the HCP-HP-UMEI wethers. In addition, the apparent retention time of the rumen digesta were not different between wethers fed unrestricted ME intake treatments (Table 4) as

expected due to the similar DM digestibility of the diets fed to these wethers. Together, these data indicate that physical distension mechanisms were unlikely to be suppressing feed intake in the wethers in the present experiment. Whilst the apparent retention time determined here was not under steady-state conditions, and hence does not meet the strict mathematical requirements of such a calculation, the method used does identify relative differences.

Overall, an animal model of metabolic regulation of voluntary feed intake was achieved in young wethers fed diets deficient in CP and P that are representative of the concentration of these nutrients in the pastures grazed by ruminants in northern Australia. The successful establishment of this animal model allowed for the further analysis of gene responses within different tissues of wethers that were both hungry and satiated, and satiated due to different nutrient intakes, which is important for understanding underlying physiological pathways that influence voluntary feed intake of ruminants in northern Australia.

Gene expression

Very few previous publications have used RNA-seq to measure the abundance of mRNA in the sheep hypothalamus, despite the significant body of research measuring the relative abundance of mRNA using *in situ* hybridisation, quantitative PCR and microarray techniques. Therefore, the data of the current experiment encompasses a unique perspective of the gene responses in the ARC, LHA and VMH of sheep fed both nutrient-deficient and restricted treatments.

The ARC had more DE genes than the LHA and VMH, indicative that the ARC has a more important role in the regulation of feed intake in wethers. This is consistent with the critical role of the ARC in the hypothalamus in receiving and integrating peripheral and central signals. There were also 123 DE genes in the VMH between the wethers fed the low phosphorus (HCP-LP-UMEI) and the restricted ME intake (HCP-HP-RMEI) treatments that included genes that enriched for pathways related to transmembrane transport of anions and the synaptic vesicle. Some of these DE genes (*SLC47A1, SLC5A5, BHMT,* and *OSR1*) were also identified in clusters of genes upregulated in the ARC of the HCP-LP-UMEI and LCP-LP-UMEI treatments relative to the HCP-HP-UMEI treatment. Although this might suggest differences in neural signalling in treatments with low P, an inspection of the boxplots of these genes showed significant variation between animals in the nutrient-deficient treatments and further interpretation is not possible with the available data [Supplementary Fig. S1 in Innes et al. (2023)]. Overall, the established hunger and satiety centre in the LHA and VMH, respectively, could not be confirmed from the DE genes and pathways observed in this experiment. Therefore, we have focused our interpretation of the results to the ARC in this discussion.

The top DE genes, ranked on PIF, in the ARC of the wethers included the orthologs of genes previously implicated in mammalian feed intake regulation in model species: *NPY*, *AGRP*, *CARTPT* and *POMC*. The mRNA encoding the orexigenic (i.e. appetite stimulating) *NPY* and *AGRP* genes were significantly higher in the ARC of wethers fed the restricted (HCP-HP-RMEI) treatment compared to the unrestricted (HCP-HP-UMEI) treatment, as well as both low CP treatments (LCP-HP-UMEI and LCP-LP-UMEI). Likewise, the *CARTPT* gene had the inverse response to *NPY* and *AGRP* in the same treatment groups, reflecting the anorexigenic (i.e. appetite supressing) nature of the peptides encoded by *CARTPT*. This inverse gene response in the ARC of the restricted wethers (HCP-HP-RMEI) suggests a normal, coordinated signalling from *CARTPT* and *NPY/AGRP* neurons, consistent with previously reported responses in the hypothalamus of sheep fed restricted diets (Adam et al., 2002; Relling et al., 2010). However, it is unusual that the apparently satiated wethers in the low CP treatments would have the same hypothalamic ARC gene responses as the restricted wethers that represented a typical hunger model.

The *POMC* gene, an anorexigenic gene, was only DE in the LHA between the wethers fed the HCP-HP-UMEI and the nutrient-deficient treatments; however, the response in the ARC was highly variable. The expression of *POMC* in the hypothalamus of wethers has also previously had variable responses to feed restriction in wethers (Adam et al., 2002; Relling et al., 2010). Likewise, the mRNA encoding the receptors for α-melanocyte stimulating hormone (*MC3R* and *MC4R*) were not DE, indicating the melanocortin system may have had a minor role in regulating feed intake in these wethers.

Previously, infusion of cholecystokinin (as cholecystokinin-8) or its antagonist (loxiglumide) to the lateral ventricle has implicated cholecystokinin as a potent central satiety factor in ewe lambs with depressed feed intake due to an intestinal worm (*Trichostrongylus colubrifonnis*) infection (Dynes et al., 1998). However, the *CCK* gene, and its receptor *CCKAR*, were not DE between treatments in any region of the hypothalamus in the current experiment, suggesting that this pathway may not be important in a nutrient-deficient model of feed intake regulation in ruminants.

The lack of detectable response of *NPY*, *AGRP* and *CARTPT* in the ARC of wethers fed the P-deficient treatment (HCP-LP-UMEI) could be explained by their slightly higher mean DM intake (Table 2). In P-deficient wethers, the *CARTPT* gene had a tendency to be different, and the *NPY* and *AGRP* gene were ranked highly by the PIF metric for their numerical difference. These neuropeptides may have had an acute response to the preslaughter intakes of the wethers. However, the DM intake of the wethers fed the P-deficient (HCP-LP-UMEI) treatment for the previous 24-h period before their euthanasia was not different to the wethers fed the CP-deficient (LCP-HP-UMEI) treatment (24.4 g DM/kg LW and 23.6 g DM/kg LW, respectively), which were both lower than the wethers fed the HCP-HP-UMEI treatment ($P < 0.05$; 37.2 g DM/kg LW). Likewise, in the final 2 h before euthanasia the DM intake of wethers fed the HCP-LP-UMEI treatment was lower than wethers fed the HCP-HP-UMEI treatment ($P < 0.05$; 5.69 g DM/kg LW and 8.72 g DM/kg LW, respectively). Therefore, feed intake does not explain any differences in the abundance of *NPY*, *AGRP* and *CARTPT* in the ARC between the wethers fed the HCP-HP-UMEI and HCP-LP-UMEI treatments but may indicate that short-term feeding behaviour did not influence these genes. Nevertheless, the precise timing of the euthanasia of each wether, at exactly 2 h after their final meal, was considered important in the design of this experiment to minimise differences associated with acute changes in gene expression in the hypothalamus related to feeding behaviour. It is noted that this is either not considered or not reported in the experimental designs of previously published animal models measuring treatment responses of mRNA abundance.

Nonetheless, it appears that hypothalamic circuitry (at least *AGRP*, *NPY* and *CARTPT*) normally attributed to regulating feed intake was functioning in response to ME intake. In other words, wethers that were satiated but voluntarily consuming less, and ruminants that were feed restricted, both exhibited a response in the hypothalamus representative of hunger.

The pathway terms representing genes with a lower expression in the ARC of wethers fed the LCP-LP-UMEI treatment compared to the HCP-HP-UMEI treatment included 'ATP synthesis coupled proton transport' and 'oxidative phosphorylation'. This list of DE genes included *COX5A,* which is last enzymatic step of the electron transport chain (complex IV), and genes encoding various subunits of complex V (*ATP5MC1*, *ATP5F1B*, *ATP5MC3*), which are the ATPases that generate ATP from ADP. This may be significant as oxidative phosphorylation is the main source of ATP used by the central

neurons for information processing (Hall et al., 2012). In addition, two mRNA encoding enzymes from the citric acid cycle, citrate synthase (*CS*) and oxoglutarate dehydrogenase (*OGDH*), and an enzyme from glycolysis, triosephosphate isomerase 1 (*TPI1*), also had lower expression in the ARC of wethers fed the LCP-LP-UMEI relative to the HCP-HP-UMEI treatment. These responses may also indicate a lower availability of cellular energy substrates. For example, genes encoding the enzymes glutamate dehydrogenase (*GLUD1*) and glutamic-oxaloacetic transaminase (*GOT1*), which convert glutamate to α-ketoglutarate in the mitochondria and cytoplasm, respectively, were also lower in LCP-LP-UMEI. Nevertheless, the downregulation of enzymes in the glycolysis, citric acid cycle and oxidative phosphorylation pathways appear to be unique to the LCP-LP-UMEI, despite the restricted wethers (HCP-HP-RMEI) consuming the same ME intake.

The pathway terms representing genes with a higher expression in the ARC of wethers fed the LCP-LP-UMEI treatment compared to the HCP-HP-UMEI treatment included 'cellular response to hormone stimulus' and 'methylation', indicating a potential differentiation in the signalling within the hypothalamus. The mRNA encoding various methyltransferases had higher expressions in the ARC of wethers fed the LCP-LP-UMEI treatment (*TRMT13, VIRMA, BHMT, BHMT2, ASH1L,* and *METTL17*), and included the mRNA encoding for sirtuin 1 (*SIRT1*). Sirtuin 1 is a master metabolic regulator that responds to cellular energy (NAD+) influencing several transcription factors involved in cholesterol metabolism, fat metabolism, glucose homeostasis and immune response in various tissues (Schug and Li, 2011). The *SIRT1* gene had a higher expression in the ARC of wethers fed the LCP-LP-UMEI treatment compared to the HCP-HP-UMEI treatment. This is significant because this gene been implicated in nutrient sensing in the hypothalamus, responding to feed restriction or caloric reduction models to increase hypothalamic neuronal activity (Schug and Li, 2011). The deletion of *SIRT1* on *NPY/AGRP* neurons was also associated with a decrease in feed intake in transgenic mice (Dietrich et al., 2010). However, SIRT1 has an opposite effect in peripheral tissues, such as the liver, where it promotes fatty acid oxidation and inhibits gluconeogenesis (Schug and Li, 2011). Altogether the increase of *SIRT1* in the ARC of wethers in the nutrient-deficient treatments of the current experiment reinforces hunger signalling, which did not eventuate into feeding behaviour by the wethers.

Overall, these data suggest that the neuropeptides related to feed intake regulation in the hypothalamus of wethers fed a nutrient-deficient diet (LCP-LP-UMEI) responded in the same way as restricted-fed wethers (HCP-HP-RMEI); however, it is possible that the ARC of the wethers fed the LCP-LP-UMEI treatment experienced significant changes to both the translation of mRNA and the downstream regulation of protein expression, perhaps in response to a reduction in available cellular energy substrates. If there were indeed lower levels of cellular energy substrates in the hypothalamus then the question remains why wethers fed the nutrient-deficient diets voluntarily allow this to be the case, when a feed of adequate ME content was readily available.

Various conceptual models have been proposed to explain the regulation of feed intake in ruminants, such as (1) the two-phase model proposed by Conrad et al. (1964), where a crossover between physical and metabolic mechanisms of regulation occur with increasing diet digestibility, and (2) the conceptual model by Weston (1996) where feed intake is regulated by the constant, yet variable, energy deficit hunger signal that is assessed by the animal against negative feedback (satiety) signals. The Weston (1996) model is more consistent with the current experiment where pathways related to cellular energy metabolism were downregulated in the ARC of LCP-LP-UMEI wethers, which we speculate is a variable signal related to the animal's energy deficit. In addition, the key hunger signalling (*NPY/AGRP* neurons) in the ARC seemed to be activated in these wethers. Therefore, if this variable hunger signal, related to an energy deficit, was high in the nutrient-deficient wethers that consumed less feed, then some central neural or peripheral satiety signal/s must have been able to compete with the hypothalamic hunger signal. For example, stimulation of deep cerebellar nuclei in the lateral nucleus of the cerebellum of feed-deprived mice can prevent the normal increase in feed intake in response to stimulation of *AGRP* neurons, and this satiety mechanism was associated with an increased activation of dopamine (reward) neurons in the ventral tegmental area (Low et al., 2021).

This competing signalling is also consistent with the minimum total discomfort theory, which is a development of Weston's model proposed by Forbes (2007) where the accumulation of multiple 'discomfort' signals, both physical and metabolic, are integrated by the central nervous system in an additive manner and the animal adjusts feeding behaviour to minimise this total discomfort. If the 'discomfort' was minimised in the wethers consuming nutrient-deficient diets, then it may be assumed that the metabolic consequence of them eating more of the same nutrient-deficient diet was resulting in a signal that increased the total 'discomfort' and subsequently induced satiety. Indeed, if ruminants with a low voluntary feed intake due to a nutrient-deficient diet, such as the wethers in the LCP-LP-UMEI treatment, did increase their intake of the same diet, then it is not known what the metabolic consequences of this would be for the animal or to what amount feed intake could be increased. The downregulation of enzymes in glycolysis, citric acid cycle and oxidative phosphorylation pathways appear to be unique to the LCP-LP-UMEI suggesting that increased flux (through increased nutrient intake) could not be accommodated. Overall, the evaluation of these transcriptional changes in the context of previously established theories suggests that any development of intervention strategies to stimulate feed intake in nutrient-deficient ruminants should not be directed at previously established orexigenic and anorexigenic centres of the hypothalamus, which appear to respond to total ME intake. Instead, it is proposed that further research investigates possible satiety signalling related to the metabolic consequences of increased nutrient intake in nutrient-deficient ruminants, both in peripheral tissues and the brain.

Conclusion

In conclusion, an animal model of metabolic regulation of voluntary feed intake was established in young wethers fed diets deficient in CP and/or P. Orexigenic (*NPY*, *AGRP*) and anorexigenic (*CARTPT*) genes in the ARC had a similar response in the wethers consuming nutrient-deficient treatments and those consuming a ME-restricted treatment, despite these wethers expressing behaviours that would be indicative of satiated and hungry states, respectively. Genes involved with oxidative phosphorylation (*COX5A, ATP5MC1*, *ATP5F1B*, *ATP5MC3*), glycolysis (*TPI1*) and the citric acid cycle (*CS, OGDH, GLUD1, GOT1*) were downregulated in the ARC of wethers fed a nutrient-deficient (LCP-LP-UMEI) treatment relative to the non-deficient (HCP-HP-UMEI) treatment. It was proposed that hunger appears to be a constant state driven by the extent of energy deficit and that satiety signalling reduces total DM intake to minimise total 'discomfort' and avoid a potentially negative metabolic consequence for the over-consumption of a nutrient-deficient diet.

Supplementary materials

Supplementary materials and data are deposited at https://doi.org/10.6084/m9.figshare.c.6394533.

Ethics approval

All procedures involving animals were done so in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by The University of Queensland Animal Ethics Committee (SAFS/049/19).

Data and model availability statement

The raw RNA sequence data have been deposited in NCBI's Gene Expression Omnibus and are accessible via GEO series accession number GSE221072 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221072).

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence assisted technologies in the writing process.

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Declaration of interest

None.

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