Dietary and Genetic Regulation of B12 Metabolism in Sheep

Amey Brassington

Thesis submitted to the University of Nottingham for the Degree of Doctor of Philosophy

January 2019



School of Biosciences

Sutton Bonington Campus

Loughborough

Leicestershire

LE12 5RD

Declaration

I hereby declare that this thesis is my own work and that it has not been submitted for any other degree or award. The work presented herein is my own, and where other sources of information have been used, they have been duly acknowledged. All assistance given to me during the preparation of this thesis is also acknowledged.

Amey Brassington

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors Professor. Kevin Sinclair and Dr Nigel Kendall.

Kevin – Thank you for your continuous support throughout my PhD and for always making time for me. You have taught me a lot and I think your perfectionism has rubbed off on me.

Nigel – Thank you for sharing your knowledge of trace-element deficiencies with me and for having a great sense of humour.

Thank you to all of my colleagues at the University of Nottingham and the team at Roslin including Alan Archibald, Ozzie Matika, Richard Emes and others. Dongfang, Lydia and Cat, thank you for all your help and knowledge during my experimental work.

Thank you to all of the friends I made during my time at SB. I would like to give special thanks to my fellow PhD student and close friend Connie for her continued friendship throughout my PhD. Your phone chats are second to none and you never fail to make me laugh. Laura, thank you for the tea breaks and for preparing me for what was to come.

Thank you to my family for all their love and support, for being interested in my work and for believing in me.

Lastly but certainly not least, I would like to thank my partner, Alex, for being my rock. You have supported me unconditionally throughout my PhD studies and I honestly couldn't have done this without you.

Abstract

Cobalt (Co) deficiency in sheep can result in poor animal health and performance, ultimately decreasing farm profitability. Development of strategies to improve the health and productivity of Co deficient animals at different stages of the production cycle, but particularly of rapidly growing lambs, is therefore of significant interest and major economic importance to the sheep industry.

Cobalt is an essential component of vitamin B12, and therefore the nutritional status of this trace-element can impact on one-carbon (1C) metabolism and associated pathways. A number of factors influence the prevalence and severity of Co/B12 deficiency in sheep including nutritional status, stage of production cycle and geographical location. In addition, human studies have provided clear evidence for genetic predispositions to 1C metabolic imbalances. Currently, however, knowledge of such susceptibilities in farm animals does not exist. In response to these gaps in knowledge, this thesis sought to increase our understanding of nutritional and genetic regulation of B12 metabolism in sheep through supplementation trials and genetic analyses.

The first part of this thesis (Chapters 2 and 3) addressed nutritional aspects of B12 deficiency. These studies sought to analyse the biochemical consequences of decreased B12, to formally assess the interaction between Co and Se, and to evaluate the effectiveness of a novel long-acting supplementation strategy. B12 status was determined though biochemical assessment of primary and secondary metabolite concentrations in blood. Live weights and body condition scores were recorded to assess the potential consequences of depleted B12. The efficacy of SMARTShot®, a long-acting injectable microencapsulated source of B12, compared to the commonly used Co bolus on repletion of weaned Co deficient lambs determined its usefulness as a method of repletion. This method of supplementation was found to be a beneficial option for young lambs due to its ease and speed of administration and

ability to supplement light weight lambs from 3 weeks of age. Whilst it was not possible to address the primary hypothesis of Chapter 3, that a Se deficiency masked an underlying Co deficiency, this study provided evidence of seasonal and year-to-year variation in B12 status, BCS and live-weight gains highlighting the requirement for strategic 'risk management' approaches to supplementation.

The second part of this thesis (Chapters 4 and 5) addressed the genetic aspect of B12 deficiency. The aim of Chapter 4 was to address the hypothesis that single nucleotide polymorphisms (SNPs) in genes involved in or directly related to 1C metabolism lead to inter-individual variability in metabolic responses to micronutrient deficiencies in sheep. Furthermore, as every farm has different nutritional challenges and with evidence of geographic and ethnic variation in frequencies of 1C metabolic gene polymorphisms in humans, Chapter 5 sought to characterise 1C genetic diversity between breeds.

The objectives of Chapter 4 were addressed in a series of analyses which included depleting the Co status of weaned lambs by feeding a Co and S deficient barley-based diet and confirmation of status as in previous chapters. From these animals, DNA samples were collected for genotyping and liver samples were collected for quantification of metabolite concentrations through GCMS, HPLC and LC-MS/MS. An Illumina Infinium® iSelect® Custom Array with 4,576 probes capturing SNPs in 115 1C metabolism and related genes, and 108 related epigenetic regulators was constructed and used to genotype the collected DNA. Following this, a pipeline of bioinformatic analyses was developed, including a genome-wide association study (GWAS), facilitating identification and prioritisation of functionally significant SNPs. 33 SNPs were found to be significantly associated with concentrations of biochemical parameters related to 1C metabolism in the study population of 360 Texel lambs. Of particular biological interest were two SNPs in *CUBN;* a gene fundamental in the transport of B12 for provision to the methionine cycle and propionate pathways.

Chapter 5 investigated population differentiation of four commercially important breeds in the UK; Texel, Suffolk, Bluefaced Leicester and Swaledale, for SNPs in 1C related genes, with specific focus on the 33 functionally significant SNPs identified in Chapter 4. Of particular significance, Swaledale sheep differed genetically from the other three breeds, predominantly for functionally significant SNPs in *CUBN* identified in Chapter 4. Furthermore, interbreed variation in allele frequency was also observed for SNPs in important 1C regulatory genes including *MTHFR, MTHFD1, MTR, MUT* and *MAT1.*

Overall, outcomes of the studies in Chapters 4 and 5 provide an unprecedented understanding of the functional significance of complex relationships between 1C metabolites and variants in 1C metabolic genes, providing a basis for further study into judicious targeted supplementation and selection for genetic tolerance to Co deficiency in breeding programmes.

In summary this thesis confirms that B12 deficiency in sheep varies with respect to geographical location, stage of production cycle and nutritional availability partly as a consequence of seasonal and inter-year variability. Furthermore, the studies detailed in this thesis represent the first investigation of genetic influences on 1C metabolism in sheep, providing evidence for a possible genetic predisposition to 1C metabolic imbalances, resulting in inter-individual and inter-breed variation in the regulation of 1C and other B12 associated metabolic pathways.

Abbreviations

µg	Micrograms
µl	Microlitres
µM	Micromolar
µmols	Micromoles
10-fTHF	10-formyltetrahydrofolate
1C	One-carbon
3'/5'UTR	3-prime / 5-prime untranslated region
5, 10-CH=THF	5, 10-methenyltetrahydrofolate
5, 10-CH2THF	5, 10-methylenetetrahydrofolate
5-mTHF	5-methyltetrahydrofolate
aB12	Adenosyl-B12
ACN	Acetonitrile
ADP	Adenosine diphosphate
AHCY/SAHH	S-adenosyl-L-homocysteine hydrolase
AHVLA	Animal health and Veterinary Laboratories Agency
ALDH4A1	Aldehyde dehydrogenase 4 family member A1
ANOVA	Analysis of variance
APHA	Animal and Plant Health Agency
ASMTL	Acetylserotonin O-methyltransferase Like
Asn	Asparagine
Asp	Aspartate
ATIC	Phosphoribosylaminoimidazole-carboxamide formyltransferase
B	Boron
B12	Vitamin B12
B2	Vitamin B2
B6	Vitamin B6
B9	Vitamin B9 / Folic acid
BCKDC	Branched chain α-keto acid dehydrogenase
BCS	Body condition score
BFL	Bluefaced Leicester
BGT1	Betaine/GABA transporter

BHMT	Betaine-homocysteine S-methyltransferase
Ca	Calcium
CA3	Carbonic anhydrase 3
Cbl	Cobalamin
CBL	Cystathionine beta-lyase
CBS	Cystathionine beta-synthase
CH_3	Methyl
CHDH	Choline dehydrogenase
CHOL	Choline
Cit	Citrulline
Cl	Chlorine
Co	Cobalt
COQ5	Coenzyme Q5 methyltransferase
CP	Crude protein
CTGL	Cystathionine gamma-lyase
Cth	Cystathionine
CTH	Cystathionine γ-lyase
Cu	Copper
CUBN	Cubilin
CV	Coefficient of variation
Cys	Cysteine
dADP	Deoxyadenosine diphosphate
dATP	Deoxyadenosine triphosphate
dGDP	Deoxyguanosine diphosphate
dGTP	Deoxyguanosine triphosphate
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DM	Dry matter
DMG	Dimethylglycine
DNA	Deoxyribonucleic acid
DNMT1/3A/3B/3L	DNA methyltransferases
DPEP1	Dipeptidase 1
DPH5	Diphthamide Biosynthesis 5
dSAM	Decarboxylated S-adenosylmethionine

dTMP	Thymidine monophosphate
dTTP	Thymidine triphosphate
dUDP	Deoxyuridine diphosphate
dUMP	Deoxyuridine monophosphate
EI	Electron impact
FA	Folic acid
FAD	Flavin adenine dinucleotide
Fe	Iron
FMOC	9-fluorenylmethyl chloroformate
FPGH	Folypolyglutamate hydrolase
FR	Folate receptor
F_{ST}	Fixation index
FW	Fresh weight
g	Grams
GABA	Gamma-aminobutyric acid
GAD1	Glutamate Decarboxylase
GART	Phosphoribosylglycinamide formyltransferase
GC-MS	Gas chromatography-mass spectrometry
GCPII	Glutamate carboxypeptidase
GCS	Glycine cleavage system
GCSH	Glycine cleavage system protein H
GDP	Guanosine diphosphate
Glu	Glutamate
Gly	Glycine
GNMT	Glycine N-methyltransferase
GSH	Glutathionine
GSHPx	Glutathione peroxidase
GWAS	Genome wide association analysis
h	Hour
h^2	Heritability
Hcy	Homocysteine
HDACs	Histone deacetylases
HILIC-MS/MS	Hydrophilic interaction liquid chromatography - tandem mass spec

His	Histidine
HMT	Histone methyltransferase
HPLC	High performance liquid chromatography
HSG	High sugar grass
Hse	Homoserine
I	Iodine
ICP-MS	Inductively coupled plasma mass spectrometry
Ile	Isoleucine
IMP	Inosine monophosphate
IMPDH1	Inosine monophosphate dehydrogenase 1
K	Potassium
Kb	Kilobases
kg	Kilograms
KMT	Histone lysine N-methyltransferase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
lcRNAs	Long non-coding RNAs
Leu	Leucine
LH	Lithium heparin
LOD	Limit of detection
LOQ	Limit of Quantification
LWG	Live weight gain
M	Molar
m^6A	N6-methyladenosine
MAF	Minor allele frequency
MAT1A/2A/2B/I/III	Methionine adenosyltransferases
mB12	methylB12
MCEE	Methylmalonyl-CoA epimerase
MDS	Multi-dimensional scaling
Met	Methionine
METTL	Methyltransferase like
mg	Milligrams
Mg	Magnesium
Min	Minutes
miRNA	Micro-RNA

MMA	Methylmalonic acid
Mn	Manganese
MnO	Manganese oxide
MTA	5’-methylthiodenosine
mTHF	methyltetrahydrofolate
MTHFD1/2	Methylene-tetrahydrofolate dehydrogenase
MTHFR	5, 10-methylenetetrahydrofolate reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase
MUT	Methylmalonyl-CoA mutase
Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NCGD	Neutral Cellulase Gammanase Digestibility
NDF	Neutral detergent factor
NH3	Ammonia
nm	Nanometers
nM	Nanomolar
NME	Nucleoside diphosphate kinase
nmol	Nanomoles
NOX4	NADPH Oxidase 4
NSUN6	NOP2/sun domain 6
NTDs	Neurotubular defects
OCFA	Odd-chain fatty acids
ODC	Ornithine decarboxylase
OMT	O-methyltransferases
Orn	Ornithine
P	Phosphorus
PC	Phosphatidylcholine
PCA	Principal component analysis
PCCA/B	Propionyl-CoA carboxylase
PCFT	Proton-coupled folate carrier
PCMT1	Protein-L-isoaspartate (D-aspartate) O-methyltransferase
PE	Phosphatidylethanolamine

PEMT	Phosphatidylethanolamine methyltransferase
Phe	Phenylalanine
PL	Pyridoxal (B6)
pM	Picomolar
pmol	Picomoles
PPA	Propionic acid
PRMT	Protein arginine methyltransferase
PRPP	Phosphoribosyl pyrophosphate
Put	Putrescine
QC	Quality control
RFC1/SLC19A1	Reduced folate carrier/Succinate CoA ligase
RNA	Ribonucleic acid
S	Sulphur
SA	Succinic acid
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAMDC	S-adenosylmethionine decarboxylase
SAR	Sarcosine
Se	Selenium
SED	Standard error of differences
SEM	Standard error of means
Ser	Serine
SETD1B	SET domain containing 1B
SHMT	Serine hydroxymethyltransferase
SMS	Spermine synthase
SNP	Single nucleotide polymorphism
SO4	Sulphate
Spd	Spermidine
Spm	Spermine
SRM	Spermidine synthase
SUF	Suffolk
SWA	Swaledale
TAU	Taurine
TCA	Tricarboxylic acid

TCN2	Transcobalamin II
TET	ten-eleven translocation
TEX	Texel
THF	Tetrahydrofolate
Thr	Threonine
TMG	Trimethylglycine/betaine
TPMT	TRNA Methyltransferase
Trp	Tryptophan
TYMS	Thymidylate synthase
Tyr	Tyrosine
U/L	Units per litre
UDP	Uridine diphosphate
UK	United Kingdom
UMP	Uridine monophosphate
V_a	Additive variance
Val	Valine
V_d	Dominance variance
V_e	Environmental variance
V_g	Genetic variance
VIDA	Veterinary Investigation Diagnosis Analysis
V_p	Phenotypic variance
WT	Wild type
XMP	Xanthine monophosphate
Zn	Zinc
α-KB	Alpha-ketobutyrate
αKγMB	Alpha-keto-gamma-methiolbutyrate

List of figures

Figure 1.1. Relative Co topsoil concentrations in England and Wales taken from the UK soil observatory……..….8

Figure 1.2. Monthly trends of Co deficiency diagnosis in sheep from VIDA records over four consecutive years; 2012 to 2015……..……..…9

Figure 1.3. One-carbon metabolic pathways……..…….16

Figure 1.4. Methyl-trap hypothesis; the effect of B12 deficiency on the folate cycle ……..…..……..18

Figure 1.5. Propionate metabolism……21

Figure 1.6. Polyamine pathway……..……..22

Figure 1.7. Phosphatidylcholine pathway……..23

Figure 1.8. Nucleotide biosynthesis……24

Figure 1.9. Development of working hypothesis. Flow diagram outlining the studies in this thesis……35

Figure 2.1. Growth rate from birth to weaning of all lambs on the participating monitor farm and cohort of 26 lambs used in the current study. (Median and interquartile ranges with 10^{th} and 90^{th} percentiles)……..…..39

Figure 2.2. The effect of microencapsulated B12 injection ● and Co bolus ● on blood metabolite concentrations; serum B12 (A) and plasma Hcy (B), plasma MMA (C) and plasma SA (D). → depicts time of treatment. For B12 (A), interaction (P<0.001; SED=329) between treatment and time. For Hcy (B), effect of time (P=0.007; SED=0.57). For MMA (C), effect of treatment (P=0.014; SED=3.5) and time (P<0.001; SED=2.7). For SA (D), effect of time (P=0.015; SED=0.3)……..……..48

Figure 2.3. Effect of B12 injection o and Co bolus ● on dry matter (DM) intake (g/kg $M^{0.75}$) over time. → depicts time of treatment. Effect of time (P<0.001; SED=4.9)……..49

Figure 2.4. Effect of microencapsulated B12 injection ● and Co bolus ● on lamb live-weight. Effect of time (P<0.001; SED=0.46)……50

Figure 2.5. Comparison of trace-element supplementation strategies. Taken from Grace and Knowles (2012). —— Cobaltised drench (7 mg Co as $CoCl_2$), ····· Soluble B12 injection (2 mg B12), - - - - Co bolus (10g bolus as 30% CoO:70% Fe), ——— Long-acting B12 injection (6 mg B12)……..……52

Figure 3.1. Lamb plasma homocysteine concentrations at four weeks post weaning (data from unpublished observations of Dodd and Sinclair (2014)). Means (—) and reference intervals for deficient (- - -) and adequate (- - -) B12 status are included. Supplementation was provided at weaning. Supplementation with Se-I alone led to increased (P=0.006; SED=1.5) plasma homocysteine concentrations……..……..…...61

Figure 3.2. Eight-week and weaning (~12 weeks) plasma selenium concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for sufficient (- - -) and deficient (- - -) status are given. (Grace, 2006)……..68

Figure 3.3. Eight-week and weaning (~12 weeks) plasma cobalt concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for deficient (- - -) and sufficient (- - -) status are given. (Suttle, 2010) For ewes (A) effect of time (P=0.035; SED=0.43)…….69

Figure 3.4. Eight-week and weaning (~12 weeks) serum vitamin B12 concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for deficient (- - -) and adequate (- - -) status are given (Suttle, 2005)……..…...69

Figure 3.5. Ewe weights (A) and ewe BCS (B) (median, interquartile ranges and 1-99th percentiles)……..70

Figure 3.6. Eight-week and weaning (12 weeks) concentrations of serum B12 in ewes (A) and lambs (B), plasma homocysteine in ewes (C) and lambs (D) and plasma methylmalonic acid (E) and succinic acid (F) in ewes. Mean values (—), and deficient (- - -) and marginal (- - -) reference ranges are given. For ewe serum B12 (A), interaction (P=0.037; SED=219) between B12 treatment and time. For ewe plasma SA (F), effect of B12 treatment (P=0.015; SED=1.2) and time (P<0.001; SED=1.1). For lamb serum B12 (B), effect of time (P<0.001; SED=91.8). For lamb plasma Hcy (D), effect of time (P<0.001; SED=0.346) ……75

Figure 3.7. Eight-week and weaning (~12 week), ewe (A) and lamb (B), Se concentrations. Mean values (—), and deficient (- - -) and marginal (- - -) reference ranges are included. For ewe plasma Se (A), effect of Se treatment (P=0.002; SED=0.054) and time (P<0.001; SED=0.049). For lamb plasma Se (B), effect of time (P<0.001; SED=0.024) ……..…...76

Figure 3.8. Eight-week and weaning (~12 week), ewe (A) and lamb (B), GSHPx activity. Mean values (—) and deficient reference points (- - -) are included. For ewe GSHPx (A), effect of Se treatment (P<0.001; SED=10.21) and time (P<0.001; SED=5.59). For lamb GSHPx, effect of Se treatment (P<0.001; SED=9.45) and time (P<0.001; SED=3.12)……..……...76

Figure 3.9. Co concentrations (mg/kg DM) of washed (●) and unwashed (○) grass samples taken at 0, 5, 8 and 12 weeks post lambing in year 1 of the study. (Keenan, Kendall and Sinclair (2015; unpublished data)). Effect of time (P<0.001)……82

Figure 3.10. Between-year variation in serum B12 at 8 weeks and weaning, in ewes (A) and lambs (B). For ewes (A) effect of year (P<0.004; SED=195). For lambs (B), effect of year (P<0.003; SED=200)……...84

Figure 3.11. Ewe BCS (A) at lambing, 8 weeks and weaning and ewe weight change (B) from 8 weeks to weaning (B) in Experiment 1 (**—**) and Experiment 2 (**- - -**)……..86

Figure 4.1. Total plasma Hcy concentrations in ewes offered a Control or Methyl Deficient (MD) diet (deficient in methionine, B12 and folate) (data derived from Kanakkaparambil et al., 2009) (P<0.001; SED=2.08)……...93

Figure 4.2. Liver Co concentrations for 277 lamb livers from 5 farms. Reference concentrations for sufficient (**---**) and deficient (**---**) status (Smith, 1987; Ulvund, 1990) are given. Box plots show median and interquartile ranges, with whiskers set at 5th and 95th percentiles. Effect of farm (P<0.001; SED=0.003)……..……113

Figure 4.3. Plasma Co concentrations for 83 lambs from 6 farms on arrival at the University farm. Reference concentrations for sufficient (---) and deficient (---) status are given (Suttle, 2010). Box plots show median and interquartile ranges, with whiskers set at 5th and 95th percentiles. Effect of Farm (P<0.001; SED=1.77)……..…114

Figure 4.4. Dry matter (DM) intake (g/kg M$^{0.75}$) over time. (median, interquartile ranges and, 1 and 99th percentiles). Effect of time (P<0.001; SED=1.2)……..……...115

Figure 4.5. Change in lamb weights over time. Mean weight (**—**). Effect of time (P<0.001; SED=0.25). Effect of farm (P<0.001; SED=1.5). Time by farm interaction (P<0.001; SED=1.7)…….....115

Figure 4.6. Serum B12 (A), plasma Hcy (B) and plasma MMA (C) concentrations over time. Reference concentrations for sufficient (---) and deficient (---) status are given (Suttle, 2005; Gruner et al., 2004; Kennedy et al., 1992). For B12 (A), effect of time (P<0.001; SED=13.8). For Hcy (B), effect of time (P<0.001; SED=0.3). For MMA (C), effect of time (P<0.001; SED=6.87)……..…117

Figure 4.7. Number of SNPs identified by genomic sequencing of 20 unrelated Texel ewes. Novel SNPs identified are in blue, SNPs already present in the Ensemble database are in red……120

Figure 4.8. Multi-dimensional Scaling (MDS) display of the population of 360 lambs for 3,173 1C metabolism-related SNPs that passed QC……..……123

Figure 4.9. Manhattan plots for the GWAS of sheep liver 1C metabolites. Genomic location (horizontal axis) is plotted against $-\log_{10}$ (p-value); genome wide ($P<0.05$) and suggestive thresholds are shown as black (---) and red (---) coloured lines, respectively. Chromosomes are alternatively coloured blue and green. DMG (A), TMG (B), mB12 (C), FAD (D), PL (E), PPA (F), MMA (G)……...124

Figure 4.10. Observed genotype frequencies for significant SNPs associated with (A) DMG 1=dbrs400381348 2=UoNOvar.1503; (B) TMG 1=dbrs428658243 2=dbrs400381348 3=dbrs161801525 4=dbrs161923561 5=dbrs159794405 6=UoNOvar.439; (C) mB12 1=UoNOvar.325 2=dbrs430165882; (D) FAD 1=dbrs408003853 2=dbrs162034765 3=dbrs427234880 4=dbrs414970315 5=dbrs404243017 6=dbrs424867462 7=dbrs399509316 8=dbrs404706479 9=UoNOvar.764 10=dbrs423172086; (E) PL 1=dbrs408003853 2=dbrs406023352 3=dbrs162081923 4=dbrs402697947 5=dbrs406167325 6=dbrs417907151 7=dbrs161814674; (F) PPA 1=dbrs413573281 2=dbrs413234073 3=UoNOvar.460 4=UoNOvar.463 5=dbrs419604537 6=UoNOvar.380. Wild type is in black, heterozygous in dark grey and homozygous rare in light grey……127

Figure 4.11. (Adapted from Clare et al., 2019, in press) Purine nucleotide biosynthesis. Enzymes are in boxes: GART, phosphoribosylglycinamide formyltransferase; IMPD1, Inosine Monophosphate Dehydrogenase 1; ATIC, phosphoribosylaminoimidazole-carboxamide formyltransferase; MTHFD1/2, methylene-tetrahydrofolate dehydrogenase; THF, tetrahydrofolate; 10-f-THF, 10-formyl-tetrahydrofolate; 5,10-CH=THF, 5,10-methenyl-tetrahydrofolate; 5,10-CH_2THF, 5,10-methylene-tetrahydrofolate; PRPP, phosphoribosyl pyrophosphate; IMP, Inosine monophosphate; XMP, xanthine monophosphate; GDP, guanosine diphosphate; dGDP, deoxyguanosine diphosphate; dGTP, deoxyguanosine triphosphate; ADP, adenosine diphosphate; dADP, deoxyadenosine diphosphate; dATP, deoxyadenosine triphosphate……..……139

Figure 4.12. Simplified depiction of one-carbon metabolism (adapted from Clare et al., 2019, in press). Met, Methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HCY, homocysteine; Cth, cystathionine; DMG, dimethylglycine; TMG, trmethylglycine; GLY, glycine; SAR, sarcosine; THF, tetrahydrofolate; DHF, dihydrofolate; SER, serine; 5,10-CH_2THF, 5,10-methylenetetrahydrofolate; 5-mTHF, 5-methyltetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; 10-f-THF, 10-formyltetrahydrofolate; CHOL, choline. Methylene tetrahydrofolate is underlined highlighting its position at the metabolic intersection whereby folate is either directed towards nucleotide synthesis (i.e. purines and thymidylate) or toward methylation reactions.....141

Figure 5.1. Determination of K using CV error……152

Figure 5.2. K means clustering of the 4 sheep breeds. Individuals are presented as vertical lines divided into K colours, representing constructed populations. K4 = 4 populations; POP, population; TEX, Texel; SWA, Swaledale; BFL, Bluefaced Leicester; SUF, Suffolk…….....155

Figure 5.3. Phylogenetic relationship of sheep from 4 breeds; Texel (Green), Suffolk (Blue), BFL (Turquoise) and Swaledale (Red). The unrooted tree was constructed using Figtree software as described in the methods. The horizontal axis represents the distance or dissimilarity between clusters. Each line on the vertical axis represents a sample……...156

Figure 5.4. Manhattan plots of the pairwise genome-wide autosomal F_{ST} analyses. Black threshold lines represent the 95% quantile and dashed purple lines represents 99% quantile of the F_{ST} distribution……..157

Figure 5.5. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 6 SNPs (dbrs413573281, dbrs413234073, UoNOvar.460, UoNOvar.463, dbrs419604537, UoNOvar.380) identified in Chapter 4 to be associated with varying concentrations of PPA in sheep liver in 4 sheep breeds……166

Figure 5.6. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 2 SNPs (dbrs400381348 and UoNOvar.1503) identified in Chapter 4 to be associated with varying concentrations of DMG in sheep liver in 4 sheep breeds; TEX, Texel; SUFF, Suffolk; SWA, Swaledale; BFL, Bluefaced Leicester……..….167

Figure 5.7. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 5 SNPs (dbrs428658243, dbrs161801525, dbrs161923561, dbrs159794405, UoNOvar.439) identified in Chapter 4 to be associated with varying concentrations of TMG in sheep liver in 4 sheep breeds……168

Figure 5.8. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 2 SNPs (UoNOvar.325, dbrs430165882) identified in Chapter 4 to be associated with varying concentrations of MethylB12 in sheep liver in 4 sheep breeds……169

Figure 5.9. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 10 SNPs (dbrs408003853, dbrs162034765, dbrs427234880, dbrs414970315, dbrs404243017, dbrs424867462, dbrs399509316, dbrs40470679, UoNOvar.764, dbrs423172086) identified in Chapter 4 to be associated with varying concentrations of FAD in sheep liver in 4 sheep breeds……170

Figure 5.10. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 6 SNPs (dbrs406023352, dbrs162081923, dbrs402697947, dbrs406167325, dbrs417907151, dbrs161814674) identified in Chapter 4 to be associated with varying concentrations of PL in sheep liver in 4 sheep breeds……171

List of tables

Table 1.1. Reference ranges/deficiency classifications for B12 status. (Rice et al., 1989; Kennedy et al., 1992; Gruner et al., 2004; Suttle, 2005; Furlong et al., 2010)……...12

Table 1.2. Example variants in key 1C genes associated with adverse clinical consequences……..….30

Table 2.1. Chemical and mineral composition of barley, soya and hay……..……42

Table 3.1. Combined twin-lamb weights and growth rates (mean ± s.e.m.) up to weaning……71

Table 3.2. Ewe and combined twin-lamb performance (mean ± s.e.m.) from lambing to weaning. ……....77

Table 3.3. Temperature (°C) and rain fall (mm) recorded from March to July during Experiments 1 and 2 (www.metoffice.gov.uk).....85

Table 3.4. Between year variation in combined mean (± s.e.m.) twin lamb weights (A) and growth rates (B) at 3 time points; lambing, 8 weeks and weaning. ……87

Table 4.1. Chemical and mineral composition of barley based concentrate diet and hay……..……98

Table 4.2. Mobile phase gradients for analysis of liver polyamine concentrations by HPLC ……106

Table 4.3. Mobile phase gradients for analysis of liver amino acid concentrations by HPLC ……108

Table 4.4. Limits of quantification and inter-assay CVs for all liver amino acids quantified by HPLC……108

Table 4.5. Metabolite concentrations in 360 lamb livers determined by GCMS. Concentrations are presented as nmol/g liver. MMA, methylmalonic acid; SA, succinic acid; PPA, propionic acid. ……....118

Table 4.6. Metabolite concentrations in 360 lamb livers determined by HPLC. Concentrations are presented as nmol/g liver. Ser, serine; Gly, glycine; Met, methionine; Put, putrescene; Spd, spermidine; Spm, spermine. ……118

Table 4.7. Metabolite concentrations in 360 lamb livers determined by HILIC-MS/MS. ……119

Table 4.8. Prioritisation of SNPs for retention on the Illumina Infinium iSelect® Custom Array……121

Table 4.9. Estimates of variance components and heritabilities for metabolite concentrations in sheep liver……121

Table 4.10. Significant SNPs identified in the association analysis using GenABEL……..125

Table 4.11. Significant SNPs identified using ASReml for metabolite levels in sheep liver. ……...…128

Table 5.1. Sheep breeds and numbers used in the population genetics analysis……152

Table 5.2 SNPs in metabolic genes appearing in more than one pairwise F_{ST} analysis. Breed comparisons in which each SNP was identified as being significantly different between breeds are given. From top to bottom, sub-sectioned in grey, SNPs were found to differ between breeds in 4, 3 and 2 of the 6 breed comparisons analysed. ……...…160

Table 5.3. Genetic differentiation for SNPs dbrs413573281 and dbrs413234073 in *CUBN* between breeds, expressed as the fixation index (F_{ST}; Weir and Cockerham, 1984) ……..166

Table 6.1. Possible experimental diet arrangement for proof study..…185

List of Contents

Introduction 1
CHAPTER ONE: Literature review 4
1.1 Trace-element deficiencies 5
1.2 Cobalt deficiency in sheep 5
1.3 Vitamin B12 6
1.4 Cobalt requirements of sheep and susceptibility to deficiency . 7
1.5 Assessment of status 11
1.6 Treatment of cobalt deficiency in sheep 13
1.7 One-carbon metabolism and related pathways 14
1.7.1 Methionine cycle 17
1.7.2 Folate Cycle 17
1.7.3 *Trans*sulphuration pathway 19
1.7.4 Propionate pathway 20
1.7.5 Polyamine pathway 21
1.7.6 Phosphatidylcholine pathway 22
1.7.7 Nucleotide biosynthesis 23
1.8 Nutritional regulation of one-carbon metabolism 25
1.9 Genetic regulation of one-carbon metabolism 28
1.10 Methyl group donation and epigenetics 31
1.11 Development of working hypothesis 33
CHAPTER TWO: Comparison of the efficacy of a long-acting injectable micro-encapsulated vitamin B12 supplement and intra-ruminal cobalt bolus on metabolic status and performance of growth-retarded lambs ..... .36
2.1 Introduction 37
2.2 Materials and methods 40
2.2.1 Animals, treatments and sample collection 40
2.2.2 Blood analysis 43
2.2.2.1 Vitamin B12 43
2.2.2.2 Homocysteine 43
2.2.2.3 MMA and SA 44
2.2.2.4 Statistical analysis 45
2.3 Results 46
2.3.1 Blood analysis 46
2.3.2 Intakes and weights 49

2.4 Discussion 50
2.4.1 Diagnosis of Co deficiency 50
2.4.2 Supplementation strategies 51
2.4.3 B12 injection vs Co bolus: Effect on metabolite levels 53
2.4.4 Propionate-succinate pathway 54
2.4.5 B12 injection vs Co bolus: Effect on DM intake and growth rate 55
2.5 Conclusion 57
CHAPTER THREE: The effect of B12 and Se supplementation on metabolic status and performance of ewes and their lambs 58
3.1 Introduction 59
3.2 Materials and methods 62
3.2.1 Animals, treatments and sample collection 62
3.2.1.1 Experiment 1 62
3.2.1.2 Experiment 2 63
3.2.2 Body condition scoring 63
3.2.3 Blood analysis 63
3.2.3.1 Serum B12 64
3.2.3.2 Plasma Hcy 64
3.2.3.3 Glutathione peroxidase 64
3.2.3.4 Trace-elements 65
3.2.3.5 MMA and SA 65
3.2.4 Statistical analysis 65
3.3 Results 67
3.3.1 Experiment 1 67
3.3.1.1 Plasma Se 67
3.3.1.2 Plasma Co 67
3.3.1.3 Serum B12 67
3.3.1.4 Weight and BCS 67
3.3.2 Experiment 2 72
3.3.2.1 Serum vitamin B12 72
3.3.2.2 Plasma Hcy 72
3.3.2.3 Plasma MMA and SA 72
3.3.2.4 Blood Se 73
3.3.2.5 Glutathione peroxidase in blood 73

3.3.2.6 BCS and weights 73
3.4 Discussion 78
3.4.1 Suckling vs weaned lambs 79
3.4.2 Cumulative effects of Co deficiency 81
3.4.3 Seasonal effects on herbage Co levels 81
3.4.4 Animal performance 83
3.4.5 Between-year variation 83
3.4.6 Se and Co: supplementation and metabolic demands 88
3.5 Conclusions and implications 91
CHAPTER FOUR Genetic regulation of one-carbon metabolism in sheep 92
4.1 Introduction 93
4.2 Materials and methods 95
4.2.1 Animals, sample collection and diet 95
4.2.1.1 Liver digestion and ICP-MS 96
4.2.1.2 Extraction of DNA from liver samples 96
4.2.1.3 Co deficient cohort 97
4.2.1.4 Serum B12 98
4.2.1.5 Plasma Hcy 99
4.2.1.6 Plasma MMA 99
4.2.1.7 Extraction of DNA from blood samples 99
4.2.2 Construction of Illumina Infinium® iSelect® Custom Array 100
4.2.3 Liver metabolite quantification 103
4.2.3.1 GCMS 103
4.2.3.2 HPLC 105
4.2.3.3 HILIC-MS/MS 109
4.2.4 Genetic analysis 111
4.2.4.1 Variance component analysis 111
4.2.4.2 Association analysis using whole genome (GWAS) 111
4.2.4.3 Association analysis using single SNPs 112
4.2.4.4 Statistical analyses of phenotypic data 112
4.3 Results 113
4.3.1 Liver cobalt 113
4.3.2 Plasma Cobalt 114

4.3.3 Intakes and weights 114
4.3.4 Blood metabolites 116
4.3.5 Liver metabolites 117
4.3.6 Illumina Infinium® iSelect® Custom Array 120
4.3.7 Variance component analysis 121
4.3.8 Classical multidimensional scaling 122
4.3.9 Association analysis 123
4.4 Discussion 129
4.4.1 SNP discovery 130
4.4.2 Measurement of liver metabolites 130
4.4.3 Selection of animals 131
4.4.4 Dry matter intakes and lamb growth 132
4.4.5 Co deficient diet end point 133
4.4.6 Variance component analysis 134
4.4.7 Association analysis 136
4.4.7.1 PPA with dbrs413573281and dbrs413234073 in CUBN 136
4.4.7.2 FAD and PL with dbrs408003853 in ENSOARG00000010451 137
4.4.7.3 methylB12 with UoNOvar.325 in DPEP1 137
4.4.7.4 methylB12 with dbrs430165882 in GCSH 137
4.4.7.5 DMG and TMG with dbrs400381348 in GART 138
4.4.7.6 TMG with dbrs161923561 in MTHFD1L 140
4.4.7.7 TMG with dbrs428658243 in GAD1 141
4.4.7.8 Other variant associations 142
4.4.7.9 Epigenetic regulators 143
4.5 Conclusions, implications and future research priorities 144
CHAPTER FIVE Inter-breed variability in the regulation of one-carbon metabolism in sheep 146
5.1 Introduction 147
5.2 Materials and methods 150
5.2.1 Animals, treatments and sample collection 150
5.2.2 SNP genotyping and quality control 151
5.2.3 K-means clustering 151
5.2.4 Phylogenetic analysis 152
5.2.5 Pairwise fixation index (F_{ST}) 153

5.2.6 Statistical analyses 154
5.3 Results and discussion 155
5.3.1 K means clustering 155
5.3.2 Phylogenetic tree 155
5.3.3 Pairwise F_{ST} 157
5.3.3.1 Key findings from breed comparisons 158
5.3.3.2 Summary of breed comparisons 161
5.3.3.3 33 SNPs identified in Chapter 4 164
5.3.4 Genotype frequencies of SNPs identified in Chapter 4 165
5.3.4.1 Variants associated with PPA 165
5.3.4.2 Variants associated with DMG 167
5.3.4.3 Variants associated with TMG 167
5.3.4.4 Variants associated with methylB12 169
5.3.4.5 Variants associated with FAD 169
5.3.4.6 Variants associated with PL 171
5.4 Summary of main findings 172
5.5 Conclusions and implications 174
CHAPTER SIX Discussion and Conclusions 175
6.1 Introductory comments 176
6.2 Summary of key findings 177
6.3 The bigger picture 180
6.4 General conclusion and future research priorities 183
References 187

Introduction

Cobalt (Co) deficiency in sheep is of major economic importance in several countries (Kennedy et al, 1992), with even marginal deficiencies leading to poor animal performance and ultimately decreased farm profitability (Grace, 1994). The disease manifests itself in ill-thrift and loss of appetite, which lead to decreased live weights, anaemia, increased susceptibility to infection and increased perinatal mortality in lambs (Grace, 1994; Underwood and Suttle, 1999). In Australia and New Zealand this debilitating disease in sheep is known as ‘wasting disease’ and ‘bush sickness’, and in the UK as ‘cobalt pine’ (Suttle, 2010).

Analysis of data from the National Veterinary Investigation Diagnosis Analysis (VIDA) database (records compiled from APHA and SAC centres across Great Britain) revealed that, between the years of 2011 and 2014, Co deficiency was in the top 10 most diagnosed diseases in sheep of all ages in the UK. Between July and September 2017, Co deficiency was the second most diagnosed disease, contributing to 11% of all diagnostic submissions. In lambs, such deficiencies lead to reduced growth rates by ~30% on average which equates to around £5 million loss to the industry per year for weaned, autumn finished lambs. Considering sales at other times of the year, reduced ewe productivity and increased parasitic susceptibility as a consequence of Co deficiency, further increases losses, both financially and environmentally (e.g. increased green-house gas emissions that arise as a consequence of poor productivity and extended finishing periods). Prophylactic treatment of all ewes and lambs with supplements to mitigate these effects, however, would be both wasteful and expensive for the industry and could compromise animal welfare (due to route and frequency of administration).

Dietary Co is utilised by sheep ruminal bacteria to synthesize vitamin B12. The ruminal bacteria require B12 for their own survival as well as

for the production of nutrients subsequently absorbed and utilised by the animal. An example of such a nutrient is propionic acid, a major source of energy for ruminants. In addition to its effects on ruminal bacteria, a dietary deficiency of Co will induce depletion of B12 within the animal's tissues. Consequently, activity of the B12 dependent enzymes methylmalonyl-CoA mutase (MUT; EC 5.4.9.92) and methionine synthase (MTR; EC 2.1.1.13) become compromised, resulting in a multiplicity of defects involving propionate, succinate, folates, methionine, and a variety of methyl dependent reactions.

With the potential economic losses involved, early diagnosis is essential and it is imperative that an effective method of treatment or prevention is decided on. Some of the best short term measures include supplementation strategies discussed later in this thesis. However, since mass intervention with mineral supplements is often only beneficial to a minority of the flock and since susceptibility to mineral disorders is often a heritable characteristic, a superior and longer term preventative approach may reside in the potential for genetic selection for tolerance to mineral imbalances (Suttle, 2000).

As Co is a component of vitamin B12 (McDowell, 2000), regulation of one-carbon (1C) metabolism is influenced by dietary intake of Co. In recent years, there has been renewed interest in dietary constituents that mediate 1C metabolism. Such nutrients include methionine, choline (betaine), folate (B9) and vitamins B2, B6 and B12; all of which act as essential substrates or cofactors in 1C metabolism (Mason, 2003). Evidence from human studies has shown that genetic polymorphisms in several key 1C genes alter metabolism, absorption and transport of these metabolites, increasing the risk of a variety of complex traits and disorders. By way of example, the common *MTHFR* (EC 1.5.1.20) C677T (rs1801133) polymorphism (Bailey et al., 1999) has been found to be a risk variant for idiopathic male infertility (Gong et al., 2015), recurrent pregnancy loss (Cao et al., 2013), NTDs (Yan et al., 2012) and Down syndrome (Cyril et al., 2009; Kaur and Kaur, 2016) in human populations.

Nutritional folate status has been shown to interact with this polymorphism, resulting in variable outcomes. These observations provide evidence for nutrient-gene interactions and an explanation for geographical differences in the prevalence of genetic polymorphisms; likely as a result of increased selection pressure in regions of variable nutrient availability. Investigation of the regulatory aspects of 1C metabolism and exploration of the impact of perturbations to these pathways in animals is limited to mice and has never before been studied in sheep.

Consequently, the broad aims and objectives of this thesis were to increase our understanding of dietary and genetic regulation of B12 metabolism in sheep through supplementation trials and genetic analyses.

CHAPTER ONE

Literature review

This chapter contains an overview of trace-element deficiencies in sheep, with particular focus on cobalt (Co), reviewing the current understanding of susceptibility and treatment of Co deficiency. A detailed description of the biochemistry of one-carbon (1C) metabolism and the role of Co as a component of vitamin B12 within this complex network of pathways is given. Furthermore, this chapter includes a summary of what is currently understood about the nutritional and genetic regulation of 1C metabolism, culminating in a working hypothesis and the broad aims and objectives of this thesis.

1.1 Trace-element deficiencies

Trace-elements are essential micronutrients which serve a variety of functions in animals including structural, physiological, catalytic and regulatory activities (Suttle, 2010). Involved in all major metabolic pathways, interacting with macromolecules such as enzymes and biological membranes, these micronutrients are essential for maintenance of normal metabolism and homeostatic mechanisms, and lack of them in the diet can result in disease. Functions performed by trace-elements can only take place if sufficient amounts are ingested, absorbed and retained. Several key trace-elements (i.e. Co, Cu, Se, Fe, I, Mn and Zn) have been shown to be essential for animals, each required in varying amounts depending on demand for growth, production, reproduction and to ensure immune competency (Underwood and Suttle, 1999). Sheep are frequently subject to dietary deficiencies of these trace-elements and as key components of the diet, the effects of these deficiencies on performance, health, fertility and welfare are well documented (Suttle, 2010; Kincaid, 1999; Hidiroglou, 1979; Fisher and MacPherson, 1991) particularly in New Zealand (Grace et al., 1994; 2000; 2002; 2012; Clark, 1985; Andrews, 1963).

1.2 Cobalt deficiency in sheep

Following ingestion, digestion of trace-elements can alter the proportions that are absorbed and can alter the forms in which they are utilised by the animal (Suttle, 2010). Cobalt (Co) is an essential micro-nutrient for

ruminants, the adequacy of which is necessary for ruminal bacteria to synthesise vitamin B12 (McDowell, 2000). Co was shown to be a dietary essential for sheep in 1935 by Underwood and others in Australia (Underwood, 1935). The Australians showed that a deficiency in Co caused debilitating diseases in sheep known as ‘wasting disease’. In the UK this became known as ‘pining’ and in New Zealand as ‘bush sickness’ (Suttle, 2010). The disease manifests itself as ill-thrift with loss of appetite, which leads to decreased live weight, anaemia, increased susceptibility to infection and increased perinatal mortality in lambs (Grace, 1994; Underwood and Suttle, 1999). Another consequential complication is ovine white liver disease which manifests itself as severe hepatic steatosis, with many associated complications (Sutherland et al., 1979). After determination of the structure of B12 in 1948 (Smith, 1948), Smith and co-workers discovered that injections of vitamin B12 prevented all signs of Co deficiency (Smith et al., 1952), confirming the biological relationship between Co and B12.

1.3 Vitamin B12

Vitamin B12, structurally the largest of the B-vitamins, is also commonly referred to as cobalamin (Hodgkin, 1956). This water soluble vitamin is unique among vitamins as it contains an essential trace-element; namely cobalt. Co makes up 4.4% of vitamin B12 (Underwood and Suttle, 1999). In bacteria and algae, there are several B12 dependent enzymes (Rodionov et al., 2003). In animals, however, only two enzymes utilise B12 as a cofactor; methionine synthase (MTR) (EC 2.1.1.13) and methylmalonyl-CoA mutase (MUT) (EC 5.4.99.2) (Kräutler, 2012). Present in a variety of tissues, but most active in liver, these enzymes utilise two distinct but interdependent biologically active forms of cobalamin. Methyl-cobalamin is a co-enzyme to MTR which catalyses the re-methylation of homocysteine (Hcy) to Methionine (Met) in 1C metabolism; an integrated series of metabolic pathways including the folate cycle, methionine cycle and transsulfuration pathway, which generate and deliver methyl groups for a range of key cellular processes (Lucock, 2000; Ducker and Rabinowitz, 2017). Adenosyl-cobalamin is

coenzyme to MUT in the conversion of propionate to succinate; the principal gluconeogenic precursor in sheep (O'Harte, 1989). As Co is required for the synthesis of B12, Co deficiency will therefore lead to disruption of these B12 dependent and related pathways (Kennedy et al., 1992).

1.4 Cobalt requirements of sheep and susceptibility to deficiency

Dietary trace-element requirements are difficult to determine precisely because a number of interacting factors can influence intake, absorption and utilisation (Herbert, 1972). Since Co is a component of B12, the B12 requirement of sheep is closely associated with dietary Co intake. The established dietary Co requirement for sheep to maintain optimal B12 status is between 0.1 to 0.3 mg/kg DM (Suttle, 2010). Pasture and concentrate diets containing Co concentrations below this are associated with Co deficiency in sheep (Andrews, 1965; Marston, 1970; Ulvund, 1995). For grazing animals, supply of Co is dependent on pasture content, with herbage concentrations, selective grazing habits and soil contamination influencing Co intake by the animal (Grace, 2006). Herbage concentrations are affected by forage species, environmental conditions and soil conditions in different geographical locations (Harrington, 2006; Underwood, 1977). Large parts of the UK produces pasture herbage containing less than the minimum Co requirement (Thorton and Webb, 1980), with alkaline soils (increased pH) or high soil Mn content (particularly as MnO) contributing to decreased Co uptake by plants (Suttle et al., 2003; Underwood and Suttle, 1999; Adams, 1969).

With this in mind, identification of risk areas by geochemical mapping has been advocated (Suttle, 2000). Soil Co analysis can be used to predict herbage concentrations of Co and other minerals, and hence to assess the likelihood of a deficiency in grazing sheep in that location (McLaren et al., 1987). Figure 1.1, taken from the UK soil observatory, illustrates the relative topsoil Co levels in England and Wales, highlighting areas in which susceptibility to Co may be increased. It can be seen from this heat

map that the Yorkshire Dales and northern England, Norfolk and Suffolk, as well as parts of Wales have particularly low topsoil Co concentrations, whilst central England is relatively sufficient. However, closer (higher resolution) inspection reveals pockets of deficiency within these areas.

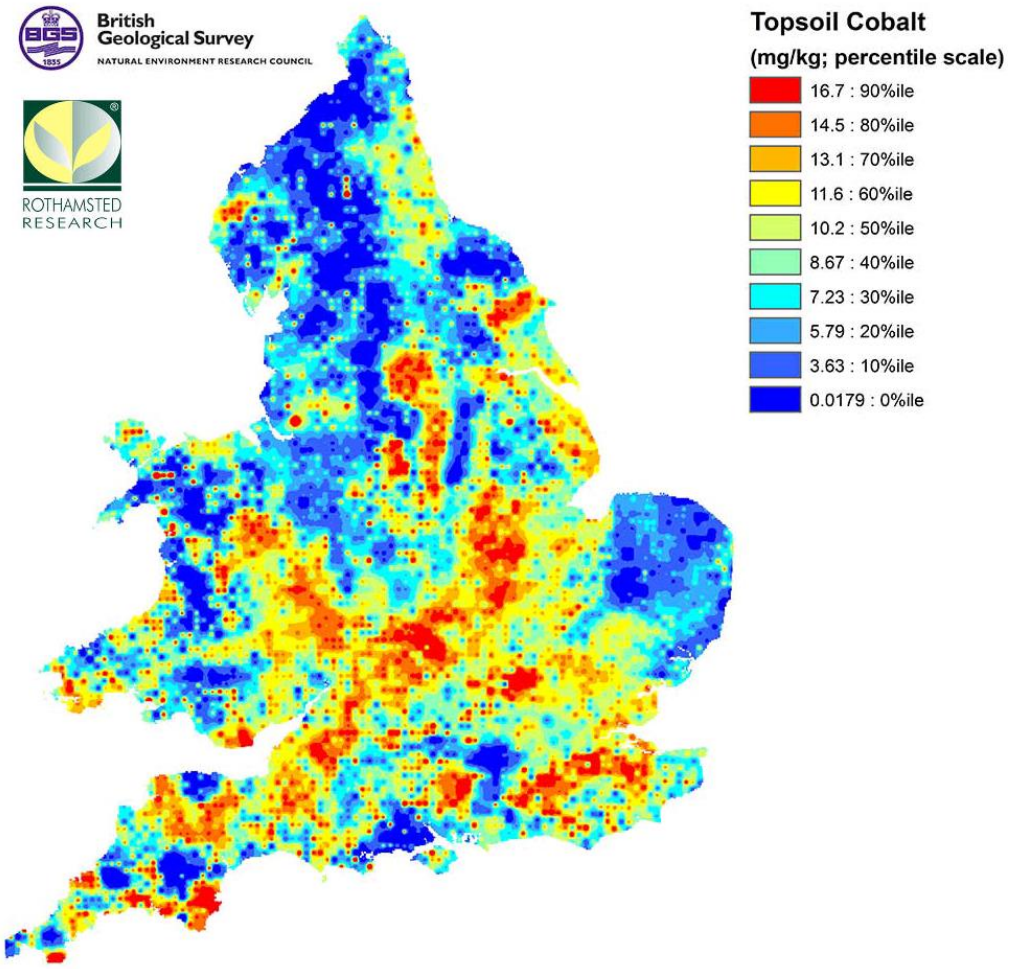


Figure 1.1. Relative Co topsoil concentrations in England and Wales taken from the UK soil observatory (http://www.ukso.org/nsi/Cobalt.html)

Seasonal weather fluctuations can also influence herbage Co availability. It is well recognised that cobalt levels in herbage are usually adequate during the autumn/winter and decline as herbage growth accelerates in the spring/summer (Andrews, 1958). As Co is not an essential nutrient for plants, high growth rates can dilute the amount of Co in the plant (Suttle, 2010). Furthermore, heavy rainfall can leach Co from the soil

decreasing availability to the plant whilst, conversely, poor drainage can increase availability (Suttle, 2010; Berrow, 1986). Monthly trends of Co deficiency diagnoses in the UK over 4 consecutive years (2012-2015) (Figure 1.2), highlights an increased incidence of Co deficiency between July and September.

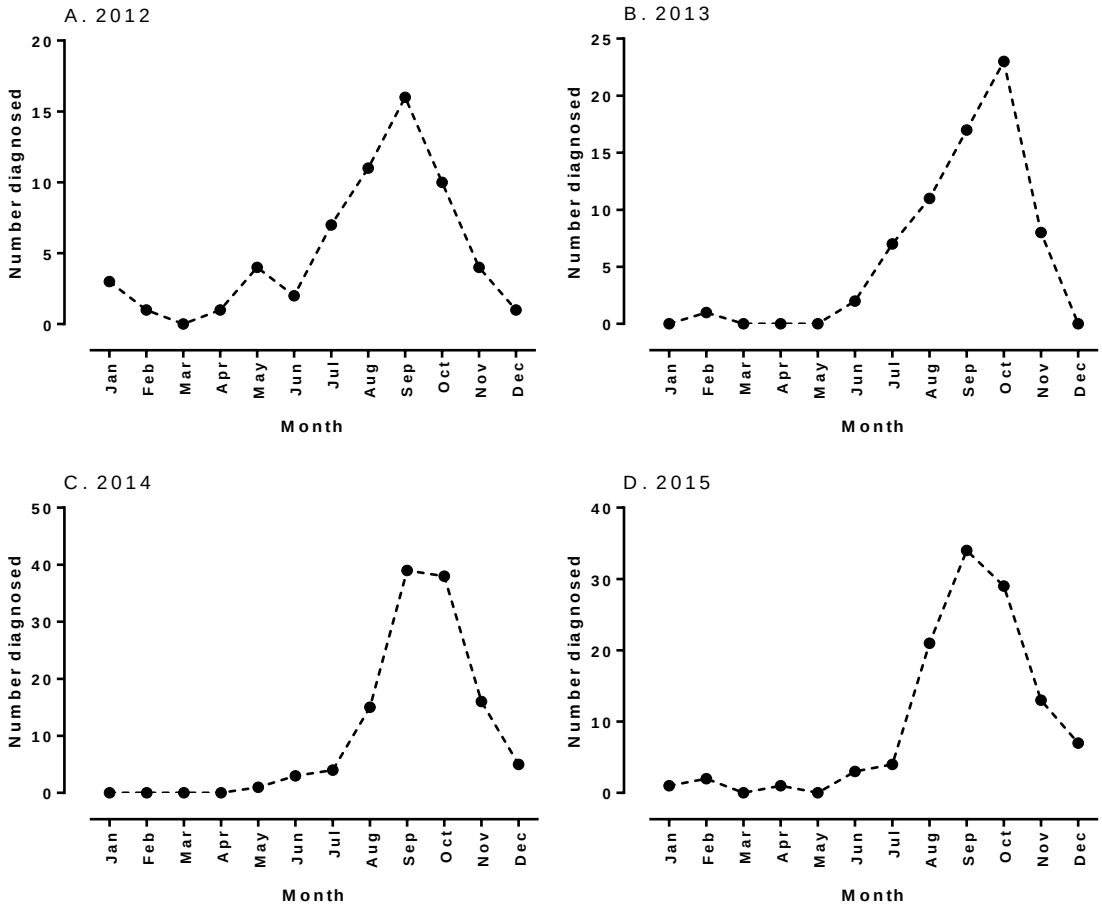


Figure 1.2. Monthly trends of Co deficiency diagnosis in sheep from VIDA records over four consecutive years; 2012 to 2015.

Diet composition and level of digestible dry matter intake can also influence minimum Co requirements of sheep (Suttle, 2010). Rumen synthesis of B12 has been shown to be decreased when sheep are fed a cereal based diet (Sutton and Elliot, 1972; Hedrich et al., 1973), and the efficiency of rumen B12 synthesis in sheep fed a predominantly barley diet is less than half that of sheep fed hay (Smith and Marston, 1970).

As well as varying supply of Co from the diet, susceptibility to deficiency is dependent on stage of production cycle. The annual trend observed in Figure 1.2 correlates with increased incidence of Co deficiency in young lambs around the time of weaning (~12 weeks old) (Clark et al., 1985; Metherell et al., 1997). Under grazing conditions, rapidly growing weaned lambs are most sensitive to Co deficiency, and experiments with sheep suggest minimum oral requirements for growing lambs of ~200 µg/day, about 10x the reported oral requirement of other species (Marston, 1970; Singh, 1995). This high requirement for Co could be due to high demand for B12 utilization in metabolism of the principal gluconeogenic precursor, propionate, for provision of energy for growth (Kennedy et al., 1994). Energy metabolism in non-ruminant species is based on glucose absorbed from the small intestine, whilst ruminants get approximately 70% of their metabolizable energy from volatile fatty acids (Bergman, 1990). In ruminants, almost all dietary carbohydrates are fermented to volatile fatty acids in the rumen. As propionate is the only major volatile fatty acid that contributes to gluconeogenesis, it is of great importance to sheep (Young, 1977). Provided with an adequate supply of dietary Co, sheep have the ability to synthesise their required B12, as long as they have a normally functioning rumen. Typically the rumen is only fully functional after ~8 weeks of age, therefore, the young sucking lamb requires a dietary source of B12 from milk (Ørskov, 1972). Although concentrations of B12 in milk decline considerably in the first few weeks of lactation (Ramos et al., 1994; Marca et al., 1996), suckling lambs can tolerate relatively low levels of B12 as they obtain glucose directly from enzymatic digestion of milk, reducing the requirements of B12 for propionate metabolism (Gruner et al., 2004). Pasture Co concentrations of 0.03-0.05 mg/kg DM may be tolerated by mature ewes, but requirements increase with the onset of lactation (Quirk and Norton, 1987; Gruner et al., 2004). At this time, increased B12 is required for propionate metabolism in the liver and to support synthesis of very-low density lipoproteins from phosphatidyl choline, facilitating mobilisation of body fats to meet energy needs (Cole et al., 2012).

1.5 Assessment of status

The signs and symptoms of Co deficiency are non-specific (i.e. a marked depression in appetite, poor growth rates, anaemia, diarrhoea and ill thriven appearance), and economically important effects on performance and health of animals can be affected before clinical signs are evident. When performance is below expectation, assessment of trace-element status is carried out to determine the presence or prevalence of a trace-element deficiency within the population.

There have been a number of different approaches to diagnosing B12 deficiency in sheep. Although useful information can be gained from assessing performance responses of animals to B12 and Co supplementation (Clark et al., 1989; Grace, 1998; McDowell, 1992), this is too time-consuming for routine diagnostic use (McQueen, 1984). Signs of Co deficiency have been observed in sheep grazing pasture Co concentrations of <0.08 mg/kg (Andrews, 1965; Marston, 1970), although, selective grazing and poor sampling techniques mean using herbage concentrations as a method for diagnosis is limited.

Cobalt concentration in blood and tissue decreases with Co deficiency (Kercher and Smith, 1956). However, with different forms of Co (i.e. various degrees of oxidation) and biologically inactive analogues of cobalamin found in circulation (Czarnek et al., 2015), measurement of Co can often result in overestimation of status. As the only metabolic role of Co is as a component of B12 (Kincaid, 1999), assessment of the biochemical consequences of decreased B12 is a superior method for diagnosing B12 deficiency (Andrews et al., 1960). Suggested reference values for classifying the B12 status of sheep are given in Table 1.1.

Table 1.1. Reference ranges/deficiency classifications for B12 status. (Rice et al., 1989; Kennedy et al., 1992; Gruner et al., 2004; Suttle, 2005; Furlong et al., 2010)

Status	Serum	Liver	Plasma	
	B12 pmol/L	B12 nmol/g	MMA µmol/L	Hcy µmol/L
Deficient	<250	<150	>13	>14
Marginal	250-350	150-300	7-14	10-14
Sufficient	>350	>300	<7	<10

When dietary supply of Co is inadequate, a series of biochemical changes take place prior to presentation of clinical symptoms of deficiency. In line with this series of changes, four successive stages of deprivation occur; depletion, deficiency, dysfunction and disease (Suttle, 2010). Each stage of this progression has an increasingly negative impact on animal health. Reduced intake of Co decreases ruminal B12 synthesis, leading to depleted levels of B12 in blood. Measurement of serum B12 concentration is considered to be the cornerstone for determining adequacy or deficiency of B12 in livestock, reflecting the amount of B12 being absorbed and indicating depletion of B12 stored in the blood (Suttle, 2010). A decline in liver B12 concentration occurs later, indicating slow mobilisation of liver stores for extra hepatic purposes. Measurement of liver B12 levels provides a more integrated picture of historical rates of absorption and better reflects body reserves (Millar and Penrose, 1980). This stage of deprivation is described as ‘Co deficiency’. Animals can persist in this deficient state for an extended period before becoming dysfunctional.

The principal source of energy for ruminants is volatile fatty acids, including propionic acid. Propionate is produced by rumen fermentation and following absorption, is converted to succinate by the B12 dependent

enzyme methylmalonyl CoA mutase (MUT). B12 deficiency inhibits this pathway and results in decreased rates of propionate clearance from the blood and accumulation of the intermediary metabolite MMA (Rice et al., 1989; Kennedy et al., 1991). Vitamin B12 is also essential for methionine synthase (MTR) activity. In Co deprived sheep, activity of this enzyme is impaired, and decreased methylation causes accumulation of Hcy in the blood and liver. This metabolic dysfunction can be further confirmed by concurrent increases in SAM relative to SAH in the liver. MMA and Hcy measures are now well recognised as the most sensitive, accurate and specific indicators of functional B12 deficiency in humans and animals (Stabler, 1996; Gruner, 2004; Elin and Winter, 2001; Stangl et al., 2000; Rice et al., 1989; Clark et al., 1985). It is unclear whether B12 deficiency in sheep results primarily from failure of the propionate-succinate pathway or the Hcy re-methylation pathway and so it is important to measure both MMA and Hcy for comparison. After mineral dependent functions become rate limited, the level of deprivation reaches the 'disease' stage and clinical symptoms begin to appear in the animal.

1.6 Treatment of cobalt deficiency in sheep

Once a trace-element deficiency has been diagnosed, or susceptible animals identified, the most effective method of treatment or prevention must be decided on (Grace, 1992). To be effective, the chosen supplementation strategy must increase and maintain the mineral status of the animal for long periods whilst also being cost effective. Topdressing pastures with Co salts or oxides aims to increase pasture Co concentrations and so provide the grazing animal with sufficient amounts of this element to meet demands for B12 synthesis. However, soil type, terrain and husbandry practices alter the amount and frequency of fertiliser required, and uncontrollable environmental factors such as increased rainfall and high levels of Mn oxides in the soil decrease herbage Co uptake, making this an unreliable method of treatment (Adams et al., 1969; Li et al., 2001). Dietary supplements such as loose mineral mixtures, licks and blocks are a simple and economical method of Co supplementation commonly used as a preventative measure.

However, uptake may be variable and consequently inadequate for some individuals during crucial periods of production when Co requirements are increased. Injections of water soluble B12 rapidly alleviate signs of depletion, however, effective for only around four weeks (Grace et al., 1998; Judson et al., 2000), this method of supplementation is a short-term solution and the need for repeated dosing can become prohibitively expensive. Effects of Co drenches are similarly short lived, lasting less than two weeks (Field et al., 1988), and route of administration makes the required frequency of dosing impractical. Intra-ruminal Co boluses, when not regurgitated, have been shown to increase and maintain B12 status for up to one year (Judson et al., 1995; Kendall et al., 2001). Boluses are commercially available and are commonly used for treatment of Co deficiency in weaned lambs and adult sheep in the UK. However, they cannot be given to young suckling lambs under the age of 10 weeks as they do not have a fully functional rumen capable of synthesising B12. Slow release of soluble B12 from a lactide-glycolide polymer microcapsule injected subcutaneously into the anterior neck is thought to be an effective alternative strategy (Grace and Lewis, 1999). Increased and maintained serum and liver B12 concentrations have been observed in ewes and lambs of different ages treated with microencapsulated B12, lasting up to 8 months (Grace and Lewis, 1999; Judson et al., 2000; Grace et al., 2003; Grace et al., 2006).

1.7 One-carbon metabolism and related pathways

One-carbon (1C) metabolism is an integrated series of metabolic pathways including the folate cycle, methionine re-methylation and transsulphuration pathways (Figure 1.3) (Xu and Sinclair, 2015). Compartmentalised in the cytoplasm, nucleus and mitochondria of cells (Stover, 2009; Stover and Field, 2011), this complex network of biochemical reactions facilitates the transfer of 1C moieties, in the form of methenyl, formyl and methyl groups, required for vital cellular processes. Such processes include molecular biosynthesis (e.g. proteins, polyamines, creatine, phospholipids), genomic maintenance via regulation of nucleotide pools, epigenetic control of gene expression by

methylation of DNA, RNA and histones, and redox defence (Lucock, 2000; Ducker and Rabinowitz, 2017). In recent years, there has been renewed interest in dietary constituents that mediate 1C metabolism. Such nutrients include folate (B9), B-vitamins (B2, B6 and B12), methionine and choline (betaine); all of which act as essential substrates or cofactors in 1C metabolism (Mason, 2003).

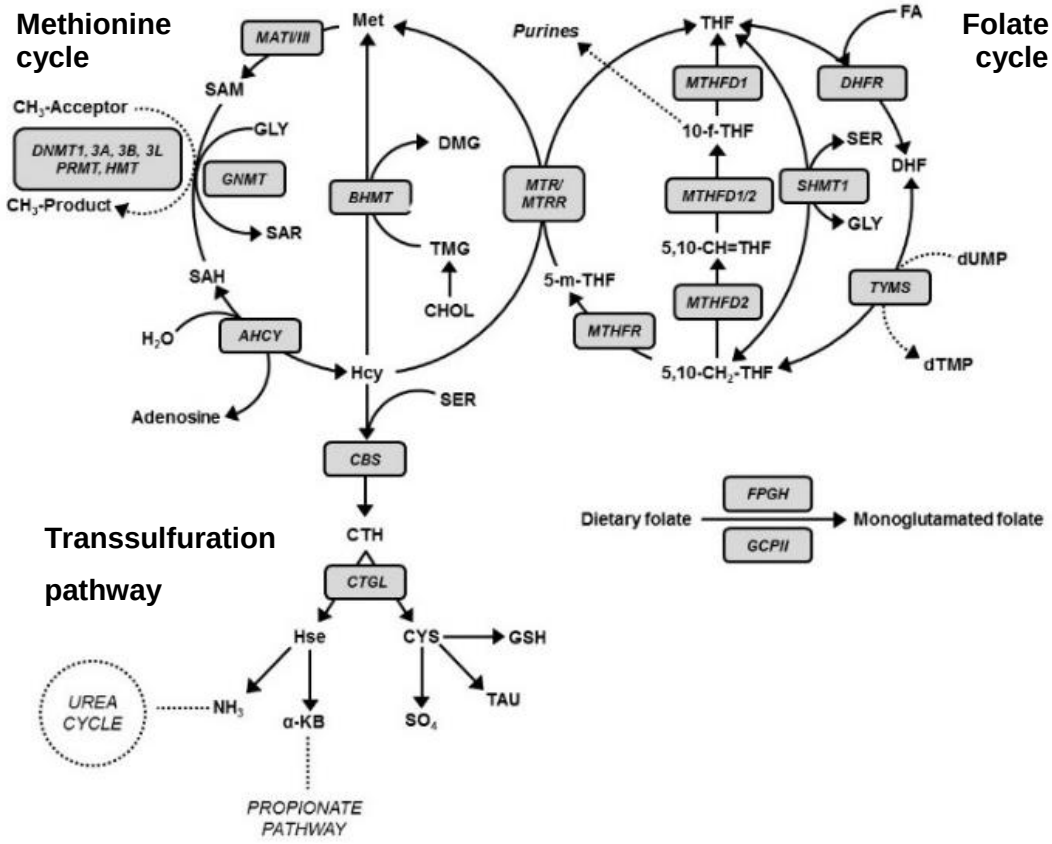


Figure 1.3. One-carbon metabolic pathways. Enzymes in shaded boxes. Key below

Folate cycle enzymes	
DHFR	dihydrofolate reductase
MTHFR	5,10-methylenetetrahydrofolate reductase
TYMS	thymidylate synthase
MTHFD1/2	methylene-tetrahydrofolate dehydrogenase
SHMT	serine hydroxymethyltransferase
FPGH	folypolyglutamate hydrolase
GCPII	glutamate carboxypeptidase

Methionine cycle enzymes	
MTR	methionine synthase
MTRR	methionine synthase reductase
MATI/III	methionine adenosyltransferase
GNMT	glycine N-methyltransferase
AHCY	S-adenosyl L-homocysteine hydrolase
CHDH	choline dehydrogenase
BHMT	betaine-homocysteine S-methyltransferase

Transsulfuration pathway enzymes	
CBS	cystathionine β-synthase
CTH	cystathionine γ-lyase

Methyltransferase enzymes	
DNMTs	DNA methyltransferases
PRMT	protein arginine methyltransferase
HMT	histone methyltransferase

Substrates	
5-mTHF	5-methyl-tetrahydrofolate
THF	tetrahydrofolate
FA	folic acid
DHF	dihydrofolate
5,10-CH2THF	5,10-methylene-tetrahydrofolate
5,10-CH=THF	5,10-methenyl-tetrahydrofolate
10-fTHF	10-formyl-tetrahydrofolate
dUMP	deoxyuridine monophosphate
dTMP	thymidine monophosphate
SER	serine
GLY	glycine
Met	methionine
SAM	S-adenosyl methionine
SAH	S-adenosyl homocysteine
Hcy	homocysteine
SAR	sarcosine
CHOL	choline
TMG	trimethylglycine/betaine
DMG	dimethylglycine
Cth	cystathionine
Hse	homoserine
CYS	cysteine
NH3	ammonia
α-KB	α-ketobutyrate
SO4	Sulphate
TAU	taurine
GSH	glutathione

1.7.1 Methionine cycle

As mentioned previously, B12 is an essential co-factor for methionine synthase (MTR) (EC 2.1.1.13) in the re-methylation of Hcy to Met (Figure 1.3). Methionine is subsequently adenylated by methionine adenosyltransferase (MAT; EC 2.5.1.6) to S-adenosylmethionine (SAM or AdoMet), a universal methyl donor involved in numerous downstream cellular reactions (Roje, 2006). SAM-dependent transmethylation plays a pivotal role in all aforementioned cellular processes, as well as reductive methylation of cob(II)alamin for MTR restoration (Ho et al., 2013). When SAM donates its labile methyl group, it becomes S-adenosylhomocysteine (SAH) (Ulrey et al., 2005). Accumulation of SAH inhibits methylation reactions from occurring, hence, the SAM:SAH ratio is often used as an indicator of methylation capacity. SAH is hydrolysed to Hcy and adenosine through a reversible reaction that favours SAH synthesis, catalysed by S-adenosylhomocysteine hydrolase (AHCY/SAHH; EC 3.3.1.1) to complete the methionine cycle (Caudill et al., 2001).

1.7.2 Folate Cycle

B12 is also required for normal folate metabolism (Figure 1.3). The relationship between vitamin B12 and folate is best explained by the ‘methyl-trap’ hypothesis (Figure 1.4). B12 deficiency can lead to lowered levels of methionine synthase, which as well as leading to an accumulation of Hcy, results in a functional folate deficiency by ‘trapping’ an increased proportion of folate as the 5-methyl derivative (Abou-Zeina, 2008).

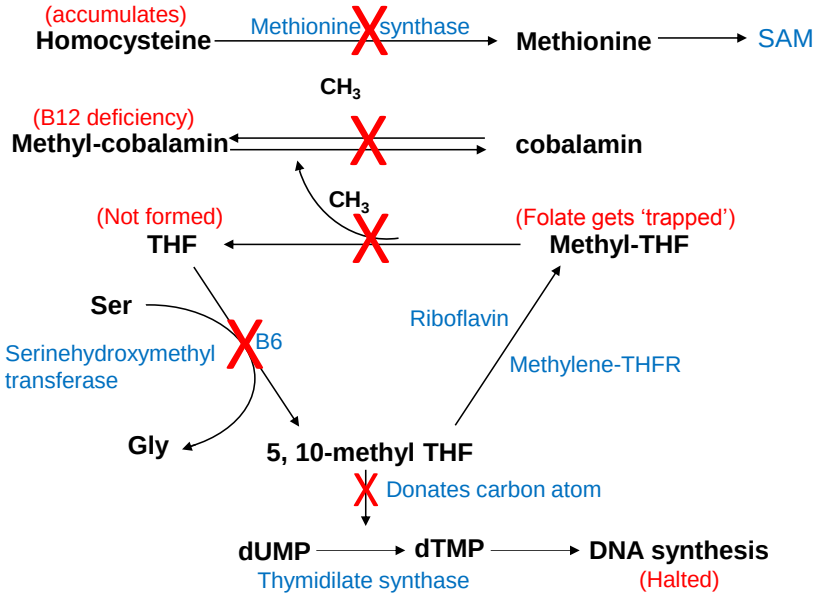


Figure 1.4. Methyl-trap hypothesis; the effect of B12 deficiency on the folate cycle. CH3, methyl group; SAM, S-adenosyl methionine; THF, tetrahydrofolate; Ser, serine; Gly, glycine; dUMP, deoxyuridine monophosphate; dTMP, thymidine monophosphate.

Folates are versatile methyl donors that carry and chemically activate 1C units (Stover, 2009). Since animals cannot endogenously synthesise folates, they are derived entirely from dietary sources (Garrett et al., 2003). Absorption of folates takes place in the small intestine (Visentin et al., 2014) where they are hydrolysed to their monoglutamated forms in the intestinal mucosa by folypolyglutamate hydrolase (FPGH; EC 3.4.19.9) and glutamate carboxypeptidase II (GCPII; EC 3.4.17.21) to facilitate absorption. Due to their hydrophilic nature, folates require functionally diverse transport systems, including the reduced folate carrier (RFC), the proton-coupled folate carrier (PCFT) and folate receptors (FR) (Zhao et al., 2009). Upon reaching target cells, folate monoglutamates are transformed to 5-methyltetrahydrolate (5-mTHF), the predominant species in non-hepatic tissues, and are subsequently polyglutamated for cellular retention and 1C coenzyme function (Xu and Sinclair, 2015). Most, if not all, reactions in the folate cycle use polyglutamated folates as substrates under physiological conditions.

Folic acid (FA), the synthetic form (Wright et al., 2007), is fully oxidised and not an active coenzyme (Shane, 2008). FA is reduced to dihydrofolate (DHF) by dihydrofolate reductase (DHFR; EC 1.5.1.3) and then the biologically active form, THF, before it can enter the folate cycle (Ducker and Rabinowitz, 2017; Wright et al., 2007). In turn, THF is converted to 5, 10-methylenetetrahydrofolate (5, 10-CH2THF) by vitamin B6-dependent enzyme, serine hydroxymethyltransferase (SHMT1; EC 2.1.2.1), and then irreversibly reduced to 5-mTHF by vitamin B2-dependent enzyme, methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20). Alternatively, 5, 10-CH2THF can be converted to 10-formyltetrahydrofolate (10-fTHF) through a series of reactions catalysed by methylenetetrahydrofolate dehydrogenases (MTHFD; EC 1.5.1.5). Under normal circumstances, SAM serves as a major regulator determining which path folate will be routed, largely through its role as an allosteric inhibitor of MTHFR. Demethylation of 5-mTHF completes the folate cycle as 1C is donated for re-methylation of Hcy to Met (Mentch and Locasale, 2016). This reaction is catalysed by B12-dependent enzyme, methionine synthase (MTR; EC 2.1.1.13) (Škovierová et al., 2016). MTR requires methionine synthase reductase (MTRR; EC 2.1.1.135), to restore MTR activity by catalysing reductive methylation of cob(II)alamin to methylcob(III)alamin, thereby maintaining adequate levels of activated vitamin B12 cofactor for Hcy remethylation (Gaughan et al., 2001; Ho et al., 2013). A parallel pathway requires betaine-homocysteine S-methyltransferase (BHMT; EC 2.1.1.5) (Finkelstein and Martin, 1984), a B6-dependent enzyme that catalyses transfer of a methyl group from betaine (TMG) to Hcy resulting in production of dimethyglycine (DMG) and methionine (Li et al., 2008).

1.7.3 *Trans*sulphuration pathway

When methionine and folate levels are sufficient, high levels of SAM stimulate Hcy degradation via a two-step transsulphuration pathway that requires vitamin B6 as a cofactor (Figure 1.3). SAM coordinates -transsulphuration by acting as an allosteric inhibitor of MTHFR and activator of cystathionine ß-synthase (CBS; EC 4.2.1.2) (Selhub, 1999;

Lucock, 2000), the first enzyme within the transsulphuration pathway that mediates condensation of serine (Ser) with Hcy to yield cystathionine (Cth). The second step, catalysed by cystathionine gamma-lyase (CTGL; EC 4.4.1.1), produces cysteine (Cys), a precursor for glutathionine (GSH) which is a major redox-regulating system in cells (Mentch and Locasale, 2016).

1.7.4 Propionate pathway

B12 is also an essential co-factor to methylmalonyl-CoA mutase (MUT; EC 5.4.99.2), involved in the metabolism of the volatile fatty acid, propionic acid, in the liver, prior to gluconeogenesis. (Figure 1.5). Propionate, methionine (Met), threonine (Thr), isoleucine (Ile), valine (Val), odd-chain fatty acids (OCFA), α-ketobutyrate (α-KB), and cholesterol all contribute to propionyl-CoA production (Tretter et al., 2016). In the mitochondria, propionyl-CoA is first carboxylated to D-methylmalonyl-CoA by propionyl-CoA carboxylase (PCC; EC 6.4.1.3) using biotin as a co-factor (Ballhausen et al., 2009). In turn, D-methylmalonyl-CoA is epimerised to its L-stereoisomer by methylmalonyl-CoA epimerase (MCEE; EC 5.1.99.1). L-methylmalonyl-CoA then undergoes an intramolecular rearrangement catalysed by the B12-dependent MUT to form succinyl-CoA which enters the tricarboxylic acid (TCA) cycle (De Vadder et al., 2016). In the absence of vitamin B12, L-methylmalonyl-CoA forms methylmalonic acid (MMA), a robust biomarker of B12 status and 1C metabolic function (Mendonça et al., 2017).

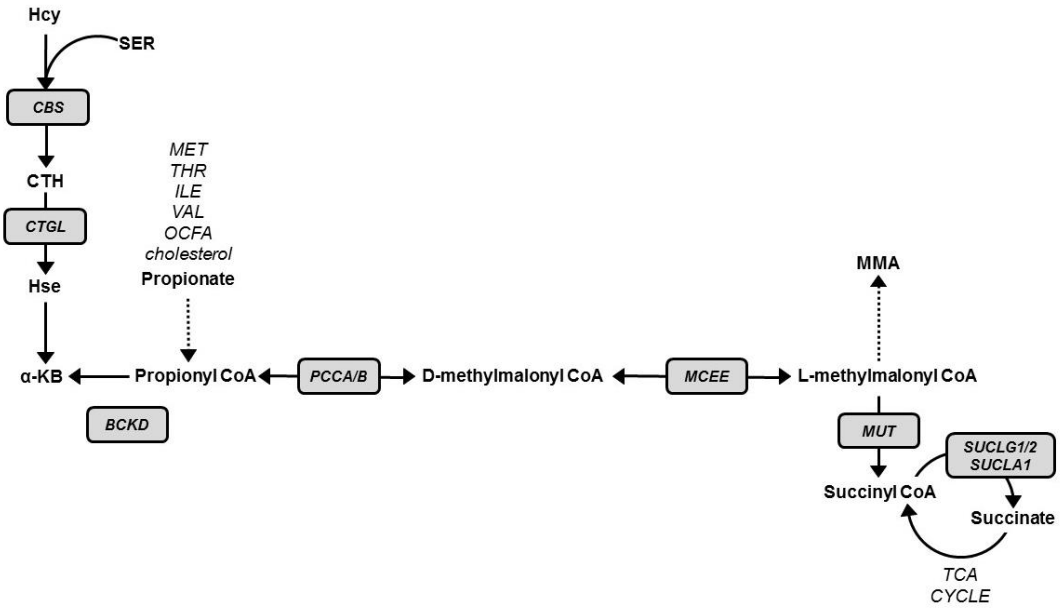


Figure 1.5. Propionate metabolism. Enzymes in shaded boxes. Key below.

Propionate pathway enzymes			
BCKDC	branched chain α-keto acid dehydrogenase		
PCCA/B	propionyl-CoA carboxylase		
MCEE	methylmalonyl-CoA epimerase		
MUT	methylmalonyl-CoA mutase		
SUGLC1/2	succinate CoA ligase		
Transsulfuration pathway enzymes			
CBS	cystathionine β-synthase		
CTH	cystathionine γ-lyase		
Substrates			
Hcy	homocysteine	THR	threonine
SER	serine	ILE	isoleucine
Cth	cystathionine	VAL	valine
Hse	homoserine	OCFA	odd-chain fatty acids
α-KB	α-ketobutyrate	MMA	methylmalonic acid
MET	methionine		

1.7.5 Polyamine pathway

Ornithine is decarboxylated to the primary polyamine, putrescine, by B6-dependent enzyme, ornithine decarboxylase (ODC; EC 4.1.1.17) (Figure 1.6) (Pegg, 2006). Putrescine is the precursor for spermidine and spermine synthesis, catalysed by aminopropylytransferases, spermidine synthase (SRM; EC 2.5.1.16) and spermine synthase (SMS; EC 2.5.1.22), respectively (Gamble et al., 2012). Although ODC is typically described as the rate-limiting enzyme in putrescine production, the supply of aminopropyl donor, decarboxylated S-adenosyl methionine, is the rate-limiting factor for higher polyamine synthesis (Pegg, 2009; 2016).

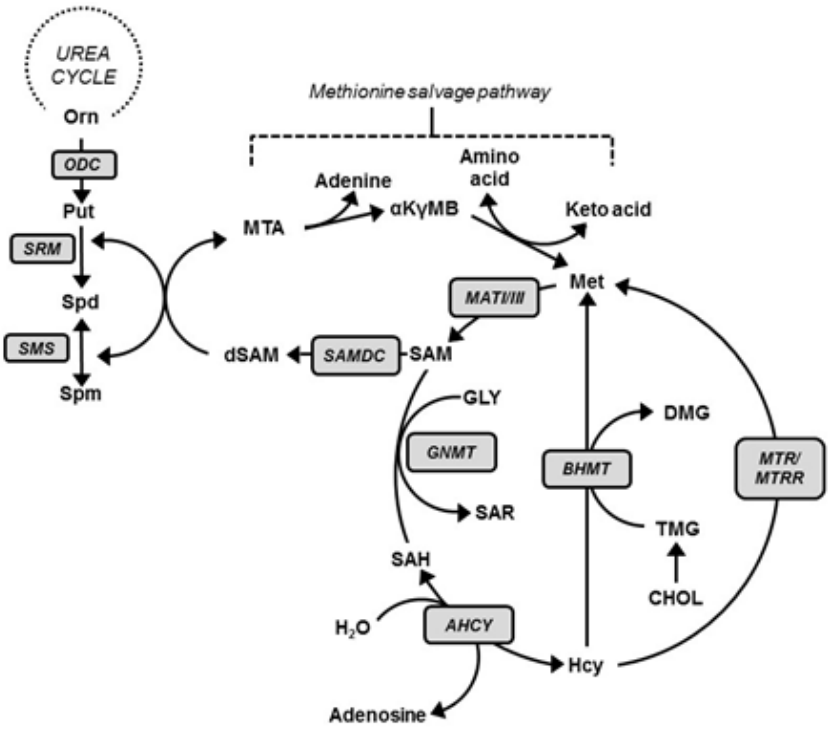


Figure 1.6. Polyamine pathway. Enzymes in shaded boxes. Key below.

Polyamine pathway enzymes	
ODC	ornithine decarboxylase
SRM	spermidine synthase
SMS	spermine synthase
SAMDC	S-adenosylmethionine decarboxylase
Methionine cycle enzymes	
MTR	methionine synthase
MTRR	methionine synthase reductase
MATI/III	methionine adenosyltransferase
GNMT	glycine N-methyltransferase
AHCY	S-adenosyl L-homocysteine hydrolase
CHDH	choline dehydrogenase
BHMT	betaine-homocysteine S-methyltransferase

Substrates			
Orn	ornithine	Met	methionine
Put	putrescine	SAM	S-adenosyl methionine
Spd	spermidine	SAH	S-adenosyl homocysteine
Spm	spermine	Hcy	homocysteine
dSAM	decarboxylated S-adenosylmethionine	SAR	sarcosine
MTA	5'-methylthiodenosine	CHOL	choline
αKγMB	α-keto-γ-methiolbutyrate	TMG	trimethylglycine/betaine
GLY	glycine	DMG	dimethylglycine

1.7.6 Phosphatidylcholine pathway

SAM also plays a role in phosphatidycholine (PC) synthesis (Figure 1.7) by donating three methyl groups to phosphatidylethanolamine (PE) via phosphatidylethanolamine methyltransferase (PEMT; EC 2.1.1.17) (Vance, 2013), an enzyme expressed principally in liver (Kanno et al., 2007). These glycerophospholipids are the most abundant in animal

tissues and are important constituents of lipoproteins in cell membranes, facilitating lipid transport (Wright et al., 2004).

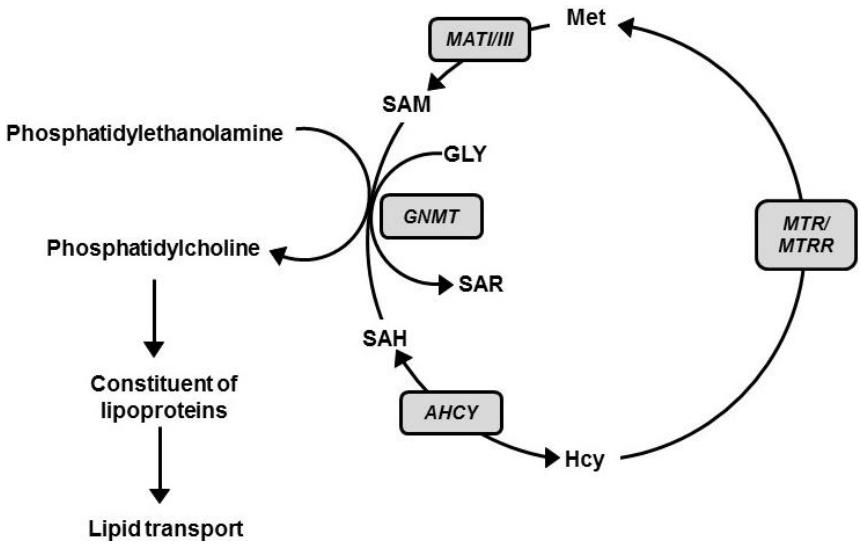


Figure 1.7. Phosphatidylcholine pathway. Enzymes in shaded boxes. Key below.

Phosphatidylcholine pathway enzymes			
PEMT	phosphatidylethanolamine methyltransferase		
MATI/III	methionine adenosyltransferase		
GNMT	glycine N-methyltransferase		
AHCY	S-adenosyl L-homocysteine hydrolase		
CHDH	choline dehydrogenase		
BHMT	betaine-homocysteine S-methyltransferase		
Substrates			
PE	phosphatidylethanolamine	Hcy	homocysteine
PC	phosphatidylcholine	SAR	sarcosine
GLY	glycine	CHOL	choline
Met	methionine	TMG	trimethylglycine/betaine
SAM	S-adenosyl methionine	DMG	dimethylglycine
SAH	S-adenosyl homocysteine		

1.7.7 Nucleotide biosynthesis

Major reactions of purine and thymidylate synthesis are shown in Figure 1.8. Inosine-monophosphate (IMP) is the central intermediate for generating purines. Generation of IMP from phosphoribosyl-pyrophosphate (PRPP) uses various multifunctional enzymes. Reactions catalysed by phosphoribosylglycinamide formyltransferase (GART; EC 2.1.2.2) and phosphoribosylaminoimidazolecarboxamide formyltransferase (ATIC; EC 2.1.2.3) provide C2 and C8 atoms for purine ring synthesis using 10-fTHF as a 1C donor. IMP oxidation to xanthine monophosphate (XMP) is the subsequent step for guanine and adenine

nucleotide biosynthesis. The central precursor for generating pyrimidines is uridine monophosphate (UMP), synthesis of which does not require 1C cofactors. UMP is converted to deoxyuridine monophosphate (dUMP) via uridine diphosphate (UDP) by *nucleoside diphosphate kinase* (NME; EC 2.7.4.6). Using 5,10-CH_2THF as the substrate, *thymidylate sythetase* (TYMS; EC 2.1.1.45) converts dUMP to thymidylate (dTMP) by transferring methyl groups and is oxidised to DHF. Subsequently, DHF is reduced to THF, enclosing the metabolic loop (Shuvalov et al., 2017).

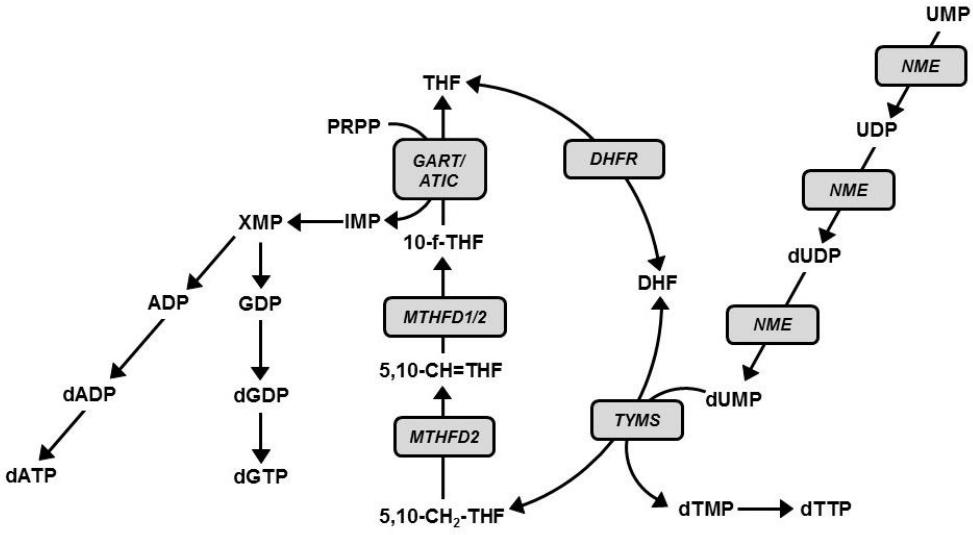


Figure 1.8. Nucleotide biosynthesis. Enzymes in shaded boxes. Key below.

Nucleotide biosynthesis enzymes			
GART	phosphoribosylglycinamide formyltransferase		
ATIC	phosphoribosyl-aminoimidazole-carboxamide formyltransferase		
NME	nucleoside diphosphate kinase		
Folate cycle enzymes			
MTHFD1/2	methylene-tetrahydrofolate dehydrogenase		
DHFR	dihydrofolate reductase		
TYMS	thymidylate synthase		
Substrates			
DHF	dihydrofolate	ADP	adenosine diphosphate
THF	tetrahydrofolate	dADP	deoxyadenosine diphosphate
10-fTHF	10-formyl-tetrahydrofolate	dATP	deoxyadenosine triphosphate
5,10-CH=THF	5,10-methenyl-tetrahydrofolate	UMP	uridine monophosphate
5,10-CH_2THF	5,10-methylene-tetrahydrofolate	UDP	uridine diphosphate
PRPP	phosphoribosyl pyrophosphate	dUDP	deoxyuridine diphosphate
IMP	Inosine monophosphate	dUMP	deoxyuridine monophosphate
XMP	xanthine monophosphate	dTMP	thymidine monophosphate
GDP	guanosine diphosphate	dTTP	thymidine triphosphate
dGTP	deoxyguanosine triphosphate		

1.8 Nutritional regulation of one-carbon metabolism

As previously mentioned, folic acid, choline, betaine, B12, B6 and B2 are coenzymes which participate in 1C metabolism (Mason, 2003). Indeed, all of these nutrients are integral to the proper functioning of 1C metabolism. Consequently, lack of dietary provision or excess of these essential micro-nutrients can result in variable inter-individual phenotypic responses.

In 1C metabolism and associated pathways, enzymatic transfer of methyl groups via the universal methyl donor, S-adenosylmethionine (SAM) is critical to many cellular processes. Such critical processes include DNA and RNA synthesis, DNA and histone methylation, phospholipid biosynthesis, and creatine and protein synthesis (Lucock, 2000; Ducker and Rabinowitz, 2017; Stipanuk, 2004). Deficiencies in dietary constituents that mediate 1C metabolism often lead to decreased levels of SAM, therefore diminishing methylation capacity. As such, interest in recent years has been growing, particularly in relation to the possibility that a range of well characterised and debilitating disorders and diseases, impairing lifelong health and wellbeing in humans and animals, may be in part explained by inadequate intake of these vitamins. In animals (ruminant and non-ruminant species), such conditions include anorexia, anaemia and metabolic complications associated with hepatic steatosis leading to compromised immune function, unthriftiness and impaired fertility (Suttle, 2010). In humans, 1C associated pathologies also include non-communicable developmental and age-related diseases such as non-alcoholic fatty liver disease and associated metabolic syndrome, various forms of cancer, vascular disease, cognitive decline, age-onset frailty syndrome, impaired fertility and neural tube defects (NTDs) (Ducker and Rabinowitz, 2017; Stover, 2011; Li et al., 2016)

A central focus of human research into one-carbon metabolism has been the link between dietary intake of vitamins and brain development and

function, with emerging evidence highlighting the negative consequences of nutritional deficiencies on cognitive and motor functions (Black, 2008). By way of example, it is well established that folic acid supplementation decreases the prevalence of neural tube defects (NTDs); a common congenital malformation in humans resulting from failure of neural tube closure during embryogenesis (Imbard et al., 2013). Despite the prevalence of these defects varying greatly with respect to geographic region and ethnicity, NTDs are one of the most frequent congenital malformations (Au et al., 2010). Consequently, in 1998 the United States and Canada introduced a policy of mandatory fortification of enriched cereal grain products with folic acid with the aim of reducing the incidence of these defects. Introduction of this policy led to a 19-32% decline in the prevalence of NTDs since its implementation (Crider et al., 2011), demonstrating the significance of the association. Since then, it is estimated that more than 81 countries have implemented similar interventions, although there is general resistance within Europe. Recent debates have centred on the optimal timing and amount of folate required, and fortification strategies (Rosenberg, 2005) with concerns revolving around the negative effects of unmetabolised folic acid (Smith et al., 2016).

Importantly in the context of this thesis, there is accumulating evidence surrounding the effects of simultaneous B12 and folate deficiencies on disease etiology due to their shared metabolism (Beaudin and Stover, 2009; Lucock, 2000; Malloy et al., 2008). In 1C metabolism, methylB12 (methylcobalamin) acts as a conduit between 5-methylTHF and methionine in cobalamin-dependent methionine synthase. Described by the ‘methyl-trap hypothesis’ (Abou-Zeina, 2008), decreased availability of B12 impairs the activity of the B12 dependent enzyme MTR, which traps an increased proportion of folate as the 5-methyl derivative, resulting in increased Hcy (Figure 1.4).

Imbalance of folate and B12 in human diets can be illustrated by the global prevalence of B12 deficiency in humans being greater than that of

folate. This variation can in part be explained by dietary preferences in different countries and regions. Estimates of B12 deficiency prevalence ranges between <10% and >50% with incidence B12 deficiency most frequent in Central and Latin America, the Indian subcontinent and China (McLean et al., 2008; Brito et al., 2015; Soofi et al., 2017). Furthermore, in the UK, serum B12 concentrations have been recorded (EPIC-Oxford study) to be sufficient (>150 pM) in 98% of omnivores and 76% of vegetarians but in only 27% of vegans, 52% of whom were categorised as deficient (<118 pM) (Gilsing et al., 2010). In contrast, folate status was sufficient (>6.3 nM) in all three groups.

Vitamin B12 and folate deficiency are also inextricably linked to vitamin B6 and B2 deficiency. Vitamin B6 is involved as a cofactor in over 100 enzyme reactions including amino acid metabolism. Particularly, as illustrated in Figure 1.3, B6 is required for the conversion of homocysteine to cysteine (transsulfuration pathway) and the successive synthesis of glutathione, as well as for the interconversion of THF and 5, 10-methyleneTHF (folate cycle). Vitamin B2 (FAD; flavin adenine dinucleotide) is cofactor to the enzyme responsible for regulating of the flow of folates between the production of nucleotides and the supply of methyl groups for methionine synthesis.

As well as these key vitamin co-enzymes, other nutrients and dietary components can also affect 1C metabolism indirectly. 1C metabolism is inextricably linked to trace-element utilization. As the main focus of this thesis, the importance of Co for the intra-ruminal synthesis of vitamin B12 has already been discussed (Section 1.2). Another important trace-element is zinc. Dietary zinc intake may modulate DNA methylation via its impact upon enzymes involved in 1C metabolism. For example, BHMT is a major Zn metalloenzyme which regulates the remethylation of Hcy to methionine (Figure 1.3) (Millian and Garrow, 1998; Mathers and Ford, 2009). Furthermore, cystathionine-β-synthase (CBS), the enzyme which catalyses the first step of the transsulfuration pathway (Figure 1.3), converting Hcy to cystathionine, is also a Zn metaloloenzyme. A dietary

deficiency in Zn, therefore, leads to elevated serum Hcy concentrations (Jing et al., 2015). Evidence from animal studies has shown that zinc deficiency alters brain structure and function, influencing long-term growth and intellectual performance (Chowanadisai et al., 2005).

Another key trace-element involved in 1C metabolism is selenium (Se). In rats, dietary Se has been reported to cause an imbalance in the methionine cycle by decreasing homocysteine concentrations (Davis & Uthus, 2003). The proposed mechanism is via the conversion of homocysteine to selenohomocysteine, cystathionine, selenocystathionine, glutathione and selenocysteine, thus reducing homocysteine availability for methionine synthesis, leading to reduced global DNA methylation (Davis et al., 2000).

1.9 Genetic regulation of one-carbon metabolism

Whilst many developmental, age related and chronic diseases arise as a direct consequence of dietary deficiencies at different stages of life, there is clear evidence for genetic predisposition to metabolic imbalances. In humans, several polymorphic variants in 1C related genes have been shown to be associated with defects in enzymes which utilise these nutrients, altering metabolism, absorption and transport, and increasing disease risk. However, current knowledge on the genotype/phenotype relationships of the inherited causes of deficiency does not fully explain the variability of their clinical and metabolic manifestations. Whilst the effects of polymorphisms in genes functionally related to 1C metabolism have been extensively studied in human populations and have been shown to be associated with a variety of complex traits and disorders, animal work has been limited to gene knock-ins and knock-outs in mice and, to date, there have not been any such studies in farm animals.

Genetic variants in 1C and related metabolic pathways can influence methyl group supply and subsequently result in aberrant methylation causing disruption to DNA synthesis and repair, increased DNA damage and gene mutations. Furthermore, there has been evidence for variant-

nutrient interactions. A notable example is the *MTHFR* (EC 1.5.1.20) C677T (rs1801133) polymorphism (Bailey et al., 1999) which is a well characterised risk variant in humans for NTDs (Yan et al., 2012), recurrent pregnancy loss (Cao et al., 2013), idiopathic male infertility (Gong et al., 2015), and Down syndrome (Cyril et al., 2009; Kaur and Kaur, 2016). The presence of the T-allele in combination with variable folate status alters the balance in use of 5, 10-CH2THF between synthesis of methionine and dTMP. If folate status is adequate, methyl groups are diverted towards the synthesis of DNA rather than methionine (Lucock and Yates, 2005). If folate status is inadequate, however, uracil may be misincorporated into DNA, causing genomic instability. Stabilisation of the catalytic activity of this enzyme by improving its ability to bind to its cofactor, flavin adenine dinucleotide (FAD), can be achieved by increasing folate status (Guenther et al., 1999). Dietary levels of riboflavin (B2), a precursor to FAD are therefore important in individuals with the C677T variant (McNulty et al., 2006). Collectively, these observations provide a possible explanation for the north south gradient in prevalence of the C677T genotype in Europe, where T-allele frequency is lower in folate deplete northern (~8%) than folate-replete southern (~18%) countries (Eussen et al., 2013; de Bree et al., 1997; Park et al., 2012), suggesting selection pressure against the T allele in regions where low folate intakes prevail.

In addition to the C677T polymorphism, other MTHFR variants including A1298C, have also been identified as potential modulators of a variety of disorders whose development depends on folate/homocysteine imbalance. Furthermore, genetic variants within coding regions of other 1C related genes have also been found to be associated with a variety of complex traits and disorders. Examples are given in Table 1.2.

Table 1.2. Example variants in key 1C genes associated with adverse clinical consequences

Gene	Variant	Clinical consequence (↑ increased risk)	Reference
Methionine synthase reductase (MTRR)	A66G	Hyperhomocysteinemia ↑cardiovascular disease ↑NTDs	Gaughan et al., 2001
Methionine synthase (MTR)	A2756G	↑Orofacial clefts ↑NTDs	Mostowska et al., 2006 Cao et al., 2018
Methylene tetrahydrofolate dehydrogensase1 (MTHFD1)	G1958A	↑NTDs ↑Second trimester pregnancy loss	Prasoona et al., 2016 Parle-McDermott et al., 2005
Glycine N methyl-transferase (GNMT)	C1298T	Hyperhomocysteinemia	Beagle et al., 2005
S-adenosylhomocysteine hydrolase (AHCY)	G336A A428G	↑congenital myopathy, delayed development	Baric et al., 2005
Betaine-homocysteine S-methyltransferase (BHMT)	G742A	↑NTD ↑Down syndrome	Boyles et al., 2006 Jaiswal et al., 2017
*Reduced folate carrier (RFC1/*SLC19A1*)*	A80G	↑Autism polyglutamation of MTX	Laverdiere, 2002 Dervieux, 2004
Transcobalamin II (TCN2)	C776G	↑Cleft lip	Martinelli, 2006
Cystathionine-β-synthase (CBS)	*C699T*	↑Ischemia, late onset preeclampsia	Koning et al., 2013
Serine hydroxymethyltransferase (SHMT)	*C1420T*	↑NTDs	Prasoona et al., 2017

1.10 Methyl group donation and epigenetics

Dietary exposures can have consequences for health for many years to come, raising the question of how such exposures are 'remembered' and how they result in altered disease risk. There is growing evidence to suggest that epigenetic mechanisms may mediate the effects of nutrition and may be causal in the development of common complex diseases. Epigenetics can be defined as heritable changes in gene expression that do not involve changes to the underlying DNA sequence. These changes result in a phenotypic change without a change in genotype occurring, in turn providing an additional level of gene regulation (Hemberger et al., 2009). These epigenetic changes include DNA methylation, histone modifications and non-coding miRNA, of which DNA methylation is the most extensively studied (Anderson et al., 2012), and occur not only during cellular differentiation in embryonic and foetal development but also in later life.

Methylation relies upon availability of methyl groups from SAM (Murín et al., 2017). Other methyl donors (e.g. choline/betaine, folate and Met), enter 1C metabolism at different sites and are ultimately converted to SAM (Pauwels et al., 2017). Methyl groups are transferred from SAM to various biomolecules, including DNA, RNA, proteins (histones) and secondary metabolites, by SAM-dependent methyltransferase enzymes. SAM-dependent methylation of DNA is catalysed by DNA methyltransferases (DNMTs; EC 2.1.1.37). Although there are five family members (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L), only DNMT1, DNMT3A and 3B possess methyltransferase activity (Portela and Esteller, 2010). DNMT1 maintains DNA methylation during mitosis, whilst DNMT3A and 3B are responsible for *de novo* methylation patterns established during early embryogenesis (Jin and Robertson, 2013). Although DNMT3L lacks inherent methyltransferase activity, due to absence of the conserved catalytic domain common to DNMTs (Chédin et al., 2002), it plays an essential role in germ line DNA methylation via allosteric co-operation with DNMT3 family enzymes (Hata et al., 2002).

DNMT2 also lacks DNA methyltransferase activity but has been reported to methylate tRNA (Goll et al., 2006).

Covalent post-translational histone modifications impact gene expression by remodelling chromatin structure or recruiting histone modifiers. Histone methylation occurs primarily on the side chains of lysine and arginine residues of histone tails and is performed by histone lysine N-methyltransferases (KMTs) and histone arginine N-methyltransferases (PRMTs), respectively (Morera et al., 2016). Lysine may be mono-, di- or trimethylated on its amine group, whereas arginine may be mono- or dimethylated on its guanidyl group (Lan and Shi, 2009; Ng et al., 2009). These marks play both a repressive and facilitative role in gene transcription depending on the site and degree of methylation (Bannister et al., 2002). Trimethylation of H3K27, H3K9, and H3K79, for example, are marks linked to genomic repression (Barski et al., 2007). Monomethylation of lysine 9 on histone 3 (H3K9), H3K27, H4K20, H3K79 and H2BK5, on the other hand, are marks linked to genomic activation (Barski et al., 2007).

RNA methylation can also regulate gene expression. As observed in DNA, methylation can occur at cytosine residues in RNA, however, knowledge about the prevalence and transcriptome-wide distribution of 5-methylcytosine (5mC) in RNA is limited and requires further verification (Liu and Jia, 2014). Amort et al. (2017) recently identified and mapped 5mC modifications in the epitranscriptome of murine brain and embryonic stem cells, presenting the first comprehensive dataset of cytosine methylation in mRNA. To date, N^6-methyladenosine (m^6A) is the best studied RNA modification, occurring in various eukaryotic RNA molecules, specifically messenger RNA (mRNA) and long non-coding RNAs (lcRNAs) (Willyard, 2017). N^6-methyladenosine methylation is catalysed by a multicomponent complex, comprising methyltransferase like 3 (METTL3) and METTL14 enzymes, alongside auxiliary proteins, such as WTAP in mammals (Lee et al., 2014). Broad roles of m^6A include regulating cellular pluripotency, programming and differentiation;

transcript stability, splicing, and protein translation; and the cell stress response. Recent work undercovers a protective function of m^6A in DNA damage repair (Yue et al., 2015; Xiang et al., 2017), indicating RNA methylation offers more than just epigenetic regulation.

The response of methylation to environmental signals indicates that it must be biochemically reversible (Szyf et al., 2016). An example of epigenetic reversibility is the reprogramming of the developing germline and early embryo, whereby genome-wide DNA demethylation and extensive chromatin remodelling facilitates erasure of epigenetic memory (Hajkova, 2011). Various epigenetic 'erasers' have been identified, including histone deacetylases (HDACs) and demethylases (e.g. LSD1) (Gillette and Hill, 2015). DNA methylation may be passively depleted through DNA replication or actively removed by ten-eleven translocation (TET) enzymes and thymine DNA glycosylase-mediated base excision repair (Kohli and Zhang, 2013). Two m^6A demethylases have been identified; fat mass and obesity associated protein (FTO) and alkB homology 5 (ALKBH5) (Batista, 2017). These mechanisms facilitate turnover of methyl groups, highlighting the dynamic and flexible nature of epigenetic gene regulation.

1.11 Development of working hypothesis

The literature reviewed above provides clear evidence that Co deficiency in sheep and consequently B12 metabolism is influenced by geographical location, nutritional status and genetics. A number of different approaches can be used to diagnose Co/B12 deficiency in sheep. Whilst arguably one of the best indicators of nutritional imbalances is the presence or absence of individual responses in health or performance to specific mineral interventions, lack of clinical and pathological specificity between mineral deficiencies and other nutrient insufficiencies can make diagnosis difficult. Therefore, assessment of the biochemical consequences of decreased B12 is superior. Once a trace-element deficiency has been diagnosed, or susceptible animals identified, the most effective method of treatment or prevention must be

decided on. Some of the best short term measures include supplementation strategies. The first part of this thesis (Chapters 2 and 3) therefore sought to assess the effectiveness of novel long-acting supplementation strategies.

Since mass intervention with mineral supplements is often only beneficial to a minority of the flock and since susceptibility to mineral disorders is often a heritable characteristic, a superior and longer term preventive approach may reside in tapping the potential for genetic improvement in tolerance of mineral imbalances. The effects of genetic polymorphisms on regulation of 1C metabolic pathways have been studied extensively in human populations and have been shown to be associated with a variety of complex traits and disorders. Animal work, on the other hand, has been limited to gene knock-ins and knock-outs in mice, and there have not been any such studies in farm animals. Nevertheless, the understanding gained from human studies can aid investigation of the regulatory aspects of 1C metabolism and exploration of the impact of perturbations to these pathways in sheep. A wide variety of environmental, dietary and lifestyle factors converge to modify 1C metabolism which in turn can influence the provision of methyl donors and thus epigenetic regulation of gene expression. The latter part of this thesis (Chapters 4 and 5), therefore, sought to provide unprecedented understanding of the functional significance of complex relationships between 1C metabolites and variants in 1C metabolic genes. Furthermore, as every farm has different nutritional challenges, Chapter 5 sought to characterise 1C genetic diversity between breeds. In summary, therefore, this thesis aims to increase understanding of dietary and genetic regulation of B12 metabolism in sheep through supplementation trials and genetic analyses.

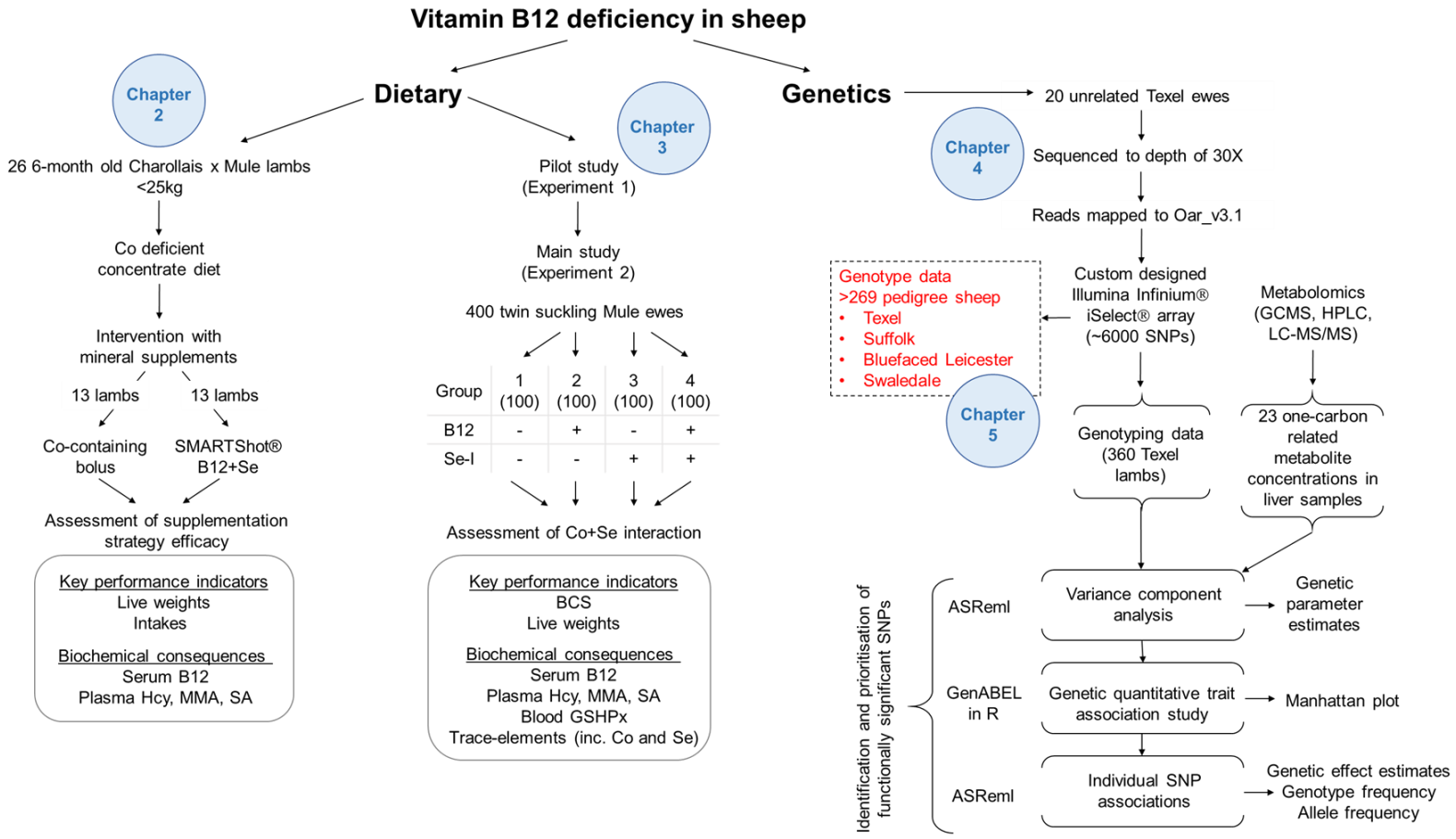


Figure 1.9. Development of working hypothesis. Flow diagram outlining the studies in this thesis.

CHAPTER TWO

Comparison of the efficacy of a long-acting injectable micro-encapsulated vitamin B12 supplement and intra-ruminal cobalt bolus on metabolic status and performance of growth-retarded lambs

2.1 Introduction

Cobalt (Co) deficiency is a recognised problem in sheep, particularly in rapidly growing lambs (Grace, 1994). As Co is essential for the microbial synthesis of vitamin B12 in the rumen, deficiency leads to disruption of important B12 dependent pathways. The two enzymes that utilise B12 as a cofactor are methylmalonyl CoA mutase (EC 5.4.99.2) and methionine synthase (2.1.1.13). The former catalyses the conversion of methylmalonyl-CoA to succinyl-CoA, an important step prior to gluconeogenesis, whereas the latter acts to re-methylate homocysteine (Hcy) in the terminal step of methionine synthesis. As the only source of B12 in ruminants, dietary deficiency of Co therefore disturbs normal energy and protein metabolism, consequently having a marked effect on animal performance and health (Rice et al., 1989; Kennedy et al., 1992).

The effects of deficiency can be mitigated by effective supplementation. To be effective, the supplement must increase and maintain the mineral status of the animal for long periods. Several strategies to prevent Co deficiency are available, each with its own benefits and limitations. Topdressing pastures with Co aims to provide the grazing animal with sufficient amounts of this element to meet demands of B12 synthesis. Environmental factors such as increased Mn in the soils and increased rainfall, however, can reduce the uptake of Co by the plant and therefore by the animal (Li et al., 2001). Injections with water soluble B12 are a quick acting yet short term solution, effective for only around 4 weeks (Grace et al., 1998; Judson et al., 2000). Effects of Co drenches are similarly short lived, lasting less than two weeks (Field et al., 1988). Intra-ruminal Co boluses, when not regurgitated, have been shown to increase and maintain B12 status for up to one year (Judson et al., 1995). Although commercially available and commonly used for the treatment of Co deficiency in weaned lambs and adult sheep, boluses cannot be given to lambs younger than 10 weeks of age because the animals are too small and do not have a functional reticulorumen capable of synthesising B12 from elemental Co. As an alternative strategy, a long-acting injectable

lactide-glycolide microencapsulated vitamin B12 has been developed (Grace and Lewis, 1999). This product is administered by subcutaneous injection into the anterior neck and has been shown to increase and maintain serum and liver vitamin B12 concentrations in lambs for up to 8 months (Grace and Lewis, 1999; Judson et al., 2000). Studies with this long-acting B12 supplement to date, however, have been conducted predominantly in New Zealand and Australia, with young (3-6 weeks) (Grace and Lewis, 1999; Grace et al., 2003) and weaned (Grace and West, 2000) lambs at pasture, and there is a dearth of information on its efficacy for the intensively reared (indoor) lamb. To date, no study has assessed its efficacy with the bottom quartile of growing lambs. It could be possible that these lambs will respond differently to supplementation due to reduced rumen function or impaired intermediary metabolism preventing the lamb from reaping the full benefits of additional provision of B12.

In every sheep flock, there is a proportion of lambs that start life behind the majority in terms of health and weight that never subsequently compensate. Under good husbandry these animals, once identified (usually around 8 weeks or weaning), would ideally be separated from the rest and preferentially fed and supplemented. Due to their reduced growth rates and additional costs of feeding and repeated supplementation, these lambs are often considered to be economic liabilities. Strategies to improve the productivity of these animals is therefore of particular interest to the sheep industry.

In the current study, we aimed to assess the effectiveness of microencapsulated B12, compared to the commonly used Co bolus, on repletion of weaned Co deficient lambs offered an intensive cereal-based diet. We investigated the metabolic consequences of Co depletion and responses to supplementation in 6 month old lambs weighing less than 25 kg by analysis of recognised biomarkers in blood in conjunction with assessments of feed intake and growth rate. Figure 2.1 illustrates the low growth rates of the cohort of lambs selected for this study from birth to

weaning, in comparison with growth rates of all other lambs on the monitor farm in the same year.

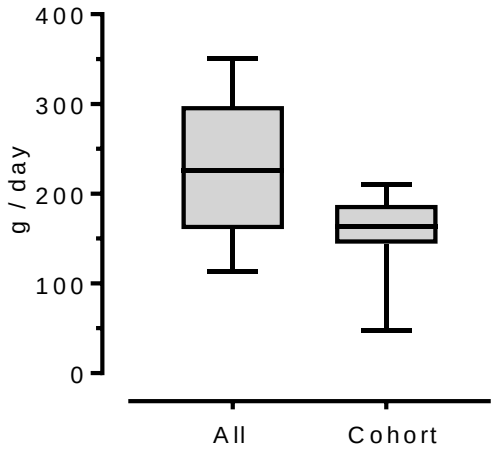


Figure 2.1. Growth rate from birth to weaning of all lambs on the participating monitor farm and cohort of 26 lambs used in the current study. (Median and interquartile ranges with 10th and 90th percentiles)

2.2 Materials and methods

2.2.1 Animals, treatments and sample collection

All procedures were conducted in accordance with the requirements of the UK Animal Scientific Procedures Act (1986) and investigations were approved by the University of Nottingham Animal Welfare and Ethical Review Board.

Twenty six, 6-month old Charollais x Mule (Bluefaced Leicester x Swaledale) lambs, weighing <25kg, were obtained from a monitor farm in Leicestershire, participating in an AHDB funded study. Before commencement of the current study, animals were adjusted to a Co deficient concentrate diet (90% Barley, 10% Soya) for 4 weeks. Details of diet composition are provided in Table 2.1. Following routine screening for a full spectrum of trace-elements (e.g. Mg, P, Ca, Na, Cu, Se, and Cl) before the start of the study, the lambs were identified to be marginally deficient in Se and so were treated with a subcutaneous injection of Se (Deposel®). Their status with respect to other trace-elements was normal, and Se rapidly returned to normal. However, three animals were removed from the study as they were found not to be thriving following introduction to the experimental unit.

At the start of the study, animals were penned individually and were randomly allocated to one of two treatment groups (13 lambs per group initially). No control group was included because the level of deficiency that would be reached by the end of the study without treatment would not be ethical. For the duration of the study (16 weeks), animals were fed a Co deficient barley-based diet ad libitum with a fixed rate of Co-deficient hay of 200 g/day (Table 2.1). Animals had continual access to water at all times. The two supplement treatments were administered after 9 weeks on study. Group 1 were treated with an intra-ruminal, Co-containing bolus (Zincosel, Telsol Limited, Denbigh, UK), administered orally using a balling gun. Each bolus contained 15.2% w/w zinc, 0.15%

w/w Se as Na_2SeO_4, 0.5% w/w Co. This Se-containing bolus was selected for this study because the monitor farm in question had a previously documented history of Se deficiency. Group 2 was treated with a 2 ml subcutaneous injection of SMARTShot® B12+Se into the anterior neck. SMARTShot® B12+Se is an oily suspension containing 3 mg/ml hydroxocobalamin hydrochloride and 12 mg/ml Se as barium selenite encapsulated in lactide/glycolide co-polymer for extended release. The manufacturer's recommended dose for lambs is 0.5-1 ml, although dose can be tailored to suit the length of efficacy required. A dose of 2 ml was given to lambs in this study to ensure longer efficacy. The Se containing SMARTShot® B12+Se supplement was selected to match the Se received in the Zincosel bolus. Both supplements cost ~£1 per dose.

Individual intakes of the Co deficient barley-based diet were recorded at 3 and 4 day intervals, along with hay fed at a fixed rate of 200 g/day. Lamb live-weights were recorded fortnightly.

Table 2.1. Chemical and mineral composition of barley, soya and hay

Nutrient	Barley	Soya	Hay
DM, g/kg DM	852.7	886.6	847.1
CP, g/kg DM	116	504	58
Ash, g/kg DM	-	-	65.7
NDF, g/kg DM	146	66	566
NCGD, % DM	88.7	92.8	59.6
Starch, g/kg DM	555	5.57	18.6
Macro minerals, g/kg DM			
P	3.2	7.41	1.47
K	5	22.4	13.4
Na	0.06	0.11	0.1
Mg	1.04	2.63	1.04
Ca	0.43	2.57	5.43
S	1.05	3.25	0.5
Micro minerals, mg/kg DM			
B	<2.45	46	10.4
Cu	3.9	16.3	3.87
I	105	113	325
Mn	9.71	37.7	55.9
Zn	26.5	34.2	10.4
Se	0.02	0.23	0.02
Co	<0.1	0.14	0.13

DM=Dry Matter; CP=Crude Protein; NDF=Neutral Detergent Fibre; NCGD=Neutral Cellulase Gammanase Digestibility

2.2.2 Blood analysis

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma-Aldrich, Company Ltd, Gillingham, Dorset).

Blood samples were collected from lambs weekly, with two additional samples collected in the two days following treatment, by superficial venepuncture into Lithium heparin (LH) tubes and vacuum tubes containing no additive. Samples were centrifuged at 4250 x g for 15 min. Whole blood, plasma and serum fractions were stored at -20°C until analysed.

2.2.2.1 Vitamin B12

Serum B12 analysis was conducted using the ADVIA Centaur ® CP VB12 assay at the AHVLA laboratory, Shrewsbury, Shropshire, UK. The ADIVA Centaur ® VB12 is a competitive immunoassay using direct chemiluminescent technology. Vitamin B12 from the serum sample competes with B12 labelled with acridinium ester in the Lite Reagent for a limited amount of purified intrinsic factor, which is covalently coupled to paramagnetic particles in the Solid Phase. The Assay uses Releasing Agent (sodium hydroxide) and DTT to release the B12 from the endogenous binding proteins in the sample and, to prevent rebinding after Solid Phase, cobinamide is added to the sample. An inverse relationship exists between the B12 in the sample and the relative light units detected by the system. A sample volume of 100 µl is required for this assay. The limit of detection was 33 pmol/L, and coefficients of variation (CVs) for low, medium and high QCs were 12.2, 12.4 and 12.4% respectively.

2.2.2.2 Homocysteine

Plasma Hcy was determined using an Imola Autoanalyser (RX imola; Randox Laboratories Ltd., Antrim, U.K.). The kit for Hcy was supplied by Randox Laboratories (catalogue no. HY4036). In this method, bound or dimerised Hcy (oxidised form) is reduced to free Hcy, which then reacts

with serine catalysed by cystathionine beta-synthase (CBS) to form cystathionine. Cystathionine in turn is broken down by cystathionine beta-lyase (CBL) to form Hcy, pyruvate and ammonia. Pyruvate is then converted by lactate dehydrogenase to lactate with nicotinamide adenine dinucleotide (NADH) as coenzyme. The rate of NADH conversion to NAD+ is directly proportional to the concentration of Hcy (ΔA340 nm). The limit of detection was 1.74 µmol/L and upper limit was 47.9 µmol/L. CVs for low, medium and high QCs were 9.32, 6.77 and 5.40% respectively.

2.2.2.3 MMA and SA

Plasma methylmalonic acid (MMA) and succinic acid (SA) concentrations were determined by Gas Chromatography with Mass Spectroscopic detection (GC-MS) following derivatisation and extraction. The method used was based on that of Kanakkaparambil et al. (2009), with the following modifications. Briefly, 50 µl plasma and 5 µl internal standard (4-chlorobutyric acid (CBA), 250 µM) were added to 250 µl 12% BF3-Methanol in a 2.5 ml screw capped glass vial, vortexed for 30 s and heated at 95°C for 15 min on a heating block. After cooling, 250 µl distilled water and 250 µl dichloromethane (CH2Cl2) was added to the vial and vortexed for 30 s. This was then centrifuged for 8 min at 2500 x g and 4°C to separate the layers. The lower dichloromethane layer was transferred to a screw capped auto-sampler vial with insert for GC-MS analysis. The method used a DB-WAX column (cross-linked polyethylene glycol; J&W Scientific Agilent technology) (30 mm x 0.25 mm; 0.15 µm film thickness). The carrier gas (He) was set at a constant flow rate of 1.0 ml/min. The injection volume was 1 µl for SCAN mode, for qualification, and SIM (selected ion monitoring) mode for quantification, both using splitless mode. The injection port and MS selective detector interference temperatures were 260°C and 280°C respectively. The chromatograph was programmed for an initial temperature of 50°C for 2 min, increased to 150°C at 8°C min^{-1}, then increased to 220°C at 100°C min^{-1} and held for 5 min at the final temperature. The MS was tuned regularly and operated in electron impact (EI) ionization mode with the ionization

energy of 70eV. SCAN mode measured at m/z: 20-500 and SIM ions were set at 105 (for CBA) and 115 (for MMA, and SA). The same method was used to produce a calibration curve for MMA and SA using standards at concentrations ranging from 0.156 µmol/L to 20 µmol/L. Calibration was carried out by comparison of peak areas of CBA with MMA and SA in the standards and the final results expressed in µmol/l plasma. The limit of detection was 0.15 µM for both and CVs for low, medium and high QCs were 0.44, 1.03, 0.83% respectively for MMA and 1.19, 1.11 and 2.33% respectively for SA.

2.2.2.4 Statistical analysis

All statistical analyses were performed within the GenStat statistical package (17th Edition, VSN International, 2011). Blood metabolite concentrations, total DM intakes and lamb weights were analysed by 'repeated measures' ANOVA (i.e. from data recorded and samples collected weekly). For the most part, data are presented as means with standard errors of means (S.E.M.). For presentation purposes, however, dry matter intakes (expressed on a metabolic live-weight basis) are presented as box plots, depicting median and inter-quartile ranges, with whiskers set at 1st and 99th percentiles.

2.3 Results

For presentation purposes, time-points indicate weekly blood sample collections, with the exception of time-points 10 and 11, which illustrate samples taken 1 and 2 days after treatment respectively.

2.3.1 Blood analysis

Figure 2.2A shows the mean serum B12 concentrations of lambs in both treatment groups over the duration of the study. Mean concentrations of serum B12 decreased over time ($P<0.001$) from 442 pmol/L at time-point 1 to 113 pmol/L at time-point 9. Immediately after treatment (time-point 10), a significant difference ($P<0.001$) in serum B12 concentrations between the two treatment groups was observed. Lambs treated with B12 showed a marked increase in serum B12 concentration (rising to 1,785 pmol/L), whilst only a small increase was observed in Co treated lambs (to 143 pmol/L). Serum B12 concentrations of lambs treated with Co bolus continued to rise steadily for the remainder of the study to a final concentration of 598 pmol/L. Conversely, after the sudden spike was observed in the B12 injected animals, serum B12 decreased to 709 pmol/L at time-point 12 (4 days post injection) followed by an increase to 3,380 pmol/L at time-point 14 (16 days post injection), with a final decrease towards the end of the trial (to 1,717 pmol/L). Overall, however, B12 injection increased and maintained serum B12 concentrations above levels seen in Co treated animals at all time-points. Variation between animals within the B12 treatment group was greatest at time-points 10 and 14.

Plasma Hcy concentrations of all animals increased ($P<0.001$) from time-point 1 to 9, peaking at 11.7 µmol/L (Figure 2.2B). Immediately after treatment, plasma Hcy concentrations decreased ($P<0.001$) to 10.4 µmol/L and 10.8 µmol/L for B12 and Co treated animals respectively. Plasma Hcy continued to decrease ($P<0.007$) for the remainder of the study to a final concentration of 8.3 µmol/L (B12 treated lambs) and 9.0

µmol/L (Co treated lambs). The difference in Hcy concentrations between the two treatment groups was not significantly different.

Figure 2.2C illustrates the decrease ($P<0.001$) observed in plasma MMA concentration over time from time-point 9 to 14 in both treatment groups. From time-point 11 onwards, B12 injected animals had significantly lower mean MMA concentrations than Co treated animals ($P<0.02$). As was seen with plasma Hcy, levels of MMA decreased ($P<0.001$) in both groups immediately post treatment. However, at time-point 11, whilst levels in B12 treated animals continued to decrease quickly, Co treated animals maintained their MMA levels until time-point 12, after which a decrease was observed. Final concentrations of plasma MMA in B12 and Co treated lambs were 0.8 µmol/L and 3.7 µmol/L respectively.

There was no significant difference in SA concentrations between the two treatment groups, however, mean SA concentrations fluctuated ($P=0.015$) over time (Figure 2.2D).

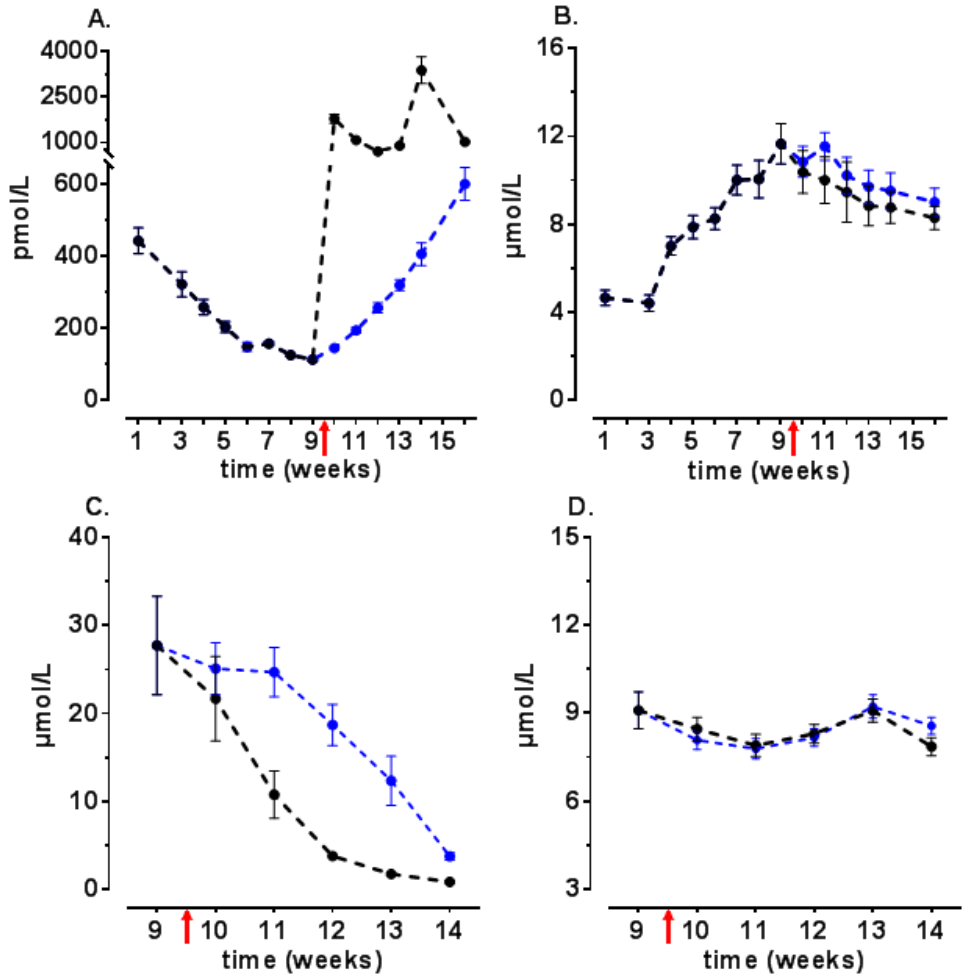


Figure 2.2. The effect of microencapsulated B12 injection ● and Co bolus ● on blood metabolite concentrations; serum B12 (A) and plasma Hcy (B), plasma MMA (C) and plasma SA (D). → depicts time of treatment. For B12 (A), interaction ($P<0.001$; SED=329) between treatment and time. For Hcy (B), effect of time ($P=0.007$; SED=0.57). For MMA (C), effect of treatment ($P=0.014$; SED=3.5) and time ($P<0.001$; SED=2.7). For SA (D), effect of time ($P=0.015$; SED=0.3).

2.3.2 Intakes and weights

DM intakes fluctuated over time (P<0.001), however, no difference between treatment groups was observed (Figure 2.3).

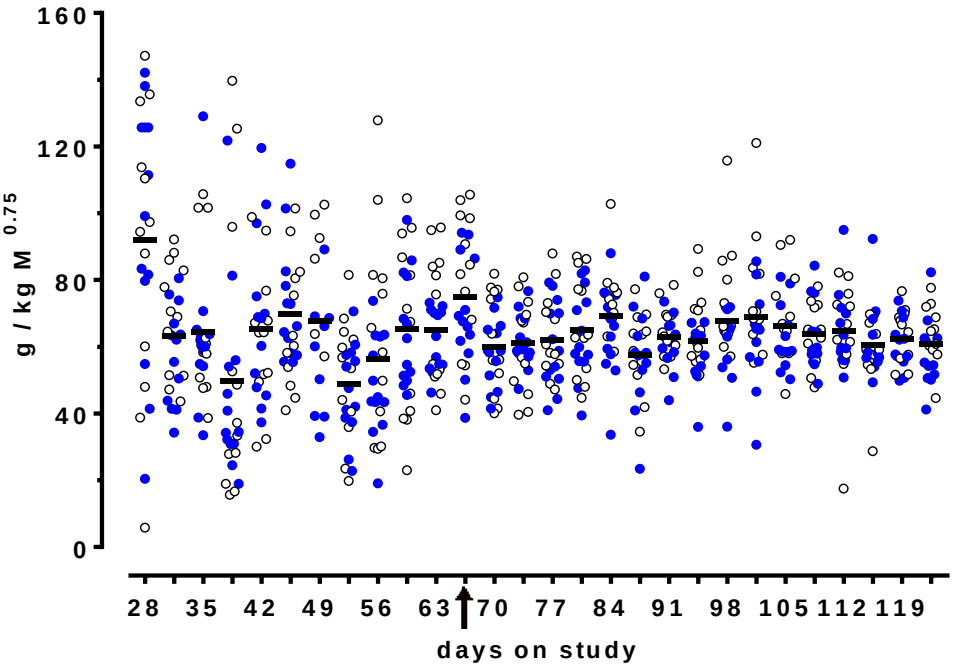


Figure 2.3. Effect of B12 injection o and Co bolus ● on dry matter (DM) intake (g/kg $M^{0.75}$) over time. → depicts time of treatment. Effect of time (P<0.001; SED=4.9).

Lamb weights increased with time (P<0.001), from a mean of 23 kg at the start of the study to a mean of 34 kg for both B12 and Co treated animals by the end of the study. Mean growth rate was 90 g/lamb/day. There was no treatment effect on lamb growth rate (Figure 2.4).

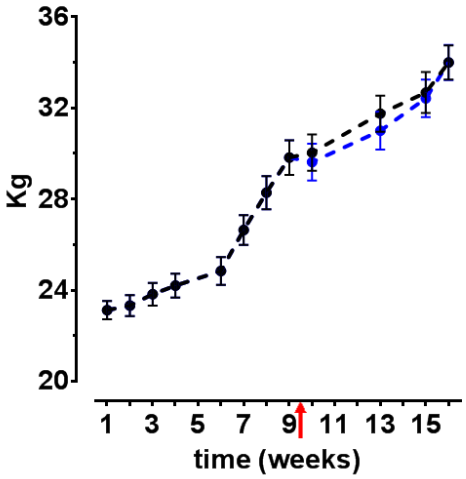


Figure 2.4. Effect of microencapsulated B12 injection ● and Co bolus ● on lamb live-weight. Effect of time (P<0.001; SED=0.46)

2.4 Discussion

In every sheep flock, there is a proportion of lambs that start life behind the majority in terms of health and weight, that never subsequently compensate. This study aimed to assess the effectiveness of SMARTShot® long-acting injectable lactide-glycolide microencapsulated B12, compared to the commonly used Co bolus, on repletion of growth retarded Co deficient lambs offered an intensive Co deficient cereal-based diet. Treatment with the long acting B12 injection improved the B12 status of Co deficient lambs more than treatment with Zincosel oral Co bolus for at least 6 weeks, however, no effect of treatment with either of the supplements was observed on intake or growth rate. It could be suggested that a more prolonged metabolic abnormality is needed to precipitate impaired performance and hence to see an effect of treatment on weight gain and feed intake (Kennedy et al., 1992).

2.4.1 Diagnosis of Co deficiency

In sheep, serum B12 concentrations are indicative of B12 status (Suttle, 2005). In the UK, suggested thresholds for B12 adequacy and deficiency are >350 pmol/L and <250 pmol/L respectively (Suttle, 2005). In the current study, serum B12 concentrations in all lambs fell to below the threshold for deficiency by 9 weeks when fed a barley-based Co deficient

diet. Biomarkers of metabolic disturbances offer a further means of detecting early stages of deficiency and dysfunction (Hannibal et al., 2016). The increase in plasma Hcy from 4.6 µmol/L (i.e. sufficient status) to 11.6 µmol/L (i.e. deficient status) (Kennedy, 1992; Vellema, 1999) is in agreement with the decreasing serum B12 concentrations. This indicates that there was a dysfunction in methylation due to Co deficiency and confirms that the lambs became B12 deficient by Week 9 of the study.

2.4.2 Supplementation strategies

When a deficiency has been identified, a suitable supplementation strategy must be implemented. A key consideration is the duration of efficacy of available supplements. Chemical form, physical presentation and route of administration can all have an effect on whether the supplement is long acting with slower absorption, providing extended supplementation, or short acting with fast absorption and quick utilisation (Grace and Knowles, 2012). A comparison of the effects of a Co oral drench, a soluble B12 injection, a Co containing intraruminal bolus and a long acting vitamin B12 injection on the serum B12 concentrations of sheep can be seen in Figure 2.5 taken from a publication by Grace and Knowles (2012).

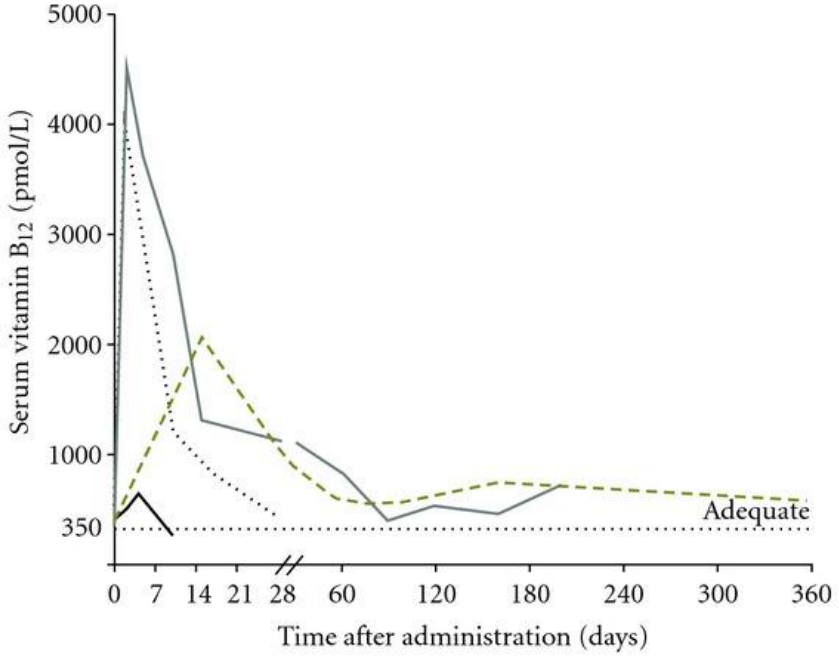


Figure 2.5. Comparison of trace-element supplementation strategies. Taken from Grace and Knowles (2012). ——Cobaltised drench (7 mg Co as $CoCl_2$), ····· Soluble B12 injection (2 mg B12), - - - - Co bolus (10g bolus as 30% CoO:70% Fe), — Long-acting B12 injection (6 mg B12)

Figure 2.5 shows that sustained adequate B12 status (>350 umol/L) is achieved with intra ruminal Co bolus and B12 injections formulated for slow and controlled release, whilst effects of the oral Co drench and soluble B12 injection were comparatively short lived.

Long-acting trace-element supplements reduce the need for repeated administration. Intra-ruminal Co boluses (also called pellets or bullets) have been shown to be effective for 12 months (Judson et al., 1995). However, safe and quick administration has its challenges and regurgitation is common. In New Zealand, boluses are no longer widely used, and long acting injectable products are preferred (Grace and Knowles, 2012). A long acting injectable lactide-glycolide microencapsulated vitamin B12 has been developed (SmartShot®). This is particularly suited to lambs which, at a young age, are too small to

receive a bolus and are very sensitive to B12 deficiency due to high metabolic demands associated with growth.

2.4.3 B12 injection vs Co bolus: Effect on metabolite levels

In the current study, efficacy of this B12 injection in comparison with an intra-ruminal Co bolus (Zincosel) was assessed in terms of changes in concentrations of serum B12, associated metabolic biomarkers, weight gains and feed intakes. It is well recognised that increased Co intake or injection with B12 results in elevated serum B12 (Somers and Gawthorne, 1969). Following treatment (Week 10), significant increases in serum B12 were seen in both treatment groups. This supports previous work with weaned and young lambs (4-5 weeks old) (Clark et al., 1989; Grace and West, 2000). Both treatment strategies increased serum B12 levels, however, the more marked increase seen in B12 treated animals immediately after treatment and higher levels at all subsequent time-points could indicate more rapid mobilisation of the B12 from the injection site, directly into the blood. The comparatively lower levels of serum B12 in Co bolused lambs at all time-points after treatment could be attributed to the different route of administration. Following ingestion, Co is incorporated into vitamin B12 by rumen bacteria. Intrinsic factor then binds to the B12 in the upper small intestines and delivers it to the distal ileum for absorption. Consequently, only 3-13% of ingested Co is incorporated into B12 (Smith and Marston, 1970). As it is difficult to titrate levels of two completely different forms of supplementation, doses of the supplements used in the current study are industry recognised levels which have been shown to raise status above the marginal band (Suttle, 2010).

As well as Hcy, accumulation of MMA in plasma, from a failure in propionate metabolism, provides an early warning sign of Co deficiency leading to dysfunction in sheep (O'Harte et al., 1989; Rice et al., 1989) and is commonly used to assess B12 status. The threshold for MMA indicating adequate B12 status in barley fed lambs is suggested to be

~10 µmol/L (O'Harte et al., 1989). At Week 9 (immediately before treatment), mean MMA concentrations were 27.7 µmol/L, indicating B12 deficiency. Following treatment, MMA concentrations in both groups decreased and continued to do so until the end of the study, with lower levels seen in B12 injected animals at all time-points, corroborating the serum B12 results. A similar trend was seen in the Hcy results, providing further validation of the effect of each treatment on B12 status.

In the development of a long-acting B12 injection to prevent Co deficiency in sheep, Grace and Lewis (1999) found that B12 injection significantly increased and maintained serum B12 concentrations for at least 210 days, compared with untreated controls. Judson et al (2000) also found similar results. As the current study only continued for 30 days after treatment, the length of efficacy of the two treatments could not be determined. However, as lamb B12 status was still improving (i.e. B12 increasing and MMA and Hcy decreasing) when the study ended, is probable that the B12 status of these animals would have continued to improve or be maintained.

2.4.4 Propionate-succinate pathway

Depression in the rate of clearance of propionate is an early consequence of B12 deficiency in sheep (Smith and Marston, 1971). Elevated propionate in the blood of B12 deficient animals is associated with a decreased rate of acetate clearance by the TCA cycle. Such a failure to clear volatile fatty acids has been suggested to lead to a depression in appetite and ultimately a loss of weight (Smith and Marston, 1971). Although propionate levels in blood were not measured, succinate levels were unaffected by supplementation. Despite high levels of MMA prior to treatment, and a decrease in MMA in both groups following treatment, succinate levels only fluctuated slightly around a mean of 8.5 µmol/L. Combined with the lack of any measurable change in DM intake or differences in growth rates before and after treatment, it is likely that the B12 deficiency was not sustained for long enough to have

a detrimental effect on the propionate-succinate pathway and to cause loss of appetite.

2.4.5 B12 injection vs Co bolus: Effect on DM intake and growth rate

As there was no difference in DM intake between the two treatment groups, overall mean intakes of all lambs on study were reported in Figure 2.3 to illustrate the effect of time. Variation in DM intake between individual lambs was greatest at the start of the study and decreased with time. This reduced variability and oscillation in intakes decrease as lambs became acclimatised to concentrate feeding indoors. Although lambs on the current study were fed the Co deficient barley based diet and hay for 4 weeks prior to the commencement of the study, these results suggest that acclimatisation to a concentrate diet takes much longer.

New Zealand studies relating changes in serum B12 concentration to growth have demonstrated in lambs that a B12 concentration <220 pmol/L will evoke a response in terms of growth rates to B12 supplementation (Clark et al., 1989). In 2002, in a study with 4-6 week old lambs, Grace et al. (2003) reported that growth rates of Co deficient lambs were significantly improved by injection with a long-acting B12. In the study by Grace and West (2000), a marked growth response to injectable microencapsulated B12 was also observed in 4-5 week old Co deficient lambs. In the same study, however, the growth of weaned lambs (ages 18-20 weeks) was not improved by B12 injection (Grace and West, 2000). This was corroborated in the assessment of a new long acting B12 injection for prevention of Co deficiency by Judson et al. (2000), who reported that no body-weight response to treatment was recorded in weaned whether lambs. It seems, therefore, that a growth-rate response to B12 supplementation is more marked in Co deficient young lambs (3-6 weeks of age) than in weaned lambs. The results of the current study, using weaned lambs, are in line with these findings. Long acting B12 treated lambs showed no significant improvement in weight gain following treatment when compared with Co bolused lambs.

Consequently there is no perceived beneficial effect of one method of supplementation over the other when administered after weaning. As the new long-acting vitamin B12 formulation can be easily injected at docking time (3 weeks) and will remain effective beyond weaning (Grace and Lewis, 1999), whereas Co bullets cannot be administered to lambs younger than 10 weeks of age, use of a microencapsulated vitamin B12 supplement early in life could improve the growth of lambs who start behind the majority in terms of weight.

2.5 Conclusion

Although in practice the use of a Co bolus has its limitations, for the purpose of the current study, it was evident that when given directly to weaned lambs, the bolus was an effective treatment for Co deficiency and improved the B12 status of the lambs. However, improvement of B12 status was greater in lambs injected with the long-acting B12 supplement, and as a strategy to prevent or treat Co deficiency, SMARTShot ® offers advantages over the intra-ruminal Co bolus. Co deficient lambs can be injected at 3 weeks of age, whereas the Co bolus must be given to lambs weighing >20 kg that have a functioning rumen for synthesis of B12. A further advantage of the B12 injection is the route of administration, as giving an injection in the anterior neck is likely to be quicker and easier than administering a bolus, and there is rapid mobilisation of B12 directly into the blood.

Based on earlier trials, the calculated release rate of vitamin B12 from a 6 mg depot is 20 µg/day (Grace and Lewis, 1999). Given that the daily B12 requirement of a 25 kg sheep is 11 µg/day (~325 pmol/kg LW) (Smith and Marston, 1970), the increase in serum B12 seen with B12 treatment in the current study was as expected and it is likely that, if the study was prolonged, this improved B12 status would have been maintained. As conversion of nutrient intake to live weight gain becomes less efficient with age, the first 12 weeks of growth is critical for total life time growth of the lamb. It would therefore be more beneficial to supplement the lighter lambs in the flock earlier in life, with this long acting microencapsulated B12 injection, to compensate for their poor start and prevent growth retardation.

CHAPTER THREE

The effect of B12 and Se supplementation on metabolic status and performance of ewes and their lambs

3.1 Introduction

When performance of sheep is below expectation, an assessment of trace-element status is commonly carried out to evaluate the presence and prevalence of deficiencies within the flock (Kincaid, 1999). Lack of clinical and pathological specificity between mineral deficiencies and other nutrient insufficiencies can make diagnosis difficult, meaning trace-element deficiencies often go undetected as a consequence of more general nutritional inadequacies (Suttle, 2010). Pre-clinical diagnosis of a specific mineral deficiency via detection of biochemical changes in the fluids of animals, which accompany mineral deprivation, is therefore highly valuable.

Cobalt (Co) and selenium (Se) are essential trace-elements for animals. Superficially, sheep with Se deficiency exhibit similar symptoms to those seen with Co deficiency, such as infertility, perinatal mortality, ill thrift and anaemia (Marston, 1970; Andrews, 1964; Underwood and Suttle, 1999). These similarities arise because Se and Co are metabolically associated. Both play critical roles in 1C metabolism and related pathways (Chapter 1, Section 1.8), and as a result, sub-optimal intake can have detrimental effects on global DNA methylation and gene expression. The inability to distinguish between Co and Se deficiencies from external symptoms alone highlights the importance of not viewing Co and Se in isolation. Assessment of the status of one should be made in conjunction with the other, alongside other contributing factors. From a practical point of view, it is important to understand the effects of Se deficiency or responses to Se supplementation on vitamin B12 status of sheep and, conversely, to have knowledge of whether Co deficiency or supplementation with Co or B12 could affect Se status. It has previously been found that Co deficiency in sheep can increase their susceptibility to Se toxicity (Gardiner, 1966). Moreover, better responses of Se deficient animals to Se supplementation have frequently been seen in animals grazing Co deficient pastures (Drake et al., 1960).

The objective of the initial study in this chapter was to make a biochemical assessment of the trace-element status of ewes and lambs in a flock with reduced performance, suspected of being deficient in Co and Se. A previous unpublished study by Dodd and Sinclair (2014), on the monitor farm in question, assessed a small cohort of lambs at four weeks post-weaning. They observed that when sub-clinically Se deficient lambs (<0.25 µmol/L) (Grace et al., 2006) were supplemented with Se at weaning, not only was the Se deficiency corrected but homocysteine (Hcy), a biomarker of vitamin B12 deficiency, increased, revealing an underlying B12 deficiency (Figure 3.1). Furthermore, when supplemented with Se and Co together, Hcy levels declined to within the B12 sufficient range. This indicated that there was both a Se and Co issue on the farm, and that Se deficiency may mask an underlying Co deficiency. Such relationships have not previously been reported. Studies reported in this chapter, therefore, sought to investigate this relationship further. It began with a second pilot study undertaken in the subsequent year (grazing season) in order to confirm and extend these original observations to an earlier stage of the production cycle (i.e. from lambing to weaning). Body condition scores (BCS) and live weights were recorded in a larger group of animals from which the cohort reported here was sampled for blood metabolite analyses. The second follow-up study sought to formally assess the interaction between Se and Co, and to test the hypothesis that Se deficiency can mask an underlying Co deficiency by assessing responses to supplementation.

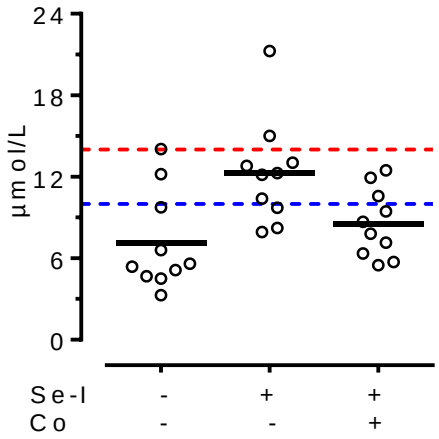


Figure 3.1. Lamb plasma homocysteine concentrations at four weeks post weaning (data from unpublished observations of Dodd and Sinclair (2014)). Means (**—**) and reference intervals for deficient (**- - -**) and adequate (**- - -**) B12 status are included. Supplementation was provided at weaning. Supplementation with Se-I alone led to increased (P=0.006; SED=1.5) plasma homocysteine concentrations.

3.2 Materials and methods

3.2.1 Animals, treatments and sample collection

All procedures were conducted in accordance with the requirements of the UK Animal Scientific Procedures Act (1986) and investigations were approved by the University of Nottingham Animal Welfare and Ethical Review Board.

The two experiments reported in this study were conducted on a single monitor farm in Leicestershire. That is, this farm was one of several participating in a larger AHDB funded study investigating trace-element status and key-performance indicators in commercial flocks of sheep in England. Ewes were housed from January in each year, sheared and fed a total mixed ration based on high-sugar ryegrass and red clover until lambing. From lambing, all animals were grazed on two high sugar grass (HSG) pastures until the end of the study.

The genotype of ewes in both experiments was the English Mule (i.e. Bluefaced Leicester x Swaledale), aged 4-6 years, with the majority aged 4 years. Male lambs were castrated shortly after birth. Sire breeds were Suffolk, Charollais, Abermax and Charollais x Suffolk.

3.2.1.1 Experiment 1

For this pilot study, 100 twin suckling ewes and their lambs were recruited at parturition. Ewe BCS and lamb weights were recorded at this time. In addition, ewe BCS and ewe and lamb weights were recorded at ~8 weeks and again at weaning (~12 weeks). The number of animals used for blood analyses was based on 'Power analyses' of Kendall (2014) for sentinel animals under commercial conditions. This study recommended a minimum number of 4 per management group/animal type for nutritional diagnostic sampling. In the current study, 6 ewes and 6 lambs (one from each set of twins) per field were randomly selected for sequential blood sampling.

3.2.1.2 Experiment 2

The effects of supplemental Selenium-Iodine (Se-I) and vitamin B12 (B12) on ewe and lamb performance from parturition to weaning were assessed in 400 twin suckling Mule ewes randomly allocated to one of four treatments in a 2 x 2 factorial arrangement. These groups included (a) non-treated Controls (C), (b) subcutaneous B12 (B12; 1 ml 3 mg/ml SMARTShot® Prime Lamb) alone, (c) Se-I bolus (Se-I; 10.5 g Animax Tracesure ®) alone, and (d) a group to which both supplements were administered (B12+Se-I). SMARTShot® Prime Lamb is an oil suspension containing 3 mg/ml hydroxocobalamin hydrochloride encapsulated in lactide/glycolide co-polymer for extended release. 1 ml volume administered per ewe in this study follows manufacturer's recommendations. One Animax Tracesure® Se/I bolus per ewe (following manufacturer's recommendations) was administered orally using a balling gun. Each bolus contained 100 mg Selenium and 660 mg Iodine. Treatments were administered immediately prior to turn-out within 2-3 days of lambing. Ewe BCS and lamb weights were recorded at parturition. In addition, ewe BCS and lamb weights were recorded at ~8 weeks and again at weaning (~12 weeks). Blood was collected by superficial venepuncture from 12 ewes and 12 lambs (one from each set of twins), randomly selected from each of the four treatment groups, at 8 weeks and again at weaning.

3.2.2 Body condition scoring

The method and grading of body condition scoring was based on that described by Russel et al. (1969). Units of body condition ranged from 0 (emaciated) to 5 (obese) and individual animals were scored to the nearest 0.25 unit. Body condition scoring was unbiased and carried out blind.

3.2.3 Blood analysis

Blood samples in both studies were collected from ewes and lambs at 8 weeks and at weaning by superficial venepuncture into Lithium heparin

(LH) vacuum tubes (plasma) and vacuum tubes containing no additive (serum). Samples were centrifuged at 4,250 x g for 15 min. Whole blood, plasma and serum fractions were stored at -20°C until analysed.

3.2.3.1 Serum B12

Serum B12 analysis was conducted using the ADIVA Centaur CP VB12 assay at the AHVLA laboratory, Shrewsbury, Shropshire, UK as described in Chapter 2, Section 2.2.2.1. The limit of detection was 33 pmol/L, and coefficients of variation (CVs) for low, medium and high QCs were 12.2, 12.4 and 12.4% respectively.

3.2.3.2 Plasma Hcy

Plasma homocysteine (Hcy) was determined using an Imola Autoanalyser (RX imola; Randox Laboratories Ltd., Antrim, U.K.) as described in Chapter 2, Section 2.2.2.2. The kit for Hcy was supplied by Randox Laboratories (catalogue no. HY4036). The limit of detection was 1.74 µM and upper limit was 47.9 µmol/L. CVs for low, medium and high QCs were 9.32, 6.77 and 5.40% respectively.

3.2.3.3 Glutathione peroxidase

Whole blood samples were analysed for glutathione peroxidase (GSHPx) (an enzyme containing four atoms of selenium) using a Randox Imola Autoanalyser (RX imola) and Randox glutathione peroxidase (Ransel) assay kit (catalogue number RS505) (Randox Laboratories Ltd., Antrim, U.K). The method is based on that of Paglia and Valentine (1967). GSHPx catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase and NADPH, the oxidised Glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured. The limit of detection was 75 U/L and upper limit was 925 U/L. CVs for low and high QCs were 7.30% and 4.37% respectively.

3.2.3.4 Trace-elements

Inductively coupled plasma mass spectrometry (ICP-MS) was used to analyse trace-elements, including Se, in blood samples. Multi-element analysis was undertaken using ICP-MS (Thermo-Fisher iCAP-Q) with a 'Flatopole collision cell' (charged with 7% H_2 in helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. Samples and calibration standards were diluted (0.5 ml into 20 ml) in a diluent containing 0.1 % non-ionic surfactant ('Triton X-100' and 'anti-foam-B', Sigma-Aldrich, UK), 2% methanol and 1% HNO_3 (Trace analysis grade, Fisher Scientific, UK), including the internal standards; Sc (50 µg/l), Ge (20 µg/l), Rh (10 µg/l) and Ir (5 µg/l). Samples were introduced via a covered auto-sampler (Cetac ASX-520) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific; 1 ml/min). Calibration standards were in the range 0–100 µg L^{-1}.). Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific).

3.2.3.5 MMA and SA

Plasma methylmalonic acid (MMA) and succinic acid (SA) concentrations were determined by Gas Chromatography with Mass Spectroscopic detection (GC-MS) following derivatisation and extraction as described in Chapter 2, Section 2.2.2.3. The limit of detection was 0.15 µM for both and CVs for low, medium and high QCs were 0.44, 1.03, 0.83% respectively for MMA and 1.19, 1.11 and 2.33% respectively for SA.

3.2.4 Statistical analysis

All statistical analyses were performed within the GenStat statistical package (17th Edition, VSN International, 2011). Blood metabolite concentrations were analysed by 'repeated measures ANOVA' (i.e. from samples collected at 8 weeks and Weaning) blocked by field in both experiments. For Experiment 2, other terms fitted to these models were the two supplementary treatments (i.e. 'B12' and 'Se-I'). Lamb weights and growth rates were analysed by General Linear Regression where, in addition to 'field' and the two supplementary treatment terms (i.e. 'B12'

and ‘Se-I’; Experiment 2), additional terms fitted to these models were ‘sire breed’ and ‘combined sex’. The latter term accounted for the fact that lambs were reared in one of the three following gender combinations: female:female, female:male and male:male. Validity of analyses (i.e. normality, homogeneity of variance and additivity) was confirmed from residual plots. For presentational purposes, metabolite (e.g. Co, Se) means and individual values scattered around these means are presented (using Graphpad Prism, Version 7.01) together with standard (industry recognised) reference ranges. Animal weight and BCS data are presented as adjusted means with standard errors of differences (S.E.D., ANOVA) or means (S.E.M., Regression).

3.3 Results

3.3.1 Experiment 1

3.3.1.1 Plasma Se

Mean plasma Se concentrations of ewes and lambs at 8 weeks and weaning were within the sufficient range (>0.25 µmol/L) (Grace et al., 2006) (Figure 3.2). At both time points, lamb plasma Se was lower than ewe plasma Se. Ewe and lamb plasma Se concentrations were both lower at weaning compared to at 8 weeks, however the decrease was not statistically significant.

3.3.1.2 Plasma Co

Mean plasma Co concentrations of ewes decreased (P=0.035) from marginal status at 8 weeks (3-5 nmol/L) to deficient status at 12 weeks (<3 nmol/L) (Figure 3.3). At both time points, mean lamb plasma Co levels were within the adequate range (>5 nmol/L), although some individual lambs had marginal/low levels. Lamb Co levels were lower at 12 weeks compared to 8 weeks, however the difference was not statistically significant.

3.3.1.3 Serum B12

There was no significant difference in mean serum B12 concentrations from 8 weeks to weaning in ewes or lambs (Figure 3.4). At 8 weeks, the majority of ewes had serum B12 concentrations below the adequate threshold (>350 pmol/L) with many in the deficient range (<250 pmol/L) (Suttle, 2005). By weaning, all ewes except one had sufficient serum B12 levels. All lambs had serum B12 concentrations in the marginal or deficient range, with means of 246 pmo/L and 230 pmol/L at 8 weeks and weaning respectively.

3.3.1.4 Weight and BCS

Ewes in the study maintained their body condition over the lambing season, remaining close to a BCS of 2.5 throughout. Mean condition

scores were 2.6 at lambing, 2.5 at 8 weeks and 2.4 at weaning (Figure 3.5B). This was also reflected in the ewe weights (Figure 3.5A), with a mean loss of only 0.7 kg from 66.4 kg to 65.7 kg, from 8 weeks to weaning.

Combined lamb weights and growth rates were measured at three time-points; birth, 8 weeks and weaning (Table 3.1). There was no significant effect of 'sire breed' or 'combined sex' on lamb weights or growth rates. There was a significant effect of time on lamb weight and growth rate ($p<0.001$), with highest growth rates during the period from birth to 8 weeks.

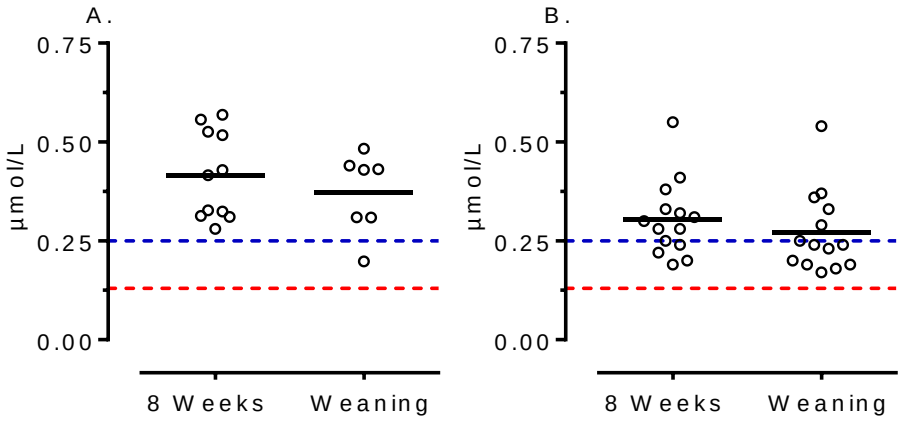


Figure 3.2. Eight-week and weaning (~12 weeks) plasma selenium concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for sufficient (- - -) and deficient (- - -) status are given (Grace, 2006).

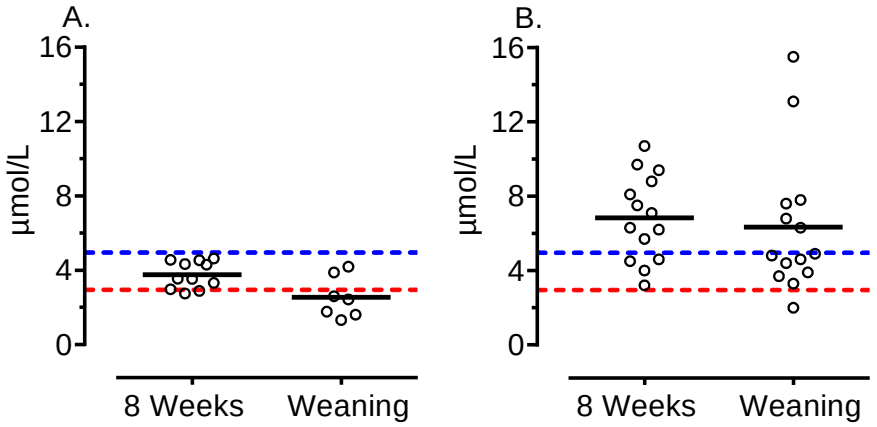


Figure 3.3. Eight-week and weaning (~12 weeks) plasma cobalt concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for deficient (- - -) and sufficient (- - -) status are given (Suttle, 2010). For ewes (A) effect of time (P=0.035; SED=0.43).

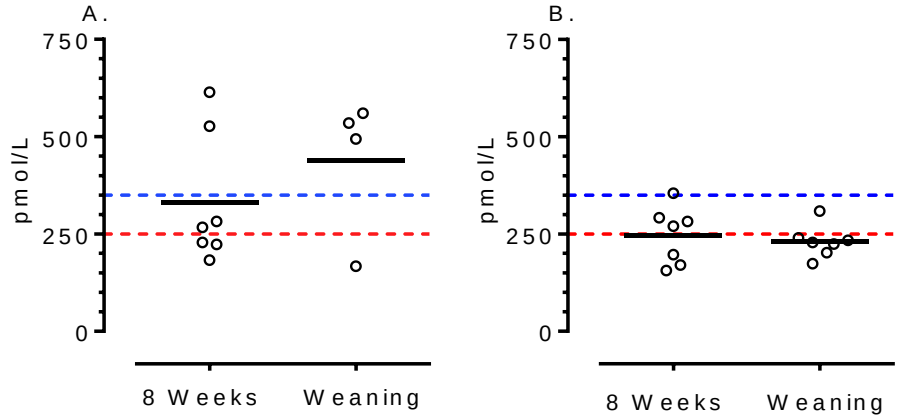


Figure 3.4. Eight-week and weaning (~12 weeks) serum vitamin B12 concentrations in ewes (A) and lambs (B). Mean values are included (—). Reference points for deficient (- - -) and adequate (- - -) status are given (Suttle, 2005).

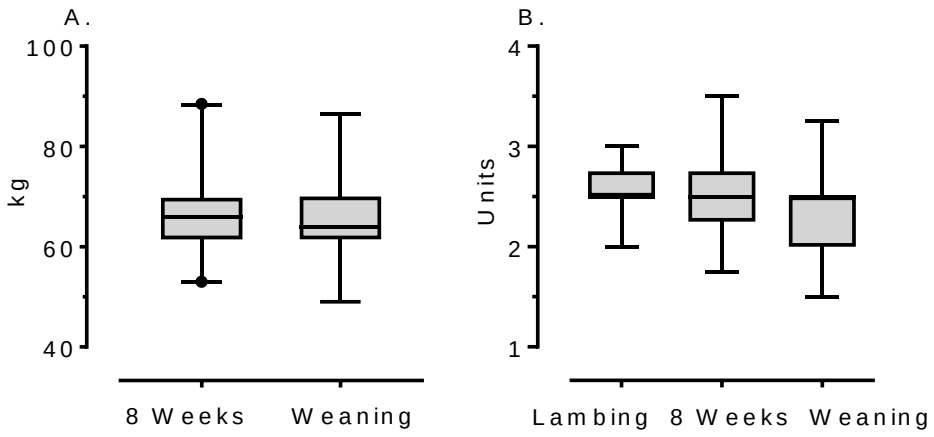


Figure 3.5. Ewe weights (A) and ewe BCS (B) (median, interquartile ranges and 1-99th percentiles).

Table 3.1. Combined twin-lamb weights and growth rates (mean ± s.e.m.) up to weaning.

			Combined lamb weight (kg)				Combined lamb growth (g/day)			
		n	Birth	8 weeks	weaning	Sig (P)	Birth-8 weeks	8 weeks-wean	Birth-wean	Sig (P)
	Total	100	12.1 ± 0.1[a]	49.2 ± 0.5[b]	63.8 ± 0.5[c]	<0.001	586 ± 7.3[d]	412 ± 10.2[e]	525 ± 5.7[f]	<0.001
Sire breed	**Abermax**	40	12.0 ± 0.2	49.0 ± 0.8	62.9 ± 0.9		584 ± 11.5	399 ± 16.3	519 ± 9.1	
	Charollais	42	12.1 ± 0.2	48.5 ± 0.8	64.1 ± 0.9		573 ± 11.3	431 ± 16.3	526 ± 9.0	
	Charollais x Suffolk	18	12.3 ± 0.3	51.5 ± 1.2	65.5 ± 1.3		622 ± 17.4	400 ± 24.6	542 ± 13.7	
Combined sex	**FF**	20	12.0 ± 0.3	48.5 ± 1.1	63.6 ± 1.2		576 ± 16.1	426 ± 23.4	523 ± 13.1	
	MM	27	12.1 ± 0.2	48.9 ± 0.7	63.4 ± 0.7		582 ± 10.0	408 ± 14.2	522 ± 7.9	
	MF	53	12.2 ± 0.3	50.3 ± 1.0	65.0 ± 1.1		600 ± 14.2	410 ± 20.1	532 ± 11.2	
Significance (P)	**Sire breed**	100	0.603	0.205	0.392		0.156	0.576	0.513	
	Combined sex	100	0.842	0.421	0.467		0.476	0.811	0.768	

n = number of ewes (lamb pairs); Means with different superscripts are different at P<0.05

3.3.2 Experiment 2

3.3.2.1 Serum vitamin B12

Serum vitamin B12 results for ewes and lambs are illustrated in Figure 3.6A and 3.6B respectively. All animals were considered to be sufficient at both time points. Ewes supplemented with B12 and B12+Se maintained a significantly higher concentration of B12 in serum than ewes in other treatment groups throughout the experimental period ($P<0.001$), although to a lesser extent at weaning. A significant effect of time on ewe and lamb serum B12 concentrations in all treatment groups ($P<0.001$) was seen, with a decrease at weaning. There was no significant effect of ewe treatment on lamb serum B12 concentrations.

3.3.2.2 Plasma Hcy

Mean plasma Hcy concentrations in ewes (Figure 3.6C) did not change significantly from 8 weeks to weaning and indicated B12 sufficiency (<10 µmol/L). Lamb plasma Hcy decreased significantly ($P<0.001$) from 8 weeks to weaning (Figure 3.6D), from levels indicating marginal/sufficient status (<14 µmol/L) to sufficient (<10 µmol/L). No effect of treatment on ewe or lamb plasma Hcy was observed at either time point.

3.3.2.3 Plasma MMA and SA

Mean plasma MMA concentration of ewes (Figure 3.6E) in the control group were slightly higher than those of ewes in the B12 treated group, although the difference was not considered to be significant, and all levels were within the sufficient range (<5 µmol/L). No significant effect of time on plasma MMA concentrations was seen. However, slightly higher concentrations were observed at weaning compared to 8 weeks.

There was a significant effect of B12 treatment ($P=0.015$) and time ($P<0.01$) on ewe plasma SA concentrations, with higher concentrations of SA in B12 treated ewes and decreased SA concentrations in all ewes at weaning compared to at 8 weeks (Figure 3.6F).

3.3.2.4 Blood Se

Mean Se concentrations in the blood of ewes in all treatment groups at 8 weeks and at weaning were within the sufficient range (>0.25 µmol/L) (Figure 3.7). Se levels in lambs were considered sufficient at 8 weeks and marginal/sufficient (>0.13 µmol/L) at weaning. There was an effect of time (P<0.001) on both ewe and lamb Se concentrations with lower concentrations recorded at weaning compared to 8 weeks. In addition to this, an effect of Se-I treatment (P<0.002) on plasma Se concentrations in ewes was observed. No effect of treatment on lamb plasma Se concentrations was detected.

3.3.2.5 Glutathione peroxidase in blood

All animals had sufficient GSHPx levels at 8 weeks and at weaning (>50 U/ml PCV) (Figure 3.8). An effect of time (P<0.001) and Se treatment (P<0.001) on blood GSH-Px concentrations was seen in ewes and lambs. Animals treated with Se had increased GSHPx levels compared to Controls and B12-only treatment groups. As with Se concentrations, ewe and lamb GSHPx concentrations were lower at weaning compared to 8 weeks in all treatment groups.

3.3.2.6 BCS and weights

Ewe BCS was recorded at 3 time points; lambing, 8 weeks post lambing and at weaning (Table 3.2). Retrospective analyses indicated that, before any treatment was administered, BCS of ewes in the Se supplemented groups were slightly higher (P<0.019) than Control ewes and B12-only supplemented groups. The Control and B12-only supplemented groups had mean BCS of 2.67 and 2.68 respectively, and Se supplemented and Se+B12 supplemented groups had BCS of 2.73 and 2.75 respectively. A significant effect of B12 supplementation on ewe BCS at 8 weeks (P<0.008) and on change in BCS from lambing to 8 weeks (P<0.005) was observed, with those in B12 supplemented groups losing more condition than other groups. Mean BCS at lambing, 8 weeks and at weaning were 2.71, 1.56 and 1.84 respectively. The condition lost after lambing (-1.15

units) was only slightly regained by weaning (+0.28 units). Although ewes weren't weighed at lambing, ewe weight change from 8 weeks to weaning reflected the trend seen in BCS, with an increase ($P<0.001$) of 4.5 kg.

No significant effect of ewe treatment on lamb weights or growth rates was seen.

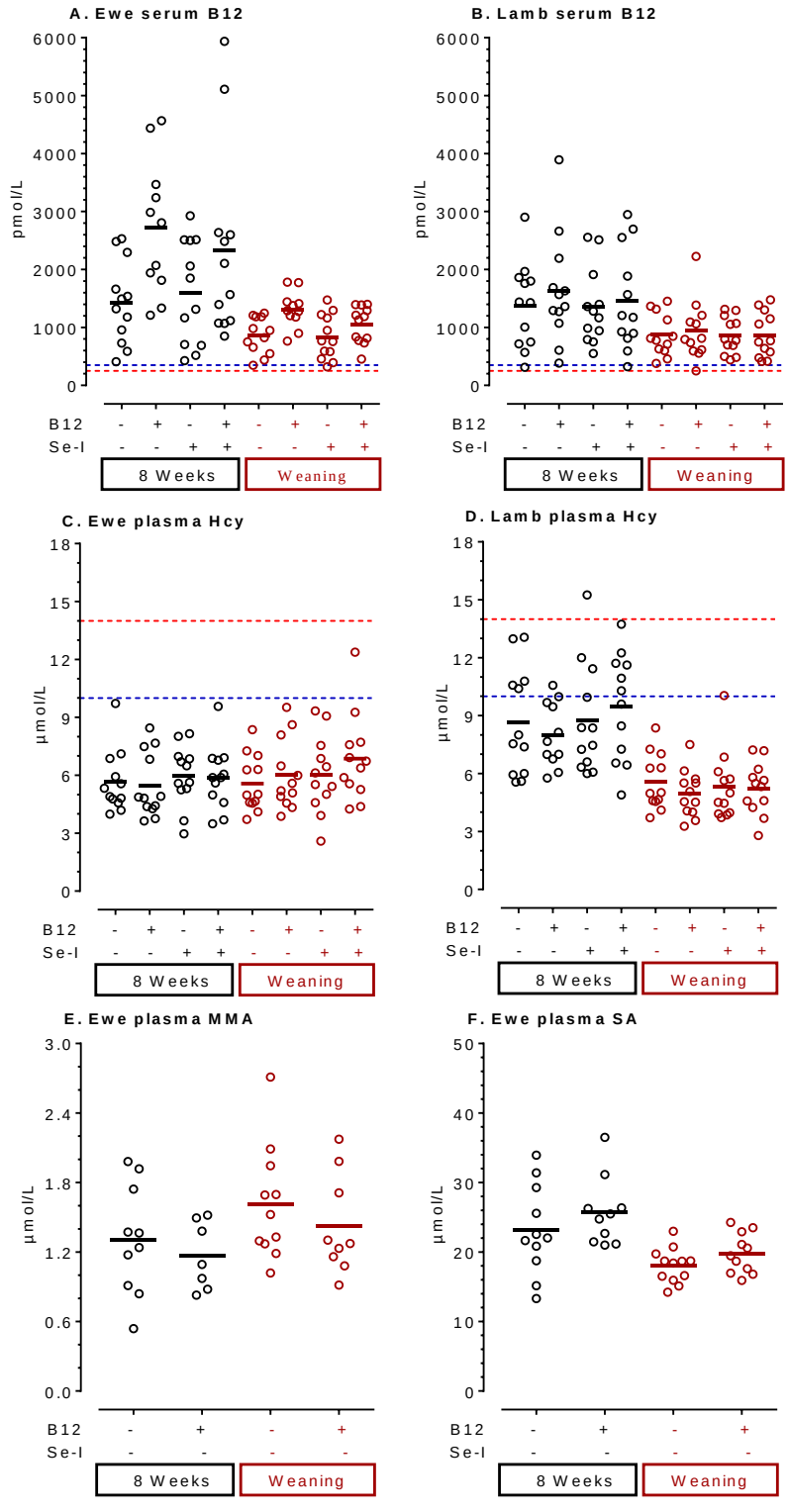


Figure 3.6. Eight-week and weaning (12 weeks) concentrations of serum B12 in ewes (A) and lambs (B), plasma homocysteine in ewes (C) and lambs (D) and plasma methylmalonic acid (E) and succinic acid (F) in ewes. Mean values (—), and deficient (- - -) and marginal (- - -) reference ranges are given. For ewe serum B12 (A), interaction (P=0.037; SED=219) between B12 treatment and time. For ewe plasma SA (F), effect of B12 treatment (P=0.015; SED=1.2) and time (P<0.001; SED=1.1). For lamb serum B12 (B), effect of time (P<0.001; SED=91.8). For lamb plasma Hcy (D), effect of time (P<0.001; SED=0.346).

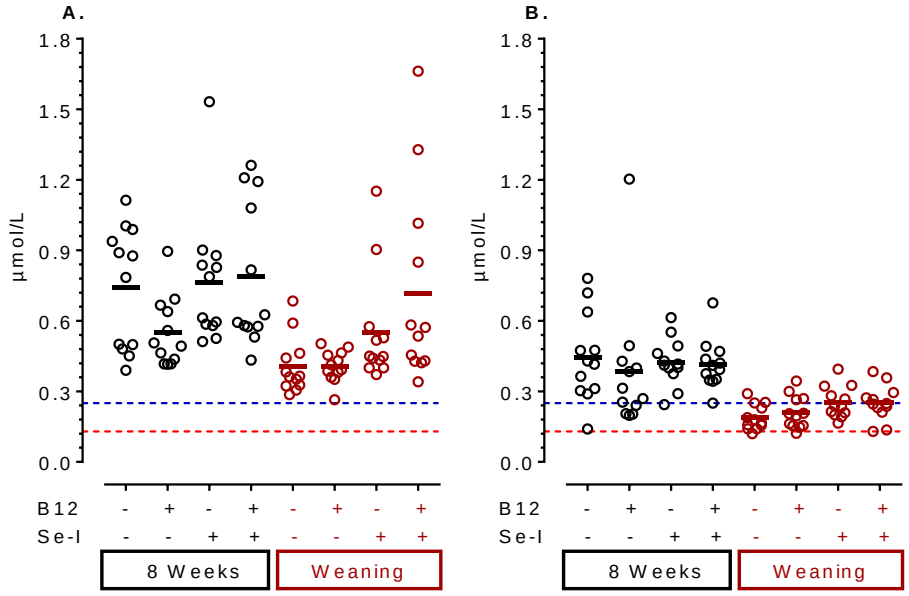


Figure 3.7. Eight-week and weaning (~12 week), ewe (A) and lamb (B), Se concentrations. Mean values (—), and deficient (- - -) and marginal (- - -) reference ranges are included. For ewe plasma Se (A), effect of Se treatment (P=0.002; SED=0.054) and time (P<0.001; SED=0.049). For lamb plasma Se (B), effect of time (P<0.001; SED=0.024)

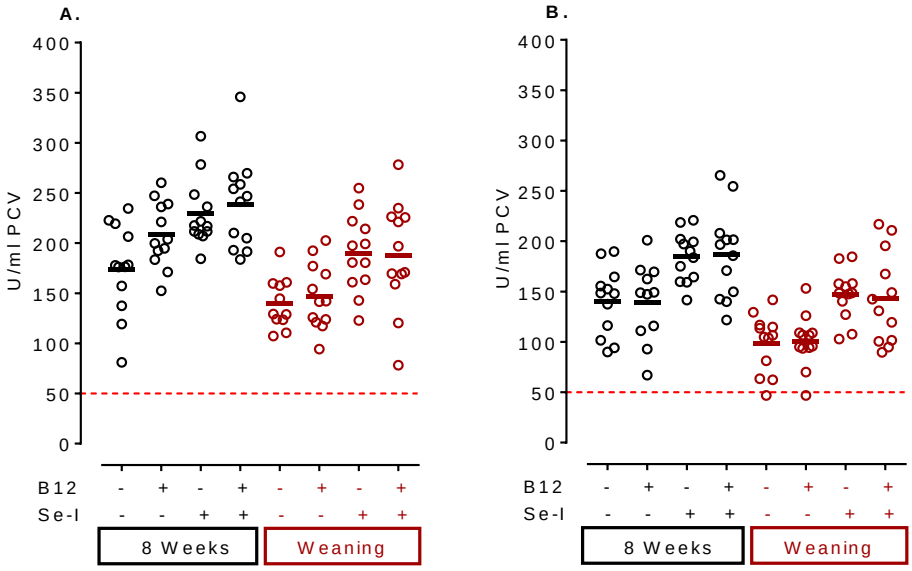


Figure 3.8. Eight-week and weaning (~12 week), ewe (A) and lamb (B), GSHPx activity. Mean values (—) and deficient reference points (- - -) are included. For ewe GSHPx (A), effect of Se treatment (P<0.001; SED=10.21) and time (P<0.001; SED=5.59). For lamb GSHPx, effect of Se treatment (P<0.001; SED=9.45) and time (P<0.001; SED=3.12)

Table 3.2. Ewe and combined twin-lamb performance (mean ± s.e.m.) from lambing to weaning.

Se-I	**-**	**-**	**+**	**+**	**Significance (P)**		
B12	**-**	**+**	**-**	**+**	**Se-I**	**B12**	**Se-I x B12**
Number of ewes (n)	100	100	100	100			
A. Ewe Body Condition (BCS) (units)							
Lambing	2.67 ± 0.02	2.68 ± 0.03	2.73 ± 0.03	2.75 ± 0.03	0.019	-	-
8 weeks	1.61 ± 0.03	1.51 ± 0.03	1.60 ± 0.03	1.53 ± 0.03	-	0.008	-
Weaning	1.86 ± 0.03	1.80 ± 0.04	1.87 ± 0.04	1.84 ± 0.04	-	-	-
∆ BCS lambing-8 weeks	-1.06 ± 0.03	-1.17 ± 0.04	-1.13 ± 0.03	-1.22 ± 0.04	-	0.005	-
∆ BCS 8 weeks-weaning	0.25 ± 0.02	0.29 ± 0.03	0.28 ± 0.02	0.31 ± 0.03	-	-	-
∆ BCS lambing-weaning	-0.81 ± 0.04	-0.88 ± 0.04	-0.86 ± 0.04	-0.91 ± 0.04	-	-	-
B. Ewe weight (kg)					-	-	-
8 weeks	55.7 ± 0.58	53.4 ± 0.67	54.8 ± 0.59	54.4 ± 0.69	-	-	-
weaning	59.5 ± 0.55	57.9 ± 0.63	59.1 ± 0.56	59.5 ± 0.65	-	-	-
8 weeks-weaning LWG	4.1 ± 0.34	4.2 ± 0.38	4.5 ± 0.35	4.7 ± 0.39	-	-	-
C. Ewe Live-weight change (g/day)							
8 weeks-weaning	127 ± 9.3	131 ± 10.3	141 ± 9.6	145 ± 10.3	-	-	-
D. Combined lamb weight (kg)							
Birth	10.3 ± 0.11	10.3 ± 0.13	10.3 ± 0.11	10.7 ± 0.13	-	-	-
8 weeks	38.6 ± 0.43	37.9 ± 0.49	38.1 ± 0.44	38.0 ± 0.50	-	-	-
Weaning	51.2 ± 0.56	50.6 ± 0.65	51.0 ± 0.58	50.7 ± 0.66	-	-	-
E. Combined lamb growth (g/day)							
Birth-8 weeks	467 ± 6.8	462 ± 7.8	462 ± 6.9	454 ± 7.9	-	-	-
8 weeks-Weaning	390 ± 9.5	392 ± 10.9	400 ± 9.9	393 ± 11.2	-	-	-
Birth-Weaning	439 ± 5.9	438 ± 6.7	440 ± 6.0	434 ± 6.8	-	-	-

3.4 Discussion

The first experiment in the current study confirmed the findings of Dodd and Sinclair (2014) in the original pilot study, indicating both a Se and Co deficiency issue on the current monitor farm. Results of Experiment 1 showed that Se and B12 were depleted and that the B12 status of the flock was marginal (Grace et al., 2006, Suttle, 2005). Despite this, ewe and lamb performance did not appear to be adversely affected and although mean lamb growth rates declined from 8 weeks to weaning, 250 g/lamb/day from birth to weaning is still considered to be a respectable live weight gain (AHDB, 2014). In the pilot study, lamb trace-element status was assessed at 4 weeks post-weaning. However, Experiment 1 in the current study was undertaken with lactating ewes and suckling lambs during the early part (April to July) of the grazing season in the subsequent year. Importantly, as illustrated by Figure 3.4, lambs were B12 deficient at weaning. Hence one would predict that if B12 concentrations remained at this low level for an extended period of time, the cumulative effects of deficient B12 levels would result in lambs becoming dysfunctional and subsequently clinically deficient (Suttle, 2010), as observed in the original pilot study later in the season. Such prolonged effects have previously been described by Kennedy et al. (1992), with dysfunctional status being indicated by increased plasma concentrations of secondary metabolites, Hcy and MMA.

In Experiment 2, both serum B12 and plasma Se concentrations of Control ewes and lambs (i.e. those that did not receive supplementation) declined between 8 weeks and weaning. In contrast to the pilot study and Experiment 1, however, levels for these two nutrients were within the adequate range (Grace et al., 2006; Suttle, 2005) indicating that, for this production year, there didn't appear to be an underlying deficiency in Se or B12. This highlights the issue of year-to-year variation due to a number of factors, not least of which is weather and prevailing growing conditions (Suttle, 2010). Addressing this year-to-year variation in trace-element

status with a strategic ‘risk management’ approach to supplementation, therefore needs to be a consideration.

With the adequacy of B12 and Se status of ewes and consequently their lambs in Experiment 2, it was not therefore possible to address the primary hypothesis that a Se deficiency masked an underlying Co/B12 deficiency, although efficacy of the supplementation strategies used were confirmed.

3.4.1 Suckling vs weaned lambs

Cobalt deficiency is well recognised and documented in rapidly growing weaned lambs and breeding ewes in the late season, as this is the time of greatest vulnerability (Fisher and MacPherson, 1990; Kennedy et al., 1992; Clark, 1998; Gruner et al., 2004; Wang et al., 2006; Mitchell et al., 2007; Sinclair et al., 2007; Abou-Zeina et al., 2008). However, relatively little information on lactating ewes and suckling lambs early in the grazing season is available. In New Zealand, Gruner et al. (2004) evaluated the chronological effects of B12 deficiency and response to supplementation in ewes, suckling lambs, lambs transitioning from suckling to weaning, and weaned lambs, on farms previously identified as having a B12 deficiency issue. In these studies, body weight, serum B12 and MMA, and Co concentrations of herbage were monitored to evaluate B12 status. Individual untreated suckling lambs with serum B12 concentrations less than 200 pmol/L and MMA concentrations above 20 µmol/L (concentrations associated with decreased growth rates in weaned lambs) maintained high growth rates, as was seen in Experiment 1 of the current study. This confirms the views of Quirk and Norton (1987) that “the vitamin B12 requirements of the suckling lamb are small, as it can obtain its glucose directly from milk”. A proportion of vitamin B12 synthesised by bacteria in the ewe’s rumen is passed on to the lamb in her milk. Colostrum is a rich source of vitamin B12 (Ramos et al., 1994). However, the concentration of this vitamin in milk declines considerably during the first two (Ramos et al., 1994) to three (Marca et al., 1996) weeks of lactation, with availability declining to around 10% (<3.69

nmol/L) (Gruner et al., 2001; Grace et al., 2006). Nevertheless, provision of supplemental B12 to the suckling lamb is not essential because its B12 requirement for gluconeogenesis is relatively low.

Ruminants do not utilise sugars and carbohydrates in their feed for direct energy production as they become fermented to short-chain volatile fatty acids, predominantly propionic acid, in the rumen. Around 90% of this propionate is then converted to succinate by the liver (Brockman, 1993), in a vitamin B12 dependent pathway, prior to gluconeogenesis. Since suckling lambs do not yet have a developed rumen and more-over, suckling triggers the oesophageal groove reflex to direct milk to the abomasum (Ørskov, 1972), they obtain glucose directly from enzymatic digestion of milk, as non-ruminant animals do. At this age, there is therefore a comparatively lower demand on B12 for the conversion of propionate to succinate, prior to gluconeogenesis. Consequently, lower B12 concentrations can be tolerated by suckling lambs (Gruner et al., 2004), than by weaned lambs or adult sheep (Grace and West, 2000). A lower reference marginal band of 100-200 pmol/L has therefore been suggested for suckling lambs (Suttle, 2005).

As the lamb approaches weaning, the physiology/biochemistry of the digestive tract changes from that of a non-ruminant to ruminant. During this period, there is less dependence on lactose as a source of glucose and energy, and more of a dependence on propionic acid from rumen fermentation. The loss of vitamin B12 via the milk, combined with the delayed increase from grazing (Joyce and Rattray, 1970) and increased metabolic demands of growth can result in lower serum B12 levels accompanied by a decline in lamb growth rates, particularly on pasture that is deficient in Co. It has been estimated that a 25 kg weaned lamb requires 11 µg (325 pmol/kg LW) vitamin B12 per day to maintain normal growth rates (Smith and Marston, 1970). In the current study, the adverse effects of deficiency observed 4 weeks after weaning (in the pilot) but not prior to weaning (in Experiment 1) confirm this.

3.4.2 Cumulative effects of Co deficiency

As illustrated by Figure 3.4, serum B12 concentrations below 250 pmol/L indicated that lambs in Experiment 1 of the current study were B12 deficient at weaning. Whilst these low B12 levels were not associated with an adverse effect on growth rate by this stage, the effects of B12 deficiency observed in lambs in the pilot study (4 weeks post lambing) suggests that prolonged Co deprivation is required to reach a dysfunctional state. Suttle (2010) describes the sequence of biochemical changes preceding the appearance of clinical symptoms (i.e. reduced appetite and reduced weight gain) in ruminants deprived of Co/vitamin B12. The four phases are depletion, deficiency, dysfunction and disease. These are discussed in more detail in Section 1. Kennedy et al. (1992), whilst assessing the biochemical effects of the entire disease process, showed that prolonged Co deprivation was required for lambs to reach a dysfunctional state, indicated by increased plasma concentrations of secondary metabolites; Hcy and MMA. Furthermore, that study indicated that appetite reduction and decreased live weight gain would only be apparent after several weeks of B12 deficiency. It is therefore likely that, had Experiment 1 been prolonged beyond weaning, the cumulative effects of B12 deficiency would have been apparent later in the season whilst lambs were at grass and receiving no or little supplementation, as in the pilot study.

3.4.3 Seasonal effects on herbage Co levels

Lack of response to deficiency in the lambs in Experiment 1, whilst effects of B12 deficiency were apparent in their counterparts in the pilot study conducted later in the season, may also correlate with a marked seasonal cycle in herbage availability of Co on the farm. It is recognised that Co levels in herbage are usually adequate during the autumn/winter and decline as herbage growth accelerates in the spring. The Co status of the pasture on which animals in Experiment 1 were grazing was measured in a parallel project. The results from the analysis of washed and unwashed grass samples at 0, 5, 8 and 12 weeks post lambing are

illustrated in Figure 3.9. Unfortunately, as no pasture samples were taken during the pilot study, no year-to-year comparison can be made.

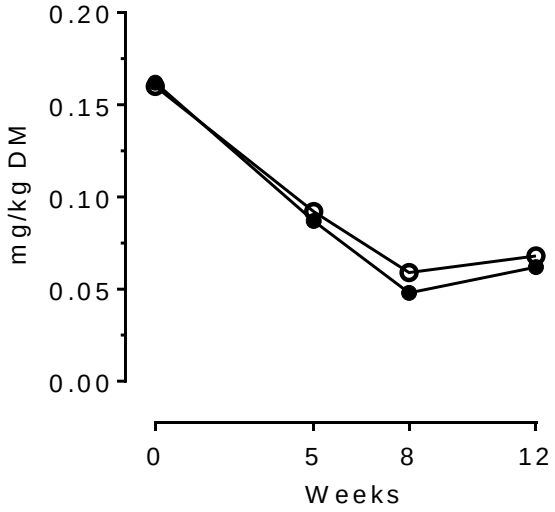


Figure 3.9. Co concentrations (mg/kg DM) of washed (●) and unwashed (○) grass samples taken at 0, 5, 8 and 12 weeks post lambing in year 1 of the study. (Keenan, Kendall and Sinclair (2015; unpublished data)). Effect of time (P<0.001)

Co concentrations in the grass decreased (P<0.001) from 0 to 8 weeks to a deficient level (<0.1 mg/kg DM), and remained deficient until 12 weeks post lambing. No significant difference in Co concentrations between washed and unwashed (soil contaminated) grass samples was detected. These observations provide a basis for the deficient status of the lambs at weaning in Experiment 1 as they become more dependent on pasture supply of Co for synthesis of vitamin B12. If Co levels in the pasture grazed during the pilot study also followed this trend, and deficient grass Co concentrations were sustained beyond weaning, this could have been a contributing factor to the resulting dysfunctional status of the lambs. Furthermore, these results could give some explanation for the marginal B12 status of the ewes at 8 weeks, as they transitioned from a Co sufficient total mixed ration (up to lambing whilst fed indoors) to this Co deficient pasture. Whilst pasture Co concentrations of 0.03-0.05 mg/kg DM may be tolerated by mature ewes for a period, requirements

increase to >0.1 mg/kg DM with the onset of lactation (Quirk and Norton, 1987, 1988; Gruner et al., 2004). This is possibly because the lactating ewe has to process more propionate to meet energy needs whilst there is also an increased demand for methionine associated with the onset of lactation. Both pathways are dependent on sufficient B12 and hence Co supply.

3.4.4 Animal performance

Despite low serum B12 concentrations, ewes in experiment 1 demonstrated their ability to maintain weight and body condition whilst raising two lambs to weaning with no negative effects on lamb live weight gain. In previous studies, it has been found that severe Co deficiency symptoms such as rapid weight loss and inability to raise a lamb were only apparent in ewes when their serum B12 concentrations fell below 150 pmol/L (Duncan et al., 1981). The probability of a live weight gain (LWG) response likely at particular serum concentrations was calculated by Clark et al. (1989) by collating data from Co supplementation trials in New Zealand. The probability increased from ~10% at serum concentrations of 300 pmol/L to 80% at <200 pmol/L. This highlights the importance of monitoring B12 status, using biochemical analysis of metabolic parameters, to enable early detection of B12 deficiency.

3.4.5 Between-year variation

Although serum B12 levels enable identification of B12 depleted and deficient animals, they alone are not sufficiently specific to accurately determine the phase of Co deprivation (O'Harte et al., 1989; Suttle, 2010) and additional metabolic factors should be taken into account. In Experiment 2, concentrations of secondary metabolites and biomarkers of B12 deficiency, Hcy and MMA were measured in addition to serum B12. In contrast to the pilot study and Experiment 1, B12 status was adequate in Experiment 2 (Suttle, 2005), indicated by B12, Hcy and MMA levels within their respective adequate ranges. This demonstrates significant year-to-year variation in B12 status in both ewes ($P<0.004$) and lambs ($P<0.003$), summarised in Figure 3.10.

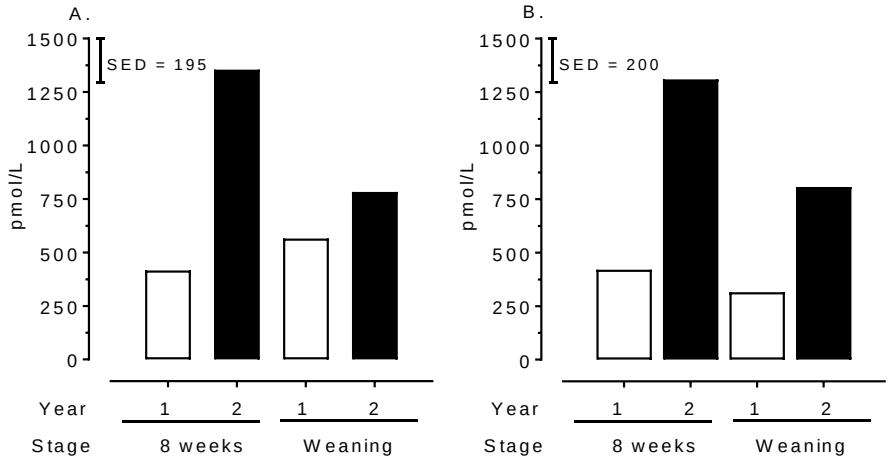


Figure 3.10. Between-year variation in serum B12 at 8 weeks and weaning, in ewes (A) and lambs (B). For ewes (A) effect of year ($P<0.004$; SED=195). For lambs (B), effect of year ($P<0.003$; SED=200)

A contributing factor to the between year variability of serum B12 could have been differences in availability of Co from pasture in the different years. Pasture quality depends on several factors, including geographical location, environmental conditions (temperature, humidity, and precipitation) and herbage composition of the sward (Underwood, 1977). As the pilot study and both experiments were conducted on the same farm, with the same species of grass, it is likely that environmental conditions were the greatest contributing factor to this variability. Co availability for plants is affected by climate. Heavy rainfall can leach Co from the soil decreasing availability to the plant whilst, conversely, poor drainage can increase availability (Suttle, 2010). Furthermore, as Co is not an essential nutrient for plants, high growth rates can dilute the amount of Co in the plant. Another contributing factor to low levels of Co in the grass can be the pH of the soil (Suttle et al, 2003). If the soil is alkaline (increased pH) the uptake of Co by plants is decreased (Underwood and Suttle, 1999).

Meteorological data from the weather station at Latitude: 52.833, Lonitude: -1.250, including rainfall and temperature, from March to July

in Experiments 1 and 2 are provided in Table 3.3. Lower temperatures and increased rainfall during Experiment 1 compared to those during Experiment 2 could have contributed to the low Co levels observed in the pasture at that time, and hence to the depleted levels of B12 in animals.

Table 3.3. Temperature (°C) and rain fall (mm) recorded from March to July during Experiments 1 and 2 (www.metoffice.gov.uk).

	Experiment 1					Experiment 2				
	March	April	May	June	July	March	April	May	June	July
Max temp (°C)	10.5	14.5	15.3	19.8	21.3	12.3	14.8	16.8	20.4	24.1
Rainfall (mm)	39	34.2	68	52.4	68.8	28	25.6	84.4	29.4	43

Between-year variation was also apparent in the BCS and weight measurements. In Experiment 2, ewes had lower body condition scores overall compared to animals in Experiment 1 (Figure 3.11A). In Experiment 2, BCS of ewes was 1 unit lower at 8 weeks and 0.5 units lower at weaning than in Experiment 1. As this does not correlate with the serum B12 results, it is likely that the limiting factor in Experiment 2 was pasture quantity and quality, rather than Co availability, whilst meeting the demands of lactation. Provision of adequate nutrition to ewes post lambing allows ewes to retain their body condition more efficiently over the season. In Experiment 1, adequate nutrition from sufficient pasture quantity provided enough energy for the animals to maintain good condition whilst raising their lambs. The ewe weights at 8 weeks and weaning showed the same trend (Figure 3.11B).

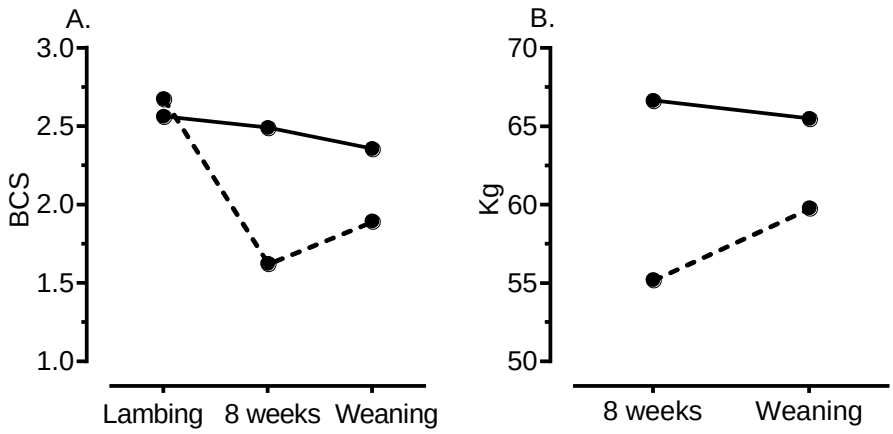


Figure 3.11. Ewe BCS (A) at lambing, 8 weeks and weaning and ewe weight change (B) from 8 weeks to weaning (B) in Experiment 1 (**—**) and Experiment 2 (**- - -**).

The lower BCS and weights of ewes in Experiment 2 were also reflected in the lamb weights (Table 3.4). In Experiment 2, ewes gave birth to lighter lambs ($P<0.001$) than in Experiment 1. This difference in lamb weight was maintained throughout the study with lambs in Experiment 2 also weighing less ($P<0.001$) at 8 weeks and at weaning than lambs in Experiment 1 due to lower ($P<0.001$) growth rates from birth to weaning.

Table 3.4. Between year variation in combined mean (± s.e.m.) twin lamb weights (A) and growth rates (B) at 3 time points; lambing, 8 weeks and weaning.

	Year				Significance (P)
	Experiment 1	**n**	**Experiment 2**	**n**	**Year**
A. Combined lamb weight (kg)					
Birth	12.73±0.22	100	10.05±0.23	319	<0.001
8 weeks	47.30±0.81	100	40.72±0.73	319	<0.001
Weaning	62.73±0.98	100	53.91±0.89	322	<0.001
B. Combined lamb growth (g/day)					
Birth - 8 weeks	568.4±13.12	97	476.2±12.71	308	<0.001
8 weeks - Wean	426.7±17.77	96	398.9±16.08	319	0.402
Birth - Wean	520.1±10.73	97	448.1±10.41	311	<0.001

3.4.6 Se and Co: supplementation and metabolic demands

The confirmation of a Se and Co deficiency issue on the current monitor farm in Experiment 1 reinforced the requirement to address the primary hypothesis that a Se deficiency could be masking an underlying Co deficiency. However, with the adequacy of B12 and Se status of ewes and consequently their lambs in Experiment 2, this was not possible.

Although the effects of supplementation on B12 status are best studied in deficient animals, some significant effects of time and treatment were still observed in Experiment 2. As expected, ewes treated with SMARTShot® B12 injection maintained a significantly higher concentration of B12 in serum than ewes in the other treatment groups throughout the experimental period, although to a lesser extent at weaning. Differences in treatment groups observed in ewe serum B12 results were not, however, reflected in the lamb results. Previous reports have shown significant increases in serum B12 concentrations in lambs from ewes supplemented during pregnancy (Gruner et al., 2004). However, despite increases in milk B12 concentrations in supplemented ewes, (Hart and Andrews, 1959; Quirk and Norton, 1987), it is likely that the majority of transfer from ewe to lamb is placental rather than via milk (Grace, 1999) as availability of supplemental B12 in milk is considered to be poor (Grace, 1999; Gruner et al., 2001). Hannam et al., (1980) reported that parental B12 supplementation of suckling lambs improved their tissue B12 status but had little effect on their serum B12 concentrations. Thus, it appears that provision of additional vitamin B12 to lambs, from increased maternal supply, results in uptake of the vitamin by tissues, thereby reducing any effect on the concentration of serum. Confirmation of this could be drawn from liver analysis. As with the B12 results, selenium status of ewes and lambs was lower at weaning compared to 8 weeks and Se supplementation increased plasma Se concentrations of ewes, but not those of lambs.

Although responses to supplementation are seen in the primary metabolites; B12 and Se, measurement of secondary metabolite concentrations are often considered to be a more reliable, though more expensive diagnostic tool (O'Harte, 1989; Gruner et al., 2004, Kennedy et al., 1991). Liver is the main site for the conversion of propionate to succinate by the B12 dependent enzyme, methylmalonyl CoA mutase (Brockman, 1993). Lack of B12 prevents the action of this enzyme and results in increased of MMA and decreased succinate. Elevated plasma MMA above 13 µmol/L is indicative of B12 deficiency (Suttle, 2005). In Experiment 2, MMA was only measured in ewe plasma as it is considered an unreliable measurement of B12 status in lambs due to lack of propionate load (Gruner, 2004). Ewes maintained MMA concentrations <2 µmol/L throughout Experiment 2, well below the critical concentration of >5 µmol/L suggested for a production response in grass fed sheep (O'Harte et al., 1989; Wiese, 2007; Rice et al., 1989). Despite sufficient concentrations, MMA and SA measurements verified the serum B12 results, confirming their suitability as diagnostic tools. MMA levels increased from 8 weeks to weaning, and B12 supplemented animals had lower MMA than those in the control groups. Furthermore, succinate levels were decreased when MMA levels were increased.

As discussed in Chapter 1, the two biochemical pathways which utilise vitamin B12 are linked. Whilst it is still unclear whether vitamin B12 deficiency in sheep results primarily from failure of the propionate-succinate pathway or the Hcy re-methylation pathway, it is important to measure metabolic parameters of both when assessing B12 status. Despite the lack of any time effect in ewes, higher Hcy concentrations were seen in lambs at 8 weeks compared to weaning, although levels indicated that they were not metabolically stressed. As this time effect is not a reflection of ewe nutrition, the high Hcy levels observed in lambs at 8 weeks probably reflects a change in composition of their diet, moving from a predominantly protein based milk diet to a forage diet as they transition from pre-ruminant to ruminant. The suckling lamb obtains key amino acids, specifically methionine, from high protein milk when

suckling, meaning there is reduced need for remethylation of Hcy to methionine at this time, resulting in accumulation of Hcy.

In addition to direct measurement of Se in blood, a functional assessment of Se status by measurement of GSHPx (EC 1.11.1.9) in whole blood can also be used. In Experiment 2, ewes and lambs supplemented with Se had increased GSHPx compared to those not given Se. Erythrocytes are packed full of this Se containing enzyme, and 75-85% of the Se in ovine erythrocytes is associated with GSHPx (Verde et al., 1995). Significant correlations have been reported for blood Se and erythrocyte GSHPx activity in sheep (Pavlata, 2012; Oh and Pope, 1976; Whanger et al., 1977; Verde, 1995). Since GSHPx becomes a component of erythrocytes already at the stage of erythropoiesis, and erythrocytes have a long half-life, its activity in whole blood indicates the long-term Se status, while blood plasma Se concentration rather reflects the actual status (Gerlof, 1992), although GSHPx levels have been shown to respond to selenium supplementation (Beilstein and Whanger, 1983).

3.5 Conclusions and implications

Following observations of a pilot study by Dodd and Sinclair (2014) identifying a Co and Se deficiency issue on the current monitor farm, the primary aim of the current study was to address the hypothesis that a Se deficiency could mask an underlying Co deficiency. Whilst Experiment 1 confirmed this thought, the adequate status of ewes and lambs in Experiment 2 prevented the hypothesis from being addressed. The seasonal and year-to-year variation in B12 status, BCS and live-weight gains observed, highlights the difficulties associated with undertaking a study of this kind. When relying on a pasture based system rather than conducting a study under controlled conditions, external factors such as pasture quality and quantity, influenced by environmental conditions, can affect results. Addressing this variation in trace-element status with a strategic 'risk management' approach to supplementation, therefore needs to be a consideration. Further investigation into the causes of inter-year variation using forage, blood and flock performance data over several years, could aid decisions on frequency and timing of supplementation strategies on farm. Furthermore, identification of individual animals that are vulnerable to deficiencies could provide a basis for targeted supplementation.

CHAPTER FOUR

Genetic regulation of one-carbon metabolism in sheep

4.1 Introduction

Whilst effective supplementation strategies such as those mentioned in Chapter 2 can mitigate the effects of trace-element deficiencies, the apparent seasonal and inter-year variability in trace-element status observed in Chapter 3 highlights the requirement for strategic 'risk management' approaches to supplementation. Furthermore, considerable inter-individual heterogeneity in terms of metabolic responses to specific diets are frequently observed in dietary studies with outbred populations of animals. This is illustrated in Figure 4.1 for the intermediary metabolite, Hcy, in ewes from a single breed offered either a normal or methyl-deficient diet (Kanakkaparambil et al., 2009). Although a number of environmental factors can contribute to this variability, these are minimised in controlled nutritional studies such as the aforementioned study, therefore highlighting a possible underlying genetic contribution. Identification of individual animals that are genetically predisposed to be susceptible to dietary deficiencies could therefore provide a basis for judicious targeted supplementation and selection of genetic tolerance in breeding programmes. This, in turn, would lead to improved animal health and welfare (by reducing the suffering associated with chronic trace-element deficiencies), and provide financial benefits for the sheep industry, both in terms of reduced costs (associated with prophylactic supplementation) and improved productivity (i.e. increased birth and growth rates).

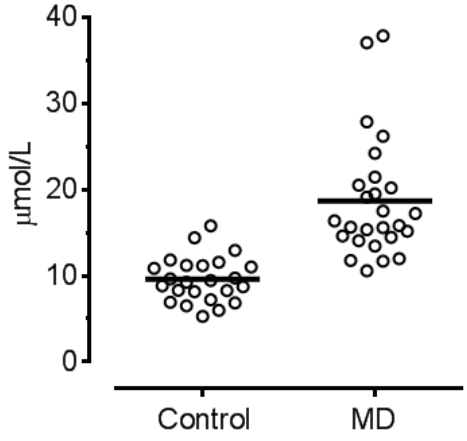


Figure 4.1. Total plasma Hcy concentrations in ewes offered a Control or Methyl Deficient (MD) diet (deficient in methionine, B12 and folate) (data derived from Kanakkaparambil et al., 2009) (P<0.001; SED=2.08)

One-carbon (1C) metabolism comprises a network of interrelated biochemical reactions that facilitate the transfer of 1C units (methyl groups) required for vital cellular processes such as DNA, RNA and protein methylation and synthesis, which in turn impacts both genomic maintenance and epigenetic control of gene expression (Lucock, 2000; Ducker and Rabinowitz, 2017). Disruptions to 1C metabolic pathways are associated with many diseases and, in recent years, there has been renewed interest in dietary components that mediate 1C metabolism. Such nutrients include folate (B9), other B-vitamins (B2, B6 and B12), methionine and choline (betaine); all of which act as essential substrates or cofactors (Mason, 2003). Whilst 1C related nutrient status is associated with several disease outcomes including anorexia, anaemia, compromised immune function, unthriftiness and impaired fertility in both ruminant and non-ruminant species (Suttle, 2010), there is clear evidence for genetic predisposition to these metabolic imbalances.

In humans, polymorphic variants of genes encoding enzymes related to key 1C metabolite concentrations (e.g. elevated plasma Hcy) have been identified, and are associated with increased disease risk. However, studies have mostly focussed on the effects of only a small number of genes and functional polymorphisms; the C677T (rs1801133) polymorphism in *MTHRF* (EC 1.5.1.20) being a notable example (Bailey et al., 1999; Ulvik et al., 2007; Gellekink et al., 2005; Malloy, 2004). No such studies have been conducted with farm animals. Therefore, the objective of the current study was to address the hypothesis that single nucleotide polymorphisms (SNPs) in genes involved in or directly related to 1C metabolism lead to inter-individual variability in metabolic responses to micronutrient deficiencies in sheep. To extend the project further, variants in genes encoding epigenetic regulators that utilise S-adenosylmethionine (SAM) as the primary methyl donor were also considered.

4.2 Materials and methods

A total of 360 lambs were used in this study. Population-based genome wide association studies, using a large number of SNP markers (i.e. a 50K or 500K SNP chip), usually require a large sample size to increase statistical power and decrease variance (Teo, 2008). Conversely, evidence has shown that a smaller sample size is required when testing more common SNPs with stronger effect sizes (Hong and Park, 2012), and when using a candidate gene approach, focussing on variants in genes that are directly associated with a measurable trait (Witte, 2010). Therefore, in the current study, the use of a custom array with fewer than 5000 SNP probes for gene variants directly involved in the regulation of measurable intermediary 1C metabolites, permitted the use of a smaller sample size. Advice from geneticists at the University of Edinburgh provided further confirmation of the suitability of this sample size for the study.

4.2.1 Animals, sample collection and diet

All animal procedures were conducted in accordance with the requirements of the UK Animal Scientific Procedures Act (1986) and investigations were approved by the University of Nottingham Animal Welfare and Ethical Review Board.

367 pure bred Texel lambs, from 11 farms in the UK, were recruited for this study. Analysis of data from the National Veterinary Investigation Diagnosis Analysis (VIDA) database (records compiled from APHA and SAC centres across Great Britain) revealed a prevalence of cobalt deficiency of around 7% (inter year range = 5-12%) in the national flock between July and September each year. With this knowledge and the requirement for a larger proportion of Co deficient animals within the study population, 90 lambs from 6 of the 11 farms were taken to the University farm at 12 weeks of age to be fed a cobalt deficient diet (Section 4.2.1.3). The remaining 277 lambs were slaughtered between May and September at regional abattoirs as part of the normal meat

production cycle and liver samples were collected. Liver samples were frozen in liquid nitrogen immediately after collection and stored at -80°C until analysed. Analysis of liver Co concentrations in the 277 lambs slaughtered at regional abattoirs was undertaken by ICP-MS to assess trace-element status and confirm the proportion of Co deficient lambs across the five farms represented. For the cohort of lambs fed at Nottingham, plasma Co concentrations were determined on arrival, and one month later (see Section 4.2.1.3).

4.2.1.1 Liver digestion and ICP-MS

Liver samples were freeze dried and 0.1g transferred to acid digestion vessels. Liver samples were wet-digested in 3 ml 70% HNO_3 (Fisher Scientific, UK), 2 ml 30% H_2O_2, (Fisher Scientific, UK) and 3 ml Milli-Q water (18.2 MΩ cm) firstly at room temperature for 1 h , followed by 45 min in a Multi-wave 3000 microwave (Anton Paar, UK). Post-digestion samples were washed from the digestion vessels into universal tubes with sequential aliquots (3 ml and 4 ml) of Milli-Q water. Blanks and reference materials (standard reference material 1577c, Bovine Liver, National Institute of Standards and Technology, US) were run with each digestion batch. Trace-element concentrations (including Co) in the liver samples were then determined by inductively coupled plasma mass spectrometry (ICP-MS). The method used was as described in Chapter 3, Section 3.2.3.4.

4.2.1.2 Extraction of DNA from liver samples

Purification of genomic DNA from liver samples was undertaken using a QIAGEN® AllPrep DNA/RNA Mini Kit. Samples were first lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer, to immediately inactivate DNases and RNases and ensure isolation of intact DNA and RNA. The lysate was then passed through an AllPrep DNA spin column. This column, in combination with the high-salt buffer, allowed selective and efficient binding of genomic DNA. The column was washed and pure DNA eluted.

4.2.1.3 Co deficient cohort

On arrival at the university farm, 90 twelve-week old lambs were adjusted to a concentrate diet by feeding Co deficient (<0.09 mg/kg DM) and sulphur (S) deficient (<1 mg/kg DM) barley and hay for 4 weeks. A blood sample was taken on arrival to assess trace-element status (principally Co) prior to commencement of the study. Plasma trace-element concentrations (including Co) were determined by ICP-MS analysis of a 0.5 ml plasma sample (no sample preparation required) using the method described in Chapter 3, Section 3.2.3.4.

At the start of the study, lambs were penned in pairs by farm origin, sex and weight. Animals had continual access to water at all times. Seven animals were removed from the study as they were found not to be thriving following introduction to the experimental unit. This is quite common for newly weaned lambs. For the duration of study (13 weeks), animals were fed a Co and S deficient barley-based diet *ad libitum* with a fixed rate of Co-deficient hay of 200 g/lamb/day. The concentrate component of the diet was composed of 30% oat feed, 25% maize, 42% barley and 3% urea, and was based on that fed previously (Sinclair et al., 2007; Kanakkaparambil et al., 2009). Details of diet composition are provided in Table 4.1. Individual intakes of the Co deficient barley-based diet were recorded at 3 and 4 day intervals, along with hay fed at a fixed rate of 200 g/day. Lamb live-weights were recorded fortnightly. Blood samples were collected from lambs weekly by superficial venepuncture into Lithium heparin (LH) tubes and vacuum tubes containing no additive. Samples were centrifuged at 4,250 x g for 15 min. Whole blood, plasma and serum fractions were stored at -20°C until analysed. The experimental end-point for this study was when lamb serum B12 remained below 200 pmol/l for ≥6 weeks with plasma Hcy rising for ≥4 weeks to ≥9 µmol/l. At this point, lambs were slaughtered and liver samples collected, immediately frozen in liquid nitrogen and stored at -80°C until analysed.

Table 4.1. Chemical and mineral composition of barley based concentrate diet and hay

Nutrient	Concentrate diet	Hay
Macro minerals, g/kg DM		
P	2.2	1.6
K	4.9	12.3
Na	0.07	0.8
Mg	0.8	1.2
Ca	0.5	3.7
S	0.8	1.0
Micro minerals, mg/kg DM		
B	4.9	8.6
Cu	2.5	4.8
Mn	14.6	133.4
Zn	18.0	23.2
Se	0.06	0.03
Co	0.02	0.2

4.2.1.4 Serum B12

Serum B12 analysis was undertaken weekly using the IMMULITE® 2000 Vitamin B12 immunoassay at SRUC, Edinburgh. The IMMULITE® 2000 Vitamin B12 is a solid-phase, competitive chemiluminescent enzyme immunoassay involving an automated alkaline denaturation procedure. Briefly, the serum sample is treated with dithiothreitol (DTT) and sodium hydroxide/potassium cyanide solution (NaOH/KCN) in a reaction tube containing no beads. After a 30 min incubation, the treated sample is transferred to a second reaction tube containing B12 coated polystyrene beads and hog intrinsic factor (HIF). During this subsequent 30 min incubation, B12 released from endogenous binding proteins during sample treatment competes with immobilised B12 for binding with HIF.

Alkaline phosphatase-labelled anti-hog intrinsic factor is then introduced which binds to any HIF that is immobilised on the B12-coated beads during the final 30 min incubation. Unbound enzyme conjugate is removed by centrifugal wash. The bound label is then quantified using the dioxetane substrate to produce light. Light is emitted when the chemiluminescent substrate reacts with the alkaline phosphatase label bound to the bead. The amount of light emitted is proportional to the amount of B12 present in the sample. This light emission is detected by the Photomultiplier Tube (PMT) and results are calculated for each sample. A sample volume of 75 µl is required for this assay. The reportable range was 111-738 pmol/L with an intra-assay coefficient of variation (CV) for low, medium and high QCs of 13.0, 7.0 and 6.2% respectively and inter-assay CV% for low medium and high QCs of 15.0, 6.0 and 7.9% respectively.

4.2.1.5 Plasma Hcy

Plasma Hcy was determined immediately upon collection using an Imola Autoanalyser (RX imola; Randox Laboratories Ltd., Antrim, U.K.) as described in Chapter 2, Section 2.2.2.2. The kit for Hcy was supplied by Randox Laboratories (catalogue no. HY4036). The limit of detection was 1.74 µM and upper limit was 47.9 µmol/L. CVs for low, medium and high QCs were 9.32, 6.77 and 5.40% respectively.

4.2.1.6 Plasma MMA

Plasma methylmalonic acid (MMA) concentrations were determined by Gas Chromatography with Mass Spectroscopic detection (GC-MS) following derivatisation and extraction as described in Chapter 2, Section 2.2.2.3. The limit of detection was 0.15 µM and CVs for low, medium and high QCs were 0.44, 1.03, 0.83% respectively.

4.2.1.7 Extraction of DNA from blood samples

1.5 ml of anti-coagulated blood was transferred to a 15 ml falcon tube containing 7.5 ml water and inverted quickly 6 times to lyse the red blood cells. 1 ml 10x Phosphate Buffered Saline (PBS; without calcium and

magnesium; Sigma Aldrich®) was added to each sample and inverted to mix. The samples were centrifuged at 2,500 rpm for 3 min and supernatant discarded. The pellet was re-suspended in 1 ml 1x PBS (without calcium and magnesium; Sigma Aldrich®) by pipetting and then transferred to a 1.5 ml tube. The sample was centrifuged at 14,500 rpm for 2 min and the supernatant discarded. DNA was extracted from the pellet using the method of Montgomery and Sise (1990) with slight modification. The white blood cell pellet was thawed at room temperature for 5 min and re-suspended by pipetting in 716 µl 19 mM Tris/0.1 mM EDTA solution and 4 µl Proteinase K (20 mg/ml) added. To each sample, 40 µl 0.5 M EDTA was added and inverted to mix. 40 µl 10% SDS was then added, inverted to mix and incubated overnight in a rotating cabinet at 55°C. A further 1.5 µl proteinase K (20 mg/ml) was added to each sample and incubated for a further 2 h in a rotating cabinet at 55°C. After cooling at room temperature for 5 min, 360 µl 5 M NaCl was added, mixed by inverting and left at room temperature for 10 min before centrifuging at 13,400 rpm for 5 min. 600 µl sample was transferred to an Eppendorf containing 1.2 ml 100% ethanol and inverted until DNA strand visible. DNA strand was transferred to a 1.5 ml Eppendorf LoBind tube containing 0.5 ml 70% ethanol, centrifuged at 13,400 rpm for 4 min and ethanol removed. The DNA pellet was re-suspended in 80 µl 19 mM Tris/0.1 mM EDTA solution and stored at -20°C.

4.2.2 Construction of Illumina Infinium® iSelect® Custom Array

Twenty unrelated Texel ewes were sequenced to a depth of 30x in 2 pools at Edinburgh Genomics. Custom array design was then undertaken at the University of Nottingham. Reads from the sequencing analysis were mapped to the reference sheep genome assembly (Oar_v3.1) and ~15 million variants were identified. Of these, ~3 million were novel SNPs and ~12 million were already present in the Ensembl genome database. Selection of SNPs for retention on the custom designed array prioritised those present within 223 1C and related gene transcripts (including those encoding for selected epigenetic regulators) identified using online databases and analyses. These databases included HymanCyc

(www.humancyc.org) Encyclopaedia of human genes and metabolism, The Biological General Repository for Interaction Datasets (BioGRID; www.thebiogrid.org), GeneMANIA (www.genemania.org) for functional association analysis, Gene Ontology enrichment analysis (www.geneontology.org/page/go-enrichment-analysis) and information in current published literature. Further SNP retention criteria was based on SNPs located within or 3 Kb upstream of coding or untranslated regions of priority genes. An Illumina Infinium® iSelect® Custom Array was constructed with 4,576 probes capturing SNPs in 115 1C metabolism and related genes, and 108 related epigenetic regulators as well as 33 control SNPs. The custom designed array was then used to SNP profile the total 360 Texel-sheep at Edinburgh Genomics.

Prior to genotyping, DNA samples were quantified using an Invitrogen Molecular Probes® Quant-IT^{TM} PicoGreen® dsDNA Assay Kit following the recommended protocol (ThermoFisher). The PicoGreen® assay is seen as a more reliable method for determining double stranded DNA (dsDNA) concentrations for genotyping than more commonly used techniques such as determination of absorption at 260 nm (A_{260}). The major disadvantages of absorbance methods are the relatively large contribution of nucleotides, single stranded nucleic acids and proteins to the signal, the inability to distinguish between DNA and RNA, and the relative insensitivity of the assay. Conversely, the PicoGreen® assay which is selective for dsDNA, does not show significant fluorescence enhancement in the presence of proteins and allows the detection and quantification of DNA concentrations as low as 5.0 ng/ml DNA. DNA samples run on agarose gels and stained with ethidium bromide were further analysed using the Invitrogen^{TM} E-Gel^{TM} Electrophoresis system and BioDoc-It® 220 Imaging System.

Genotyping was carried out using the Illumina® Infinium® HD Assay Ultra protocol. DNA samples were thawed to room temperature. 20 µl MA1 reagent was added to each well of a MSA3 plate, followed by 4 µl 50 ng/µl DNA sample. 4 µl 0.1N NaOH was then added to each well. The

plate was covered, vortexed for 1 min and centrifuged at 280 x g for 1 min before incubating at room temperature for 10 min. 34 µl MA2 reagent was added to each well to neutralise the sample, followed by 38 µl MSM (Multi-Sample Amplification Master Mix). The plate was then sealed, vortexed and centrifuged at 280 x g for 1 min. Denatured DNA was then isothermally amplified by incubating in the Illumina Hybridization Oven for 20-24 h at 37°C. The amplified DNA was then fragmented in a controlled enzymatic process by centrifuging the plate at 50 x g for 1 min, adding 25 µl FMS reagent to each well, vortexing for 1 min and then centrifuging at 50 x g for 1 min. The sealed plate was then incubated at 37°C for 1 h. 50 µl PM1 reagent was then added to each well. The plate was sealed, vortexed at 1,600 rpm for 1 min, incubated at 37°C for 5 min and centrifuged at 50 x g at 22°C for 1 min. 155 µl 100% 2-propanol was added to each sample, and fragmented DNA collected by centrifugation at 4°C and 3000 x g for 20 min. The supernatant was discarded and the pellets allowed to air dry at room temperature for 1 h. The precipitated DNA pellet was re-suspended in 23 µl RA1 reagent (hybridisation buffer) and incubated in the Illumina hybridisation oven at 48°C for 1 h. The BeadChip was prepared for hybridization in a capillary flow-through chamber. Sample plates were heated to 95°C for 20 min to denature the DNA and then cooled at room temperature for 30 min. Samples were then applied to the BeadChip. The loaded BeadChip was incubated in the Illumina Hybridization Oven for 16 h at 48°C to allow hybridization of the DNA to the BeadChips. Unhybridized and non-specifically hybridized DNA was washed away using RA1 reagent, in preparation for staining and extension.

XC1 and XC2 reagents were added to condition the BeadChip surface for the extension reaction. TEM reagents were dispensed into the Flow-Through Chambers to perform single-base extension of primers hybridized to DNA on the BeadChip. This reaction incorporated labelled nucleotides into the extended primers. 95% formamide/1 mM EDTA was added to remove the hybridized DNA. After neutralization using the XC3 reagent, the labelled extended primers underwent a multi-layer staining

process on the Chamber Rack. Next, the Flow-Through Chambers were disassembled and the BeadChips washed in PB1 reagent, and then coated with XC4 reagent and dried.

The Illumina iScan Reader was used to scan the BeadChip, using a laser to excite the fluorophore of the single-base extension product on the beads. Light emissions from these fluors were then recorded in high-resolution images of the BeadChip sections. Data from these images was analysed using Illumina's GenomeStudio Genotyping Module.

4.2.3 Liver metabolite quantification

For the same 360 Texel sheep-liver samples, 23 1C and related metabolites were quantified by GCMS, HPLC and LC-MS/MS.

4.2.3.1 GCMS

Liver methylmalonic acid (MMA) and succinic acid (SA) concentrations were determined by Gas Chromatography with Mass Spectroscopic detection (GCMS) following derivatisation and extraction. The method used was based on that of Kanakkaparambil et al. (2009), with the following modifications. Briefly, 250 µl 80% MeOH was added to 50 mg frozen liver, homogenised for 2 min and cooled on ice for 10 min. Following this, the sample was centrifuged for 15 min at 14,500 x g to form a pellet and 200 µl liver homogenate was transferred to a 2.5 ml screw capped glass vial. To this, 4 µl internal standard (1 mM 4-chlorobutyric acid (CBA) in 1 mM HCl) followed by 250 µl 12% BF_3-Methanol were added, vortexed for 1 min and heated at 95°C for 15 min on a heating block. After cooling, 250 µl cold distilled water and 250 µl cold dichloromethane (CH_2Cl_2) were added to the vial, vortexed for 30s and centrifuged for 10 min at 14,500 x g to separate the layers. The lower dichloromethane layer was transferred to a screw capped glass auto-sampler vial with insert for GCMS analysis. The GCMS method used was the same as described in Chapter 2, Section 2.2.2.3.The limit of detection was 0.75 nmol/g for both MMA and SA and inter-assay CVs were 8.4% for MMA and 11.0% for SA.

Liver propionic acid (PPA) concentrations were determined by GCMS following derivatisation and extraction. The method was based on that of Pouteau et al., (2001) with the following modifications. Briefly, 750 µl 5-Sulfosalicylic acid (SSA, 0.04 mg/ml) was added to150 mg frozen liver, homogenised for 2 min and then cooled on ice for 10 min. The sample was, centrifuged for 15 min at 14,500 x g to form a pellet and 200 µl liver homogenate was transferred to a 2.5 ml screw capped glass vial. To this, 20 µl internal standard (MBA, 400 µM), 3.5 µl HCl (37%) and 1 ml diethylether were added, vortexed for 2 min and centrifuged for 10 min at 14,500 x g to separate the layers. 600 µl of the upper layer was transferred to a screw capped glass vial containing 3.5 µl 1-(tert-butyldimethylsilyl)imidazole (TMDMSIM, 97%), vortexed for 2 min and heated at 60°C for 30 min. After cooling, the sample was analysed by GCMS. The method used a DB-5MS column (J&W Scientific Agilent technology, 30 m x 0.25 mm; 0.25 µm film thickness). The carrier gas (He) was set at a constant flow rate of 1.3 ml/min. The injection volume was 5 µl for SCAN mode, for qualification, and SIM (selected ion monitoring) mode for quantification, both using splitless mode. The injection port and MS selective detector interference temperatures were 260°C and 250°C respectively. The chromatograph was programmed for an initial temperature of 40°C for 1 min, increased to 60°C at 70°C min^{-1}, increased to 110°C at 15°C min^{-1}, then increased to a final temperature of 250°C at 70°C min^{-1}. MS was tuned regularly and operated in electron impact (EI) ionization mode with the ionization energy of 70eV. SCAN mode measured at m/z: 30-300 and SIM ions were set at 159 (for MBA) and 131 (for PPA). The same method was used to produce a calibration curve for PPA using standards at concentrations ranging from 19.5 nmol/g to 5µmol/g. The limit of detection was 19.5 nmol/g. CVs for low, medium and high QCs were 10.4, 6.3 and 6.5% and the inter-assay CV was 4.7%.

4.2.3.2 HPLC

Liver polyamine (Put, Spd and Spm) concentrations were determined by High-performance liquid chromatography (HPLC) following extraction and derivatisation. The method was based on those of Magnes et al. (2014) and Sabri et al. (1989) with the following modifications. Briefly, 500 µl cold distilled water and 40 µl cold perchloric acid (4 M) were added to 50 mg frozen liver, homogenised for 2 min and cooled on ice for 10 min. The sample was then centrifuged for 15 min at 14,500 x g to form a pellet and 400 µl liver homogenate transferred to an Eppendorf® tube. To adjust the pH to 9.0, 28 µl potassium hydroxide (KOH, 4M) was added and vortexed for 30s. The sample was then centrifuged for 10 min at 14,500 x g to form a pellet and 20 µl homogenate transferred to an Eppendorf® tube for derivatisation as follows. To the 20 µl sample, 12 µl borate buffer (0.2 M) and 4 µl 1, 6-diaminohexane (DAH, 100 µM) were added and vortexed for 30s followed by the addition of 40 µl 9-fluorenylmethyl chloroformate (FMOC, 0.01 M in acetone). The sample was vortexed for 1 min and derivatisation allowed to proceed for 10 min at room temperature. 48 µl glycine reagent (0.04 M glycine in 0.2 M sodium borate, pH 9.0) was added, vortexed for 30 s and left at room temperature for a further 2 min. Finally, 40 µl dilution buffer (0.015 M NaOAc in HPLC grade acetonitrile) was added, briefly vortexed and 100 µl transferred to a glass vial containing 400 µl dilution buffer for subsequent analysis by HPLC. The method used an Agilent 1100 HPLC. 10 µl sample was injected onto the Zorbax C18 HPLC column (250 x 4.6 mm, 5 µm) protected by a 5 µm guard column, maintained at 40°C. The chromatograph consisted of a binary solvent delivery system equipped with an injector and a gradient controller. Pump settings: Flow rate 1.2 ml/min, stop time 30 min, post run time 10 min. Mobile phase A: 0.05 M NaOAc: ACN (80:20, v/v, pH 4.2). Mobile phase B: 0.05 M NaOAc: ACN (5:95, v/v, pH 4.2). Mobile phase gradients are in Table 4.2. A fluorometer was used to monitor the elution of polyamine derivatives from the column (excitation wavelength=264, emission wavelength=310). Limit of

detection was 0.625 nmol/g for all three polyamines. Inter-assay CVs for Put, Spd and Spm QCs were 2.08, 3.09 and 3.24% respectively.

Table 4.2. Mobile phase gradients for analysis of liver polyamine concentrations by HPLC

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	50	50
5	50	50
28	0	100
28.5	0	100
29	50	50
30	50	50

Liver amino acid concentrations were determined by HPLC with online derivatisation. 20 primary amino acids were quantified including methionine, glycine and serine involved in 1C metabolism. The method was based on Henderson et al. (2000) with the following modifications. Briefly, 150 µl 80% MeOH was added to 150 mg frozen liver and homogenised for 2 min. The sample was centrifuged for 15 min at 14,500 x g, 120 µl liver homogenate was transferred to a 2.5 ml screw capped glass auto-sampler vial and 3 µl Norvaline (NVA, 40 mM) added. The method used an Agilent 1100 HPLC. Sample was injected onto a ZORBAX Eclipse-AAA column (4.6 x 150 mm; 3.5 µm), maintained at 40°C.

Injection program was as follows:

1) Draw 2.5 µl from vial 1 (borate buffer, 0.5 M, pH 8.0)
2) Draw 0.5 µl from sample
3) Mix 3 µl “in air”, max speed, 2x
4) Wait 0.5 min
5) Draw 0 µl from vial 2 (needle wash using water in uncapped vial)
6) Draw 0.5 µl from vial 3 (10 mg/ml OPA + 20 mg/ml MPA, in ACN)
7) Mix 3.5 µl “in air”, max speed, 6x
8) Draw 0 µl from vial 2 (needle wash using water in uncapped vial)
9) Draw 0.5 µl from vial 4 (ACN)
10) Mix 4 µl “in air”, max speed, 6x
11) Draw 0 µl from vial 6 (needle wash using ACN in uncapped vial)
12) Draw 16 µl from vial 5 (water)
13) Mix 10 µl “in air”, max speed, 2x
14) Inject 10 µl

The chromatograph consisted of a binary solvent delivery system equipped with an injector and a gradient controller. Pump settings: Flow rate 1.75 ml/min, stop time 30 min, post run time 0 min. Mobile phase A: 40 mM Na_2HPO_4, pH 7.8. Mobile phase B: ACN: MeOH: water (45:45:10, v/v/v). Mobile phase gradients are in Table 4.3. A fluorometer was used to monitor the elution of polyamine derivatives from the column (excitation wavelength=340, emission wavelength=450). Limits of quantification and inter-assay CVs for all amino acids quantified are given in Table 4.4.

Table 4.3. Mobile phase gradients for analysis of liver amino acid concentrations by HPLC

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	100	0
2	100	0
24	56	44
25	0	100
26	0	100
27	100	0
30	100	0

Table 4.4. Limits of quantification and inter-assay CVs for all liver amino acids quantified by HPLC

Amino Acid	LOQ (nmol/g)	Inter-assay CV (%)
Asp	31.25	14.7
Glu	1250	3.9
Asn	31.25	2.5
Ser	31.25	5.5
Gln	15.6	5.1
His	15.6	6.1
Gly	1250	5.7
Thr	15.6	1.9
Cit	31.25	4.8
Tyr	15.6	18.8
Cys	62.5	6.6
Val	15.6	4.3
Met	15.6	7.2
Trp	15.6	6.7
Phe	15.6	4.7
Ile	31.25	2.7
Leu	31.25	2.1
Cys	31.25	3.2

4.2.3.3 HILIC-MS/MS

Liver folate and B-vitamin concentrations were determined by Hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS) following extraction. The following methods were undertaken in collaboration with a fellow PhD student (Miss Constance Clare).

Folate method: 250 µl potassium phosphate extraction buffer (pH 7) containing 100 nM internal standard (MTX and SAH-d4) was added to 50 mg frozen liver. Homogenisation was achieved using a TissueLyser (Retsch Qiagen) with two disruption steps at 25 Hz for 2.5 min with freezing of samples on ice in between. Homogenate was heated at 100°C for 2 min to deactivate the conjugate enzyme and then rapidly cooled on ice. Samples were briefly vortexed and centrifuged at 9000 x g for 10 min (at 4°C). 100 µl of supernatant was transferred to an amber Eppendorf® Safe-Lock microcentrifuge tube, and 75 µl of potassium phosphate extraction buffer and 25 µl rat serum added. Following gentle mixing of the homogenate, the tube was incubated at 37°C for 2 h to allow enzymatic cleavage of glutamate residues from folates, catalysed by γ-glutamyl hydrolase (GGH). Samples were heated at 100°C for 1 min to deactivate GGH and then rapidly cooled on ice. Following centrifugation at 10,000 x g for 5 min (at 4°C), 100 µl of supernatant was transferred to a 0.5 ml Microcon-10kDa centrifugal filter unit with ultracel-10 membrane and further centrifuged at 16,000 x g for 30 min (at 4°C). The filtrate was transferred to an amber HPLC vial for LC-MS/MS analysis.

B-vitamin method: 300 µl extraction buffer (50% ACN, 0.1% L-ascorbic acid, 1% acetic acid and 0.1% MCE) containing 1 µM internal standard mix (B_1-$^{13}C_3$, PN-d_3 and 8-Br-cAMP) were added to 50 mg frozen liver. Homogenisation was achieved using a TissueLyser (Retsch Qiagen) with two disruption steps at 25 Hz for 2.5 min with freezing of samples on ice in between. The tubes containing homogenate were centrifuged at 13,000 x g for 15 min (at 4ºC) and 100 µl of supernatant was transferred

to an amber Eppendorf® Safe-Lock microcentrifuge tube. For deproteination of supernatant, 200 µl of ice-cold ACN was added and vortexed for 10s. Following centrifugation at 16,000 x g for 15 min (at 4^{o}C), supernatant was transferred to an amber HPLC vial for LC-MS/MS analysis.

Chromatographic separation was performed on a Sequant ZIC- pHILIC column (150 x 4.6 mm, 5 µM) with a guard column (Sequant ZIC-pHILIC, 20 x 2.1 mm, 5 µM) maintained at 45^{o}C. The auto-sampler was maintained at a temperature of 4^{o}C. Sample injection volume was 10 µl and flow rate was 0.4 ml/min. Mobile phases for the folate method were composed of aqueous ammonium carbonate (pH 9.1) for eluent A and 100% ACN for eluent B. Mobile phases for the B-vitamin method were composed of aqueous ammonium formate (adjusted to pH 3.5 with formic acid) for eluent A and 100% ACN for eluent B. Gradient elution was carried out by the following program: isocratic hold at 80% (eluent B) for 1 min, linear gradient from 80% to 5% over 5 min, followed by linear gradient back to 80% over 2 min, and isocratic hold at 80% for 5 min. The total run time was 13 min.

LC-MS/MS analyses were initially performed using an LC-10AD system (Shimadzu, Kyoto, Japan) equipped with a SIL-HTC autosampler coupled to ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex) tandem mass spectrometer outfitted with an electrospray ion source (Turbo Ion Spray™; Applied Biosystems, Foster City, CA). Analyst software (Applied Biosystems/MDS Sciex) was used for HPLC system control, data acquisition and processing. Source conditions were set as follows; ion source temperature (450^{o}C), ion spray voltage (5000 V), curtain gas (20 psi), collision gas (8 psi), ion source gas 1 (20 psi), ion source gas 2 (20 psi) and the activated interface heater.

4.2.4 Genetic analysis

4.2.4.1 Variance component analysis

Data on 23 1C liver metabolite concentration traits for 360 Texel lambs were analysed using mixed linear models (ASReml 4.1, 2015) (www.vsni.co.uk) to estimate genetic parameters. These traits were log transformed to normalise the distribution prior to further analyses. The model fitted accounted for the fixed effects of sex (male and female), farm (location) and breed (Texel and Texel cross) with the first three principal components (PC) used as a substitute for the effect of line. Animal was fitted as a random effect in this model.

4.2.4.2 Association analysis using whole genome (GWAS)

All liver samples were genotyped with the Illumina Infinium® iSelect® Custom Array for 4,576 single-nucleotide polymorphisms (SNPs). Quality control measures were applied to retain SNPs with a minor allele frequency (MAF) greater than 0.01 and call rates greater than 0.90, after which 3,173 SNPs remained for further analysis.

Association analysis was performed using the GenABEL package (a package for genome-wide association analysis) (Aulchenko et al., 2007) in R environment (http://www.r-project.org). Visualisation of population stratification was done using Classical Multi-dimensional Scaling (MDS) to explore population substructure and to verify the genetic homogeneity of the sample prior to analysis. The first three principal components (PC) were obtained for use as covariates to account for the presence of population stratification using GenABEL (Aulchenko et al., 2007). These models were similar to those in the variance analyses described above (Section 4.2.4.1) and used a mixed model function (i.e. an mmscore function (Chen and Abecasis, 2007)). After Bonferroni correction for multiple testing, P values for the genome-wide ($P<0.05$) and the suggestive (i.e. one false positive SNP out of all 3,173 SNPs) significance thresholds were $\leq 1.19 \times 10^{-4}$ and $\leq 2.38 \times 10^{-3}$, respectively.

4.2.4.3 Association analysis using single SNPs

Individual significant SNPs from the GWAS were further verified and genetic effects estimated in a single marker association analysis using ASReml. This was achieved by fitting a single SNP as a fixed effect in the model already described previously. The estimation of the genetic effect and proportion of variance explained by each SNP were calculated as follows: additive effect, a = (AA – BB)/2; dominance effect, d = AB – [(AA + BB)/2]; proportion of phenotypic variance due to SNP = [2pq(a + d(q – p))^2]/VP, where AA, BB and AB represent the predicted trait values for each SNP genotype from the analysis, p and q the SNP allele frequencies and VP the total phenotypic variance of each trait derived without SNP genotypic effects in the model.

4.2.4.4 Statistical analyses of phenotypic data

All statistical analyses were performed within the GenStat statistical package (17th Edition, VSN International, 2011). Liver and plasma Co concentrations were analysed by ANOVA. For presentation purposes, Liver and plasma Co concentrations are presented as box plots, depicting median and inter-quartile ranges, with whiskers set at 5th and 95th percentiles.

Dry matter (DM) intakes, lamb weights and blood metabolite concentrations were analysed by 'repeated measures ANOVA'. For the most part, data are presented as means with standard errors of means (S.E.M). For presentation purposes, however, DM intakes (expressed on a metabolic live-weight basis) are presented as box plots, depicting median and inter-quartile ranges, with whiskers set at 1st and 99th percentiles.

4.3 Results

4.3.1 Liver cobalt

Liver Co concentrations for the 277 lamb livers obtained at abattoirs from 5 farms are shown in Figure 4.2. There was a significant difference in Co concentration between farms (P<0.001). The majority of livers from farms 1, 2, 3 and 5 had marginal/sufficient liver Co concentrations (0.03-0.05 mg/kg FW; Smith, 1987; Ulvund, 1990) with means of 0.06, 0.06, 0.05 and 0.04 mg/kg FW respectively. The bottom 5% of each farm was within the deficient range (<0.03 mg/kg FW). Almost all liver samples from farm 4 were considered to be Co deficient, with a mean Co concentration of 0.017 mg/kg FW for this farm. Overall, 19% were considered to be deficient, 30% marginal and 51% sufficient. No effect of sex was observed.

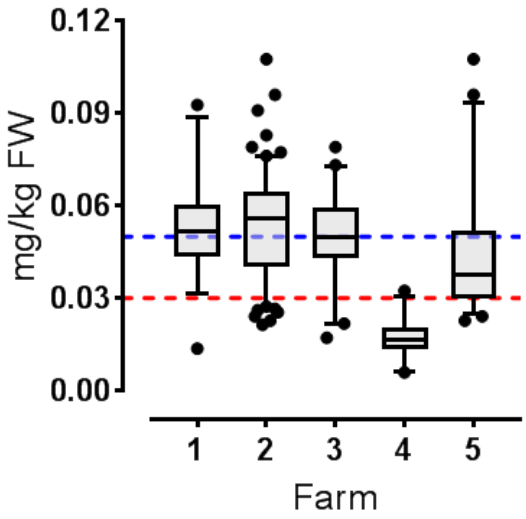


Figure 4.2. Liver Co concentrations for 277 lamb livers from 5 farms. Reference concentrations for sufficient (---) and deficient (---) status (Smith, 1987; Ulvund, 1990) are given. Box plots show median and interquartile ranges, with whiskers set at 5th and 95th percentiles. Effect of farm (P<0.001; SED=0.003).

4.3.2 Plasma Cobalt

Plasma Co concentrations for 83 lambs from 6 farms on arrival at the University farm are shown in Figure 4.3. There was a significant difference in Co concentration between farms (P=0.002). The majority of lambs from farms 1, 3, 4 and 6 were considered to have adequate plasma Co levels, with mean concentrations of 7.5, 10.5, 9.0 and 15.8 µmol/l respectively. Lambs from farm 5 were considered to be marginally deficient (4.5 µmol/l) and lambs from farm 2 considered to be deficient (2.8 µmol/l). Overall, 28% were considered to be deficient, 19% marginal and 53% sufficient. No effect of sex was observed.

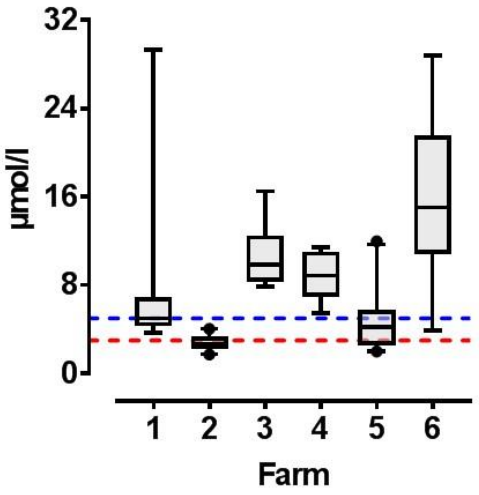


Figure 4.3. Plasma Co concentrations for 83 lambs from 6 farms on arrival at the University farm. Reference concentrations for sufficient (---) and deficient (---) status are given (Suttle, 2010). Box plots show median and interquartile ranges, with whiskers set at 5th and 95th percentiles. Effect of Farm (P<0.001; SED=1.77)

4.3.3 Intakes and weights

Dry matter (DM) intakes for the 83 lambs fed at Nottingham fluctuated over time (P<0.001) but generally increased (Figure 4.4). There was no effect of sex or farm of origin on DM intake.

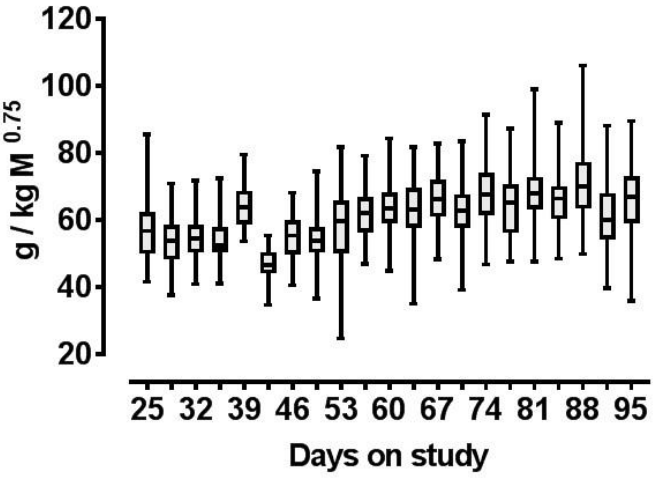


Figure 4.4. Dry matter (DM) intake (g/kg $M^{0.75}$) over time. (median, interquartile ranges and, 1st and 99th percentiles). Effect of time (P<0.001; SED=1.2).

Lamb weights (Figure 4.5) decreased from the start of the study until Day 14, after which lamb weights increased over time (P<0.001). There was no effect of sex on lamb weight, however there was a significant difference (P<0.001) in lamb weights between farms.

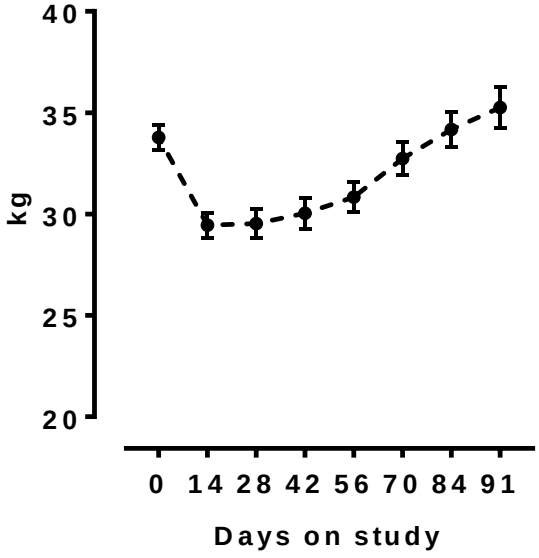


Figure 4.5. Change in lamb weights over time. Mean weight (—). Effect of time (P<0.001; SED=0.25). Effect of farm (P<0.001; SED=1.5). Time by farm interaction (P<0.001; SED=1.7)

4.3.4 Blood metabolites

Figure 4.6 shows the mean serum B12 (A), plasma Hcy (B) and plasma MMA (C) concentrations in 83 Texel lambs on a Co deficient diet offered over a 13-week period. Mean concentrations of serum B12 decreased over time ($P<0.001$) from 397 pmol/L at Week 2 to 115 pmol/L at Week 10, plateauing for the remaining weeks on study. Mean serum B12 levels were considered to be sufficient (>350 pmol/L) at the start of the study and to be deficient (<250 pmol/L) (Suttle, 2005) from Week 4 onwards. Plasma Hcy concentrations increased ($P<0.001$) from 5.87 µmol at Week 1 to 9.43 µmol/L at Week 13, with weekly undulations in concentrations over the course of the study. Final Hcy concentrations in all lamb livers indicated marginal B12 deficiency. Plasma MMA levels at week 6 were considered to be sufficient (<7 µmol/L). An increase ($P<0.001$) in MMA was observed from week 6 to week 12 with a final concentration of 21.3 µmol/L indicating B12 deficiency.

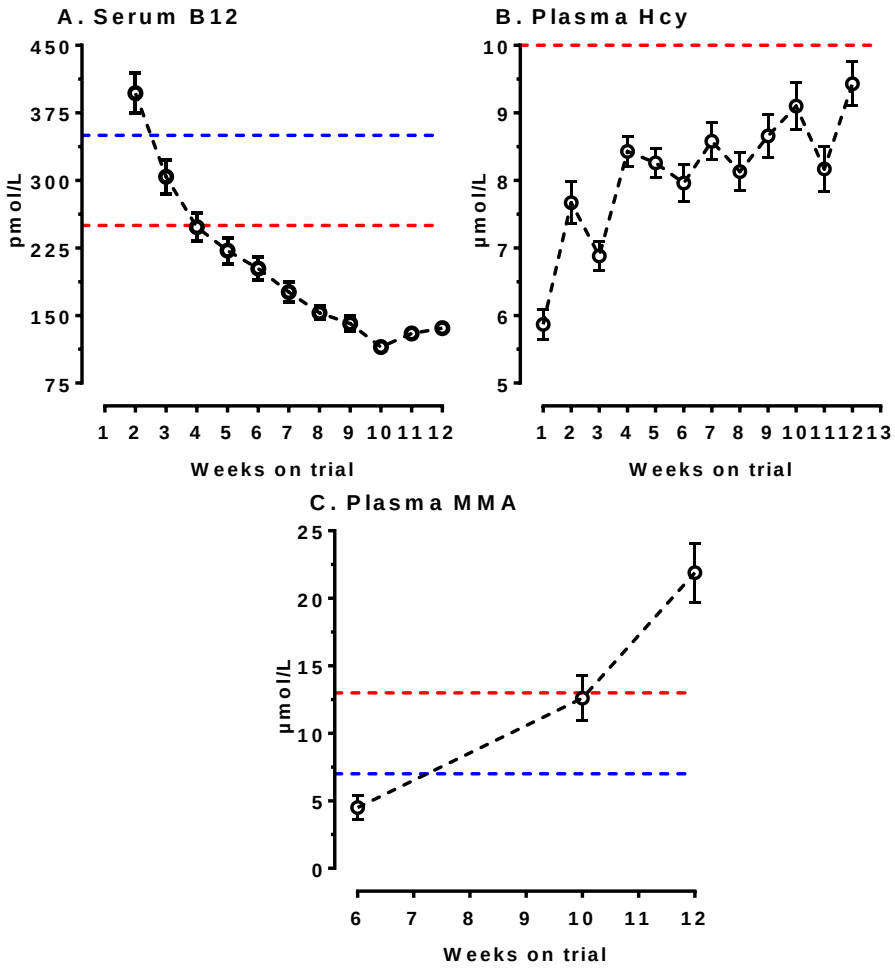


Figure 4.6. Serum B12 (A), plasma Hcy (B) and plasma MMA (C) concentrations over time. Reference concentrations for sufficient (---) and deficient (---) status are given (Suttle, 2005; Gruner et al., 2004; Kennedy et al., 1992). For B12 (A), effect of time (P<0.001; SED=13.8). For Hcy (B), effect of time (P<0.001; SED=0.3). For MMA (C), effect of time (P<0.001; SED=6.87).

4.3.5 Liver metabolites

Data on the 23 one-carbon liver metabolite concentrations for 360 Texel lamb livers, determined by GCMS, HPLC and LC-MS/MS, are presented in Tables 4.5 – 4.7. These data were used for the genome-wide association analysis.

Table 4.5. Metabolite concentrations in 360 lamb livers determined by GCMS. Concentrations are presented as nmol/g liver. MMA, methylmalonic acid; SA, succinic acid; PPA, propionic acid.

	MMA	SA	PPA
Maximum	1771.6	8709.3	567.0
Minimum	1.7	72.5	21.8
Range	1769.9	8636.8	545.2
Mean	43.1	1587.9	172.3
Median	10.2	1053.7	138.1
St. Dev.	134.9	1495.7	103.0

Table 4.6. Metabolite concentrations in 360 lamb livers determined by HPLC. Concentrations are presented as nmol/g liver. Ser, serine; Gly, glycine; Met, methionine; Put, putrescene; Spd, spermidine; Spm, spermine.

	Ser	Gly	Met	Put	Spd	Spm
Maximum	2118.3	10956.6	100.0	234.7	806.2	1139.6
Minimum	71.6	1122.7	1.0	3.7	1.7	2.1
Range	2046.6	9833.9	99.0	231.1	804.5	1137.4
Mean	353.8	3323.4	38.2	25.6	73.1	147.6
Median	246.1	3227.2	39.1	15.0	12.0	46.1
St. Dev.	329.8	1068.0	20.2	28.4	141.0	233.3

Table 4.7. Metabolite concentrations in 360 lamb livers determined by HILIC-MS/MS. Concentrations are presented as nmol/g liver. THF, tetrahydrofolate; mTHF, 5-methyltetrahydrofolate; CH2THF, 5, 10-methenyltetrahydrofolate; SAM, S-adenosyl methionine; SAH, S-adenosyl-L-homocysteine; Hcy, homocysteine; FAD, Flavin adenine dinucleotide (B2); PL, pyridoxal (B6); mB12, methyl-cobalamin; aB12, adenosyl-cobalamin; Cth, cystathionine; SAR, sarcosine; DMG, dimethylglycine; TMG, trimethylglycine.

	THF	mTHF	CH2THF	SAM	SAH	Hcy	FAD	PL	mB12	aB12	Cth	SAR	DMG	TMG
Maximum	5961.6	99511.6	5276.0	21745.8	98863.1	207035.2	92801.5	874.8	945.0	2350.4	309.5	1112.2	42.5	3849.7
Minimum	9.8	209.4	39.5	127.2	3418.4	948.5	78.9	51.6	6.2	12.0	0.4	56.9	0.1	75.3
Range	5951.8	99302.2	5236.5	21618.6	95444.8	206086.7	92722.5	823.2	938.8	2338.4	309.1	1055.2	42.5	3774.4
Mean	487.8	9373.6	616.3	3481.5	29634.2	54665.5	20294.0	235.3	176.1	400.3	35.7	244.8	2.9	936.9
Median	326.4	2897.3	471.3	2674.7	18207.5	46402.7	18342.6	211.5	154.0	342.4	20.8	170.2	1.3	919.2
St. Dev.	640.5	14621.9	559.5	2764.9	25405.0	36709.9	19772.9	111.1	116.5	328.5	51.2	196.9	4.5	515.0

4.3.6 Illumina Infinium® iSelect® Custom Array

Genomic sequencing of 20 unrelated Texel ewes identified ~15 million SNPs, of which ~3 million were novel and ~12 million were already present in the Ensemble database (Figure 4.7). Newly identified SNPs have since been deposited into the publically accessible Ensembl database.

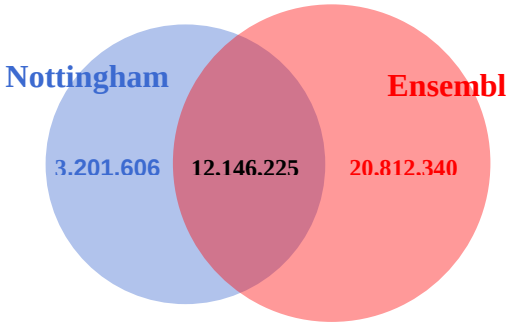


Figure 4.7. Number of SNPs identified by genomic sequencing of 20 unrelated Texel ewes. Novel SNPs identified are in blue, SNPs already present in the Ensemble database are in red.

Prioritisation of SNPs within genes related to 1C metabolism identified 223 transcripts of interest and 48,978 SNPs of interest. Of these SNPs, 80.4% were intron variants and 10.2% were downstream variants. The remaining 4,576 SNPs adhered to the SNP retention criteria of SNPs located within or 3 Kb upstream of coding or untranslated regions of priority genes (Table 4.8).

Table 4.8. Prioritisation of SNPs for retention on the Illumina Infinium iSelect® Custom Array

Transcripts of interest	223	
Total SNPs of interest	48,978	
intron variant	39,400	80.4%
downstream gene variant	5,002	10.2%
upstream gene variant	3,043	6.2%
synonymous variant	523	1.07%
3 prime UTR variant	524	1.07%
missense variant	336	0.69%
splice region variant, intron variant	81	0.17%
5 prime UTR variant	39	0.08%
splice region variant, synonymous variant	18	0.04%
missense variant, splice region variant	5	0.01%
splice donor variant	3	0.01%
stop gained	2	0.004%
splice acceptor variant	2	0.004%
Total	**4576**	

*SNPs retained on custom designed array are in green

4.3.7 Variance component analysis

A moderately high heritability estimate (0.50) was found for liver mB12 levels (Table 4.9). Heritability estimates for DMG, TMG, FAD, PL, PPA and MMA were moderate (0.19-0.24). All other metabolites analysed had low heritability estimates with high standard errors (se).

Table 4.9. Estimates of variance components and heritabilities for metabolite concentrations in sheep liver

	DMG	TMG	mB12	FAD	PL	PPA	MMA
V_a	0.012	0.006	0.033	0.008	0.006	0.007	0.012
V_p	0.064	0.030	0.065	0.039	0.027	0.036	0.051
h^2	0.20	0.21	0.50	0.20	0.23	0.19	0.24
se	0.099	0.094	0.170	0.092	0.096	0.091	0.104

Log transformed concentrations. V_a, additive variance; V_p, phenotypic variance; h^2, heritability; se, standard error.

4.3.8 Classical multidimensional scaling

Visualisation of population stratification (i.e. the presence of a systematic difference in allele frequencies between breed subpopulations), using Classical (metric) Multi-dimensional Scaling (MDS), for the 3,173 SNPs on the custom array that passed quality control, highlighted evidence of clustering (Figure 4.8). The main purpose of MDS is to visualise the structure of data by plotting multivariate data points in two dimensions in order to reveal the relative distance between observations (Borg and Groenen, 2005). The distance between each pair of points (denoted below as i and j) can be plotted on a scatter plot and are computed numerically using the Euclidean distance formula (Sneath and Sokal, 1973).

$$d_{ij} = \sqrt{\sum_{k=1}^{p} \left(x_{ik} - x_{jk} \right)^2}$$

In this formula, p is the number of dimensions, dij is the distance, and xjk is the data value of the ith row and kth column. The resulting dissimilarity matrix is an n x n inherently symmetrical matrix (so that δij = δji) with a dimension equal to the number of individuals in the data set. That is, although points are only plotted in two-dimensional space (dimension 1 and 2 on Figure 4.8), the formula computes the distance in p-dimensional space, where p can be greater than two.

Principal component analysis (PCA) is a fast and effective way to diagnose population structure (Price et al., 2006; Patterson et al., 2007) and was used to account for this structure in successive analyses in the current study. PCA transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. The first

three principal components (PC) were calculated in R and used as covariates to account for the presence of population stratification.

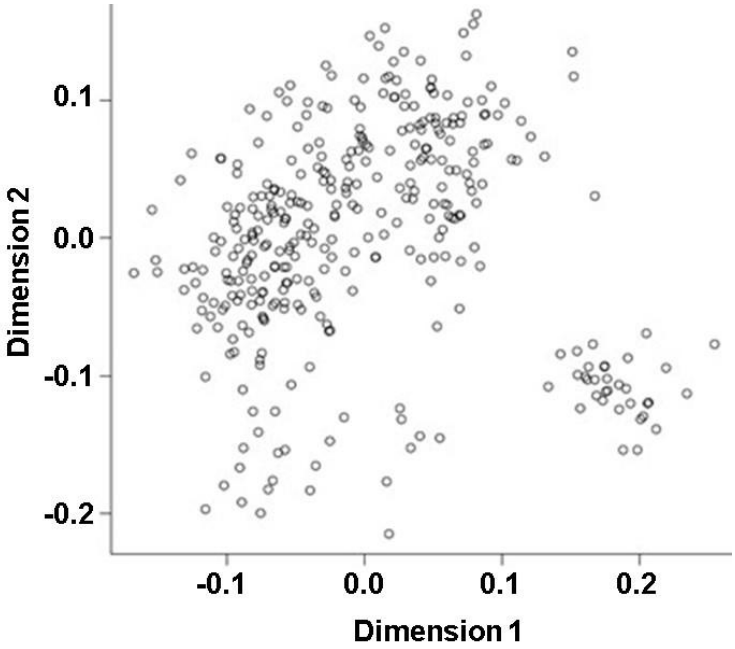


Figure 4.8. Multi-dimensional Scaling (MDS) display of the population of 360 lambs for 3,173 1C metabolism-related SNPs that passed QC.

4.3.9 Association analysis

Association analysis using GenABEL identified 47 SNPs that reached the suggested significance threshold (i.e. one false positive SNP out of all 3,173 SNPs) for the following seven metabolite traits: dimethylglycine (DMG), trimethylglycine (TMG), methyl-cobalamin (mB12), flavin adenine dinucleotide (B2) (FAD), pyridoxal (B6) (PL), propionic acid (PPA) and methylmalonic acid (MMA) (Table 4.10). One of these SNPs (dbrs408003853) reached the genome-wide significance threshold (P=0.0001) for its association with FAD. Manhattan plots of the results can be seen in Figure 4.9.

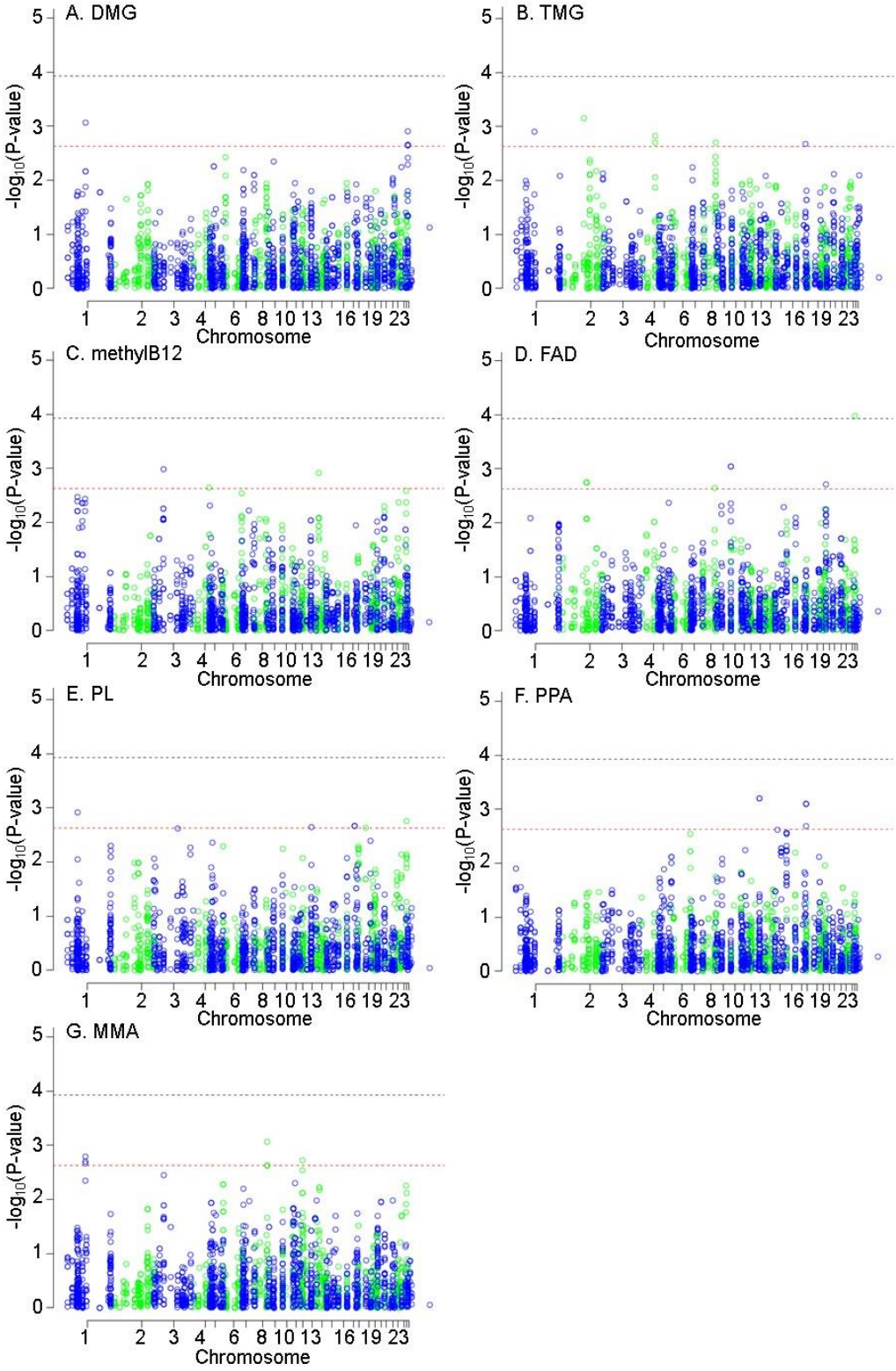


Figure 4.9. Manhattan plots for the GWAS of sheep liver 1C metabolites. Genomic location (horizontal axis) is plotted against $-\log_{10}$ (p-value); genome wide ($P<0.05$) and suggestive thresholds are shown as black (----) and red (---) coloured lines, respectively. Chromosomes are alternatively coloured blue and green. DMG (A), TMG (B), mB12 (C), FAD (D), PL (E), PPA (F), MMA (G)

Table 4.10. Significant SNPs identified in the association analysis using GenABEL

Metabolite	SNP	Chromosome	Position	P Value [a]
DMG	dbrs400381348	1	120209916	0.0009
	UoNOvar.1502	27	641201	0.0012
	dbrs421182980	27	622843	0.0022
	dbrs426885293	27	624671	0.0022
	dbrs401301776	27	624709	0.0022
	UoNOvar.1503	27	641212	0.0023
TMG	dbrs428658243	2	137750688	0.0007
	dbrs400381348	1	120209916	0.0013
	dbrs161801525	4	92644557	0.0015
	dbrs161923561	8	74706411	0.0020
	dbrs159794405	4	92644611	0.0020
	UoNOvar.439	17	62446221	0.0021
mB12	UoNOvar.919	3	57227259	0.0010
	UoNOvar.325	14	14071663	0.0012
	dbrs430165882	4	111638230	0.0022
FAD	dbrs408003853*	26	25493422	0.0001
	dbrs162034765	9	89665751	0.0009
	dbrs427234880	9	89669365	0.0009
	dbrs414970315	2	158702820	0.0018
	dbrs404243017	2	158702887	0.0018
	dbrs424867462	2	158703956	0.0018
	dbrs399509316	2	158704023	0.0018
	dbrs404706479	2	158704234	0.0018
	UoNOvar.764	21	5537402	0.0019
	dbrs423172086	8	73779282	0.0023
PL	dbrs401096164	1	76391726	0.0012
	dbrs408003853	26	25493422	0.0018
	dbrs406023352	17	53070682	0.0021
	dbrs162081923	17	53077690	0.0021
	dbrs402697947	17	53085110	0.0021
	dbrs406167325	13	32144910	0.0023
	dbrs417907151	18	66851941	0.0023
	dbrs161814674	3	137012638	0.0024
PPA	dbrs413573281	13	30417200	0.0006
	dbrs413234073	13	30417347	0.0006
	UoNOvar.460	17	72162062	0.0008
	UoNOvar.463	17	72165713	0.0008
	dbrs419604537	17	72167518	0.0008
	dbrs409777556	17	72076119	0.0021
	UoNOvar.380	15	28643171	0.0024
MMA	dbrs424129033	8	78545905	0.0009
	UoNOvar.94	1	120225428	0.0016
	dbrs162301008	12	37346009	0.0019
	dbrs419713876	1	120209909	0.0020
	dbrs410062706	1	120207769	0.0021
	dbrs399095809	8	78546752	0.0023
	dbrs415258627	8	78546371	0.0024

[a]The genome-wide significance threshold corresponded to a p value less than 1.19×10^{-6} and the suggestive significance threshold corresponded to a p value less than 2.38×10^{-3}. SNPs marked with an asterisk* are significant at the genome-wide threshold.

Summary statistics for significant SNPs associated with these seven traits, when fitted individually, are given in Table 4.11. Minor allele frequencies for all significant SNPs ranged from 0.04 to 0.49. The observed genotype frequencies are shown in Figure 4.10. Within the study population, there were no lambs with homozygous rare genotypes for SNP2 associated with DMG, SNP1 associated with TMG, SNPs 1 and 2 associated with mB12, SNPs 4, 5, 6, 7 and 8 associated with FAD and SNP6 associated with PPA. The effects of the significant SNPs explained 1.8 – 13.6 % of the phenotypic variance of the metabolite levels in the lamb livers. Metabolites with the most significant P-values were FAD (P=0.0001) and PPA (P=0.0006) for SNPs dbrs408003853 associated with FAD and dbrs413573281 and dbrs413234073 associated with PPA. The allele substitution effects of these SNPs were -0.0869 (P=0.0012) for FAD and -0.0542 (P=0.0561) for both SNPs associated with PPA. Interestingly, SNP dbrs408003853, found to have genome-wide significant association with FAD concentrations, was also found to be significantly associated with PL (P=0.0018). Of further interest, SNP dbrs400381348, was also found to be significantly associated with more than one metabolite, namely DMG (P=0.0009) and TMG (P=0.0013).

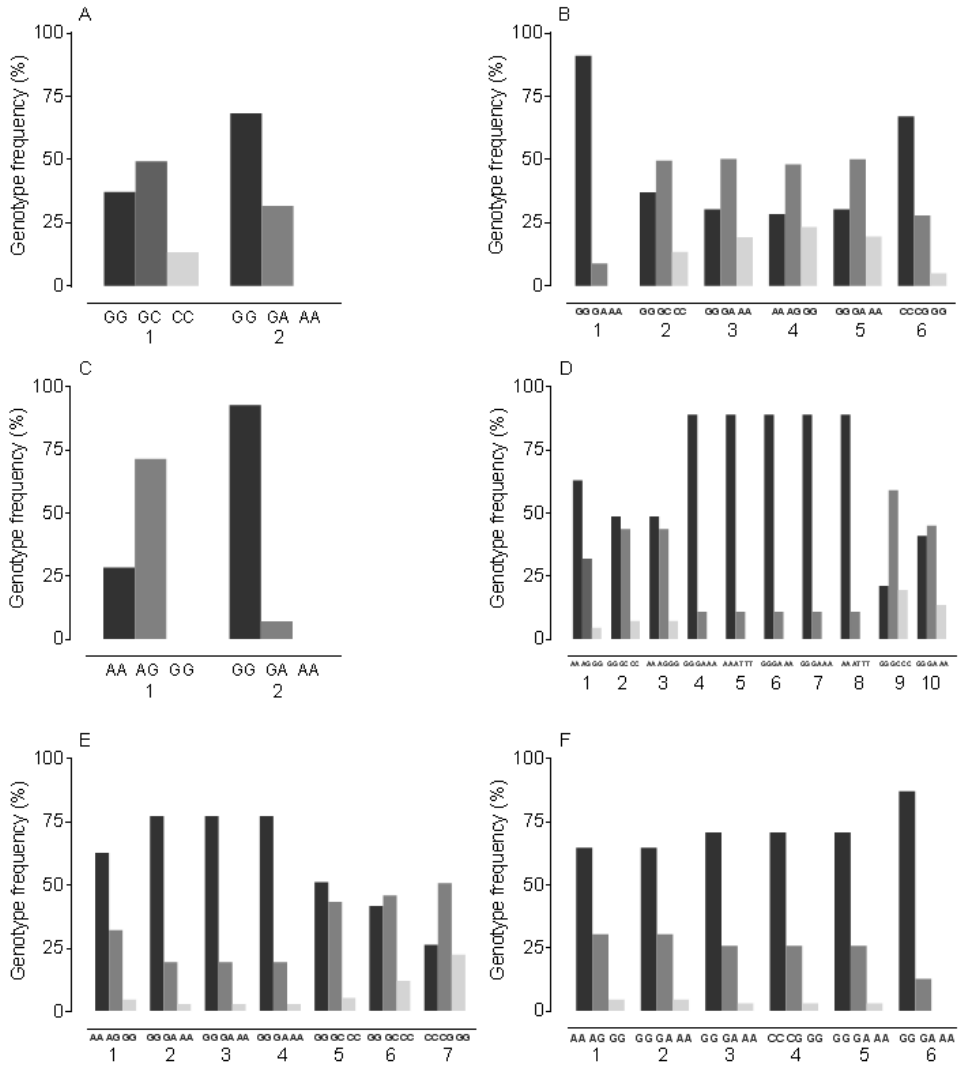


Figure 4.10. Observed genotype frequencies for significant SNPs associated with (A) DMG 1=dbrs400381348 2=UoNOvar.1503; (B) TMG 1=dbrs428658243 2=dbrs400381348 3=dbrs161801525 4=dbrs161923561 5=dbrs159794405 6=UoNOvar.439; (C) mB12 1=UoNOvar.325 2=dbrs430165882; (D) FAD 1=dbrs408003853 2=dbrs162034765 3=dbrs427234880 4=dbrs414970315 5=dbrs404243017 6=dbrs424867462 7=dbrs399509316 8=dbrs404706479 9=UoNOvar.764 10=dbrs423172086; (E) PL 1=dbrs408003853 2=dbrs406023352 3=dbrs162081923 4=dbrs402697947 5=dbrs406167325 6=dbrs417907151 7=dbrs161814674; (F) PPA 1=dbrs413573281 2=dbrs413234073 3=UoNOvar.460 4=UoNOvar.463 5=dbrs419604537 6=UoNOvar.380. Wild type is in black, heterozygous in dark grey and homozygous rare in light grey.

Table 4.11. Significant SNPs identified using ASReml for metabolite levels in sheep liver.

Metabolite	SNP	Location Chr (bp)	Gene	Variant	GWAS p-value	Additive effects (p-value)	Phenotypic (%) variance	p	q
DMG	dbrs400381348	(1)120209916	GART	5-prime UTR	0.0009	0.0647 (0.02)	1.8	0.62	0.38
	UoNOvar.1503	(27)641212	ASMTL	Upstream	0.0023	-0.0932 (0.006)	4.0	0.84	0.16
TMG	dbrs428658243	(2)137750688	GAD1	Upstream	0.0007	0.1156 (0.001)	3.8	0.96	0.04
	dbrs400381348	(1)120209916	GART	5-prime UTR	0.0013	0.0507 (0.005)	3.0	0.62	0.38
	dbrs161801525	(4)92644557	IMPDH1	synonymous	0.0015	-0.0458 (0.003)	3.4	0.56	0.44
	dbrs161923561	(8)74706411	MTHFD1L	synonymous	0.0020	-0.0423 (0.006)	2.9	0.53	0.47
	dbrs159794405	(4)92644611	IMPDH1	synonymous	0.0020	-0.0442 (0.004)	3.2	0.55	0.45
	UoNOvar.439	(17)62446221	COQ5	Upstream	0.0021	0.0504 (0.04)	2.8	0.81	0.19
mB12	UoNOvar.325	(14)14071663	DPEP1	3-prime UTR	0.0012	-0.1394 (0.003)	13.6	0.64	0.36
	dbrs430165882	(4)111638230	GCSH	Upstream	0.0022	-0.2375 (0.003)	6.0	0.96	0.04
FAD	dbrs408003853*	(26)25493422	ENSOARG 00000010451	3-prime UTR	0.0001	-0.0869 (0.001)	8.9	0.79	0.21
	dbrs162034765	(9)89665751	CA3	synonymous	0.0009	0.0696 (0.003)	13.4	0.71	0.29
	dbrs427234880	(9)89669365	CA3	synonymous	0.0009	0.0696 (0.003)	5.6	0.71	0.29
	dbrs414970315	(2)158702820	MMADHC	Upstream	0.0018	-0.1272 (0.002)	4.4	0.94	0.06
	dbrs404243017	(2)158702887	MMADHC	Upstream	0.0018	-0.1272 (0.002)	4.4	0.94	0.06
	dbrs424867462	(2)158703956	MMADHC	Upstream	0.0018	-0.1272 (0.002)	4.4	0.94	0.06
	dbrs399509316	(2)158704023	MMADHC	Upstream	0.0018	-0.1272 (0.002)	4.4	0.94	0.06
	dbrs404706479	(2)158704234	MMADHC	Upstream	0.0018	-0.1272 (0.002)	4.4	0.94	0.06
	UoNOvar.764	(21)5537402	NOX4	Upstream	0.0019	-0.0600 (0.003)	4.4	0.51	0.49
	dbrs423172086	(8)73779282	PCMT1	Upstream	0.0023	0.0492 (0.01)	2.2	0.64	0.36
PL	dbrs408003853	(26)25493422	ENSOARG 00000010451	3-prime UTR	0.0018	-0.0575 (0.01)	2.9	0.79	0.21
	dbrs406023352	(17)53070682	SETD1B	synonymous	0.0021	0.0713 (0.01)	5.7	0.87	0.13
	dbrs162081923	(17)53077690	SETD1B	synonymous	0.0021	0.0713 (0.01)	5.7	0.87	0.13
	dbrs402697947	(17)53085110	SETD1B	Upstream	0.0021	0.0713 (0.01)	5.7	0.87	0.13
	dbrs406167325	(13)32144910	NSUN6	Missense	0.0023	0.0545 (0.02)	4.6	0.73	0.27
	dbrs417907151	(18)66851941	TRMT61A	Missense	0.0023	-0.0414 (0.01)	2.5	0.65	0.35
	dbrs161814674	(3)137012638	KMT2D	synonymous	0.0024	-0.0439 (0.003)	3.6	0.52	0.48
PPA	dbrs413573281	(13)30417200	CUBN	synonymous	0.0006	-0.0542 (0.05)	4.8	0.80	0.20
	dbrs413234073	(13)30417347	CUBN	synonymous	0.0006	-0.0542 (0.05)	4.8	0.80	0.20
	UoNOvar.460	(17)72162062	TRMT2A	Missense	0.0008	-0.0756 (0.02)	5.5	0.84	0.16
	UoNOvar.463	(17)72165713	TRMT2A	Upstream	0.0008	-0.0756 (0.02)	5.5	0.84	0.16
	dbrs419604537	(17)72167518	TRMT2A	Upstream	0.0008	-0.0756 (0.02)	5.5	0.84	0.16
	UoNOvar.380	(13)30417200	KMT2A	missense	0.0024	-0.1033 (0.003)	3.6	0.94	0.06

All SNPs are significant at the suggestive threshold. SNPs marked with an asterisk* are significant at the genome-wide threshold. Additive effects are logged values and denote the increase or decrease in metabolite concentration with p values indicating the significance of the change. Phenotypic variance: % proportion of phenotypic variance explained by SNPs. p and q: allelic frequencies. Metabolic genes are highlighted in green. Epigenetic regulatory genes are highlighted in red.

4.4 Discussion

This study represents the first investigation of genetic influences on one-carbon (1C) metabolism in sheep. In the current study, sheep are the species of clinical interest for nutrient deficiencies. However, they also serve as an outbred model for humans, facilitating the study of nutrient by gene-variant interactions on health and development. Metabolites serve as good intermediate traits for genome-wide association studies (GWAS). Blood metabolites have the potential to be used to discover genetic determinants of intermediary metabolism.

Considerable inter-individual heterogeneity, in terms of metabolic responses to specific diets, are frequently observed in studies with outbred populations of animals such as sheep, as discussed in the introduction to this chapter. The current study therefore aimed to identify single nucleotide polymorphisms (SNPs) in genes involved in, or directly related to 1C metabolism that contribute to this inter-individual variability. 33 SNPs were found to be significantly associated with the concentration of 1C metabolites in the study population of 360 Texel lambs. Of these, 21 were within 1C metabolism genes and 12 were within epigenetic regulatory genes. The strongest association ($P<0.001$) was observed between the dbrs408003853 variant of ENSOARG00000010451 and concentrations of Flavin adenine dinucleotide (FAD); a form of vitamin B2. This association is also of biological interest as the gene (ENSOARG00000010451) in which this 3-prime UTR polymorphism resides, although uncharacterised, has been suggested to play a role in Flavin adenine dinucleotide binding [Source: UniProtKB; W5PL89_SHEEP]. Of further biological interest, polymorphisms dbrs413573281 and dbrs413234073 were shown to be associated with variations in propionate concentrations. These SNPs reside in the Cubilin (*CUBN*) gene which acts as a receptor for intrinsic factor-vitamin B12 complexes facilitating B12 uptake (Christensen et al., 2013). Whilst the other SNPs identified were associated statistically with 1C metabolites,

they were each less closely related to the specific metabolite, and so their biological significance is uncertain at this time.

The findings of this study contribute to a greater understanding of the role of inherited variation in blood metabolic diversity in sheep, providing a basis for further study into judicious targeted supplementation and selection of genetic tolerance to Co deficiency in breeding programmes. Furthermore, this study provides information on hundreds of single genotype-metabolite associations, greatly expanding knowledge of 1C metabolic intermediates.

4.4.1 SNP discovery

The study reported in this chapter is timely given the recent release of the sheep genome Oar v3.1 (Jiang et al., 2014) and ongoing annotation of this sequence. Full genome sequencing of 20 unrelated pure Texel ewes, carried out at Edinburgh Genomics, identified ~15 million SNPs of which ~3 million were novel. These SNPs have all been deposited into publically accessible databases (i.e. Ensembl), contributing to resources critical to the discovery and interpretation of the genetic underpinnings of essential biological processes, principally 1C metabolism.

4.4.2 Measurement of liver metabolites

One-carbon metabolism is an integrated series of metabolic pathways including the folate cycle, methionine re-methylation and transsulphuration pathways comprising numerous metabolites (Clare et al., 2019, in press). These metabolites span a variety of compound classes with significant differences in size and polarity, across a wide range of concentrations. As a consequence, no single analytical method is able to accommodate the chemical diversity of 1C related metabolites. For this reason, GCMS, HPLC and LC-MS/MS methods (described in Section 4.2) were developed during this study, to measure a total of 23 metabolites (Tables 4.5-4.7) known to play key roles in 1C metabolism. Methods for measurement of these metabolites in sheep liver samples have not previously been reported. The outcome of these analyses,

combined with genotypic analysis, has enabled the exploration of the causal determinants of varying metabolite levels, hypothesised to play a role in susceptibility to cobalt deficiency in sheep.

4.4.3 Selection of animals

Data from the National VIDA database revealed a prevalence of cobalt deficiency of around 7% (inter year range = 5-12%) in the national flock between July and September each year. With this in mind and the requirement for a larger proportion of Co deficient animals in the study population to enable assessment of the contribution of genetics on susceptibility to trace-element deficiencies, approximately one quarter of the study population was taken to the University farm at ~12 weeks of age to be fed a Co deficient diet. Plasma samples were taken on arrival at the farm and Co concentrations determined by ICP-MS. Whilst 28% (above national average) of the population were considered to be deficient in Co, the remaining 72% had marginal or sufficient Co concentrations, highlighting the need to make these animals deficient. Liver samples were taken from the remaining 277 lambs at slaughter and analysed for Co concentrations by ICP-MS. Results revealed that 19% of these lambs were considered to be Co deficient. This proportion of deficient animals is above the national average. Of these deficient animals, 64% were from farm 4, with a mean Co concentration of 0.017 mg/kg FW for this farm. When compared to mean plasma Co concentrations of 0.06, 0.06, 0.05 and 0.04 mg/kg FW for farms 1, 2, 3 and 5 respectively inter-farm differences ($P<0.002$) in Co levels are highlighted. With the 81% of the 277 lambs found to have marginal or sufficient liver cobalt concentrations, the addition of 83 Co deficient animals by feeding a controlled Co deficient diet increased the proportion of deficient lambs in the study to 37%. Whilst significant differences in Co concentrations were observed between farms, no such differences were observed between sexes. This is not surprising perhaps as lambs in this study were pre-pubertal and out of season. Furthermore, many of the male lambs had been castrated.

4.4.4 Dry matter intakes and lamb growth

Although not the primary focus of the current study, dry matter (DM) intakes and weights of the 83 lambs offered a Co deficient diet were recorded over 13 weeks, as a consequence of long-term Co deficiency is depressed appetite leading to anorexia (Suttle, 2010). Whilst DM intake (Figure 4.4) fluctuated over the 13 week period ($P<0.001$), there was no significant change from the start to the end of the study. It is likely that the diet-induced B12 deficiency was not sustained for long enough to have had a detrimental effect on the propionate-succinate pathway that can lead to loss of appetite (Kennedy et al., 1994; Smith and Marston, 1971; Farningham and Whyte, 1993). No differences in DM intakes were observed between lambs from different farms and variation in intakes between lambs over the course of the study remained constant, indicating that the acclimatisation to their new surroundings and to a concentrate diet for 4 weeks prior to feeding the Co deficient concentrate diet was successful.

Lamb weights were recorded fortnightly (Figure 4.5). Due to late arrival of lambs from farms 4, 5 and 6, mean lamb weight at the start of the study only incorporates data from farms 1, 2 and 3, and mean lamb weights from Day 14 onwards incorporates data for all 6 farms. As there was an effect of farm ($P<0.001$) on lamb weights, with farm 6 providing much lighter lambs than the other 5 farms, the decrease ($P<0.001$) in mean lamb weight from the start of the study (33.8 kg) until Day 14 (29.5 kg) can therefore be explained by this. From Day 14 to the end of the study, lamb weights increased ($P<0.001$) from 29.5 kg to 34.6 kg as would be expected with lambs of this age. The lack of a reduction in growth rate towards the end of the study indicates that, as was seen with DM intakes, B12 deficiency was not sustained long enough to impair lamb performance (Kennedy et al., 1992).

4.4.5 Co deficient diet end point

Criteria for the study endpoint were based on past publications. According to Suttle (2010) the sequence of pathophysiological changes in livestock given an inadequate mineral diet embraces the process of depletion, deficiency, dysfunction and ultimately disease. As the only metabolic role of Co is as a component of vitamin B12 (Smith et al., 2018), a reduction in Co intake from the diet decreases ruminal B12 synthesis, depleting blood B12 levels. Although concentrations of Co in liver and plasma can be used in the assessment of status (McDowell, 1992), non-functional Co can occur in various degrees of oxidation as well as a component of B12, leading to overestimation of B12 status. Measurement of serum B12 is therefore considered to be the cornerstone for determining B12 status in livestock, reflecting the amount of B12 being absorbed and indicating depletion of B12 stored in the blood (Suttle, 2010). A decline in liver B12 concentrations occurs later, and better reflects body reserves (Millar and Penrose, 1980). Decreased liver Co concentrations indicate that the animal has reached the stage of deprivation known as 'Co deficiency'. Animals can persist in this deficient state for an extended period before becoming dysfunctional. Depleted levels of B12 inhibit the B12 dependent enzyme methylmalonyl-CoA mutase in the propionate-succinate pathway, resulting in decreased rates of propionate clearance from the blood and accumulation of the intermediary metabolite MMA (Rice et al., 1989; Kennedy et al., 1991). B12 is also essential for the activity of methionine synthase in the conversion of Hcy to methionine. In Co deprived sheep, activity of this enzyme is also impaired and Hcy accumulates. The inhibition of key metabolic pathways, resulting in elevated concentrations of MMA and Hcy in plasma in the early stages of Co deficiency in sheep, precedes the onset of clinical signs of the disease (Rice et al., 1989). Concentrations of MMA and Hcy >14 µmol/l and >10 µmol/l respectively are now well recognised as accurate and specific indicators of a dysfunctional state of Co deprivation in sheep (Gruner, 2004; Rice et al., 1989; Clark et al., 1985). As it is still unclear whether B12 deficiency in sheep results

primarily from failure of the propionate-succinate pathway or the Hcy re-methylation pathway, measurement of both intermediary metabolites was undertaken in this study. As intended, lamb B12 status declined when they were fed the Co deficient diet. B12 concentrations fell from sufficient (>350 pmol/l) to deficient (<250 pmol/l) levels within 4 weeks and continued to fall for the following 6 weeks until concentrations reached the limit of detection of the analytical method. This decline in status was corroborated by the rise in Hcy over the duration of the study and the rise in MMA from Week 6. As mentioned previously, the state of 'deficiency' can persist for an extended period before entering a dysfunctional state. Therefore, it was decided that the experimental end point would be when serum B12 was within the deficient range for ≥6 weeks and also when there was a consistent increase in Hcy and MMA for ≥4 weeks.

4.4.6 Variance component analysis

Variance component methods have long been used in human quantitative genetics as well as in agricultural genetics and animal breeding (Almasy and Blangero, 2010). This method of analysis is designed for genetic analysis of continuously varying quantitative traits (Falconer and Mackay, 1996), hence justifying their use in the current study to analyse the strength of genetic effects on metabolite concentrations. Selection of covariates can have a large effect on the outcome of variance component analysis, and accounting for non-genetic sources of variation can magnify the genetic signal. Consequently, consideration must be given to the possibility that inclusion of certain covariates could account for genetic variance as well as environmental. In the current study, non-genetic sources of variation were accounted for by including sex (male and female), farm (location) and breed (Texel and Texel-cross) as covariates. As 1C metabolism is a cycle of metabolites which are all interlinked, it is possible that levels of one metabolite could 'normalise' the statistical signal of another. However, as all metabolite 'traits' are themselves genetically influenced, including metabolites used as covariates, this raises the possibility that one may correct not only for environmental variables but also for genetic factors. In the current study,

closely related metabolites were introduced as covariates to assess their potential effects on the association between individual SNPs and intermediary metabolites of primary interest. However, these covariates had no significant effect on the outcome of our analyses.

As variance component analysis assumes normal distribution of the data, metabolite concentrations were log transformed to normalise the distribution prior to further analyses. The distribution of the data was then determined by calculating the mean and variance of each metabolite. The source of this overall (phenotypic) variance (Vp) was divided into genetic (Vg) and environmental (Ve) components and genetic variance further sub-divided into additive (Va) and dominance (Vd) variance. Calculation of the heritability (h^2) of each trait (i.e. the extent to which metabolite concentrations were determined by genes transmitted from the parents) was calculated as the proportion of Vp attributable to Va (Falconer and Mackay, 1996). For 7 liver metabolites, it was found that estimated heritability explained >19% of inter-individual variation with a moderately high heritability estimate (0.50) for liver mB12 levels and moderate (0.19-0.24) heritability estimates for DMG, TMG, FAD, PL, PPA and MMA. All other metabolites analysed had very low heritability estimates with high standard errors (se). These results show that genetic differences have influenced the variation in mB12, DMG, TMG, FAD, PL, PPA and MMA concentrations between lambs in this study. Therefore, a more detailed investigation into the role of the genes and SNPs involved in the variation of these metabolite concentrations was subsequently undertaken.

Before analysis of genotype-metabolite associations was carried out, it was important to ascertain whether there were any confounding effects of a structured population. Some evidence of clustering was identified by Classical Multi-dimensional Scaling (MDS) (Borg and Groenen, 2005) (Figure 4.8). As these lambs originally came from 11 farms, it was inevitable that there would be some related ancestry. By performing principal component analysis (PCA), it was possible to account for the presence of population stratification prior to subsequent analysis.

4.4.7 Association analysis

Genome-wide significant associations of P <1.19 x 10^{-6} (corresponding to P <0.05 after Bonferroni correction for 421 independent loci) and P <2.38 x 10^{-3} (assuming one false-positive SNP out of all SNPs tested) were identified in association analyses for log-transformed liver DMG, TMG, mB12, FAD, PL, PPA and MMA concentrations. When fitted individually, 33 significant SNP-metabolite associations were identified, 21 of these were within 12 1C metabolic genes and 12 of the SNPs were within 8 epigenetic regulators. Some of these associations also appear to have significant biological importance.

4.4.7.1 PPA with dbrs413573281and dbrs413234073 in CUBN

CUBN gene encodes the protein cubilin, a multi-ligand hydrophobic protein which binds to intrinsic factor-cobalamin (Cbl-IF) complexes with high affinity facilitating B12 uptake. Variants of the *CUBN* gene have been shown in human studies to be associated with lower B12 levels due to decreased binding and transport of B12 in the ileum (Quadros, 2010; Kozyraki and Cases, 2013). This selective intestinal B12 malabsorption has been shown to be associated with megaloblastic anaemia (Aminoff et al., 1999; Gräsbeck, 2006). In the current study, when compared to wild-type genotypes, subjects with mutant genotypes for dbrs413573281 and dbrs413234073 polymorphisms in CUBN were found to have decreased liver propionate concentrations. B12 is an essential co-factor to methylmalonyl-CoA mutase (MUT; EC 5.4.99.2), involved in the metabolism of propionate in the liver, prior to gluconeogenesis (Ballhausen et al., 2009; De Vadder et al., 2016). The reduction in B12 transport associated with variants of this gene could, therefore, lead to reduced B12 availability for this reaction, and alter the conversion of propionate to succinate in the liver. With this in mind, the genetic association of SNPs in *CUBN* with varying propionate concentrations are biologically plausible. However, further research is required to gain a better understanding of the mechanism.

4.4.7.2 FAD and PL with dbrs408003853 in ENSOARG00000010451

The 3'UTR variant, dbrs408003853, of the ENSOARG00000010451 gene, localised on OAR26 between positions 25493375-25522452, was found to be associated with varying concentrations of flavin adenine dinucleotide (FAD) and pyridoxal (PL). The SNP was found to contribute 8.9% of the variance of FAD and just 2.9% of the variance of PL. Although uncharacterised, a suggested molecular function of this gene is flavin adenine dinucleotide binding [Source: UniProtKB; W5PL89_SHEEP]. As this is only a suggested function of this protein, it is not possible to confirm with certainty whether the genetic association of this SNP with FAD concentrations in sheep liver has any biological significance. However, confirmation of the function of this gene would merit further investigation into the association. No direct relationship between the SNP and PL are known.

4.4.7.3 methylB12 with UoNOvar.325 in DPEP1

The primary function of Dipeptidase 1 (DPEP1; EC 3.4.13.19) is to hydrolyse various dipeptides in renal metabolism, specifically the potent antioxidant; glutathione (Kozak and Tate, 1982). In the current study, when compared to the wild-type individuals (AA), the UoNOvar.325 heterozygous (AG) genotype subjects possessed lower liver concentrations of methylB12. A biological relationship between methylB12 and DPEP1 has not previously been recognised, however, could possibly be explained by the relationship between glutathione and methylB12 (Watson et al., 2004). It has previously been suggested that excess glutathione reacts with B12 to form glutathionylcobalamin which could play a role in the biochemistry of B12-dependent processes such as methionine biosynthesis (Xia et al., 2004).

4.4.7.4 methylB12 with dbrs430165882 in GCSH

Glycine, the simplest amino acid composed of only 2 carbons and an amino group, is a precursor for the synthesis of purines, proteins, glutathione, and 1C units in 1C metabolism (Shuvalov et al., 2017).

Degradation of glycine to produce ammonia, carbon dioxide and a carbon unit for the methylation of THF to methyleneTHF is brought about in a reversible reaction catalysed by the mitochondrial glycine cleavage system (GCS) (Kikuchi et al., 2008). The GCS is composed of four mitochondrial protein components: P-protein (a pyridoxal phosphate-dependent glycine decarboxylase), T-protein (a tetrahydrofolate-requiring amino-methyltransferase), L-protein (a lipoamide dehydrogenase) and H-protein (a lipoic acid-containing protein) (Douce et al., 2001). The protein encoded by the GCSH gene, found to be genetically associated with methylB12 concentrations in this study, is the H protein. This protein interacts with the three other GCS proteins and is responsible for transferring the methylamine group of glycine from the P protein to the T protein. In the 1C metabolic pathway (Figure 4.12), methyleneTHF is positioned at the metabolic intersection whereby folate is either directed towards nucleotide synthesis (i.e. purines and thymidylate) or toward methylation reactions. MethyleneTHF is therefore closely associated with the regeneration of methionine from Hcy; a reaction which requires methylB12 as a cofactor. It has previously been implied that under normal conditions, GCS produces methyleneTHF at a rate of ~20 times that required for Hcy re-methylation (Lamers, 2009). In the current study populations there were no homozygous AA (mutant) genotypes. However, compared to wild-type individuals (GG), subjects with heterozygous genotype (GA) possessed lower liver concentrations of methylB12. This observation poses the question of whether the upstream variant (dbrs430165882) of GCSH could be biologically related to fluctuations in methylB12 through its association with Hcy remethylation and requires further research.

4.4.7.5 DMG and TMG with dbrs400381348 in GART

Although found to be associated (P=0.0009) with varying concentrations of DMG and TMG in the current study, the protein encoded by the GART gene (EC 2.1.2.2); phosphoribosylglycinamide formyltransferase, does not have any known direct biological relationship with these 2 metabolites. GART is a trifunctional polypeptide required for *de novo*

purine biosynthesis. Inosine-monophosphate (IMP) is the central intermediate for generating purines (Figure 4.11). Generation of IMP from phosphoribosyl-pyrophosphate (PRPP) uses various multifunctional enzymes (Shuvalov et al., 2017). Reactions catalysed by GART provide C2 and C8 atoms for purine (adenine and guanine) ring synthesis using 10-fTHF as a 1C donor.

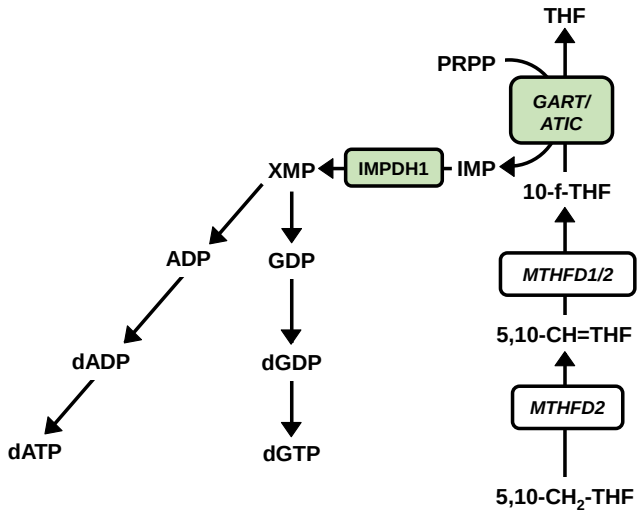


Figure 4.11. (Adapted from Clare et al., 2019, in press) Purine nucleotide biosynthesis. Enzymes are in boxes. Key below.

Purine nucleotide biosynthesis enzymes			
GART	phosphoribosylglycinamide formyltransferase		
IMPD1	Inosine Monophosphate Dehydrogenase 1		
ATIC	phosphoribosylaminoimidazole-carboxamide formyltransferase		
MTHFD1/2	methylene-tetrahydrofolate dehydrogenase		
Substrates			
THF	tetrahydrofolate	GDP	guanosine diphosphate
10-fTHF	10-formyltetrahydrofolate	dGDP	deoxyguanosine diphosphate
5,10-CH=THF	5,10-methenyltetrahydrofolate	dGTP	deoxyguanosine triphosphate
5,10-CH_2THF	5,10-methylenetetrahydrofolate	ADP	adenosine diphosphate
PRPP	phosphoribosyl pyrophosphate	dADP	deoxyadenosine diphosphate
IMP	Inosine monophosphate	dATP	deoxyadenosine triphosphate
XMP	xanthine monophosphate		

An oversimplified description of the function of 1C metabolism is that it can be perceived as serving two critically important functions: biological re-methylation and the synthesis of nucleotides. Whilst GART is involved in purine synthesis, TMG (Betaine) serves as a methyl donor (becoming DMG) in the reaction converting Hcy to Methionine, catalysed by the

enzyme betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5). With the complexity of 1C metabolic pathways, many other enzymes are involved in regulating the stages of the pathway between 10-fTHF and, TMG and DMG. Therefore, whilst the dbrs400381348 polymorphism in GART has been found to be genetically associated with TMG and DMG concentrations in the sheep livers, the biological significance of this association is uncertain.

4.4.7.6 TMG with dbrs161923561 in MTHFD1L

A gene encoding for an enzyme involved in folate-mediated 1C metabolism, shown to have genetic association with TMG concentrations in sheep liver in the current study, is methylenetetrahydrofolate dehydrogenase 1 Like (MTHFD1L; EC 6.3.4.3). Folate-mediated 1C metabolism is highly compartmentalised in eukaryotes, with THF-dependent enzymes found in the mitochondria, cytoplasm and nuclei (Stover, 2009). Mitochondrial 1C metabolism converts 1C donors such as serine and glycine to formate, which is exported and incorporated into the cytoplasmic 1C pool to provide 1C units for cytoplasmic processes such as de novo purine biosynthesis and the methyl cycle. The interconversion 1C derivatives of THF involves sequential 5, 10-methylenetetrahydrofolate dehydrogenase, 5, 10-methenyl tetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase activities. The MTHFD1L gene product; 10-formyl-THF synthetase, is a mono-functional enzyme which catalyses the final step in the mammalian mitochondrial pathway converting 10-fTHF to THF and formate (Figure 4.12) (Prasannan et al., 2003). Whilst the methionine cycle and folate pathways are closely related, there is no known direct relationship between MTHFD1L and TMG.

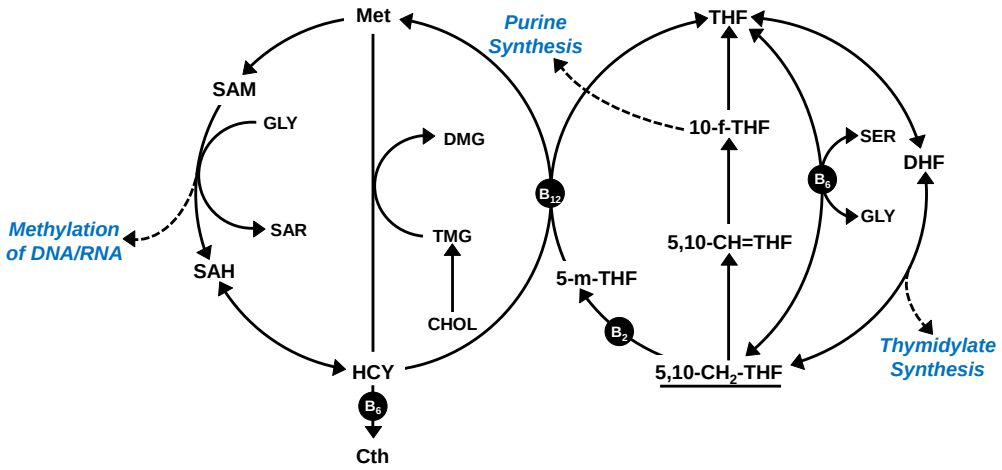


Figure 4.12. Simplified depiction of one-carbon metabolism (adapted from Clare et al., 2019, in press). Enzymes are in boxes. Key below. Methylene tetrahydrofolate is underlined highlighting its position at the metabolic intersection whereby folate is either directed towards nucleotide synthesis (i.e. purines and thymidylate) or toward methylation reactions.

Substrates			
Met	Methionine		
SAM	S-adenosylmethionine	THF	tetrahydrofolate
SAH	S-adenosylhomocysteine	DHF	dihydrofolate
HCY	homocysteine	SER	serine
Cth	cystathionine	5,10-CH_2THF	5,10-methylenetetrahydrofolate
DMG	dimethylglycine	5-mTHF	5-methyltetrahydrofolate
TMG	trimethylglycine	5,10-CH=THF	5,10-methenyltetrahydrofolate
GLY	glycine	10-fTHF	10-formyltetrahydrofolate
SAR	sarcosine	CHOL	choline

4.4.7.7 TMG with dbrs428658243 in GAD1

Glutamate Decarboxylase (GAD1; EC 4.1.1.15) catalyses the decarboxylation of glutamate (involved in the transsulfuration pathway of 1C metabolism) to GABA, the chief inhibitory neurotransmitter in the mammalian central nervous system (Roberts, 1974; Krnjevic, 2004). TMG, also known as betaine, is an important methyl donor found principally in the liver (Obeid, 2013). The biological relationship between betaine and GABA is not well understood. However, it has recently been suggested that cellular uptake of GABA is mediated by the same sodium- and chloride-dependent transporter as betaine; betaine/GABA transporter (BGT1) (Kempson et al., 2014). Physiological roles of BGT1 are still being debated and most studies are concerned with its function

and regulation in the kidney medulla. In the mouse, hepatocytes express high levels of both BGT1 and betaine homocysteine-S-methyltransferase (BHMT1) (Zhou, 2012; Finkelstein et al., 1971). Thus, liver BGT1 is ideally localized for importing betaine from the circulation for hepatocyte metabolism (Craig, 2004; Burg and Ferraris, 2008). The contribution of BGT1 in the accumulation of betaine in the liver, however, has not yet been determined. The association between GAD1 and betaine through BGT1 is speculative, and would require further investigation.

4.4.7.8 Other variant associations

Another gene found to be associated with varying concentrations of TMG in this study was Inosine Monophosphate Dehydrogenase 1 (IMPDH1; EC 1.1.1.205). This gene is involved in purine biosynthesis (Figure 4.11). However, once again, the biological significance of the relationship with TMG is uncertain.

Coenzyme Q5 Methyltransferase (COQ5; EC 2.1.1.201) was also found to be associated with TMG concentrations. COQ5 is involved in the biosynthesis of ubiquinone. No direct biological associations between COQ5 and TMG have previously been reported.

Carbonic Anhydrase 3 (CA3; EC 4.2.1.1) is a zinc-containing enzyme that catalyses the reversible hydration of carbon dioxide (Lindskog, 1997). Although found to be associated with FAD concentrations in the current study there is no known direct biological association.

Methylmalonic Aciduria and Homocysteinuria, CblD Type (MMADHC) gene encodes for a protein involved in the early stages of B12 metabolism (Froese et al., 2015). Polymorphisms in this gene can result in decreased levels of adenosylcobalamin and methylcobalamin, and consequently increased MMA and Hcy. With this is mind, it was expected that associations between SNPs in this gene and MMA, Hcy, AdoB12 or MeB12 would be observed in this study. This was not the case and 5

polymorphisms in this gene were found to be genetically associated with FAD. There is no known direct biological explanation for this.

NOX4 (EC 1.6.3) gene encodes a member of the NOX-family of enzymes that function as the catalytic subunit of the widely expressed NAPDH- and oxygen dependent oxidoreductase complexes, involved in immune response (Nisimoto et al., 2014). Although found to be associated in the current study, NOX4 and FAD have no known direct biological association.

4.4.7.9 Epigenetic regulators

In the current study, 12 associations between epigenetic regulatory genes and 1C metabolites were observed (Table 4.11). As the primary aim of this study was to assess inter-individual variability in 1C metabolites in relation to 1C metabolic genes, the effects of these epigenetic regulatory genes was beyond the scope of the immediate study and hence are only briefly discussed below.

An upstream variant of acetylserotonin O-methyltransferase Like (ASMTL), was found to be associated with DMG, contributing to 4% of the variation of this metabolite. O-methyltransferases (OMTs) constitute a large family of enzymes that facilitate the transfer of a methyl group to the oxygen atom of a variety of secondary metabolites (Lam et al., 2007). As with all of the methyltransferases mentioned below, SAM is utilised as a methyl donor by this methyltransferase and is converted to SAH. Another O-methyltransferase is protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1). In the current study, an upstream variant of this gene was found to be associated with FAD, accounting for 2.2% of the variation observed. Six gene variants were identified as having an association with PL. These included 2 synonymous and 1 upstream variant of SET domain containing 1B (SETD1B), a missense variant of NOP2/sun domain 6 (NSUN6) and TRMT61A, and a synonymous variant of KMT2D. SET1B and KMT2D encode proteins that are components of histone methyltransferase complexes that produce trimethylated histone

H3 at Lys4 (Lee et al., 2007; Lv et al., 2018). NSUN6 and TRMT61A are RNA methyltransferases, playing important roles in RNA stability, splicing, and epigenetic mechanisms (Schapira, 2016). Another tRNA methyltransferase gene, TRNA Methyltransferase 2 Homolog A (TRMT2A), was also shown to be associated with varying PPA concentration in sheep liver. Furthermore, another variant of KMT2D was also found to be associated with PPA.

4.5 Conclusions, implications and future research priorities

In the current study, 33 SNPs in genes involved in or directly related to 1C metabolism were found to be significantly associated with inter-individual variability in concentrations of 1C metabolites in the study population of 360 Texel lambs. Of these SNPs, 21 were within 1C metabolism genes and 12 were within epigenetic regulatory genes. Whilst it has not been possible to determine a definitive list of 'low-risk' and 'at-risk' allele variant combinations for susceptibility to B12 deficiency, several of the identified SNP associations appear to have significant biological importance. One of the most important interactions identified was that of CUBN with PPA. CUBN encodes the protein cubilin, a multi-ligand hydrophobic protein which binds to intrinsic factor-cobalamin (Cbl-IF) complexes with high affinity facilitating B12 uptake. Involved in B12 transport, this gene is fundamental in the provision of B12 for the methionine cycle and propionate pathway. Assessing these associations at a whole animal level is a benefit of this study, highlighting intuitive adaptive responses to limited B12 availability in the diet. The change in PPA levels as a result of altered B12 transport is perhaps an example of such an adaptation. Another biologically interesting association, which was also the strongest association identified, was that of the ENSOARG00000010451 dbrs408003853 polymorphism, which significantly decreased liver concentrations of FAD. Interestingly, although this gene is uncharacterised, the resulting protein has been suggested to have FAD binding activity. Confirmation of this function and of the significance of the association of ENSOARG00000010451 with FAD requires further investigation. Other associations were less directly

biologically associated and confirmation of the speculative relationships discussed in this chapter also require further investigation. It was anticipated that some key 1C genes such as MTHFR, MTR and BHMT would be significantly associated with their directly related metabolites such as SAM, methionine and Hcy. The lack of these findings could be due to the study population size being too small, or a lack of a large enough proportion of Co deficient animals within the population.

The data derived in this study is valuable in gaining a better understanding of genetic determinants of circulating metabolites and how genetic variation influences metabolism. As the only comprehensive investigation of genetic influences on 1C metabolism in sheep to date this study also provides a pipeline of analysis of these genetically influenced variations. Furthermore, not only is the sheep a major livestock animal, but it is also a species of clinical interest and an excellent model organism for human genetic studies. Future research priorities should be focussed on confirmation and validation of the functional significance of SNPs identified in the current study.

CHAPTER FIVE

Inter-breed variability in the regulation of one-carbon metabolism in sheep

5.1 Introduction

The effects of polymorphisms in genes functionally related to one-carbon (1C) metabolism have been extensively studied in human populations and have been shown to be associated with a variety of complex traits and disorders. Some examples are given in Chapter 1, Section 1.9. The genotype frequencies of some of these polymorphisms in human populations are known to vary substantially across different geographical regions and ethnic groups, and to also be affected by nutritional differences. A notable example is the MTHFR C677T polymorphism; a common alteration which has been shown to increase the risk of neural-tube defects and vascular dementia in humans, particularly in the presence of impaired folate status (van der Put et al., 1997; Yoo et al., 2000; Ueland et al., 2001; Liew et al., 2015). The frequency of the MTHFR 677T allele is often reported to be low in African populations, intermediate in Europe and high in Asian and Central and Southern American populations (Wilcken et al., 2003). There is also evidence to suggest geographical gradients among Chinese Han populations (north to south decrease) (Yang et al., 2013). No such studies have been conducted with Sheep.

Through their domestication and subsequent selection, sheep have adapted to thrive in a diverse range of environments. As a consequence, susceptibility to trace-element deficiencies have been altered. An example of such effects can been seen in relation to copper. Sheep, in general, are intolerant of dietary copper excess due to their impaired ability to excrete copper in bile, and consequently liver copper accumulation with ensuing toxicity is well recognised. Texel sheep have been shown to be highly susceptible to Cu accumulation in the liver. Intermediate risk breeds include Dorset and Suffolk, and the most resistant breeds include Romanov and Finnish Landrace sheep (van der Berg et al., 1983; Littledike et al., 1993). The North Ronaldsay breed are particularly copper-sensitive (Simpson et al., 2006). This genotype of sheep and their cross-breeds absorb more dietary copper than other

breeds of domesticated sheep, indicating that this trait is heritable (Wiener et al., 1977). Genetic factors must ultimately underlie these heritable inter-breed differences in responses to copper, and it is likely that there are also breed differences in response to other trace-elements. Whilst no studies have been carried out to assess sheep breed differences for other trace-elements, significant effects of breed on serum copper and selenium levels in goats have previously been reported (Gopalakrishnan et al., 2011).

Every farm has different nutritional challenges. Therefore, characterisation of genetic diversity between breeds in relation to trace-element deficiencies (i.e. cobalt) is a key aspect of developing sustainable breed improvement strategies (Groeneveld et al., 2010). Genotyping for susceptibility traits for trace-element deficiencies would allow farmers to select animals that suit their specific situation, and elucidating their genetic diversity would be beneficial in improving breeding and management strategies.

As the only known function of cobalt (Co) in sheep is as a component of vitamin B12, alterations to B12 dependent pathways (1C metabolism and propionate metabolism) can alter the susceptibility of sheep to Co deficiency. The possibility of having a better understanding of the inter-breed variability in distribution of polymorphisms within 1C related genes, and the frequency of functionally significant SNPs between and within these breeds, would aid the evaluation of dietary requirements and supplementation strategies and provide a basis for the selection of genetic tolerance to trace-element deficiencies in breeding programmes. Furthermore, following the study carried out in Chapter 4 with Texel sheep, comparison of breed differences could highlight alternative breeds for use in successive GWAS studies to identify different or additional nutrient-gene interactions. It would be useful to ascertain whether, in addition to the small proportion of deficient animals used, selection of the Texel breed in Chapter 4 contributed to the lack of biologically significant interactions identified.

The study reported in this chapter, therefore, aimed to investigate the population differentiation of 4 commercially successful breeds in the UK; Texel, Suffolk, Bluefaced Leicester and Swaledale, for SNPs in 1C related genes, with specific focus on the 33 functionally significant SNPs identified in Chapter 4.

5.2 Materials and methods

5.2.1 Animals, treatments and sample collection

Nasal swab samples were taken from 269 pure-bred unrelated sheep of 4 breeds using a Performagene™ DNA collection kit (DNA Genotek, Inc). The 4 breeds included; Texel (n=79), Suffolk (n=61), Bluefaced Leicester (n=58), and Swaledale (n=70). Texel was selected because of its popularity as a terminal sire in the UK and because of the use of this breed in Chapter 4. Suffolk, another commonly used terminal sire, was selected as a native British Breed. One of the most commercially successful breeds and now one of the most prolific lowland sheep, the North of England Mule, is produced by crossing the Swaledale with the Bluefaced Leicester, hence these 2 breeds were also selected for this study.

Following collection, DNA was immediately stabilised by mixing with the Performagene™ solution. Nasal swabs were kept at ambient temperature prior to analysis. DNA was extracted from a 0.5 ml aliquot of the Performagene™ nasal samples according to the DNA Genotek protocol for manual purification of DNA (PD-PR-083), as follows. Samples were vortexed and then incubated at 50°C for 2 h. After removal of the nasal swab sponge, 500 µl sample was transferred to a microcentrifuge tube. To this, 20 µl of PG-L2P purifier was added, vortexed and incubated on ice for 10 min to remove impurities. The sample was centrifuged at 15000 x g at room temperature for 15 min and the supernatant transferred to a microcentrifuge tube and centrifuged again at 15000 x g for 5 min. The supernatant was transferred to a microcentrifuge tube, 25 µl 5M NaCl added and inverted 10 times. 600 µl 100% ethanol was added, inverted 10 times and left at room temperature for 10 min. The sample was then centrifuged at 15000 x g at room temperature for 2 min and the supernatant discarded. The DNA pellet was washed with 250 µl 70% ethanol and then re-dissolved in 500 µl TE (10 mM Tris-HCl, 10 µM EDTA, pH 8.0) buffer, and left at room

temperature overnight. RNA was removed by double RNase digestion as follows. To the 500 µl dissolved DNA in TE buffer, 5 µl Ribonuclease A (1 mg/ml) and 5 µl Ribonulcease T1 (6000 U/ml) was added and incubated for 30 min at 37°C. 10 µl NaCl (5 M) and 1 ml Ethanol (95%) was added, mixed well and incubated at room temperature for 10 min. DNA precipitates were collected by centrifugation at 13000 x g for 2 min at room temperature. The supernatant was discarded and DNA pellet re-dissolved in 500 µl TE buffer (10 mM Tris-HCl, 10 µM EDTA, pH 8.0).

5.2.2 SNP genotyping and quality control

All DNA samples were genotyped at Edinburgh Genomics using the Illumina Infinium® iSelect® Custom Array for 4576 1C related single-nucleotide polymorphisms (SNPs), using the methods described in Chapter 4, Section 4.2.2. Quality control measures were applied to retain SNPs with a minor allele frequency (MAF) greater than 0.01 and call rates greater than 0.90, after which 4104 SNPs remained for further analysis.

5.2.3 K-means clustering

Initially, SNPs were pruned using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink) and those in linkage disequilibrium, with r^2>0.1, were identified and dropped from further analyses. r^2 reflects the extent to which a particular allele at one locus is inherited together with a particular allele at another locus. Windows of 50 kb were used and an overlap of 10 kb allowed. Following this, 3052 SNPs remained. K-means clustering was performed using the software 'Admixture' (http://www.genetics.ucla.edu/software/admixture) and the cluster output plotted using STRUCTURE software in R to confirm the 4 distinct breed populations. Admixture software is a tool for maximum likelihood estimation of the number of individual ancestral populations underlying the genetic diversity. K-means clustering allows the assignment of individuals based on their genetic similarities. K = 3, 4, 5, 6, 7, 8 and 9 were tested, where k is the number of populations. A cross-validation (CV error) value was recorded for each test to identify the optimum population number (Figure 5.1). Lower CVs are optimum. K = 4

produced the lowest CV value of 0.48, and the 4 breeds were clearly differentiated. The sheep breeds and numbers of samples within each are given in Table 5.1. This analysis was conducted at the University of Edinburgh.

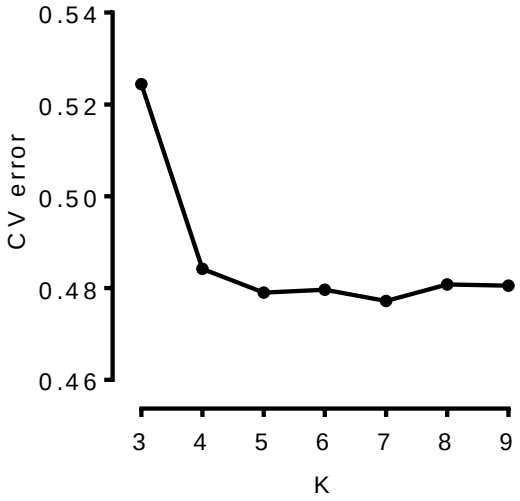


Figure 5.1. Determination of K using CV error

Table 5.1. Sheep breeds and numbers used in the population genetics analysis

Breed	Number
Texel	79
Swaledale	70
Bluefaced Leicester	58
Suffolk	61

5.2.4 Phylogenetic analysis

A phylogenetic tree was used to further confirm the clusters and to illustrate genetic distances between individuals. This methodology allows for the construction of dendrograms that show the genetic similarity among individuals with no assumptions concerning previously established populations. Using the genotypic data, a distance matrix was

created using Plink (https://www.cog-genomics.org/plink/1.9/distance) which gave pair-wise distances between samples. Using R (http://www.r-project.org), hierarchical clustering was applied using the complete linkage method (also known as furthest neighbour or maximum method) to find the maximum distance between points belonging to two different clusters. The resulting data was then used to plot a dendrogram using the Figtree software (http://tree.bio.ed.ac.uk/software/figtree/).

5.2.5 Pairwise fixation index (F_{ST})

Another strategy for extracting information from individual genotypes is the analysis of inter-individual distances based on the proportion of shared alleles. Allele frequencies were calculated in the R environment (http://www.r-project.org), and pairwise fixation index (F_{ST}) between the 4 breeds (Texel, Suffolk, Swaledale and BFL), was calculated using the Wright's F_{ST} estimator (Wright, 1921). A cumulative value of the relative marker positions by chromosome was used as the genomic value. F_{ST} values were calculated in sliding windows of 5 SNPs. The upper 1% and 5% were arbitrarily chosen as thresholds.

The fixation index (F_{ST}) was developed by Wright (1921) as a tool for describing the partitioning of genetic diversity within and among populations. F_{ST} accounts for the effect of inbreeding within samples, the quantity of which is defined in terms of a correlation coefficient. The general formulae is $F_{ST} = \frac{\sigma_S^2}{\sigma_T^2} = \frac{\sigma_S^2}{p(1-p)}$, where p is the average frequency of an allele in the total population, σ^2_S is the variance in the frequency of alleles in different sub-populations and σ^2_T is the variance of allele frequency in the total population. The F_{ST} is therefore defined as the 'correlation between two alleles chosen at random within subpopulations relative to alleles in the total population' (Wright, 1951; 1965).

5.2.6 Statistical analyses

Genotype frequencies for the 33 significant SNPs identified in Chapter 4 were determined for the 4 breeds (Texel, Suffolk, Swaledale and Bluefaced Leicester) using Statistical Analysis Software (SAS).

5.3 Results and discussion

5.3.1 K means clustering

Figure 5.2 illustrates population structure provided by STRUCTURE software, assuming 4 hypothetical populations (K=4) (based on Figure 5.1). K-means clustering analysis was used to confirm the allocation of samples to the 4 breeds, by partitioning the 268 samples into clusters in which each observation belongs to the cluster with the nearest mean. K=4 resulted in the optimum fit, with 4 breeds clearly differentiated, confirming the correct allocation of the samples. The assignment of individuals to groups in STRUCTURE methods, is based on their genetic similarities, thereby providing information about the number of ancestral populations underlying the observed genetic diversity (Grasso et al., 2014). Indeed, some features of the three native British breeds were present in the Texel cluster, however, were with low ancestry coefficient.

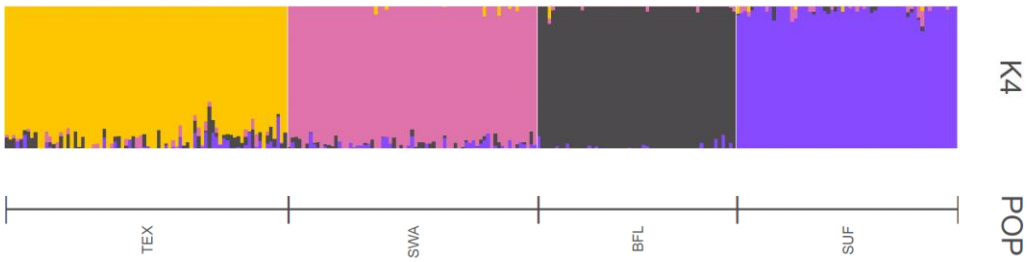


Figure 5.2. K means clustering of the 4 sheep breeds. Individuals are presented as vertical lines divided into K colours, representing constructed populations. K4 = 4 populations; POP, population; TEX, Texel; SWA, Swaledale; BFL, Bluefaced Leicester; SUF, Suffolk.

5.3.2 Phylogenetic tree

The phylogenetic tree (Figure 5.3), used to resolve the population genetic structure, illustrates the similarities and differences in 1C genetic characteristics between the 4 breeds, and highlights the clustering by breed. Each joining (fusion) of two clusters is represented on the graph by the splitting of a horizontal line into two horizontal lines. The horizontal position of the split, shown by the short vertical bar, gives the distance

(dissimilarity) between the two clusters. Four well defined clusters can be identified by four branches occurring at similar horizontal distances. Sheep within each breed were relatively homogeneous, however, breed differences are clear cut and some differentiation of the Texel sheep from the other 3 native British breeds can be seen. If this analysis were to be carried out using the commercially available Ovine 50k SNP chip which contains 54,241 SNPs that are uniformly widespread across the whole Ovine genome, even greater breed differences could be expected. However, as the SNPs on the custom array were in genes specific to 1C metabolic pathways, involved in vital cellular processes which are essential to growth or development, it is perhaps unsurprising that they are relatively similar.

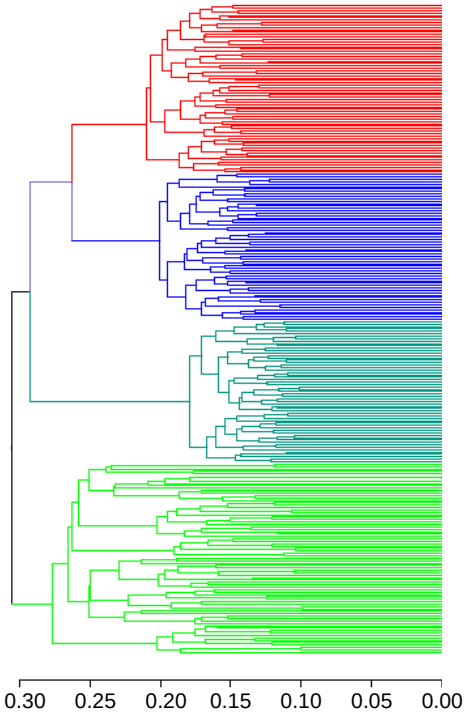


Figure 5.3. Phylogenetic relationship of sheep from 4 breeds; Texel (Green), Suffolk (Blue), BFL (Turquoise) and Swaledale (Red). The unrooted tree was constructed using Figtree software as described in the methods. The horizontal axis represents the distance or dissimilarity between clusters. Each line on the vertical axis represents a sample.

5.3.3 Pairwise F_{ST}

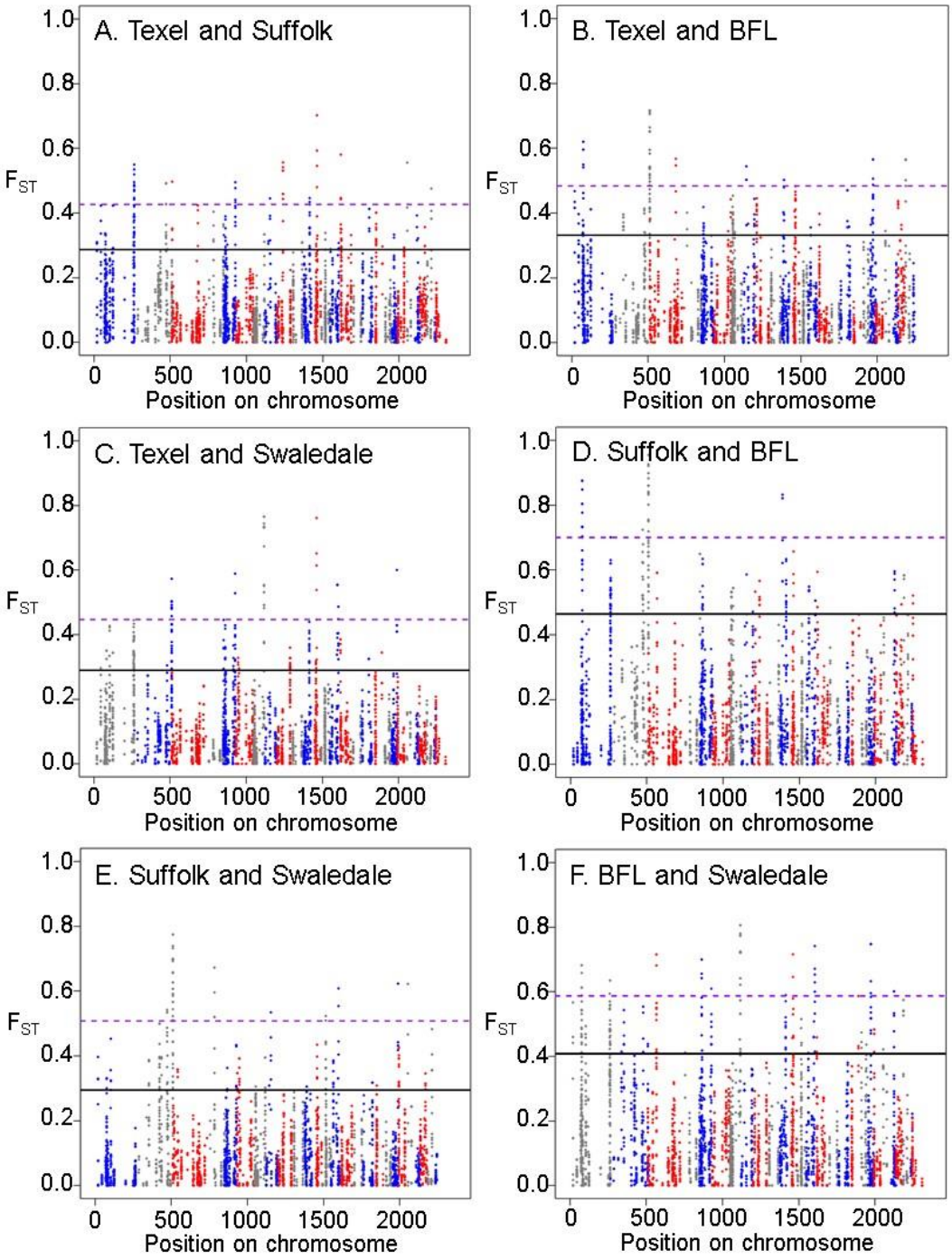


Figure 5.4. Manhattan plots of the pairwise genome-wide autosomal F_{ST} analyses. Black threshold lines represent the 95% quantile and dashed purple lines represents 99% quantile of the F_{ST} distribution.alternate colours represent chromosomes.

Pairwise F_{ST} breed comparison results are presented in Figure 5.4 as Manhattan plots. These Manhattan plots can be used to identify regions of the genome that have been the target of selection and that have resulted in population becoming genetically differentiated over time. F_{ST} is directly related to the variance in allele frequency and, conversely, to the degree of resemblance among individuals within populations. This statistic can therefore be defined as the proportion of genetic diversity due to allele frequency differences among populations. When considering 2 subpopulations (i.e. pairwise), the F_{ST} value will be 0 if the allele frequencies within each population are identical and 1 if allele frequencies within the two subpopulations are totally different. Hence, the larger the F_{ST} value, the more different the two populations are. If natural selection favours one allele over the others at a particular locus in some populations, the F_{ST} at that locus will be larger than loci in which among-population differences are purely a result of genetic drift (random fluctuations in allele frequencies over time that are due to chance alone). SNPs falling into the 99^{th} percentile F_{ST} window were considered to be significant and were viewed as candidates of 1C differentiation between breeds (Appendix 1 Tables 1-6). There were some biologically interesting genes within this window, as discussed below. Whilst some of the SNP variants were only found to be significantly different in one pairwise breed comparison, some were identified in more than one comparison. These are depicted in Table 5.2, sub-sectioned into those identified in 4 breed comparisons, 3 breed comparisons or 2 breed comparisons.

5.3.3.1 Key findings from breed comparisons

Table 5.2 provides a precis of information detailed in Appendix 1 Tables 1-6, and highlights breed differences for variants in key 1C genes which appeared in more than one breed comparison. An overview of the key finding is provided below, followed by a more general discussion of these findings.

Texel vs BFL

Highly differentiated regions were identified on chromosomes 2, 20 and 25. Amongst other genes, these regions contain genes encoding two B12 dependent enzymes; methionine synthase (MTR) and methylmalonyl-CoA mutase (MUT). The former is involved in folate/methionine metabolism and the latter in propionate metabolism. Whilst some variants in MUT also differed between Swaledale and BFL, variants in MTR did not differ between the other breeds. Located on chromosome 2, numerous variants in aldehyde dehydrogenase 4 family member A1 (ALDH4A1) differed between Texel and BFL as well as several others,

Texel vs Suffolk

Highly differentiated regions were found on chromosomes 12 and 25. Amongst other genes, these regions contained (methylene tetrahydrofolate reductase (*MTHFR*) and methionine adenosyltransferase 1A (*MAT1A*), involved in the folate and methionine cycles respectively. MTHFR also differed in the comparisons of Texel with Swaledales and BFL with Swaledales.

Texel vs Swaledale

Highly differentiated regions were identified on chromosomes 2, 7, 12. Amongst other genes, these regions contained methylenetetrahydrofolate dehydrogenase (*MTHFD1*), methylene tetrahydrofolate reductase (*MTHFR*) involved in the folate cycle, and aldehyde dehydrogenase 4 family member A1 (*ALDH4A1*), which was also found in several other breed comparisons.

Suffolk vs BFL

Highly differentiated regions were identified on chromosome 2. Variants in *ALDH4A1* located on chromosome 2 were of particular interest due to their presence in the results of several other pairwise comparisons.

Table 5.2. SNPs in metabolic genes appearing in more than one pairwise F_{ST} analysis. Breed comparisons in which each SNP was identified as being significantly different between breeds are given. From top to bottom, sub-sectioned in grey, SNPs were found to differ between breeds in 4, 3 and 2 of the 6 breed comparisons analysed.

4 Comparisons			
Gene	**SNP (variant)**		**Breed comparisons**
ALDH4A1	dbrs398747325 (5' UTR) dbrs398315711 (upstream) dbrs399876942 (synonymous) dbrs414019897 (5'UTR)	dbrs421014314 (missense) dbrs425592173 (synonymous) dbrs426161601 (upstream) UoNOvar.594 (synonymous)	TEX vs BFL TEX vs SWA SUFF vs BFL SUFF vs SWA
3 Comparisons			
Gene	**SNP (variant)**		**Breed comparisons**
ALDH4A1	dbrs404102797 (upstream) dbrs405220014 (upstream) dbrs426517658 (upstream)	dbrs426859693 (upstream) dbrs427880280 (upstream)	TEX vs BFL TEX vs SWA SUFF vs SWA
	dbrs405910531 (upstream) dbrs408384461 (upstream) dbrs408465439 (upstream)	dbrs418449012 (upstream) dbrs429989460 (upstream)	TEX vs BFL SUFF vs BFL SUFF vs SWA
MTHFR	dbrs160775144 (synonymous)	dbrs160775146 (missense)	TEX vs SUFF TEX vs SWA BFL vs SWA
SLC19A1	dbrs415564973 (upstream)		TEX vs SUFF SUFF vs BFL BFL vs SWA
2 Comparisons			
Gene	**SNP (variant)**		**Breed comparisons**
ALDH4A1	dbrs413017578 (upstream)	UoNOvar.592 (upstream)	TEX vs BFL SUFF vs SWA
	dbrs404058123 (upstream) dbrs425688145 (upstream)	dbrs415575413 (upstream)	SUFF vs BFL SUFF vs SWA
MTHFD1	dbrs417369843 (upstream) dbrs413879483 (upstream) dbrs414517250 (upstream) dbrs419191996 (upstream) dbrs420883701 (upstream) dbrs424495782 (upstream)	dbrs428708652 (upstream) dbrs430066170 (upstream) dbrs398475224 (upstream) dbrs402994374 (upstream) dbrs413194343 (upstream)	TEX vs SWA BFL vs SWA
DPH5	dbrs417887819 (upstream) dbrs418648546 (upstream) dbrs161767028 (upstream) dbrs404927371 (upstream)	dbrs406781512 (upstream) dbrs411081297 (upstream) dbrs421429336 (upstream)	SUFF vs BFL BFL vs SWA
MUT	dbrs162200463 (upstream) dbrs418774463 (upstream)	dbrs411838155 (upstream)	TEX vs BFL BFL vs SWA
MTHFR	dbrs416523788 (upstream)	dbrs402145672 (missense)	TEX vs SUFF TEX vs SWA
TPMT	dbrs403061814 (3'UTR)		TEX vs SWA SUFF vs SWA
GOT1	dbrs405881066 (upstream)		TEX vs SUFF SUFF vs SWA
ENSOARG 00000014803	dbrs398777693 (upstream)		TEX vs SWA BFL vs SWA
AGL	dbrs401096164 (upstream)		TEX vs BFL BFL vs SWA

TEX, Texel; SUFF, Suffolk; SWA, Swaledale; BFL, Bluefaced Leicester

Suffolk vs Swaledale

The comparison of Suffolks with Swaledales also highlighted breed differences for variants in *ALDH4A1*, as seen in several other breed comparisons.

BFL vs Swaledale

Highly differentiated regions were evident on chromosomes 7, 12 and 20. Amongst other genes, these regions contained MTHFD1, MUT, MTHFR. MTHFD1 was also found to differ between Texel and Swaledale, MUT was also observed to differ between BFL and Texel and MTHFR was different between Texel and Suffolk, and Texel and Swaledale.

5.3.3.2 Summary of breed comparisons

When Texels were compared to Suffolks, there were highly differentiated regions on chromosomes 12 and 25. Amongst other genes, these regions contained *MTHFR* and *MAT1A* which are of particular biological interest. F_{ST} values for these differentiations were high, ranging between 0.442 and 0.702. MTHFR is a key enzyme in 1C metabolism, facilitating the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a reaction required for the multistep process of converting Hcy to methionine. MAT1A is essential for the subsequent conversion of methionine to SAM, the universal methyl donor. The allele frequency of the upstream gene variant of MAT1A was different only between these two breeds. Conversely, two variants of *MTHFR* (one synonymous, the other missense), found to be different between Texels and Suffolk, were also different between Texel and Swaledale, and BFL and Swaledale. Whilst the synonymous variant (dbrs160775144) does not change the encoded amino acid, the missense variant (dbrs160775146) causes a non-synonymous substitution, resulting in a change in the amino acid sequence. In human studies, the missense 677 C>T allele variant of the MTHFR gene has been a focus of increasing interest (Wilcken et al., 2003; Shane et al., 2018; Huang et al., 2018; Del Gobbo et al., 2018). This variant of the gene has been linked with a range

of conditions, including adverse birth outcomes (including birth defects), pregnancy complications, cancers and cardiovascular diseases (Ueland et al., 2001; Turgal et al., 2018; Gonzales et al., 2018). Whilst some research has been found to be inconclusive or conflicting, there has been recent interest in exploring the geographical and ethnic distribution of the T allele and associated genotypes. The frequency of the homozygous TT genotype has been found to be high in Asian and Central and Southern American populations, moderate in European populations and low in populations of African ancestry. Whilst the two MTHFR variants found to be variable between breeds in the current study are not the 677 C>T variant, the strong evidence for breed variation in this missense variant suggests that dbrs160775146 in particular could have an effect on interbreed differences in metabolic outcomes. Some ethnic differences have also been observed for variants of MAT2A and MAT2B isotopes of the MAT gene in human populations (Nordgren et al., 2011), however, these are much less extensively studied and no ethnic/breed differences for the MAT1A isotope have previously been reported.

In the comparison of Texels and Swaledale, as well as variants in the *MTHFR* gene located on chromosome 12, chromosomes 2 and 7 were found to have highly differentiated regions. Chromosome 7 contains the *MTHFD1* gene of particular biological interest. *MTHFD1* encodes a protein with three distinct enzymatic activities; 5, 10-methylenetetrahydrofolate dehydrogenase, 5, 10-methenyl tetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase. Each of these activities catalyses one of three sequential reactions in the interconversion of 1C derivatives of THF which are substrates for methionine, thymidylate, and de novo purine syntheses. 11 upstream gene variants of *MTHFD1* were also found to be different between BFL and Swaledale. Whilst these 11 SNPs were not in the top 1% of F_{ST} values in the comparison of Suffolks with Swaldales, the F_{ST} values were still moderately high, ranging between 0.201 and 0.395. Cumulatively, this indicates that Swaledales are different from the other 3 breeds. Similar to the *MTHFR* 677 C>T variant, ethnic variation has

been observed in human studies with the *MTHFD1* 1958 G>A variant (Binia et al., 2014). This gene variant has been found to be associated with birth defects (such as Orofacial clefts) and pregnancy complications (Wu et al., 2015; Parle-McDermott et al., 2005). Similar to the *MTHFR* 677 C>T variant, *MTHFD1* 1958 G>A has been shown to vary across different geographical regions and ethnic groups. The risk-associated genotype (AA) has been found in particularly high frequency in Indigenous American populations, and much less frequently in people of Asian and African ancestry (Binia et al., 2014). Swaledale sheep mostly inhabit the more mountainous areas of Great Britain, particularly the Yorkshire Dales from where they originate, whilst BFL are an upland breed and Suffolks and Texels are lowland breeds. These differences in habitats could have implications for genetic adaptations leading to breed differences in *MTHFD1* gene variants.

When comparing genotype frequencies between Texels and BFL, highly differentiated regions were identified on chromosomes 2, 20 and 25. Amongst other genes, these regions contained *MTR* and *MUT* of particular biological interest. *MTR* codes for the B12 dependent enzyme; methionine synthase, essential for the transfer of a methyl group from methylB12 to Hcy in the synthesis of methionine. *MUT* encodes for another B12 dependent enzyme; methylmalonyl-CoA mutase, responsible for the conversion of methylmalonyl CoA to succinate in the propionate pathway. BFL and Swaledales were also highly differentiated for SNPs in the *MUT* gene, with F_{ST} values of 0.63 to 0.75. Furthermore, although not in the top 1% of F_{ST} scores, BFL and Suffolk were also moderately different for the same variants in *MUT* (F_{ST} 0.26 to 0.33). This suggests that BFL are different from the other 3 breeds for these *MUT* variants. Defects in the *MUT* gene cause methylmalonic aciduria, clinically manifesting as lethargy, respiratory distress, recurrent vomiting, dehydration, and hypotonia (Zwickler et al., 2008). For several variants of this gene, a North-South gradient has been observed in Chinese populations (Liu et al., 2012). With this in mind, it seems plausible that

breed differences for variants in this gene may occur due to stratification of the UK sheep industry.

The two *MTR* variants found to differ between Texels and BFL were splice region variants and were not different between the other breeds. In human populations, variants of *MTR* have been shown to be associated with increased risk of prostate cancer, coronary heart disease, Neurotubular defects and orofacial clefts. (Qu et al., 2016; Zhao et al., 2014; Cao et al., 2018; Mostowska et al., 2006). These associated risks have been studies in different ethnic populations, however, unlike with some of the other genes mentioned above, no inter-ethnic variation has been reported.

Whilst not one of the core genes of interest, *ALDH4A1* on chromosome 2 occurred with high F_{ST} values in the comparisons of Texel with BFL, Texel with Swaledale, Suffolks with BFL, and Suffolks with Swaledales. This gene encodes an enzyme which catalyses the second step of the proline degradation pathway, converting pyrroline-5-carboxylate to glutamate. The majority of the SNPs identified were upstream gene variants.

5.3.3.3 33 SNPs identified in Chapter 4

None of the 33 SNPs found to be significant in Chapter 4 had FST values within the 99th percentile, and the majority had FST<0.25 (Appendix 2, Table 1). The *CUBN* gene variants, found to be significantly associated with varying concentrations of PPA, were the most biologically interesting of all 33 variants. When comparing frequencies of the two CUBN variants (Table 5.3), low to moderate genetic differentiation was observed (FST = 0.01-0.28) between breeds. Swaledale and BFL had the greatest differentiation, with FST = 0.28 and 0.26 respectively for the two CUBN variants, closely followed by Texel and Swaledale (FST = 0.21 for both) and the Suffolk and Swaldale (FST = 0.13 and 0.14 respectively). This indicated that Swaledales are the most differentiated from the other three breeds for the two SNPs in *CUBN*. The greatest similarities were seen

between Texel and BFL (FST = 0.03 and 0.01), Texel and Suffolk (FST = 0.05 for both), and Suffolk and BFL (FST = 0.08 and 0.09). In the UK, the sheep industry is set out in a 'stratified system'. This unique system plays on the strengths of the different breeds of sheep in different environments, dividing breeds into three tiers: hill, upland and lowland. Swaledale sheep are a typical hill breed, adapted to living in the harsh hill conditions, whilst BFL are an upland breed, and Texel and Suffolk are lowland breeds. Genetic differences in population structure and variation detected in allele frequencies, could be explained by the effects of differences in environment and habitat on selection.

5.3.4 Genotype frequencies of SNPs identified in Chapter 4

Figures 5.5 – 5.10 show the respective genotype frequencies of the four sheep breeds for the 33 SNPs identified in Chapter 4 as being functionally associated with varying concentrations of 1C metabolites. Although some genotype frequencies appear to differ greatly between breeds, F_{ST} values for these gene variants in the pairwise F_{ST} analyses were moderate to low. For purposes of continuity in the following sections, homozygous wild-type are denoted as AA, heterozygous as AB, and homozygous mutants as BB.

5.3.4.1 Variants associated with PPA

Proportions within the four breed populations of the different variant genotypes found to be associated with PPA are illustrated in Figure 5.5. For the two *CUBN* variants, no BFLs had the BB genotype; instead, 90% were AA and 10% AB. Conversely, the other three breed populations were composed of all three genotypes, though these differed in proportions. As identified in the pairwise F_{ST} analyses in Section 5.3.3.1, the frequency of the variant genotypes in the Swaledale population differed from the other breeds. The B allele was the most frequent in this population, resulting in over half of Swaledales being BB, and only 6% AA. For all other SNPs found to be associated with PPA in Chapter 4, 100% of BFL had the AA genotype. BB genotypes in the other breeds were very low frequency or not present at all.

Table 5.3. Genetic differentiation for SNPs dbrs413573281 and dbrs413234073 in *CUBN* between breeds, expressed as the fixation index (F_{ST}; Weir and Cockerham, 1984)

dbrs413573281 CUBN	F_{ST}			
	Texel	Suffolk	Swaledale	BFL
Texel	0			
Suffolk	0.05	0		
Swaledale	0.21	0.13	0	
BFL	0.03	0.08	0.28	0

dbrs413234073 CUBN	F_{ST}			
	Texel	Suffolk	Swaledale	BFL
Texel	0			
Suffolk	0.05	0		
Swaledale	0.21	0.14	0	
BFL	0.01	0.09	0.26	0

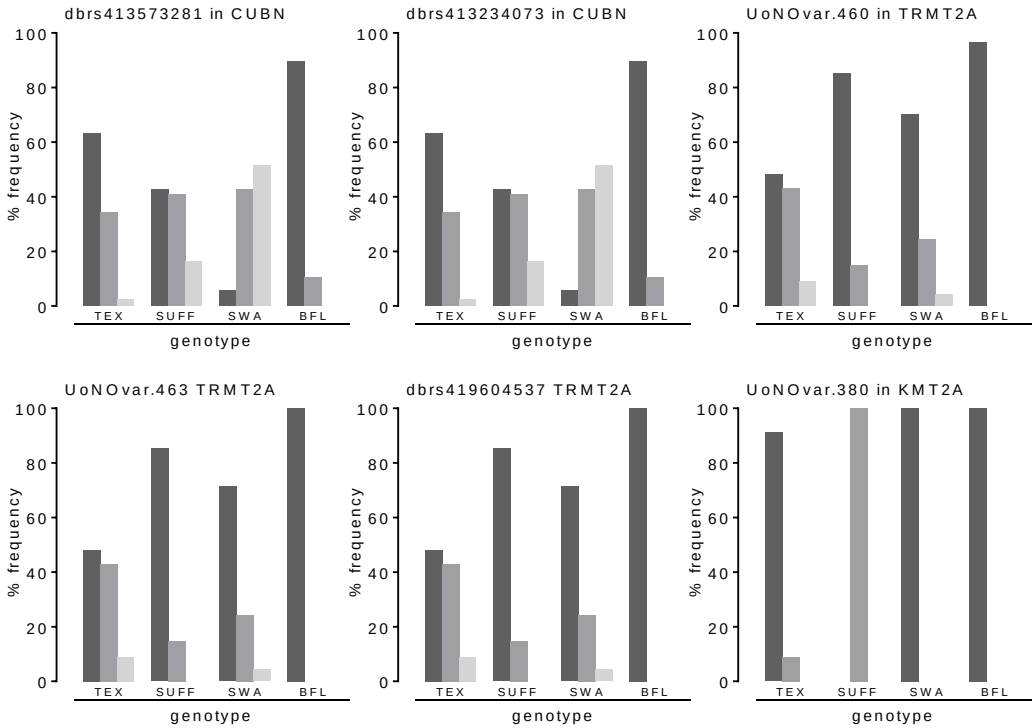


Figure 5.5. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 6 SNPs (dbrs413573281, dbrs413234073, UoNOvar.460, UoNOvar.463, dbrs419604537, UoNOvar.380) identified in Chapter 4 to be associated with varying concentrations of PPA in sheep liver in 4 sheep breeds.

5.3.4.2 Variants associated with DMG

The results for the SNPs associated with DMG (Figure 5.6) showed that for the dbrs400381348 SNP in *GART*, the A allele was the most frequent and, consequently, in Suffolk, Swaledale and BFL sheep, the AA genotype was the most frequent genotype. In Texels, the AB genotype dominated. The BB genotype was only identified in Texel and Swaledale sheep, and was present in a much larger percentage of the Texel than the Swaledale population (30% and 1% respectively). No breeds had the BB genotype for the UoNOvar.1503 variant of *ASMTL*, suggesting that the mutant form of this variant could be very unfavourable or possibly lethal. AA and AB genotypes were distributed similarly amongst the four breeds.

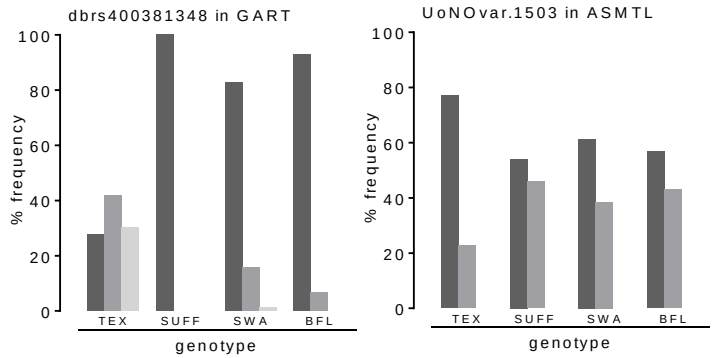


Figure 5.6. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 2 SNPs (dbrs400381348 and UoNOvar.1503) identified in Chapter 4 to be associated with varying concentrations of DMG in sheep liver in 4 sheep breeds; TEX, Texel; SUFF, Suffolk; SWA, Swaledale; BFL, Bluefaced Leicester.

5.3.4.3 Variants associated with TMG

Frequencies of SNPs associated with TMG (Figure 5.7) varied between breeds. For the *GAD1* dbrs428658243 SNP, Swaledale, Suffolk and BFL populations were 100% AA genotype, whilst 87% of the Texel population were AA and the remaining 13% were AB. No animals were of the BB genotype. Rodent studies suggest that *GAD1* is important in maintaining GABA levels in the brain, and that *GAD1* knockout is usually lethal (Asada et al., 1996). As no sheep (regardless of breed) with the mutant

genotype were present in the total study population, it is possible that the BB genotype is lethal in sheep as well. Three genotypes for the *IMPDH1* dbrs161801525 and dbrs159794405 SNPs were fairly evenly distributed between and within the 4 breeds. The BB mutant genotype of dbrs161923561 in *MTHFD1L* was present in 95% of the BFL population; the highest of all four breeds. No BFL sheep had the AA genotype. Conversely, the AA genotype for this SNP was present in 33% of the Texel sheep population. Only 22% of Texels had the BB genotype; the lowest proportion of all four breeds. Similar to the BFL, the frequency of the BB genotype in Suffolk and Swaledale populations was high. The BB variant of UoNOvar.439 in *COQ5* was only present in a small proportion of Texel, Suffolk and Swaledale sheep and was not present in BFL. Whilst the BFL population was 100% AA, the remaining three breed populations contained all three genotypes.

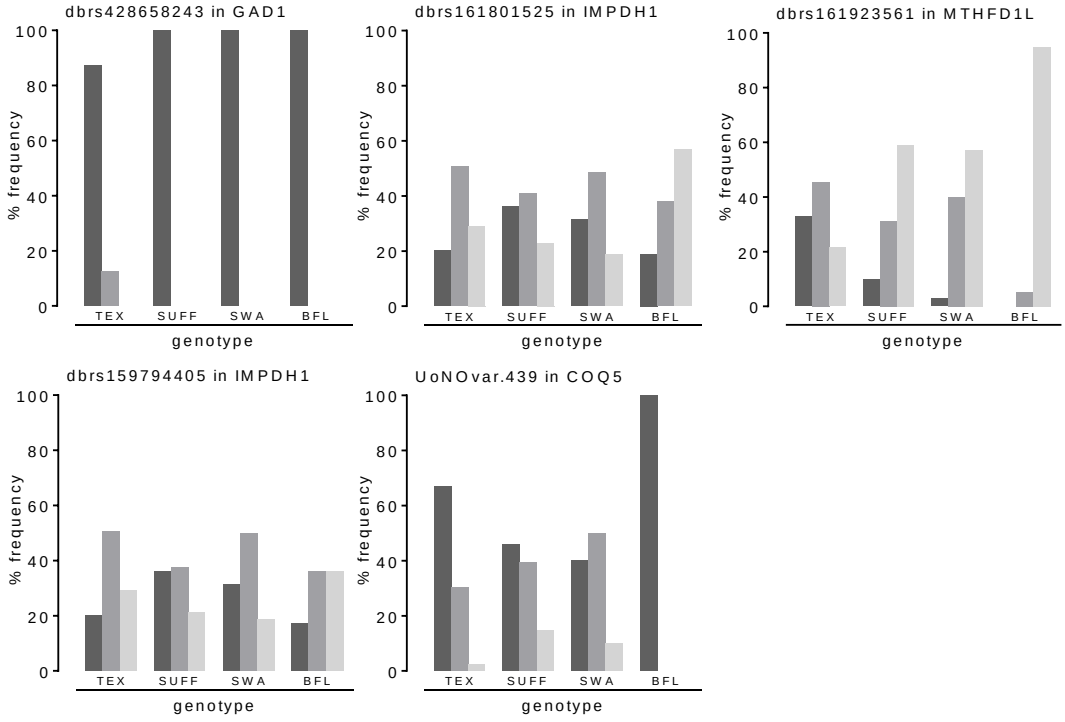


Figure 5.7. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 5 SNPs (dbrs428658243, dbrs161801525, dbrs161923561, dbrs159794405, UoNOvar.439) identified in Chapter 4 to be associated with varying concentrations of TMG in sheep liver in 4 sheep breeds.

5.3.4.4 Variants associated with methylB12

With regard to SNPs in Chapter 4 associated with methylB12 (Figure 5.8), BFL sheep differed from the other three breeds. With respect to both the *DPEP1* and *GCSH* gene variants, 100% of the BFL population were AA genotype. The AA genotype of *GCSH* also made up the largest proportion of the other three breeds. Suffolk sheep were 100% heterozygous (AB) for the *DPEP1* SNP, with a large proportion of Texels and Swaledales also having this genotype. No BB genotypes were identified.

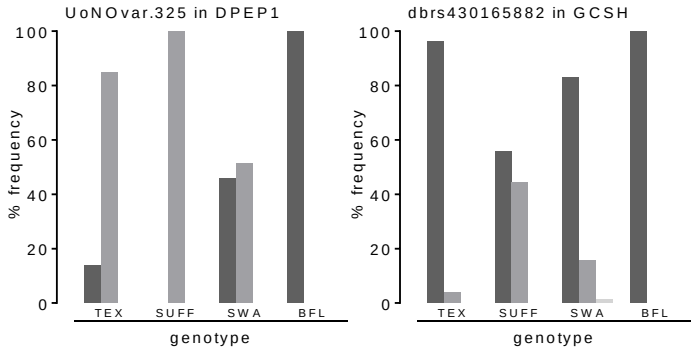


Figure 5.8. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 2 SNPs (UoNOvar.325, dbrs430165882) identified in Chapter 4 to be associated with varying concentrations of MethylB12 in sheep liver in 4 sheep breeds.

5.3.4.5 Variants associated with FAD

For all variants associated with FAD (Figure 5.9), the A allele was once again predominant. A high proportion or all BFL sheep had AA genotypes. On the other hand, the other three breeds displayed a more even distribution of genotypes. For the dbrs408003853 variant in ENSOARG00000010451, which is likely to have some direct biological relationship with FAD, the three genotype frequencies were similarly distributed among Texel, Suffolk and Swaledale sheep; with a low frequency of the BB genotype. BFL was different, however, with 100% AA genotype and no AB or BB.

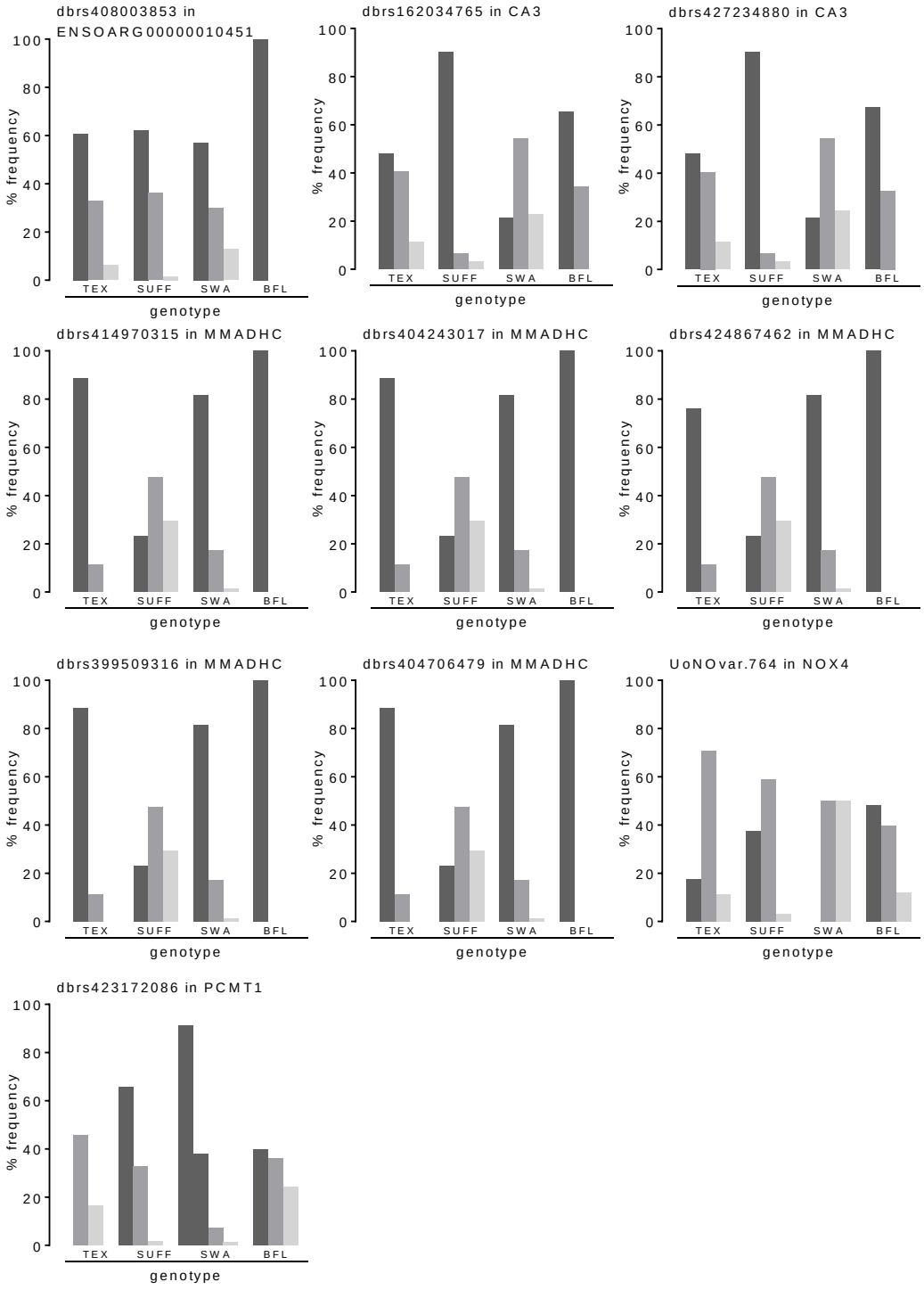


Figure 5.9. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 10 SNPs (dbrs408003853, dbrs162034765, dbrs427234880, dbrs414970315, dbrs404243017, dbrs424867462, dbrs399509316, dbrs40470679, UoNOvar.764, dbrs423172086) identified in Chapter 4 to be associated with varying concentrations of FAD in sheep liver in 4 sheep breeds.

5.3.4.6 Variants associated with PL

As seen with several variants associated with other 1C related metabolites, BFL sheep genotype frequencies in SNPs associated with PL appeared to differ when compared to the other sheep breeds (Figure 5.10). For the three *SETD1B* variants, 100% of BFL sheep had the AA genotype. This was also the predominant genotype for these SNPs in the other three breeds. Frequencies of the three genotypes for the remaining SNPs were more evenly distributed between the breeds, but again, the B allele was much less frequent than the A allele in most cases.

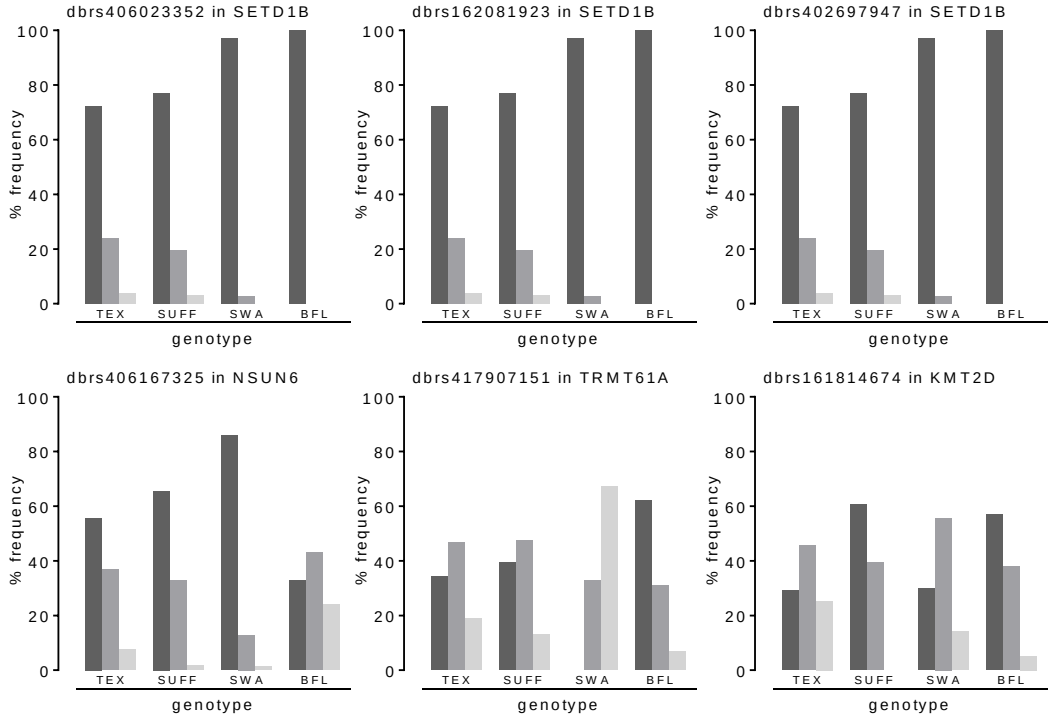


Figure 5.10. Frequency of homozygous wild type (dark grey), heterozygous (mid grey) and homozygous mutant (light grey) genotypes for the 6 SNPs (dbrs406023352, dbrs162081923, dbrs402697947, dbrs406167325, dbrs417907151, dbrs161814674) identified in Chapter 4 to be associated with varying concentrations of PL in sheep liver in 4 sheep breeds.

5.4 Summary of main findings

Whilst the phylogenetic analysis in the current study appeared clear-cut, indicating that Texels differ from the three native British breeds, the genetic effects associated with each SNP were relatively small. As a result, different inter-breed variations were observed for each gene variant in the F_{ST} analyses. Key genes that differed between the breeds included *MTHFR, MTHFD1, MAT1A, MTR* and *MUT.* Although these were not found to be significantly associated with varying 1C metabolite concentrations in the GWAS analyses reported in Chapter 4, human studies have shown that variants in these genes lead to numerous metabolic disorders and diseases including adverse birth outcomes, pregnancy complications, cancers and cardiovascular diseases (He and Shen, 2017; Parle-McDermott et al., 2005; Cao et al., 2018; Zhao et al., 2014; Gonzalez et al., 2018), and are commonly associated with varying 1C metabolite concentrations due to their critical role in the associated pathways (Steluti et al., 2017; Chien et al., 2015). Furthermore, variants of these genes in humans have previously been reported to vary with ethnicity and geographical location (Yang et al., 2013; Wilcken et al., 2003). With this in mind, interbreed differences observed in the current study are likely, despite their lack of functional significance in Chapter 4. It is possible that the relatively small proportion of Co deficient animals in the study population in Chapter 4 prevented identification of significant metabolite associations and were these limitations addressed, their significance would be appreciated. Furthermore, breed differences in variant genotype frequencies highlights that use of one of the other breeds may have resulted in alternative or additional nutrient-gene associations being significant.

Some of the 33 gene variants identified in Chapter 4 were moderately different between breeds. *CUBN* is a notable example. The association with PPA identified in Chapter 4 is thought to be biologically significant because of its role in B12 transport. *CUBN* encodes the protein cubilin, a multi-ligand hydrophobic protein which binds to intrinsic factor-cobalamin

(Cbl-IF) complexes with high affinity facilitating B12 uptake. In the current study, Swaledale sheep were found to differ from the other 3 breeds for the two *CUBN* variants. The UK sheep industry is structured as a 'stratified system' which plays on the strengths of different breeds, dividing them into three tiers; hill, upland and lowland. Of the four breeds in the current study, Swaledales are the only 'hill breed', adapted to survive in relatively harsh hill environments. Genetic differences in population structure and variation detected in allele frequencies could be explained by the effects of differences in environment and habitat on selection. Figure 1.1 in Chapter 1 illustrates the relative concentrations of Co in topsoils in England and Wales. It can be seen from this figure that the Yorkshire dales, in particular Swaledale, has low Co top soil concentrations, particularly in comparison with lowland areas. This provides tentative evidence for geographical differences in Co availability to grazing animals and the likelihood that adaptation of different breeds to their native environments has resulted in geographical gradients in genotypes. It may be possible, therefore, that Swaledale sheep have adapted their B12 transport pathways over time to more efficiently utilise B12 for vital cellular processes a result of cobalt challenge in their natural habitat. This is similar to the differences seen with North Ronaldsey sheep in relation to copper (Simpson et al., 2006). Studies of the apportionment of human genetic variation have established that variation between population groups is greatest when comparing different geographical locations. In the current study, as well as Swaledales, genotype frequencies within the BFL population differed from the other three breeds for several of the gene variants studied. Whilst the Texel, Suffolk and Swaledale populations often contained all three genotypes, the BFL population was frequently only of the WT genotype. Again this could be caused by adaptation of the breed to geographical influences over time.

5.5 Conclusions and implications

The current study successfully determined population differentiation for four commercially important breeds in the UK; i.e. Texel, Suffolk, Bluefaced Leicester and Swaledale for SNPs within 1C related genes, with specific focus on the 33 functionally significant SNPs identified in Chapter 4. Identification of inter-breed SNP genotype differences focussed on quantifying population structure using F_{ST} estimates. The high F_{ST} outlier SNPs that corresponded to the upper 1% of the distribution were selected as the SNPs under selection. These included variants in biologically significant genes, *MTHFR*, *MTHFD1*, *MTR*, *MUT* and *MAT1*. Of particular interest, significantly different genotype frequencies within the Swaledale population compared to the other three breeds for variants in *CUBN* were observed, pointing towards the possibility of genetic adaptation to their native environment. Determining the genetic structure of natural populations forms an important part of population genetics and has many applications in animal breeding. Whilst still in the preliminary stages, further research to validate the results of the current study and to investigate breed differences further would be beneficial within breeding programmes. Such work could lead to the decrease in incidence of deficiencies and lessen the dependence on supplementation in deficient locations.

CHAPTER SIX

Discussion and Conclusions

6.1 Introductory comments

Cobalt (Co) deficiency is a recognised problem in sheep. With each stage of progression having an increasingly negative impact on animal health and performance, farm profitability is ultimately decreased (Grace, 1994). Therefore, Co deficiency is of major economic importance (Kennedy et al., 1992). As the signs and symptoms of Co deficiency are not dissimilar to those associated with other nutritional inadequacies (Marston, 1970), economically important effects on performance and health of animals can be affected before definitive clinical signs are evident meaning early diagnosis is crucial (Underwood and Suttle, 1999). Cobalt is an essential micro-nutrient for ruminants, the adequacy of which is necessary for ruminal synthesis of vitamin B12 (McDowell, 2000). In animals, two key 1C metabolic enzymes are known to utilise B12 as a cofactor; methionine synthase (MTR) (EC 2.1.1.13) and methylmalonyl-CoA mutase (MUT) (EC 5.4.99.2) (Kräutler, 2012). These enzymes catalyse the re-methylation of homocysteine to methionine (Lucock, 2000; Ducker and Rabinowitz, 2017) and the conversion of propionate to succinate (O'Harte, 1989) respectively. With the requirement of Co for the synthesis of B12, Co deficiency therefore leads to disruption of these B12 dependent pathways resulting in adverse outcomes (Kennedy et al., 1992).

The effects of Co/B12 deficiency can be mitigated by effective supplementation through a strategic 'risk management' approach, taking influencing factors into consideration (Grace, 1992). A number of factors influence the prevalence and severity of Co deficiency in sheep including nutritional status, stage of production cycle and geographical location (Suttle, 2000). In addition, human studies have provided clear evidence for genetic predispositions to 1C metabolic imbalances, with substantial variation across different geographical regions and ethnic groups (Ulvik et al., 2007; Gellekink et al., 2005; Malloy, 2004; Parle-McDermott et al., 2005; Zhao et al., 2014). Several significant polymorphisms in one-carbon genes have been found to be associated with increased disease

risk and have been the central focus of human studies worldwide (Ducker and Rabinowitz, 2017; Stover, 2011; Li et al., 2016). Furthermore, there has been increasing interest into nutrient interactions with these SNPs (Black, 2008; Mason, 2003).

Genetic regulation of B12 metabolism in sheep has never previously been investigated. However, considerable inter-individual heterogeneity in terms of metabolic responses to specific diets are frequently observed in dietary studies with outbred populations of animals (Kanakkaparambil et al., 2009), highlighting a possible underlying genetic contribution. This thesis therefore sought to increase the understanding of nutritional/dietary and genetic regulation of B12 metabolism through supplementation trials and genetic analyses.

6.2 Summary of key findings

Cobalt (Co) deficiency is a recognised problem in sheep, particularly in rapidly growing lambs (Grace, 1994). Lambs that are born weighing less than average are often considered to be economic liabilities, due to their reduced growth rates and additional costs of feeding. The supplementation trial reported in Chapter 2 found that SMARTShot® long-acting injectable lactide-glycolide microencapsulated B12 increases B12 status and is a beneficial supplementation option for young lambs due to its ease and speed of administration and ability to supplement light weight lambs from 3 weeks of age.

Identification of animals requiring Co or B12 supplementation is commonly carried out by assessment of trace-element concentrations in blood to determine status (Suttle, 2010). Co/B12 status can be affected by a number of factors including geographical location, underlying general nutritional inadequacies, stage of production cycle (i.e. age), and seasonal and yearly changes in weather and prevailing growing conditions. Whilst it was not possible to address the primary hypothesis of Chapter 3, that a Se deficiency masked an underlying Co deficiency, efficacy of a selenium-iodine bolus and long-acting injectable B12

supplement were confirmed. Furthermore, seasonal and year-to-year variation in B12 status, BCS and live-weight gains were evident, highlighting the requirement for a strategic 'risk management' approach to supplementation. Whilst effective supplementation strategies (such as those used in Chapter 2 and 3) can mitigate the effects of Co/B12 deficiency in the flock, the apparent seasonal and inter-year variability observed in this thesis highlights the requirement for early identification of susceptible animals to enable management of risk factors through targeted supplementation. Supplementation needs to be an exercise in risk management, with the decision to supplement based on whether the likelihood of losses from a deficiency are greater than the cost of treatment. If a supplement is required and financially beneficial then it is better provided before deficiency is obvious and before a clinical effect on performance occurs. Little is currently known about this and further research is required for clarity.

Considerable inter-individual heterogeneity in terms of metabolic responses to specific diets are frequently observed in dietary studies with outbred populations of animals, as highlighted in Chapter 4, Section 4.1 (Kanakkaparambil et al., 2009). Although a number of environmental factors can contribute to this variability, there is a possible underlying genetic contribution. In corroboration with this, the effects of polymorphisms in genes functionally related to 1C metabolism have been extensively studied in human populations in relation to varying 1C metabolite concentrations and have been shown to be associated with a variety of complex traits and disorders (Malloy, 2004; Malloy et al., 2008; Ulvik et al., 2007; Qu et al., 2016; Cao et al., 2018; Steluti, 2017). Chapter 4 represents the first investigation of genetic influences on regulation of B12 metabolism in sheep. The study aimed to provide unprecedented insights into gene-nutrient interactions among determinants of 1C metabolism, not only in sheep as a species of clinical interest in their own right, but also as a model for humans. 33 SNPs were found to be significantly associated with concentrations of biochemical parameters related to 1C metabolism in the study population of 360 Texel lambs. Of

particular biological interest were two SNPs in *CUBN;* a gene fundamental in the transport of B12 for provision to the methionine cycle and propionate pathways. These polymorphisms were found to be associated with lower liver propionate concentrations; a possible direct result of altered B12 transport. Further research would, however, be required to confirm this. As the only comprehensive investigation of genetic influences on 1C metabolism in sheep to date, this study contributes to resources critical to the discovery and interpretation of the genetic underpinnings of essential biological processes, principally 1C metabolism and also provides a pipeline of analysis of genetically influenced metabolic variations.

Several variants in 1C related genes have been shown in human studies to vary substantially across different geographical regions and ethnic groups (Binia et al., 2014; Wilcken et al., 2003; Yang et al., 2013), and also to be affected by nutritional challenges. With this in mind, Chapter 5 assessed inter-breed differences in 1C metabolism associated genes. Of particular interest, Swaledale sheep were found to differ genetically from the other three breeds, particularly for the functionally significant SNPs in *CUBN* identified in Chapter 4. As Swaledale sheep mostly inhabit the more mountainous areas of Great Britain, particularly the Yorkshire Dales from where they originate, whilst BFL are an upland breed and Suffolks and Texels are lowland breeds, the findings of Chapter 5 point towards the possibility of genetic adaptation to their native environment. Interbreed variation in allele frequency was also observed for SNPs in important 1C regulatory genes including *MTHFR, MTHFD1, MTR, MUT* and *MAT1*. However, under the conditions of the studies in Chapters 4 and 5 (relatively low population number and insufficient proportion of deficient animals), it was not possible to detect significant associations of these SNPs with the metabolites measured.

6.3 The bigger picture

The outcomes of this thesis confirm that B12 deficiency in sheep varies with respect to geographical location, stage of production cycle and nutritional availability. Furthermore, there is evidence for a genetic influence on B12 deficiency through a predisposition to 1C metabolic imbalances, resulting in inter-individual variation in the regulation of 1C and other B12 associated metabolic pathways. Although the primary focus of this study was animal nutrition and genetics, many of the outcomes can be translated to humans. Not only is the sheep a major livestock animal, but it is also a species of clinical interest and an excellent model organism for human genetic studies due to their physiological parallels with humans (Pinnapureddy et al., 2015). Human studies have previously identified a number of polymorphisms in genes functionally related to 1C metabolism that are associated with a variety of complex traits and disorders (Ducker and Rabinowitz, 2017). Furthermore, there has been accumulating evidence that genotype frequencies of some of these polymorphisms in human populations vary substantially across different geographical regions and ethnic groups, and can be altered by nutritional challenges.

Inter-individual and ethnic variability in 1C regulation can arise as a direct consequence of single-nucleotide polymorphisms within 1C genes. Variants in human 1C genes are significantly important as they can increase the risk of impaired fertility, congenital malformations, certain types of cancer and other metabolic disorders and diseases (Ducker and Rabinowitz, 2017). A notable example is the commonly studied *MTHFR* C677T polymorphism which is a well-characterised risk variant for male infertility (Gong et al., 2015), recurrent pregnancy loss (Cao et al., 2013), NTDs (Yan et al., 2012), and Down syndrome (Cyril et al., 2009; Kaur, 2016). This SNP has been found to be associated with varying concentrations of folate in humans. In addition to individual differences, ethnic and geographical differences in this variant have also been

observed reflecting responses to selection pressures in regions where low folates prevail (Khoshnood et al., 2015).

Although the mechanisms of such metabolic imbalances are not yet fully understood, there is accumulating evidence to suggest that in addition to dietary deficiencies in folate, deprivation of B12 is likely to have downstream effects on linked metabolic pathways resulting in detrimental effects on human health, particularly during pregnancy and early childhood (Malloy et al., 2014). The estimated global prevalence of B12 deficiency in humans ranges from <10% to >50% in different populations, with the highest incidences of B12 deficiency recorded in Central and Latin America, the Indian subcontinent and China (McLean et al., 2008; Brito et al., 2015; Soofi et al., 2017). Whilst these geographical differences in B12 status in humans can be partly explained by dietary preferences, geographical differences in B12 status of sheep is determined by availability of Co for intra-ruminal synthesis of B12 (McDowell, 2000). Co deficiency in sheep is prevalent in the UK and is one of the most highly diagnosed diseases in sheep of all ages, contributing to 11% of all diagnostic submissions in 2017 (VIDA). Furthermore, Co deficiency is found in soils of diverse origin across the world, leading to coast and wasting diseases of sheep in Australasia and cobalt pine in sheep within the UK. Geographical mapping of Co topsoil concentrations in the UK confirm that concentrations vary with geographical location (Chapter 1 Section 1.4). Despite this, there are no previous studies assessing geographical or breed differences in the prevalence of Co/B12 deficiency.

Whilst previous studies have assessed genomic variation and population structure in different sheep breeds (Grasso et al., 2014; Dalvit et al., 2009; Edea et al., 2017), these analyses have analysed whole genome differences rather than focussing on 1C metabolism and have been focussed on production traits rather than underlying metabolic consequences. Through their domestication and subsequent selection, sheep have adapted to thrive in a diverse range of environments. As a

consequence, susceptibility to trace-element deficiencies have been altered. Whilst no previous studies have assessed the effects in relation to Co, an example of such effects can been seen in relation to copper. The North Ronaldsay breed are particularly copper-sensitive (Simpson et al., 2006). This genotype of sheep and their cross-breeds absorb more dietary copper than other breeds of domesticated sheep, indicating that this trait is heritable (Wiener et al., 1977). Furthermore, studies in Edinburgh showed that selection for high plasma Cu amongst ram sires of a sheep breed vulnerable to Cu deficiency, the Scottish Blackface, improved resistance to swayback and growth retardation after only four years (Woolliams et al., 1986), indicating a genetic influence.

With other UK livestock industries seeing rapid uptake and benefits of genomic technologies to help improve the productivity and health of animals, which will in turn, improve efficiency and profitability for farmers, it may be time for the UK sheep industry to adopt such technologies. Every farm has different nutritional challenges. Therefore, genotyping for susceptibility traits to Co deficiency and other trace-element deficiencies would allow farmers to select animals that suit their specific situation. Screening for functionally significant polymorphisms could begin with sires as a starting point, and as the technologies become more robust and cheaper, they could become more applicable to the female side to enable more rapid progression. Overall, development and industry uptake of a screening tool for identification of sheep susceptible or tolerant to B12 deficiency would enable better decisions to be made about which animals to select for breeding, helping improve the production, health and longevity of animals, which would in turn, improve efficiency and profitability for farmers.

Combining the emerging evidence from human studies with the outcomes of the comprehensive investigation of genetic influences on 1C metabolism in sheep presented in this thesis will enable a greater understanding of the role of inherited variation in blood metabolic diversity in sheep, providing a basis for further study into judicious

targeted supplementation and selection of genetic tolerance to Co deficiency in breeding programmes.

6.4 General conclusion and future research priorities

In summary, this thesis demonstrates that B12 metabolism is regulated by dietary and genetic influences. It is apparent that seasonal and inter-year variation in nutrient availability has a large impact on B12 status in animals at different stages of the production cycle. Furthermore, inter-individual and inter-breed variation in 1C metabolism and related pathways results from polymorphic differences in 1C regulatory genes. The results of this thesis therefore support and promote the importance of early identification of susceptible animals to enable effective management of B12 deficiency through targeted supplementation and selection of genetic tolerance in breeding programmes.

Future research priorities as a consequence of the outcomes of this thesis should include development and implementation of risk management strategies. Between year and seasonal variation in B12 status was particularly evident in Chapter 3 of this thesis, in addition to variation associated with geographical location, underlying general nutritional inadequacies and stage of production cycle. This highlights the requirement for early identification of susceptible animals to enable management of risk factors through targeted supplementation. Such a risk management strategy could include a framework to identify the key contributing factors, to enable management and mitigation of the effects of B12 deficiency. A possible strategy could include use of grass mineral profiling to determine requirements and optimal timing of supplements before a clinical effect on performance occurs. With the possible genetic predisposition to 1C metabolic imbalances highlighted in this thesis, additional risk management strategies could involve identification and selection of animals with genetic tolerance in breeding programmes.

Given the complexity of 1C metabolic regulation, it was not possible to achieve all of the research objectives detailed in this thesis. However,

with further investigation, the significance of the outcomes of this thesis could be realised. The study in Chapter 4 was limited by population size and a relatively small proportion of B12 deficient animals due to logistic challenges. As such, SNPs in important 1C regulatory genes identified in human studies to cause a genetic predisposition to 1C metabolic imbalances and to be associated with a variety of complex traits and disorders (Table 1.2), were not identified as being functionally associated with varying 1C metabolite concentrations in sheep. The outcomes of Chapter 5, however, demonstrate that there is clear inter-breed variation in genotype frequencies of SNPs in some of these key genes. In particular, Swaledales were found to be genetically different from the other three breeds in relation to 1C metabolism.

Future research priorities should therefore be focussed on confirmation and validation of the functional significance of the 33 SNPs identified in Chapter 4 as well as SNPs in key 1C genes found to differ between breeds in Chapter 5. Determination of metabolic consequences of significant SNPs with regard to Co/B12, through assessment of status, would also be required to specifically determine risk of susceptibility to Co/B12 deficiency. These objectives could be achieved by constructing a functional SNP chip containing the priority SNPs, to allow for selective screening of several flocks of sheep throughout the UK. Working with levy-body industry partners (AHDB Beef and Lamb, HCC and Agrisearch) and sheep breed societies would allow identification of suitable farms. The aim would be to identify 24 ‘Low-risk’ (i.e. without these priority SNP genotypes), and 24 ‘at-risk’ (i.e. with these priority SNP geneotypes) unrelated weaned lambs for a controlled study. Screening would involve collecting a single nasal swab from each sheep; a well-established routine practice for many commercial breeding flocks in the UK, and one practiced in the study detailed in Chapter 5 of this thesis. DNA would be extracted and analysed using the functional SNP chip. With suitable animals identified, it would then be possible to carry out a study with interest focussed on the effect of these variants on feed intakes, growth, animal health, liver metabolism and blood metabolism.

Blood and liver metabolites could be measured using the analytical methods developed in this thesis. A GWAS could then be carried out using the data analysis pipeline described in Chapter 4 of this thesis. The study structure would be as detailed in Table 6.1.

Table 6.1 Possible experimental diet arrangement for proof study

Diet	Control	Methyl deficient	
Genetic background	Mixed ('low' and 'at-risk)	'low-risk'	'at-risk'
No. of lambs	16 (8+8)*	16	16

*(8 'low-risk' and 8 'at-risk')

Methyl-deficinet diets would be based on those fed in Chapter 4 of this thesis, rendering the animals deficient in Co/B12. A control group (8 'low' and 8 'at-risk)' fed a methyl-sufficient diet would be included for comparison. Termination of the study would be determined when lambs fed the methyl-deficient diet reached a deficient state, with reference to previously reported ranges (Table 1.1).

Particular focus should be given to the two SNPs (dbrs413573281 and dbrs413234073) in *CUBN* discussed in Chapters 4 and 5. These polymorphisms were found to be associated with lower liver propionate concentrations; a possible direct result of altered B12 transport, and are therefore of significant biological importance. Simultaneous assessment of propionate and B12 concentrations in animals in the two study groups fed a Co deficient diet would be required to confirm this and could be carried out as part of the metabolic profiling using analytical methods developed in this thesis.

Furthermore, the outcomes of Chapter 5 raise the question of whether selection of an alternative breed for the GWAS study would result in a different outcome. It would therefore be beneficial to ascertain whether an alternative breed to Texel, for example Swaledale, could be more susceptible to changes in one-carbon metabolic pathways and therefore

be more advantageous to use in future GWAS studies. To determine this, a parallel study to that described above could be carried out with Swaledale lambs.

Although strictly beyond the scope of this thesis, which focussed primarily on variants in metabolic genes, genetic variants in several epigenetic regulators (e.g. DNMT1) have previously been found to be associated with adverse pregnancy outcomes in humans (Barišić et al., 2017; Tang et al., 2017). Knowledge of such relationships in farm animals, however, does not exist. Furthermore, at present it is not known how SNPs within 1C metabolism function to confer risks for NTDs and other developmental conditions in the embryo, foetus and adult. It would therefore be interesting to extend future proof of concept studies to investigate the effect of gene-nutrient interactions on epigenetic programming during embryonic development, and the influences of this on long-term development and offspring health in sheep.

References

Abou-Zeina HAA, Zaghawa AA, Nasr SM, Keshta HGE. 2008. Effects of Dietary Cobalt Deficiency on Performance, Blood and Rumen Metabolites and Liver Pathology in Sheep. Global Veterinaria. 2(4): 182-191

Adams SN, Honeysett JL, Tiller KG, Norrish K. 1969. Factors controlling the increase of cobalt in plants following the addition of a cobalt fertiliser. Australian Journal of Soil Research 7: 29-42

Almasy L, Blangero J. 2010. Variance component methods for analysis of complex phenotypes. Cold Spring Harb Protocols.

Aminoff M, Carter JE, Chadwick RB, Johnson C, Gräsbeck R et al. 1999. Mutations in CUBN, encoding the intrinsic factor-vitamin B12 receptor, cubilin, cause hereditary megaloblastic anaemia 1. Nature Genetics. 21(3): 309-13

Amort T, Rieder D, Wille A, Khokhlova-Cubberley D, Riml C, et al. 2017. Distinct 5-methylcytosine profiles in poly(A) RNA from mouse embryonic stem cells and brain. Genome Biology. 18: 1 1-16

Anderson OS, Sant KE, Dolinoy DC. 2012. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. Journal of Nutritional Biochemistry. 23(8):853-9

Andrews ED, Grant AB, Stephenson BJ. 1964. Weight responses of sheep to cobalt and selenium in relation to vitamin B12 and selenium concentrations in liver and kidney. New Zealand Journal of Agricultural Research. 7(1): 17-27

Andrews ED, Hart LI, Stephenson BJ. 1960. Vitamin B12 and cobalt in livers from grazing cobalt-deficient lambs and from others given Various Cobalt Supplements. New Zealand Journal of Agricultural Research. 3(2): 364-376

Andrews ED, Stephenson BJ, Anderson JP, Faithful WC. 1958. The effect of length of pasture on cobalt-deficiency disease in lambs. New Zealand Journal of Agricultural Research. 1(2): 125-139

Andrews ED. 1965. Cobalt poisoning in sheep. New Zealand Veterinary Journal. 13(4): 101-3.

Asada H, Kawamura Y, Maruyama K. Kume H, Ding R, et al. 1996 Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. Biochemical and Biophysical Research Communications. 229(3): 891–895

Au KS, Ashley-Koch A, Northrup H. Epidemiologic and genetic aspects of spina bifida and other neural tube defects. Developmental Disabilities Research Reviews. 16(1): 6-15

Aulchenko YS, Ripke S, Isaacs A, van Duijn CM. 2007. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 23(10): 1294-6

Bailey LB, Gregory JF. 1999. Polymorphisms of Methylenetetrahydrofolate Reductase and Other Enzymes: Metabolic Significance, Risks and Impact on Folate Requirements. The Journal of Nutrition. 129(5): 919-922

Ballhausen D, Mittaz L, Boulat O, Bonafé L, Braissant O. 2009. Evidence for catabolic pathway of propionate metabolism in CNS: expression pattern of methylmalonyl-CoA mutase and propionyl-CoA carboxylase alpha-subunit in developing and adult rat brain. Neuroscience. 164(2):578-87.

Bannister AJ, Schneider R, Kouzarides T. 2002. Histone methylation: dynamic or static? Cell. 109: 801-6

Barić I, Cuk M, Fumić K, Vugrek O, Allen RH, et al. 2005. S-Adenosylhomocysteine hydrolase deficiency: a second patient, the younger brother of the index patient, and outcomes during therapy. Journal of Inherited Metabolic Disease. 28: 885-902

Barišić A, Pereza N, Hodžić A, Ostojić S, Peterlin B. 2017. A Single Nucleotide Polymorphism of DNA methyltransferase 3B gene is a risk factor for recurrent spontaneous abortion. American Journal of Reproductive Immunology. 78(6)

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, et al. 2007. High-resolution profiling of histone methylations in the human genome. Cell. 129: 823-37

Batista PJ. 2017. The RNA Modification N6-methyladenosine and Its Implications in Human Disease. Genomics Proteomics Bioinformatics. 15: 154-63

Beagle B, Yang TL, Hung J, Cogger EA, Moriarty DJ, Caudill MA. 2005. The glycine N-methyltransferase (GNMT) 1289 C->T variant influences plasma total homocysteine concentrations in young women after restricting folate intake. Journal of Nutrition. 135: 2780-5

Beaudin A, Stover P. 2009. Insights into Metabolic Mechanisms Underlying Folate-Responsive Neural Tube Defects: A Minireview. Birth Defects Research Part A Clinical and Molecular Teratology. 85(4):274-84

Beilstein MA, Whanger PD. 1983. Distribution of selenium and glutathione peroxidase in blood fractions from humans, rhesus and squirrel monkeys, rats and sheep. The Journal of nutrition. 113(11): 2138-46

Berrow ML, Ure AM. 1986. Trace element distribution and mobilization in Scottish soils with particular reference to cobalt, copper and molybdenum. Environmental Geochemistry & Health. 8(1):19-24

Binia A, Contreras AV, Canizales-Quinteros S, Alonzo VA, Tejero ME et al. 2014. Geographical and ethnic distribution of single nucleotide polymorphisms within genes of the folate/homocysteine pathway metabolism. Genes & Nutrition. 9(5): 421

Black MM. 2008. Effects of vitamin B12 and folate deficiency on brain development in children. Food & Nutrition Bulletin. 29(2 Suppl): S126-31.

Borg I, Groenen PJF, 2005. Modern Multidimensional Scaling: Theory and Applications. Springer-Verlag, New York

Boyles AL, Billups AV, Deak KL, Siegel DG, Mehltretter L, et al. 2006. Neural tube defects and folate pathway genes: family-based association tests of gene-gene and gene-environment interactions. Environ Health Perspect. 114: 1547-52

Brito A, Mujica-Coopman MF, López de Romaña D, Cori H, Allen LH. 2015. Folate and Vitamin B12 Status in Latin America and the Caribbean: An Update. Food & Nutrition Bulletin. 36: S109-18

Brockman RP. 1993. Glucose and short-chain fatty acid metabolism. Quantitative Aspects of Ruminant Digestion and Metabolism, JM Forbes, editor. 249–265

Burg MB, Ferraris JD. 2008. Intracellular organic osmolytes: function and regulation. Journal of Biological Chemistry. 283: 7309–7313

Cao L, Wang Y, Zhang R, Dong L, Cui H, et al. 2018. Association of neural tube defects with gene polymorphisms in one-carbon metabolic pathway. Childs Nervous System. 34: 277-84

Cao Y, Xu J, Zhang Z, Huang X, Zhang A, et al. 2013. Association study between methylenetetrahydrofolate reductase polymorphisms and unexplained recurrent pregnancy loss: a meta-analysis. Gene. 514: 105-11

Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, et al. 2001. Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice. Journal of Nutrition. 131: 2811-8

Chedin F, Lieber MR, Hsieh CL. 2002. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. PNAS. 99: 16916-21

Chen WM, Abecasis GR. 2007. Family-based association tests for genomewide association scans. American Journal of Human Genetics. 81(5): 913-26

Chien YH, Abdenur JE, Baronio F, Bannick AA, Corrales F, et al. 2015. Mudd's disease (MAT I/III deficiency): a survey of data for MAT1A homozygotes and compound heterozygotes. Orphanet Journal of Rare Diseases. 10: 99

Chowanadisai W, Kelleher SL, Lönnerdal B. 2005. Zinc deficiency is associated with increased brain zinc import and LIV-1 expression and decreased ZnT-1 expression in neonatal rats. Journal of Nutrition. 135(5):1002-7.

Christensen KE, Deng L, Leung KY, Arning E, Bottiglieri T, et al. 2013. A novel mouse model for genetic variation in 10-formyltetrahydrofolate synthetase exhibits disturbed purine synthesis with impacts on pregnancy and embryonic development. Human Molecular Genetics. 22: 3705-19

Clare CE, Brassington AH, Kwong WY, Sinclair KD. 2019. One-carbon metabolism: Linking nutritional biochemistry to epigenetic programming of long-term development. Annual Reviews of Animal Biosciences. 15(7): 263-287

Clark RG, Wright DF, Millar KR, Rowland JD. 1989. Reference curves to diagnose cobalt deficiency in sheep using liver and serum vitamin B12 levels. New Zealand Veterinary Journal. 37(1): 7-11

Clark RG, Wright DF, Millar KR. 1985. A proposed new approach and protocol to defining mineral deficiencies using reference curves. Cobalt deficiency in young sheep is used as a model. New Zealand Veterinary Journal. 33(1-2): 1-5.

Cole LK, Vance JE, Vance DE. 2012. Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochimica et Biophysica Acta. 1821: 754-61

Craig SA. 2004. Betaine in human nutrition. American Journal of Clinical Nutrition. 80(3): 539-49.

Crider KS, Bailey LB, Berry RJ. 2011. Folic acid food fortification-its history, effect, concerns, and future directions. Nutrients. 3: 370-84

Cyril C, Rai P, Chandra N, Gopinath PM, Satyamoorthy K. 2009. MTHFR Gene variants C677T, A1298C and association with Down syndrome: A Case-control study from South India. Indian J Hum Genet. 15: 60-4

Czarnek L, Terpiłowska S, Siwicki AK. 2015. Selected aspects of the action of cobalt ions in the human body. Cent Eur J Immunol. 40(2): 236–242.

Dalvit C, De Marchi M, Zanetti E, Cassandro M. 2009. Genetic variation and population structure of Italian native sheep breeds undergoing in situ conservation. Journal of Animal Science. 87(12): 3837–3844

Davis CD, Uthus EO, Finley JW. 2000. Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rate liver and colon. Nutr. 130: 2903-2909

Davis CD, Uthus EO. 2003. Dietary folate and selenium affect dimethylhydrazine-induced aberrant crypt formation, global DNA

methylation and one-carbon metabolism in rats. J Nutr. 133(9):2907-14.

de Bree A, van Dusseldorp M, Brouwer IA, van het Hof KH, Steegers-Theunissen RP. 1997. Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr. 51: 643-60

De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. 2016. Microbiota-Produced Succinate Improves Glucose Homeostasis via Intestinal Gluconeogenesis. Cell Metab. 24: 151-7

Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, Kremer J. 2004. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 50(9):2766-74.

Douce R, Bourguignon J, Neuburger M, Rébeillé F. 2001. The glycine decarboxylase system: a fascinating complex. Trends Plant Sci. 6(4):167-76.

Drake C, Grant A, Hartley W. 1960. Selenium and animal health Part 2: The effect of selenium on unthrifty weaned lambs. New Zealand Veterinary Journal. 8(1): 7-10.

Ducker GS, Rabinowitz JD. 2017. One-Carbon Metabolism in Health and Disease. Cell Metab. 25(1): 27-42

Duncan WR, Morrison ER, Garton GA. 1981. Effects of cobalt deficiency in pregnant and post-parturient ewes and their lambs. Br J Nutr. 46(2):337-44.

Edea Z, Dessie T, Dadi H, Do KT, Kim KS. 2017. Genetic Diversity and Population Structure of Ethiopian Sheep Populations Revealed by High-Density SNP Markers. Front Genet. 8:218

Elin RJ, Winter WE, 2001. Methylmalonic Acid. Archives of Pathology & Laboratory Medicine 125(6):824-827

Eussen SJ, Nilsen RM, Midttun Ø, Hustad S, IJssennagger N, et al. 2013. North-south gradients in plasma concentrations of B-vitamins and other components of one-carbon metabolism in Western Europe: results from the European Prospective Investigation into Cancer and Nutrition (EPIC) Study*. Br J Nutr.* 110: 363-74

Falconer DS, Mackay TFC. 1996. Introduction to quantitative genetics. Longman; Essex, England

Farningham DAH, Whyte CC. 1993. The role of propionate and acetate in the control of food intake in sheep. Br. J. Nutr. 70(1):37-46

Field AC, Suttle NF, Brebner J, Gunn GW. 1988. An assessment of the efficacy and safety of selenium and cobalt included in an anthelmintic for sheep. The Veterinary record. 123(4): 97-100.

Finkelstein JD, Kyle W, Harris BJ. 1971. Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch Biochem Biophys. 146(1):84-92.

Finkelstein JD, Martin JJ. 1984. Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. *J Biol Chem.* 259: 9508-13

Fisher GEJ, MacPherson A. 1990. Serum vitamin B12 and methylmalonic acid determinations in the diagnosis of cobalt deficiency in pregnant ewes. Br. Vet. J. 146:120-128

Fisher GEJ, MacPherson A. 1991. Effect of Cobalt deficinecy in the pregnant ewe on reproductive performance and lamb viability. Research in veterinary Science. 50:319-327

Friso S, Choi SW. 2002. Gene-nutrient interactions and DNA methylation. J Nutr. 132: 2382S-87S

Froese DS, Kopec J, Fitzpatrick F, Schuller M, McCorvie TJ, Chalk R et al. 2015. Structural Insights into the MMACHC-MMADHC Protein Complex Involved in Vitamin B12 Trafficking. J Biol Chem. 290(49): 29167–29177

Furlong JM, Sedcole JR, Sykes. 2010. An evaluation of plasma homocysteine in the assessment of vitamin B12 status of pasture-fed sheep. 58(1):11-16

Gamble LD, Hogarty MD, Liu X, Ziegler DS, Marshall G, et al. 2012. Polyamine pathway inhibition as a novel therapeutic approach to treating neuroblastoma. Front Oncol. 162: 1-10

Gardiner M. 1966. Chronic Selenium Toxicity Studies in Sheep. Australian Veterinary Journal. 42(12): 442-448

Garratt LC, Ortori CA, Tucker GA, Stablitzky F, Bennett MJ, Barrett DA. 2005. Comprehensive metabolic profiling of mono- and polyglutamated folates and their precursors in plant and animal tissue using liquid chromatography/negative ion electrospray ionisation tandem mass spectrometry. Rapid Commun Mass Spectrom. 19(17):2390-8

Gaughan DJ, Kluijtmans LA, Barbaux S, McMaster D, Young IS, et al. 2001. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis. 157: 451-6

Gellekink H, den Heijer M, Heil SG, Blom HJ. 2005. Genetic determinants of plasma total homocysteine. Semin Vasc Med. 5(2): 98-109

Gerloff BJ. 1992. Effect of selenium supplementation on dairy cattle. J. Anim. Sci. 70(12):3934-40

Gillette TG, Hill JA. 2015. Readers, writers, and erasers: chromatin as the whiteboard of heart disease. Circ Res. 116: 1245-53

Gilsing AM, Crowe FL, Lloyd-Wright Z, Sanders TA, Appleby PN, et al. 2010. Serum concentrations of vitamin B12 and folate in British

male omnivores, vegetarians and vegans: results from a cross-sectional analysis of the EPIC-Oxford cohort study. Eur J Clin Nutr. 64: 933-9

Goll MG, Kirpekar F, Maggert KA, Yoder JA, Hsieh CL, et al. 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science. 311: 395-8

Gong M, Dong W, He T, Shi Z, Huang G, et al. 2015. MTHFR 677C>T polymorphism increases the male infertility risk: a meta-analysis involving 26 studies. PLoS One. 10: e0121147

Gonzales MC, Yu P, Shiao SP. 2017. MTHFR Gene Polymorphism-Mutations and Air Pollution as Risk Factors for Breast Cancer: A Metaprediction Study. Nurs Res. 66(2):152-163.

Gopalakrishnan R, Ajithkumar R. 2011. Effect of breed on serum levels of trace minerals in goats of Kerala.

Grace N. 1994. Managing trace element deficiencies: the diagnosis and prevention of selenium, cobalt, copper and iodine deficiencies in New Zealand grazing livestock. AgResearch.

Grace N. West D. Sargison N. 1998. The efficacy of a subcutaneous injection of soluble Vitamin B12 in lambs. New Zealand Veterinary Journal. 46(5): 194-196

Grace ND, Knowles S, West DM. 2006. Dose-response effects of long-acting injectable vitamin B12 plus selenium (Se) on the vitamin B 12 and Se status of ewes and their lambs. New Zealand Veterinary Journal. 54(2): 67-72

Grace ND, Knowles SO, Sinclair GR, Lee J. 2003. Growth response to increasing doses of microencapsulated vitamin B12 and related changes in tissue vitamin B12 concentrations in cobalt-deficient lambs. New Zealand Veterinary Journal. 51(2): 89–92

Grace ND, Knowles SO. 2012. Trace element supplementation of livestock in New Zealand: Meeting the challenges of free-range grazing systems. Veterinary medicine international.

Grace ND, Lewis DH. 1999. An evaluation of the efficacy of injectable microencapsulated Vitamin B12 in increasing and maintaining the serum and liver Vitamin B12 concentrations of lambs. New Zealand Veterinary Journal. 47(1): 3-7

Grace ND, West DM. 2000. An Evaluation of Long Acting Injectable Microencapsulated Vitamin B12 to Prevent Cobalt Deficiency in Grazing Ruminants. Asian-Aus. J. Anim. Sci. 13: 199–202

Grace ND. 1992. Prevention of trace element deficiencies in grazing ruminants: an evaluation of methods. Proceedings of the New Zealand Grassland Association. 54: 31-34

Grace ND. 1999. The effect of increasing the Vitamin B12 status of Romney ewes on foetal liver Vitamin B12, milk Vitamin B12 and liver Vitamin B12 concentrations in suckling lambs. New Zealand Veterinary Journal. 47(3): 97-100

Gräsbeck R. 2006. Imerslund-Gräsbeck syndrome (selective vitamin B12 malabsorption with proteinuria). Orphanet J Rare Dis. 2006; 1: 17.

Grasso AN, Goldberg V, Navajas EA , Iriarte W, Gimeno D, Aguilar I. 2014. Genomic variation and population structure detected by single nucleotide polymorphism arrays in Corriedale, Merino and Creole sheep. Genet Mol Biol. 37(2): 389–395

Gris JC, Quéré I, Monpeyroux F, Mercier E, Ripart-Neveu S, Tailland ML et al. 1999. Case-control study of the frequency of thrombophilic disorders in couples with late foetal loss and no thrombotic antecedent--the Nîmes Obstetricians and Haematologists Study5 (NOHA5). Thromb Haemost. 81(6):891-9.

Groeneveld LF, Lenstra JA, Eding H, Toro M A, Scherf B, Pilling D et al. 2010. Genetic diversity in farm animals - a review. Anim. Genet. 4:6–31

Gruner T, Sedcole JR, Furlong JM, Grace ND, Williams SD et al. 2004. Changes in serum concentrations of methylmalonic acid and vitamin B12 in cobalt-supplemented ewes and their lambs on two cobalt-deficient properties. New Zealand Veterinary Journal. 52(3): 117-128

Gruner T, Sedcole JR, Furlong JM, Grace ND, Williams SD et al. 2004. Concurrent changes in serum vitamin B_{12} and methylmalonic acid during cobalt or vitamin B_{12} supplementation of lambs while suckling and after weaning on properties in the South Island of New Zealand considered to be cobalt-deficient. New Zealand Veterinary Journal. 52(3): 129-136

Gruner TM. 2001. Vitamin B12 metabolism in sheep, Lincoln University.

Guenther BD, Sheppard CA, Tran P, Rozen R, Mathews RG, Ludwig ML. 1999. The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia. Nat. Struct. Biol. 6(4):359-65.

Hajkova P. 2011. Epigenetic reprogramming in the germline: towards the ground state of the epigenome. Philos Trans R Soc Lond B Biol Sci. 366(1575):2266-73

Hannam RJ, Judson GJ, Reuter DJ, McLaren LD, McFarlane JD. 1980. Effect of vitamin B12 injections on the growth of young Merino sheep. Australian Journal of Agricultural Research. 31(2): 347

Hannibal L, Lysne V, Bjorke-Monsen AL, Behringer S, Grunert SC et al. 2016. Biomarkers and Algorithms for the Diagnosis of Vitamin B12 Deficiency. Frontiers in molecular biosciences. 3: 27

Harrington K. Thatcher A, Kemp P. 2006. Mineral composition and nutritive value of some common pasture weeds. New Zealand Plant Protection. 59. 261-265.

Hart LI, Andrews ED. 1959. Effect of cobaltic oxide pellets on the vitamin B12 content of ewe’s milk. Nature. 184: 1242

Hata K, Okano M, Lei H, Li E. 2002. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development. 129: 1983-93

He L, Shen Y. 2017. MTHFR C677T polymorphism and breast, ovarian cancer risk: a meta-analysis of 19,260 patients and 26,364 controls. Onco Targets Ther. 10: 227–238

Hedrich MF, Elliot JM, Lowe JE. 1973. Response in vitamin B12 production and absorption to increasing cobalt intake in the sheep. Journal of Nutrition. 103:1646.

Hemberger M, Dean W, Reik W. 2009. Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. Nat Rev Mol Cell Biol. 10: 526-37

Herbert V. 1972. The five possible causes of all nutrient deficiency: illustrated by deficiencies of vitamin B12 and folic acid. Aust N Z J Med. 2(1):69-77

Hidiroglou M. 1979. Manganese in ruminant nutrition. Canadian Journal of Animal Science. 59(2): 217-236

Ho V, Massey TE, King WD. 2013. Effects of methionine synthase and methylenetetrahydrofolate reductase gene polymorphismson markers of one-carbon metabolism. *Genes Nutr.* 8: 571-80

Hodgkin DC. Kamper J. MacKay M, Pickworth J, Trueblood KN, White JG. 1956. Structure of Vitamin B12. Nature. 178(4524):64–66

Hong EP, Park JW. 2012. Sample Size and Statistical Power Calculation in Genetic Association Studies. Genomics and informatics. 10(2): 117–122.

Imbard A, Benoist JF, Blom HJ. 2013. Neural tube defects, folic acid and methylation. Int J Environ Res Public Health. 10(9):4352-89

Jaiswal SK, Sukla KK, Chauhan A, Lakhotia AR, Kumar A, Rai AK. 2017. Choline metabolic pathway gene polymorphisms and risk for Down syndrome: An association study in a population with folate-homocysteine metabolic impairment. Eur J Clin Nutr. 71: 45-50

Jiang Y, Xie M, Chen W, Talbot R, Maddox JF, Faraut T, Wu C. 2014. The sheep genome illuminates biology of the rumen and lipid metabolism. Science. 344 (6188): 1168-1173.

Jin B, Li Y, Robertson KD. 2011. DNA methylation: superior or subordinate in the epigenetic hierarchy? Genes Cancer. 2: 607-17

Jin B, Robertson KD. 2013. DNA methyltransferases, DNA damage repair, and cancer. Adv Exp Med Biol. 754: 3-29

Jing M, Rech L, Wu Y, Goltz D, Taylor CG, House JD. 2015. Effects of zinc deficiency and zinc supplementation on homocysteine levels and related enzyme expression in rats. J Trace Elem Med Biol. 30: 77-82

Joyce J, Rattray P. 1970. The intake and utilization of milk and grass by lambs. Proceedings of the New Zealand Society of Animal Production. 30: 94-105

Judson GJ, Babidge PJ, Vawser M-J, Chen ZH, Sansom LN. 2000. Long Acting Vitamin B12 Pellet for Preventing Cobalt Deficiency. Asian-Aus. J. Anim. Sci. 13: 154

Judson GJ, Woonton TR, McFarlane JD, Mitsioulis A. 1995.Evaluation of cobalt pellets for sheep. Aust. J. Exp. Agr. 35(1): 41–49

Kanakkaparambil R, Singh R, Li D, Webb R, Sinclair KD. 2009. B-Vitamin and Homocysteine Status Determines Ovarian Response to Gonadotropin Treatment in Sheep. Biol Reprod. 80: 743–752

Kanno K, Wu MK, Scapa EF, Roderick SL, Cohen DE. 2007. Structure and function of phosphatidylcholine transfer protein (PC-TP)/StarD2. Biochim Biophys Acta. 1771: 654-62

Kaur A, Kaur A. 2016. Maternal MTHFR polymorphism (677 C-T) and risk of Down's syndrome child: meta-analysis. J Genet. 95: 505-13

Kempson SA, Zhou Y, Danbolt NC. 2014. The betaine/GABA transporter and betaine: roles in brain, kidney, and liver. Front Physiol. 5:159

Kendall N, Jackson DW, Mackenzie AM, Illingworth DV. 2001. The effect of a zinc, cobalt and selenium soluble glass bolus on the trace element status of extensively grazed sheep over winter. Animal Science. 73, 163-169.

Kendall N. 2014. http://beefandlamb.ahdb.org.uk/wp-content/uploads/2014/03/74210-KPI-Sheep-Validation.pdf

Kennedy DG, Blanchflower WJ, Scott JM, Weir DG, Molloy AM, Kennedy S, Young PB. 1992. Cobalt-Vitamin B-12 Deficiency Decreases Methionine Synthase Activity and Phospholipid Methylation in Sheep. J. Nutr. 122(7): 1384-1390

Kennedy DG, Kennedy S, Blanchflower WJ, Scott JM, Weir DG, et al. 1995. Cobalt-vitamin B12 deficiency causes accumulation of odd-numbered, branched-chain fatty acids in the tissues of sheep. British Journal of Nutrition. 71: 67-76

Kennedy DG, O'Harte FP, Blanchflower WJ, Rice DA. 1991. Sequential changes in propionate metabolism during the development of cobalt/vitamin B12 deficiency in sheep. Biological Trace Element Research. 28(3): 233–241

Kercher CJ, Smith SE. 1956. The synthesis of vitamin B12 after oral and parenteral administration of cobalt to cobalt-deficient sheep. J. Anim. Sci. 15: 550-8

Khoshnood B, Loane M, de Walle H, Arriola L, Addor MC, et al. 2015. Long term trends in prevalence of neural tube defects in Europe: population based study. *BMJ*. 351: h5949

Kikuchi G, Motokawa Y, Yoshida T, Hiraga K. 2008. Glycine cleavage system: Reaction mechanism, physiological significance, and

hyperglycinemia Proc Jpn Acad Ser B Phys Biol Sci. 84(7):246-63.

Kincaid RL. 1999. Assessment of trace mineral status of ruminants: A review. Journal of Animal Science. 77: 1-10

Kohli RM, Zhang Y. 2013. TET enzymes, TDG and the dynamics of DNA demethylation. Nature. 502(7472):472-9

Koning AM, Holwerda KM, Weedon-Fekjær SM, Staff AC, Nolte IM, et al. 2013. P23 CBS gene variants are associated with conditions characterized by ischemia. *Nitric Oxide.* 31: S44-5

Kozak EM, Tate SS. 1982. Glutathione-degrading enzymes of microvillus membranes. J Biol Chem. 257(11):6322-7.

Kozyraki R, Cases O. 2013. Vitamin B12 absorption: mammalian physiology and acquired and inherited disorders. Biochimie. 95(5):1002-7.

Krnjevic K. 2004. How does a little acronym become a big transmitter? Biochem Pharmacol. 68:1549–1555

Kräutler B. 2012. Biochemistry of B12-cofactors in human metabolism. Subcell Biochem. 56:323-46.

Lam KC, Ibrahim RK, Behdad B, Dayanandan S. 2007. Structure, function, and evolution of plant O-methyltransferases. Genome. 50(11):1001-13.

Lamers Y, Williamson J, Theriague DW, Shuster JJ, Glibert LR et al. 2009. Production of 1-Carbon Units from Glycine Is Extensive in Healthy Men and Women. The Journal of Nutrition. 139(4): 666–671

Lan F, Shi Y. 2009. Epigenetic regulation: methylation of histone and non-histone proteins. Sci China C Life Sci. 52: 311-22

Laverdiere C, Chiasson S, Costea I, Moghrabi A, Krajinovic M. 2002. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. Blood. 100(10):3832-4

Laws, D and Genever, L. 2014. Growing and finishing lambs for Better Returns. EBLEX sheep BRP manual 5. www.eblex.org.uk

Lee JH, Tate CM, You JS, Skalnik DG. 2007. Identification and characterization of the human Set1B histone H3-Lys4 methyltransferase complex. J Biol Chem. 282(18):13419-28.

Lee M, Kim B, Kim VN. 2014. Emerging roles of RNA modification: m(6)A and U-tail. Cell. 158: 980-987

Li F, Feng Q, Lee C, Wang S, Pelleymounter LL, et al. 2008. Human betaine-homocysteine methyltransferase (BHMT) and BHMT2: common gene sequence variation and functional characterization. Mol Genet Metab. 94: 326-35

Li K, Wahlqvist ML, Li D. 2016. Nutrition, One-Carbon Metabolism and Neural Tube Defects: A Review. Nutrients. 8(11):741

Li Z, McLaren RG, Metherell AK. 2001. Cobalt and manganese relationships in New Zealand soils. New Zealand Journal of Agricultural Research. 44(2-3): 191-200

Liew SC, Gupta ED. 2015. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: epidemiology, metabolism and the associated diseases. Eur J Med Genet. 58(1):1-10

Lindskog S. 1997. Structure and mechanism of carbonic anhydrase. Pharmacol Ther. 74(1):1-20

Littledike ET, Young ID.1993. Effect of sire and dam breed on copper status of fat lambs. J Anim Sci. 71:774–778

Liu J, Jia G. 2014. Methylation modifications in eukaryotic messenger RNA. J Genet Genomics. 41(1):21-33

Liu MY, Liu TT, Yang YL, Chang YC, Fan YL et al. 2012. Mutation Profile of the MUT Gene in Chinese Methylmalonic Aciduria Patients. JIMD Rep. 6:55-64

Lucock M, Yates Z. 2005. Folic acid - vitamin and panacea or genetic time bomb? *Nat Rev Genet.* 6: 235-40

Lucock M. 2000. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab. 71(1-2): 121-138

Lv S, Ji L, Chen B, Liu S, Lei C, Liu X et al. 2018. Histone methyltransferase KMT2D sustains prostate carcinogenesis and metastasis via epigenetically activating LIFR and KLF4. Oncogene. 37(10):1354-1368.

Magnes C, Fauland A, Gander E, Narath S, Ratzer M. 2014. Polyamines in biological samples: rapid and robust quantification by solid-phase extraction online-coupled to liquid chromatography-tandem mass spectrometry. J Chromatogr A. 1331: 44-51

Malloy AM, Kirke PN, Brody LC, Scott JM, Mills JL. 2008. Effects of folate and vitamin B12 deficiencies during pregnancy on fetal, infant, and child development. Food Nutr Bull. 29(2 Suppl):S101-11; discussion S112-5.

Malloy AM. 2004. Folate and homocysteine interrelationships including genetics of the relevant enzymes. Curr Opin Lipidol. 15: 49-57

Molloy AM, Kirke PN, Brody LC, Scott JM, Mills JL. 2014. Effects of Folate and Vitamin B 12 Deficiencies During Pregnancy on Fetal, Infant, and Child Development. Food Nutr Bull. 29(2 Suppl):S101-11; discussion S112-5.

Marca MC, Ramos JJ, Saez T, Sanz MC, Verde MT, Fernandez A. 1996. Vitamin B12 supplementation of lambs. Small Ruminant Research. 20(1): 9-14

Marston HR. 1970. The requirement of sheep for cobalt or for vitamin B12. Br J Nutr. 24(3):615-33.

Martinelli M, Scapoli L, Palmieri A, Pezzetti F, Baciliero U, Padula E et al. 2006. Study of four genes belonging to the folate pathway: transcobalamin 2 is involved in the onset of non-syndromic cleft lip with or without cleft palate. Hum Mutat. 27(3):294.

Mason JB. 2003. Biomarkers of nutrient exposure and status in one-carbon (methyl) metabolism. J Nurt. 3(3): 941-947

McDowell 2000. Vitamins in animal nutrition: Comparative aspects to human nutrition. Academic press, San Diego, California

McDowell, LR. 1992. Minerals in animal and human nutrition. Academic press, San Diego, California

McLaren RG. 1987. The availability to pasture plants of native and applied soil cobalt in relation to extractable soil cobalt and other soil properties. Journal of the Science of Food and Agriculture. 39(2): 101-112

McLean E, de Benoist B, Allen LH. 2008. Review of the magnitude of folate and vitamin B12 deficiencies worldwide. Food Nutr Bull. 29: S38-51

McNulty H, Dowey le RC, Strain JJ, Dunne A, Ward M, et al. 2006. Riboflavin lowers homocysteine in individuals homozygous for the MTHFR 677C->T polymorphism. Circulation. 113: 74-80

McQueen IPM. 1984. The effect of stocking rate on response to cobalt supplementation in sheep. Proceedings of the New Zealand Society of Animal Production. 44: 159-161

Mendonça N, Granic A, Mathers JC, Martin-Ruiz C, Wesnes KA, et al. 2017. One-Carbon Metabolism Biomarkers and Cognitive Decline in the Very Old: The Newcastle 85+ Study. J Am Med Dir Assoc. 18: 806.e19-806.e27

Mentch SJ, Locasale JW. 2016. One-carbon metabolism and epigenetics: understanding the specificity. Ann N Y Acad Sci. 1363: 91-8

Metherell AK, Owens JL, Moore CH, MacIntosh CG. 1997. Selenium supplementation alternatives for the South Island high country. Proceedings of the new Zealand Grassland Association 58: 199-204

Millar KR, Penrose ME. 1980, A comparison of vitamin B12 levels in the livers and sera of sheep measured by microbiological and radioassay methods. N Z Vet J. 28(5):97-9.

Millian NS, Garrow TA. 1998. Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. Arch Biochem Biophys. 356(1):93-8.

Mitchell LM, Robinson JJ, Watt RG, McEvoy TG, Ashworth CJ et al. 2007. Effects of cobalt / vitamin B12 status in ewes on ovum

development and lamb viability at birth. Reproduction, Fertility and development. 19: 553-562

Molloy AM, Kirke PN, Troendle JF, Burke H, Sutton M et al., 2009. Maternal Vitamin B12 Status and Risk of Neural Tube Defects in a Population With High Neural Tube Defect Prevalence and No Folic Acid Fortification. Pediatrics. 123(3): 917–923.

Morera L, Lübbert M, Jung M. 2016. Targeting histone methyltransferases and demethylases in clinical trials for cancer therapy. Clin Epigenetics. 8: 57 1-16

Mostowska A, Hozyasz KK, Jagodzinski PP. 2006. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. Clin Genet. 69: 512-7

Murín R, Vidomanová E, Kowtharapu BS, Hatok J, Dobrota D. 2017. Role of S-adenosylmethionine cycle in carcinogenesis. Gen Physiol Biophys. 36: 513-20

Ng SS, Yue WW, Oppermann U, Klose RJ. 2009. Dynamic protein methylation in chromatin biology. Cell Mol Life Sci. 66: 407-22

Nisimoto Y, Diebold BA, Cosentino-Gomes D, Lambeth JD. 2014. Nox4: a hydrogen peroxide-generating oxygen sensor. Biochemistry. 53(31):5111-20.

Nordgren KKS, Peng Y, Pelleymounter LL, Moon I, Abo R et al. 2011. Methionine Adenosyltransferase 2A/2B and Methylation: Gene Sequence Variation and Functional Genomics. Drug Metab Dispos. 39(11):2135-2147

O'Harte FP, Kennedy DG, Blanchflower WJ, Rice DA. 1989. Methylmalonic acid in the diagnosis of cobalt deficiency in barley-fed lambs. The British journal of nutrition. 62(3): 729-38

Obeid R. 2013. The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. Nutrients. 5(9):3481-95

Oh SH, Sunde RA, Pope AL, Hoekstra WG. 1976. Glutathione Peroxidase Response to Selenium Intake in Lambs Fed a Torula Yeast-Based, Artificial Milk. J*ournal of Animal Science*. 42(4): 977–983

Ørskov ER, Mayes RW, Mann SO. 1972. Postruminal digestion of sucrose in sheep. Br J Nutr. 28(3):425-32.

Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. The Journal of laboratory and clinical medicine. 70(1), 158-69

Park JY, Nicolas G, Freisling H, Biessy C, Scalbert A, et al. 2012. Comparison of standardised dietary folate intake across ten countries participating in the European Prospective Investigation into Cancer and Nutrition. Br J Nutr. 108: 552-69

Parle-McDermott A, Pangilinan F, Mills JL, Signore CC, Molloy AM et al. 2005. A polymorphism in the MTHFD1 gene increases a mother's risk of having an unexplained second trimester pregnancy loss. Mol Hum Reprod. 11(7):477-80.

Pauwels S, Ghosh M, Duca RC, Bekaert B, Freson K et al. 2017. Maternal intake of methyl-group donors affects DNA methylation of metabolic genes in infants. Clin Epigenetics. 9: 16.

Pavlata L, Misurova L, Pechova A, Husakova T, Dvorak R. 2012. Direct and indirect assessment of selenium status in sheep – a comparison. Veterinarni Medicina. 57(5): 219–223

Pegg AE. 2006. Regulation of ornithine decarboxylase J Biol Chem.. 281: 14529-32

Pegg AE. 2009. Mammalian polyamine metabolism and function. IUBMB Life. 61(9):880-94.

Pegg AE. 2016. Functions of Polyamines in Mammals. J Biol Chem. 291: 14904-12

Pinnapureddy AR, Stayner C, McEwan J, Baddeley O, Forman J, Eccles MR. 2015. Large animal models of rare genetic disorders: sheep as phenotypically relevant models of human genetic disease. Orphanet J Rare Dis. 10: 107

Portela A, Esteller M. 2010. Epigenetic modifications and human disease. Nat Biotechnol. 28: 1057-68

Pouteau E, Meirim I, Métairon S, Fay LB. 2001. Acetate, propionate and butyrate in plasma: determination of the concentration and isotopic enrichment by gas chromatography/mass spectrometry with positive chemical ionization. J Mass Spectrom. 36(7):798-805.

Prasannan P, Pike S, Peng K, Shane B, Appling DR. 2003. Human mitochondrial C1-tetrahydrofolate synthase: gene structure, tissue distribution of the mRNA, and immunolocalization in Chinese hamster ovary calls. J Biol Chem. 278(44):43178-43187

Prasoona KR, Sunitha T, Srinadh B, Deepika ML, Kumari TM, Jyothy A. 2016. Paternal transmission of MTHFD1 G1958A variant predisposes to neural tube defects in the offspring. Dev Med Child Neurol. 58: 625-31

Prasoona KR, Tella S, Buragadda S, Tiruvatturu MK, Akka J. 2017. Interaction between Maternal and Paternal SHMT C1420T Predisposes to Neural Tube Defects in the Fetus: Evidence from Case-Control and Family-Based Triad Approaches. Birth Defects Res. 109: 1020-29

Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. 2006. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 38(8):904-9.

Qu YY, Zhou SX, Zhang X, Zhao R, Gu CY et al. 2016. Functional variants of the 5-methyltetrahydrofolate-homocysteine

methyltransferase gene significantly increase susceptibility to prostate cancer: Results from an ethnic Han Chinese population. Sci Rep. 6:36264.

Quadros EV, Lai SC, Nakayama Y, Sequeira JM, Hannibal L, Wang S et al. 2010. Positive newborn screen for methylmalonic aciduria identifies the first mutation in TCblR/CD320, the gene for cellular uptake of transcobalamin-bound vitamin B(12). Hum Mutat. 31(8):924-9

Quirk MF, Norton BW. 1987. The relationship between the cobalt nutrition of ewes and the vitamin B12 status of ewes and their lambs. Australian Journal of Agricultural Research. 38(6): 1071

Quirk MF, Norton BW. 1988. Detection of cobalt deficiency in lactating heifers and their calves. J Agric Sci. 110: 465-470

Ramos J, Saez T, Bueso JP, Sanz MC, Fernandez A. 1994. Vitamin B12 levels in ewe colostrum and milk and in lamb serum. Veterinary Research BioMed Central. 25(4): 405-409

Rice DA, McLoughlin M, Blanchflower WJ, McMurray CH, Goodall EA. 1989. Sequential changes in plasma methylmalonic acid and vitamin B12 in sheep eating cobalt-deficient grass. Biological Trace Element Research. 22(2): 153-164

Rice DA, McLoughlin M, Blanchflower WJ, McMurray CH, Goodall EA. 1989. Sequential changes in plasma methylmalonic acid and vitamin B12 in sheep eating cobalt-deficient grass. Biological trace element research. 22(2): 153-64

Roberts E. 1974. Gamma-aminobutyric acid and nervous system function – a perspective. Biochem Pharmacol. 23(19): 2637-49

Rodionov DA, Vitreschak AG, Mironov AA, Gelfand MS. 2003. Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J Biol Chem. 278(42):41148-59.

Roje S. 2006. S-Adenosyl-L-methionine: beyond the universal methyl group donor. Phytochemistry. 67: 1686-98

Rosenberg IH. 2005. Science-based micronutrient fortification: which nutrients, how much, and how to know? Am J Clin Nutr. 82(2):279-80.

Russel A. 1969. Body condition scoring of sheep. In Practice. 91-93

Sabri MI, Soiefer AI, Kisby GE, Spencer PS. 1989. Determination of polyamines by precolumn derivatization with 9-fluorenylmethyl chloroformate and reverse-phase high-performance liquid chromatography. J Neurosci Methods. 29(1):27-31.

Schapira M. 2016. Structural Chemistry of Human RNA Methyltransferases. ACS Chem Biol. 11(3):575-82

Selhub J. 1999. Homocysteine metabolism. Annu Rev Nutr. 19:217-46.

Shane B, Pangilinan F, Mills JL, Fan R, Gong T et al. 2018. The 677C→T variant of MTHFR is the major genetic modifier of biomarkers of

folate status in a young, healthy Irish population. Am J Clin Nutr. 108(6):1334-1341

Shane B. 2008. Folate and vitamin B12 metabolism: overview and interaction with riboflavin, vitamin B6, and polymorphisms. Food Nutr Bull. 29: S5-16

Shuvalov O, Petukhov A, Daks A, Fedorova O, Vasileva E, Barlev NA. 2017. One-carbon metabolism and nucleotide biosynthesis as attractive targets for anticancer therapy. Oncotarget. 8: 23955-23977

Simpson DM, Mobasheri, A, Haywood S, Beynon RJ. 2006. A proteomics study of the response of North Ronaldsay sheep to copper challenge. BMC Vet Res. 2: 36

Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, et al. 2007. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A. 104: 19351-6

Singh KK, Chhabara A.1995. Effect of dietary cobalt on ruminal vitamin B12 synthesis and rumen metabolites. Journal of Nuclear Agricultural Biology. 24: 112-116

Škovierová H, Vidomanová E, Mahmood S, Sopková J, Drgová A, et al. 2016. The Molecular and Cellular Effect of Homocysteine Metabolism Imbalance on Human Health. Int J Mol Sci. 17: 1-18

Smith EL. 1948. Purification of Anti-pernicious Anæmia Factors from Liver. Nature. 161, pages 638–639

Smith EL, Fantes KH, Ball S, Waller JG, Emery WB.1952. B12 vitamins (cobalamins). Biochem J. 52(3): 389–395.

Smith AD, Warren MJ, Refsum H. 2018. Adv Food Nutr Res. 83:215-279.

Smith AD, Refsum H. 2016. Homocysteine, B Vitamins, and Cognitive Impairment. *Annu Rev Nutr.* 17: 211-39

Smith RM, Marston HR. 1970. Production, absorption, distribution and excretion of vitamin B12 in sheep. Br J Nutr. 24: 857-877

Smith RM, Marston HR. 1971. Metabolism of propionate by pair-fed vitamin B12-deficient and vitamin B12-treated sheep. Br. J. Nutr. 26: 41-53

Smith RM. 1987. Trace Elements in Human and Animal Nutrition: Cobalt, 5th.ed.,Vol. 1, Academic Press, London, 143-183

Sneath PHA, Sokal RR. 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman, San Francisco.

Somers M, Gawthorne J. 1969. The effect of dietary cobalt intake on the plasma vitamin B12 concentration of sheep. Australian Journal of Experimental Biology and Medical Science. 47(2): 227-233

Soofi S, Khan GN, Sadiq K, Ariff S, Habib A, et al. 2017. Prevalence and possible factors associated with anaemia, and vitamin B 12 and folate deficiencies in women of reproductive age in Pakistan: analysis of national-level secondary survey data. BMJ Open. 7: e018007 1-9

Stabler SP, Lindenbaum J, Allen RH. 1996. The use of homocysteine and other metabolites in the specific diagnosis of vitamin B12 deficiency. J. Nutr. 126, 1266S–1272S

Stangl GI, Schwarz FJ, Müller H, Kirchgessner M. 2000. Evaluation of the cobalt requirement of beef cattle based on vitamin B12, folate, homocysteine and methylmalonic acid. Br J Nutr. 84(5):645-53.

Steluti J, Carvalho AM, Carioca AAF, Miranda A, Gattás GJF et al. 2017. Nutrients. 9(6):539

Stipanuk MH. 2004. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu Rev Nutr. 24:539-77

Stover PJ, Field MS. 2011. Trafficking of Intracellular Folates. Adv Nutr. 2(4): 325-331

Stover PJ. 2009. One- carbon metabolism-genome interactions in folate-associated pathologies. J Nutr. 139(2): 2402-2405

Stover PJ. 2011. Polymorphisms in 1-carbon metabolism, epigenetics and folate-related pathologies. J Nutrigenet Nutrigenomics. 4(5):293-305

Sutherland RJ, Cordes DO, Carthew GC.1979. Ovine white liver disease - an hepatic dysfunction associated with vitamin B12 deficiency. N Z Vet J. 27(11):227-32

Suttle NF. 2000. Minerals in Livestock production. Asian-Aus. J. Anim. Sci. 13: 1-9

Suttle NF, Bell J, Thornton I, Agyriaki A. 2003. Predicting the risk of cobalt deprivation in grazing livestock from soil composition data. Environmental Geochemistry and Health. 25: 33-39

Suttle N. 2005. Assessing the needs of sheep for trace elements. In Practice. British Medical Journal Publishing Group. 27(9): 474-483

Suttle N. 2010. Mineral Nutrition of Livestock.

Sutton AL, Elliott JM. 1972. Effect of ratio of roughage to concentrate and level of feed intake on ovine ruminal vitamin B12 production. J. Nutr. 102:1341-1346.

Szyf M, Tang YY, Hill KG, Musci R. 2016. The dynamic epigenome and its implications for behavioral interventions: a role for epigenetics to inform disorder prevention and health promotion. Transl Behav Med. 6: 55-62

Tang Q, Chen Y, Wu W, Ding H, Xia Y et al. 2017. Idiopathic male infertility and polymorphisms in the DNA methyltransferase genes involved in epigenetic marking. Scientific reports. 7(11219)

Teo YY. 2008. Common statistical issues in genome-wide association studies: a review on power, data quality control, genotype calling and population structure. Curr. Opin. Lipidol. 19(2): 133-43

Thornton I, Webb JS. 1980. Regional distribution of trace element problems in Great Britain. Applied soil trace elements. Chichester, Wiley, 381-439.

Tretter L, Patocs A, Chinopoulos C. 2016. Succinate, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. Biochim Biophys Acta. 1857: 1086-101

Turgal M, Gumruk F, Karaagaoglu E, Beksac MS. 2018. Methylenetetrahydrofolate Reductase Polymorphisms and Pregnancy Outcome. Geburtshilfe Frauenheilkd. 78(9):871-878

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. 2001. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci. 22(4):195-201

Ulray CL, Liu L, Andrews LG, Tollefsbol TO. 2005. The impact of metabolism on DNA methylation. Hum Mol Genet. 14(1):R139-47.

Ulvik A, Ueland PM, Fredriksen A, Meyer K, Vollset SE, Hoff G, Schneede J. 2007. Functional inference of the methylenetetrahydrofolate reductase 677C > T and 1298A > C polymorphisms from a large-scale epidemiological study. Hum Genet. 121(1): 57-64

Ulvund MJ, Pestalozzi M. 1995. Cobalt deficiency in sheep.

Ulvund MJ. 1990. Ovine white-liver disease. Manifestations of cobalt/vitamin B12 deficiency in lambs. Thesis. Norwegian Coll of Vet Med, Oslo

Underwood EJ .1977. Trace elements in human and animal nutrition. Academic press, New York, 4th Ed.

Underwood EJ, Suttle NF. 1999. The mineral nutrition of livestock. 3rd edition

Underwood EJ, Filmer JF. 1935. The determination of the biologically potent element (cobalt) in limonite. The Australian Veterinary Journal. 11(3):84-92

van der Berg R, Levels FH, van der Schee W. 1983. Breed differences in sheep with respect to the accumulation of copper in the liver. Vet Q. 5(1):26-31

van der Put NM, Thomas CM, Eskes TK, Trijbels FJ, Steegers-Theunissen RP et al. 1997. Altered folate and vitamin B12 metabolism in families with spina bifida offspring. QJM. 90(8):505-10

Vance DE. 2013. Physiological roles of phosphatidylethanolamine N-methyltransferase. Biochim Biophys Acta. 1831: 626-32

Vellema P, van den Ingh TS, Wouda W. 1999. Pathological changes in cobalt-supplemented and non-supplemented twin lambs in

relation to blood concentrations of methylmalonic acid and homocysteine. Vet Q. 21(3):93-8.

Verde MT, Sanz MC, Ramos JJ, Fernandez A, Marca MC, Saez T. 1995. Selenium and Glutathione Peroxidase Correlation in Different Blood Samples in Sheep. Journal of Applied Animal Research. 8(1): 21-27

Visentin M, Diop-Bove N, Zhao R, Goldman ID. 2014. The intestinal absorption of folates. Annu Rev Physiol. 76: 251-74

Huang X, Qin X, Yang W, Liu L, Jiang C et al. 2018. MTHFR Gene and Serum Folate Interaction on Serum Homocysteine Lowering: Prospect for Precision Folic Acid Treatment. Arterioscler Thromb Vasc Biol. 38(3):679-685.

Wang RL, Kong XH, Zhang YZ, Zhu XP, Narenbatu, Jia ZH. 2006. Influence of dietary cobalt on performance, nutrient digestibility and plasma metabolites in lambs. Animal Feed Science and Technology. 135: 346-352

Watson WP, Munter T, Golding BT. 2004. A new role for glutathione: protection of vitamin B12 from depletion by xenobiotics. Chem Res Toxicol. 17(12):1562-7.

Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution. 38(6):1358-1370.

Whanger PD, Weswig PH, Schmitz JA, Oldfield JE. 1977. Effects of selenium and vitamin E deficiencies on reproduction, growth, blood components, and tissue lesions in sheep fed purified diets. J Nutr. 107(7):1288-97.

Wiener G, Field AC, Smith C. 1977. Deaths from copper toxicity of sheep at pasture and the use of fresh seaweed.Vet Rec. 101(21):424-425

Wilcken B, Bamforth F, Li Z, Zhu H, Ritvanen A et al. 2003. Geographical and ethnic variation of the 677C>T allele of 5,10 methylenetetrahydrofolate reductase (MTHFR): findings from over 7000 newborns from 16 areas world wide. J Med Genet. 40(8):619-25.

Willyard C. 2017. An epigenetics gold rush: new controls for gene expression. Nature. 542: 406-8

Witte JS. 2010. Genome-Wide Association Studies and Beyond. Annu Rev Public Health. 31: 9-20.

Woolliams JA, Woolliams C, Suttle NF, Jones DG. 1986. Studies on lambs from lines genetically selected for low and high copper status 2. Incidence of hypocuprosis on improved hill pasture. Anim. Sci. 43(2):303-317

Wright AJ, Dainty JR, Finglas PM. 2007. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr. 98(4):667-75.

Wright MM, Howe AG, Zaremberg V. 2004. Cell membranes and apoptosis: role of cardiolipin, phosphatidylcholine, and anticancer lipid analogues. Biochem Cell Biol. 82(1):18-26.

Wright S. 1921. Systems of Mating. I. The Biometric Relations between Parent and Offspring. Genetics 6(2):111-23.

Wright S. 1965. The Interpretation of Population Structure by F-Statistics with Special Regard to Systems of Mating. Evolution. 19(3):395-420

Wright, S. 1951. The general structure of populations. Anals of Eugenics. 15, 323-354

Wu J, Chen Y, Pei J, Pan J. 2015. MTHFD1 gene polymorphisms as risk factors involved in orofacial cleft: an independent case-control study and a meta-analysis. Int J Clin Exp Med. 2015; 8(5): 7737–7745

Xia L, Cregan AG, Berben LA, Brasch NE. 2004. Studies on the Formation of Glutathionylcobalamin: Any Free Intracellular Aquacobalamin Is Likely to Be Rapidly and Irreversibly Converted to Glutathionylcobalamin. Inorg. Chem. 43 (21): 6848–6857

Xiang Y, Laurent B, Hsu CH, Nachtergaele S, Lu Z, Sheng W et al. 2017. RNA m6A methylation regulates the ultraviolet-induced DNA damage response. Nature. 543(7646):573-576.

Xu J, Sinclair KD. 2015. One-carbon metabolism and epigenetic regulation of embryo development. Reprod Fertil Dev. 27: 667-76

Yan L, Zhao L, Long Y, Zou P, Ji G, et al. 2012. Association of the maternal MTHFR C677T polymorphism with susceptibility to neural tube defects in offspring: evidence from 25 case-control studies. PLoS One. 7: e41689

Yang B, Liu Y, Li Y, Fan S, Zhi X et al. 2013. Geographical Distribution of MTHFR C677T, A1298C and MTRR A66G Gene Polymorphisms in China: Findings from 15357 Adults of Han Nationality. PLoS One. 8(3): e57917

Yoo JH, Choi GD, Kang SS. 2000. Pathogenicity of thermolabile methylenetetrahydro-folate reductase for vascular dementia. Arterioscler Thromb Vasc Biol 20:1921–1925

Young JW. 1977. Gluconeogenesis in cattle: significance and methodology. J. Dairy Sci. 60, 1–15

Yue Y, Liu J, He C. 2015. RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. Genes Dev. 29: 1343-55

Zhao JY, Qiao B, Duan WY, Gong XH, Peng QQ, Jiang SS et al. 2014. Genetic variants reducing MTR gene expression increase the risk of congenital heart disease in Han Chinese populations. Eur Heart J. 35(11):733-42

Zhao R, Matherly LH, Goldman ID. 2009. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med. 28;11:e4.

Zhou Y1, Holmseth S, Hua R, Lehre AC, Olofsson AM, Poblete-Naredo I, Kempson SA, Danbolt NC. 2012. The betaine-GABA transporter (BGT1, slc6a12) is predominantly expressed in the liver and at lower levels in the kidneys and at the brain surface. Am J Physiol Renal Physiol. 302(3):F316-28

Zwickler T, Lindner M, Aydin HI, Baumgartner MR, Bodamer OA et al. 2008. Diagnostic work-up and management of patients with isolated methylmalonic acidurias in European metabolic centres. Journal of Inherited Metabolic Disease. 31:36

Appendix 1

Table 1. 99th percentile SNPs from pairwise F_{ST} breed comparison between Texel and Suffolk sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.702	12	40020610	dbrs160775144	MTHFR	methylenetetrahydrofolate reductase	synonymous variant
0.593	12	40020689	dbrs160775146	MTHFR	methylenetetrahydrofolate reductase	missense variant
0.556	9	40872445	UoNOvar.1356	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.556	22	19760157	dbrs405881066	GOT1	glutamic-oxaloacetic transaminase 1	upstream gene variant
0.550	1	2.64E+08	dbrs428874743	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.546	12	40009379	dbrs416523788	MTHFR	methylenetetrahydrofolate reductase	upstream gene variant
0.542	9	40872131	dbrs422630111	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.535	1	2.64E+08	UoNOvar.133	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.531	9	40872385	UoNOvar.1355	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.531	9	40872232	dbrs412643355	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.519	1	2.64E+08	dbrs410615477	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.497	3	2232105	dbrs400510485	SARDH	sarcosine dehydrogenase	upstream gene variant
0.495	1	2.63E+08	dbrs415564973	SLC19A1	Solute Carrier Family 19 Member 1	upstream gene variant
0.487	1	2.64E+08	dbrs424304321	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.482	1	2.64E+08	dbrs405186770	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.482	1	2.64E+08	dbrs415672426	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.480	12	40020842	dbrs402145672	MTHFR	methylenetetrahydrofolate reductase	missense variant
0.476	25	35314831	dbrs425668423	MAT1A	methionine adenosyltransferase I	upstream gene variant

0.472	1	2.64E+08	dbrs425579738	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.467	1	2.64E+08	dbrs413839874	FTCD	Formimidoyltransferase Cyclodeaminase	upstream gene variant
0.459	9	40871064	UoNOvar.1350	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.459	9	40850699	dbrs160652929	GGH	gamma-glutamyl hydrolase	synonymous variant
0.447	1	2.64E+08	UoNOvar.131	FTCD	Formimidoyltransferase Cyclodeaminase	synonymous variant
0.445	8	37470977	dbrs402406072	COQ3	coenzyme Q3 methyltransferase	upstream gene variant
0.443	9	40871755	dbrs401558953	GGH	gamma-glutamyl hydrolase	upstream gene variant
0.442	1	2.63E+08	dbrs403440484	SLC19A1	Solute Carrier Family 19 Member 1	upstream gene variant
0.442	1	2.63E+08	dbrs412617642	SLC19A1	Solute Carrier Family 19 Member 1	upstream gene variant
0.581	15	13332553	dbrs405597416	JRKL	JRK-like	upstream gene variant
0.495	5	77864935	dbrs405592176	FBLL1	fibrillarin-like 1	upstream gene variant
0.492	2	2.09E+08	dbrs411356281	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.491	2	2.09E+08	dbrs401515390	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.491	2	2.09E+08	dbrs409019572	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.477	5	77865016	dbrs428269541	FBLL1	fibrillarin-like 1	upstream gene variant
0.446	15	13332149	dbrs160679348	JRKL	JRK-like	synonymous variant
0.446	11	47502099	dbrs426944821	FTSJ3	FtsJ homolog 3	upstream gene variant
0.442	15	13332676	dbrs193640709	JRKL	JRK-like	upstream gene variant
0.441	5	77866233	dbrs401422264	FBLL1	fibrillarin-like 1	upstream gene variant
0.434	11	47500436	dbrs161095825	FTSJ3	FtsJ homolog 3	upstream gene variant
0.433	5	77864762	dbrs402361146	FBLL1	fibrillarin-like 1	upstream gene variant
0.428	2	2.09E+08	dbrs405084479	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.427	2	2.09E+08	UoNOvar.566	METTL21A	Methyltransferase Like 21A	upstream gene variant

Table 2. 99th percentile SNPs from pairwise F_{ST} breed comparison between Texel and BFL sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.716	2	246600331	dbrs398315711	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.711	2	246600531	dbrs426161601	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.705	2	246600221	dbrs408384461	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.664	2	246602059	dbrs425592173	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant
0.652	2	246602032	dbrs414019897	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	5'UTR variant
0.652	2	246602050	UoNOvar.594	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant
0.651	2	246600603	dbrs405910531	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.620	1	76391726	dbrs401096164	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	upstream gene variant
0.595	1	76391204	dbrs425987306	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	upstream gene variant
0.595	2	246602013	dbrs398747325	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	5'UTR variant
0.583	2	246602095	dbrs399876942	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant
0.567	3	170568117	dbrs400688608	CHPT1	Choline Phosphotransferase 1	upstream gene variant
0.565	25	9499732	dbrs428838550	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	synonymous variant
0.565	20	22014880	dbrs162200463	MUT	methylmalonyl CoA mutase	upstream gene variant
0.565	20	22014820	dbrs418774463	MUT	methylmalonyl CoA mutase	upstream gene variant
0.563	25	9493566	dbrs424850025	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	synonymous variant
0.549	1	76391418	dbrs409548758	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	upstream gene variant
0.547	3	170568302	dbrs423449486	CHPT1	Choline Phosphotransferase 1	upstream gene variant
0.544	8	26562237	dbrs401063150	ENSOARG00000009927	adenosylmethionine decarboxylase 1	upstream gene variant
0.543	2	246601490	dbrs404102797	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant

0.542	2	246602981	dbrs421014314	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	missense variant
0.541	1	76391613	dbrs407100624	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	upstream gene variant
0.540	2	246600161	dbrs418449012	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.540	2	246600213	dbrs429989460	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.537	2	246601690	dbrs405220014	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.537	2	246601819	dbrs426859693	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.537	2	246601851	dbrs427880280	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.520	2	246601011	dbrs408465439	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.515	2	246601900	UoNOvar.592	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.507	2	246601570	dbrs426517658	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.506	20	22015178	dbrs411838155	MUT	methylmalonyl CoA mutase	upstream gene variant
0.502	8	26561349	dbrs399356982	ENSOARG00000009927	adenosylmethionine decarboxylase 1	upstream gene variant
0.502	8	26561761	dbrs425705340	ENSOARG00000009927	adenosylmethionine decarboxylase 1	upstream gene variant
0.501	25	9534974	dbrs415683482	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	splice region synonymous variant
0.497	2	246599490	dbrs412648337	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.497	2	246599790	dbrs413755405	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.487	20	22016618	dbrs428961712	MUT	methylmalonyl CoA mutase	upstream gene variant
0.485	2	246600646	dbrs413017578	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.485	2	246600721	dbrs402931585	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.483	25	9547354	dbrs401976569	MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	splice region intron variant
0.502	11	23062675	dbrs412025156	ENSOARG00000015825	methyltransferase like 16 (METTL16)	upstream gene variant

Table 3. 99th percentile SNPs from pairwise F_{ST} breed comparison between Texel and Swaledale sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.765	7	73462473	dbrs424495782	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.765	7	73462406	dbrs413194343	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.761	12	40020610	dbrs160775144	MTHFR	methylenetetrahydrofolate reductase	synonymous variant
0.744	7	73461982	dbrs420883701	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.734	7	73463461	dbrs402994374	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.732	7	73461925	dbrs398475224	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.732	7	73461954	dbrs413879483	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.732	7	73461337	dbrs428708652	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.731	7	73461653	dbrs430066170	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant

0.731	7	73463345	dbrs419191996	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.673	7	73462578	dbrs414517250	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.651	12	40020689	dbrs160775146	MTHFR	methylenetetrahydrofolate reductase	missense variant
0.614	12	40009379	dbrs416523788	MTHFR	methylenetetrahydrofolate reductase	upstream gene variant
0.600	20	38387709	dbrs403061814	TPMT	thiopurine S-methyltransferase	3'UTR variant
0.589	5	78072063	dbrs398777693	ENSOARG00000014803	dihydrofolate reductase	upstream gene variant
0.573	2	2.47E+08	dbrs425592173	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant
0.554	7	73461035	dbrs417369843	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.550	7	73463564	dbrs160351613	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.538	12	40020842	dbrs402145672	MTHFR	methylenetetrahydrofolate reductase	missense variant
0.531	7	73463266	dbrs410904488	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.528	5	78071508	dbrs404593738	ENSOARG00000014803	dihydrofolate reductase	upstream gene variant
0.503	2	2.47E+08	dbrs398747325	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	5'UTR variant
0.503	2	2.47E+08	dbrs414019897	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	5'UTR variant
0.503	2	2.47E+08	UoNOvar.594	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant

0.502	2	2.47E+08	dbrs398315711	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.501	2	2.47E+08	dbrs426161601	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.492	2	2.47E+08	dbrs404102797	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.491	7	73463007	UoNOvar.1276	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.491	7	73463013	UoNOvar.1278	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.484	2	2.47E+08	dbrs405220014	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.484	2	2.47E+08	dbrs426859693	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.484	2	2.47E+08	dbrs427880280	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.482	7	73462630	dbrs429989176	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.480	2	2.47E+08	dbrs399876942	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	synonymous variant
0.480	2	2.47E+08	dbrs421014314	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	missense variant
0.457	2	2.47E+08	dbrs426517658	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	upstream gene variant
0.446	12	40008809	dbrs400076665	MTHFR	methylenetetrahydrofolate reductase	upstream gene variant
0.554	14	48528814	dbrs160953982	FBL	fibrillarin	synonymous variant
0.553	14	48528820	dbrs160953983	FBL	fibrillarin	synonymous variant
0.486	14	55108157	dbrs423648417	ENSOARG00000013381	protein arginine methyltransferase 1	upstream gene variant
0.448	14	55107983	dbrs412342675	ENSOARG00000013381	protein arginine methyltransferase 1 (PRMT1)	upstream gene variant

Table 4. 99th percentile SNPs from pairwise F_{ST} breed comparison between Suffolk and BFL sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.954	2	2.47E+08	dbrs408384461	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.926	2	2.47E+08	dbrs398315711	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.921	2	2.47E+08	dbrs426161601	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.898	2	2.47E+08	dbrs405910531	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.882	2	2.47E+08	dbrs399876942	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.875	1	77535481	dbrs404927371	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.875	1	77535894	dbrs406781512	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.860	2	2.47E+08	dbrs414019897	ALDH4A1	aldehyde dehydrogenase 4 family, A1	5'UTR variant
0.860	2	2.47E+08	UoNOvar.594	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.860	2	2.47E+08	dbrs408465439	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.848	1	77534695	dbrs161767028	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.841	2	2.47E+08	dbrs421014314	ALDH4A1	aldehyde dehydrogenase 4 family, A1	missense variant
0.837	2	2.47E+08	dbrs415575413	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.832	2	2.47E+08	dbrs425592173	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.804	1	77535915	dbrs417887819	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.804	1	77536258	dbrs421429336	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.804	1	77536340	dbrs411081297	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.801	2	2.47E+08	dbrs398747325	ALDH4A1	aldehyde dehydrogenase 4 family, A1	5'UTR variant
0.777	1	77534939	dbrs418648546	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.754	2	2.47E+08	dbrs418449012	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.754	2	2.47E+08	dbrs429989460	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant

0.750	2	2.47E+08	dbrs425688145	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.732	1	77535998	dbrs418890952	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536031	dbrs429751100	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536079	dbrs408469951	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536198	dbrs399046846	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536353	dbrs404792889	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536443	dbrs423340683	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536523	dbrs402618596	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536570	dbrs418125116	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536595	dbrs429207750	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.732	1	77536625	dbrs403605828	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.719	2	2.47E+08	dbrs404058123	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.703	1	77535422	dbrs426411233	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.702	1	77536636	dbrs414664169	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.700	1	2.63E+08	dbrs415564973	SLC19A1	Solute Carrier Family 19 Member 1	upstream gene variant
0.700	1	77534494	dbrs430472614	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.832	11	23062515	dbrs400009621	ENSOARG00000015825	methyltransferase like 16 (METTL16)	upstream gene variant
0.821	11	23062632	dbrs398655478	ENSOARG00000015825	methyltransferase like 16 (METTL16)	upstream gene variant
0.821	11	23062675	dbrs412025156	ENSOARG00000015825	methyltransferase like 16 (METTL16)	upstream gene variant
0.724	2	2.09E+08	dbrs423921704	METTL21A	Methyltransferase Like 21A	upstream gene variant

Table 5. 99th percentile SNPs from pairwise F_{ST} breed comparison between Suffolk and Swaledale sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.774	2	2.47E+08	dbrs399876942	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.774	2	2.47E+08	dbrs421014314	ALDH4A1	aldehyde dehydrogenase 4 family, A1	missense variant
0.739	2	2.47E+08	dbrs425592173	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.732	2	2.47E+08	dbrs408465439	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.699	2	2.47E+08	dbrs398747325	ALDH4A1	aldehyde dehydrogenase 4 family, A1	5'UTR variant
0.699	2	2.47E+08	dbrs414019897	ALDH4A1	aldehyde dehydrogenase 4 family, A1	5'UTR variant
0.699	2	2.47E+08	UoNOvar.594	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.698	2	2.47E+08	dbrs398315711	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.697	2	2.47E+08	dbrs426161601	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.692	2	2.47E+08	dbrs415575413	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.672	4	48948857	dbrs429447122	DLD	dihydrolipoamide dehydrogenase	upstream gene variant
0.658	2	2.47E+08	dbrs408384461	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.656	2	2.47E+08	dbrs405910531	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.626	2	2.47E+08	dbrs415480557	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.626	2	2.47E+08	dbrs404841173	ALDH4A1	aldehyde dehydrogenase 4 family, A1	synonymous variant
0.623	20	38387709	dbrs403061814	TPMT	thiopurine S-methyltransferase	3'UTR variant
0.622	22	19760157	dbrs405881066	GOT1	glutamic-oxaloacetic transaminase 1	upstream gene variant
0.606	2	2.47E+08	dbrs405220014	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.606	2	2.47E+08	dbrs426859693	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.606	2	2.47E+08	dbrs427880280	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant

0.596	4	48948628	dbrs402918603	DLD	dihydrolipoamide dehydrogenase	upstream gene variant
0.591	2	2.47E+08	dbrs404102797	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.591	2	2.47E+08	dbrs426517658	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.585	2	2.47E+08	dbrs404058123	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.566	2	2.47E+08	UoNOvar.592	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.566	2	2.47E+08	UoNOvar.593	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.544	2	2.47E+08	dbrs425688145	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.534	8	37471192	dbrs425115509	COQ3	coenzyme Q3 methyltransferase	upstream gene variant
0.523	2	2.47E+08	UoNOvar.589	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.521	4	48948875	dbrs410376183	DLD	dihydrolipoamide dehydrogenase	upstream gene variant
0.519	4	48948512	dbrs417272289	DLD	dihydrolipoamide dehydrogenase	upstream gene variant
0.510	2	2.47E+08	dbrs418449012	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.510	2	2.47E+08	dbrs429989460	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.508	2	2.47E+08	dbrs413017578	ALDH4A1	aldehyde dehydrogenase 4 family, A1	upstream gene variant
0.608	14	48528820	dbrs160953983	FBL	fibrillarin	synonymous variant
0.554	14	48528814	dbrs160953982	FBL	fibrillarin	synonymous variant
0.542	2	2.09E+08	dbrs411356281	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.534	2	2.09E+08	dbrs401515390	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.534	2	2.09E+08	dbrs409019572	METTL21A	Methyltransferase Like 21A	upstream gene variant
0.523	13	28554124	dbrs406021605	SUV39H2	suppressor of variegation 3-9 homolog 2	upstream gene variant
0.509	13	28554086	dbrs416066351	SUV39H2	suppressor of variegation 3-9 homolog 2	upstream gene variant

Table 6. 99th percentile SNPs from pairwise F_{ST} breed comparison between BFL and Swaledale sheep

FST value	Chromosome	Position	SNP	Gene	Gene name	Variant
0.806	7	73462406	dbrs413194343	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.806	7	73462473	dbrs424495782	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.780	7	73461982	dbrs420883701	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.773	7	73463345	dbrs419191996	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.773	7	73463461	dbrs402994374	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.747	20	22014820	dbrs418774463	MUT	methylmalonyl CoA mutase	upstream gene variant
0.747	20	22014880	dbrs162200463	MUT	methylmalonyl CoA mutase	upstream gene variant
0.724	7	73461925	dbrs398475224	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.724	7	73461954	dbrs413879483	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant

0.721	7	73461337	dbrs428708652	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.721	7	73461653	dbrs430066170	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.716	12	40020610	dbrs160775144	MTHFR	methylenetetrahydrofolate reductase	synonymous variant
0.715	3	56179087	dbrs422369506	SUCLG1	Succinate-CoA Ligase Alpha Subunit	upstream gene variant
0.715	3	56179401	dbrs419443300	SUCLG1	Succinate-CoA Ligase Alpha Subunit	upstream gene variant
0.700	5	14636917	UoNOvar.1071	CD320	CD320 molecule	upstream gene variant
0.682	1	77535481	dbrs404927371	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.682	1	77535894	dbrs406781512	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.681	3	56179458	dbrs402079572	SUCLG1	Succinate-CoA Ligase Alpha Subunit	upstream gene variant
0.658	1	77534695	dbrs161767028	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.656	5	14636707	dbrs424181338	CD320	CD320 molecule	upstream gene variant
0.646	12	40020689	dbrs160775146	MTHFR	methylenetetrahydrofolate reductase	missense variant
0.642	5	14636637	dbrs401467672	CD320	CD320 molecule	upstream gene variant
0.641	7	73462578	dbrs414517250	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant
0.635	1	2.63E+08	dbrs415564973	SLC19A1	Solute Carrier Family 19 Member 1	upstream gene variant
0.632	20	22015178	dbrs411838155	MUT	methylmalonyl CoA mutase	upstream gene variant
0.621	7	73461035	dbrs417369843	MTHFD1	methylenetetrahydrofolate dehydrogenase 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	upstream gene variant

0.612	1	77535915	dbrs417887819	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.612	1	77536258	dbrs421429336	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.612	1	77536340	dbrs411081297	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.609	5	78072063	dbrs398777693	DHFR	dihydrofolate reductase	upstream gene variant
0.601	23	42524629	dbrs398285176	NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	upstream gene variant
0.595	20	22014717	dbrs407266367	MUT	methylmalonyl CoA mutase	upstream gene variant
0.594	1	2.61E+08	dbrs406467581	CBS	cystathionine-beta-synthase	upstream gene variant
0.589	1	77534939	dbrs418648546	DPH5	diphthamide biosynthesis 5	upstream gene variant
0.587	1	76391726	dbrs401096164	AGL	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	upstream gene variant
0.741	14	55108157	dbrs423648417	PRMT1	protein arginine methyltransferase 1	upstream gene variant
0.671	14	55108257	dbrs405830799	PRMT1	protein arginine methyltransferase 1	upstream gene variant
0.653	14	55109921	dbrs429974244	PRMT1	protein arginine methyltransferase 1	upstream gene variant
0.629	14	55107983	dbrs412342675	PRMT1	protein arginine methyltransferase 1	upstream gene variant
0.600	14	55109020	dbrs424380324	PRMT1	protein arginine methyltransferase 1	upstream gene variant
0.589	11	47502099	dbrs426944821	FTSJ3	FtsJ homolog 3	upstream gene variant

Appendix 2

Table 1. Pairwise breed comparison F_{ST} values for 33 significant SNPs identified in Chapter 4

SNP	Gene	Texel/Suffolk	Texel/Swaledale	Texel/BFL	Suffolk/Swaledale	Suffolk/BFL	Swaledale/BFL
dbrs400381348	GART	0.13	0.13	0.17	0.00	0.18	0.18
UoNOvar.1503	ASMTL	0.18	0.24	0.03	0.03	0.08	0.14
dbrs408003853	ENSOARG00000010451	0.03	0.02	0.05	0.03	0.08	0.05
dbrs162034765	CA3	0.15	0.18	0.05	0.16	0.10	0.23
dbrs427234880	CA3	0.09	0.08	0.08	0.11	0.04	0.13
dbrs414970315	MMADHC	0.18	0.07	0.04	0.20	0.25	0.19
dbrs404243017	MMADHC	0.15	0.06	0.03	0.17	0.19	0.14
dbrs424867462	MMADHC	0.21	0.00	0.01	0.19	0.23	0.01
dbrs399509316	MMADHC	0.21	0.01	0.01	0.19	0.23	0.02
dbrs404706479	MMADHC	0.24	0.05	0.03	0.29	0.23	0.10
UoNOvar.764	NOX4	0.05	0.16	0.01	0.31	0.02	0.22
dbrs423172086	PCMT1	0.10	0.09	0.10	0.01	0.08	0.11
UoNOvar.325	DPEP1	0.06	0.11	0.18	0.24	0.32	0.03
dbrs430165882	GCSH	0.25	0.13	0.11	0.06	0.16	0.04
dbrs408003853	ENSOARG00000010451	0.03	0.02	0.05	0.03	0.08	0.05

dbrs406023352	SETD1B	0.39	0.18	0.30	0.24	0.05	0.11
dbrs162081923	SETD1B	0.21	0.16	0.24	0.02	0.01	0.02
dbrs402697947	SETD1B	0.00	0.01	0.02	0.01	0.02	0.01
dbrs406167325	NSUN6	0.02	0.02	0.10	0.03	0.08	0.11
dbrs417907151	TRMT61A	0.00	0.00	0.00	0.00	0.00	0.00
dbrs161814674	KMT2D	0.03	0.00	0.13	0.03	0.07	0.11
dbrs413573281	CUBN	0.05	0.21	0.03	0.13	0.08	0.28
dbrs413234073	CUBN	0.05	0.21	0.01	0.14	0.09	0.26
UoNOvar.460	TRMT2A	0.05	0.02	0.07	0.02	0.01	0.03
UoNOvar.463	TRMT2A	0.00	0.00	0.00	0.00	0.00	0.00
dbrs419604537	TRMT2A	0.00	0.00	0.00	0.00	0.00	0.00
UoNOvar.380	KMT2A	0.04	0.05	0.06	0.02	0.04	0.02
dbrs428658243	GAD1	0.01	0.00	0.02	0.01	0.05	0.01
dbrs400381348	GART	0.13	0.13	0.17	0.00	0.18	0.18
dbrs161801525	IMPDH1	0.03	0.03	0.02	0.00	0.03	0.03
dbrs161923561	MTHFD1L	0.12	0.09	0.24	0.04	0.20	0.15
dbrs159794405	IMPDH1	0.02	0.01	0.00	0.00	0.04	0.03
UoNOvar.439	COQ5	0.02	0.03	0.03	0.01	0.05	0.04

Metabolic genes are highlighted in green. Epigenetic regulatory genes are highlighted in red.