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Low levels of sulfur and cobalt during the pre- and periconceptional periods affect the oocyte yield of donors and the DNA methylome of preimplantation bovine embryos

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Abstract

Maternal nutrition is critical in mammalian development, influencing the epigenetic reprogramming of gametes, embryos, and fetal programming. We evaluated the effects of different levels of sulfur (S) and cobalt (Co) in the maternal diet throughout the pre- and periconceptional periods on the biochemical and reproductive parameters of the donors and the DNA methylome of the progeny in *Bos indicus* cattle. The low-S/Co group differed from the control with respect to homocysteine, folic acid, B_{12}, insulin growth factor 1, and glucose. The oocyte yield was lower in heifers from the low S/Co group than that in the control heifers. Embryos from the low-S/Co group exhibited 2320 differentially methylated regions (DMRs) across the genome compared with the control embryos. We also characterized candidate DMRs linked to the *DNMT1* and *DNMT3B* genes in the blood and sperm cells of the adult progeny. A DMR located in *DNMT1* that was identified in embryos remained differentially methylated in the sperm of the progeny from the low-S/Co group. Therefore, we associated changes in specific compounds in the maternal diet with DNA methylation modifications in the progeny. Our results help to elucidate the impact of maternal nutrition on epigenetic reprogramming in livestock, opening new avenues of research to study the effect of disturbed epigenetic patterns in early life on health and fertility in adulthood. Considering that cattle are physiologically similar to humans with respect to gestational length, our study may serve as a model for studies related to the developmental origin of health and disease in humans.

Introduction

Although the genome remains relatively stable during the lifetime of an organism, independent of the environmental influences, the epigenome is modulated and highly susceptible to environmental stimuli, primarily in the initial stages of mammalian development, specifically, gametogenesis and early embryogenesis.[1,2] This modulation occurs because during this window of development, widely distributed epigenetic reprogramming involving DNA methylation and histone modifications must occur to produce a viable oocyte that can be fertilized to generate a good quality embryo.[3] Therefore, studies investigating the influence of environmental stimuli, such as nutrition and in vitro manipulations, on the epigenetic profiles of gametes and embryos are essential for improving the quality of the embryos and health of the progeny in adulthood.

In vitro embryo production (IVP), the nutrition quality and physical status of oocyte donors, in vitro manipulation of oocytes and embryos, and in vitro culture conditions, especially the culture medium composition, can strongly influence embryo quality.[4–6] In vivo endocrine and metabolic changes that can be induced by the nutritional conditions of oocyte donors are followed by alterations in follicular fluid composition, which may impair oocyte

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development and quality, as the oocyte is highly susceptible to any disturbance in its microenvironment.[7] Taken together, all these aspects, either in vivo or in vitro, may specifically alter DNA methylation patterns and, in turn, impair initial embryo development.[8,9] Moreover, aberrant establishment of the epigenetic profile during initial embryo development may result in a persistently abnormal epigenetic profile throughout development, thereby compromising the health of the fetus and progeny in adulthood.[1,10]

Both posttranslational histone modifications and DNA methylation patterns are crucial to the regulation of gene expression; S-adenosylmethionine (SAM), which is produced from methionine, is the substrate that provides methyl groups for the establishment of histone and DNA methylation patterns.[11] Methionine is a sulfur (S)-containing amino acid that can be supplied through the diet,[12] as well as through homocysteine remethylation, in which a methyl group is transferred from 5-methyltetrahydrofolate (5-methyl-THF) or from betaine, which is a by-product of choline.[13,14] In addition, B vitamins, including vitamins B_2, B_6, and B_{12}, are cofactors in the homocysteine remethylation process.[14] Ruminants usually do not consume any dietary source of vitamin B_{12} and depend entirely on the ruminal microbiota for the synthesis of this vitamin, which is dependent on continuous cobalt (Co) supplementation in the diet.[15] Therefore, the supply of SAM as a methyl donor for DNA methylation depends on the availability of all these nutrients, which are involved in one-carbon metabolism. The major function of this cycle is ensuring that cells always have an adequate supply of SAM, even when the ingestion of methyl group donors, such as methionine, betaine, or choline, is low.[16] Mammalian oocytes and embryos have been reported to express several important enzymes related to one-carbon metabolism.[17,18] This finding suggests that oocytes and preimplantation embryos can independently utilize and metabolize nutrients from one-carbon metabolism, such as methionine, choline, betaine, folate, and B vitamins.

Therefore, considering the relevance of the one-carbon cycle as a methyl donor in the epigenetic machinery, we hypothesized that manipulating specific compounds associated with this cycle in the oocyte donor diet may change the epigenetic reprogramming of the progeny, thereby helping to elucidate how maternal nutrition influences the epigenome of the progeny and their health later in life.[2,19]

Accordingly, we evaluated the effects of different levels of S and Co in the diet of the oocyte donor *Bos taurus indicus* Nellore heifers during the entire pre- and periconceptional periods on oocyte and embryo production and on the DNA methylome of F1 embryos. Moreover, we evaluated candidate differentially methylated regions (DMRs) identified in embryos in the blood and sperm cells of their counterparts in adult animals.

Materials and methods

Animals and experimental diets

The experiment was approved by the Ethics Committee on Animal Use (CEUA-Protocol no. 98/2010), School of Veterinary Medicine and Animal Science of the Universidade Estadual Paulista "Júlio de Mesquita Filho."

In this study, we used Nellore (*B. taurus indicus*) heifers (n = 20) aged approximately 30 months, with an average weight of 395.36 ± 33.3 kg and body condition scores (scale 1–5) between 3 and 4. The animals were fed in pens with widths of 80 cm per animal and allowed free access to water. We offered a diet based on sugarcane, urea, S, and mineral premixes. The animals were randomly allocated into two experimental groups of 10 animals each.

Table 1. Consumption of the experimental diets

	Diets	
	Control	Low S/Co
Dry matter consumption/ sugarcane (kg/d)	4.59	4.38
Ingredient kg/d (%)		
Sugarcane	20.674 (98.54)	19.735 (98.53)
Urea	0.188 (0.90)	0.199 (0.99)
Ventilated sulfur	0.021 (0.10)	–
Mineral premix[b]	0.097 (0.46)	0.095 (0.48)[a]

[a]Mineral premix without any source of sulfur and cobalt.
[b]Chemical composition of mineral premix is shown in Table 2.

The feed was offered to the animals twice a day and was chemically similar for both groups, except for the Co and S contents. The diet primarily consisted of sugarcane, urea, and mineral premix (SAL GRAMA NUTRIÇÃO ANIMAL®) (Table 1). The chemical composition of the mineral premix was verified through an analysis performed before the initiation of the experiment. The chemical composition of sugarcane was based on three analyses performed throughout the experiment and aimed to adjust the diets only for the maintenance of the animals (Table 2). Sugarcane was chosen as the forage due to its low content of S, Co, and protein (Table 2). Heifers were randomly assigned to one of two dietary treatments: (1) control with sugarcane (ad libitum) + urea/S (9:1) (1%) and mineral premix and (2) low S/Co with sugarcane (ad libitum) + urea (1%) and mineral premix without any source of S and Co (Tables 1 and 2). The diets were administered over 6 months. Every day prior to providing the feed, any remaining food was removed from the troughs and weighed to calculate the daily consumption. The animals were weighed every 15 days with 8 h of fasting to calculate the average daily gain (ADG) and for blood collection. At the end of the experiment, all heifers (n = 20) were inseminated with the same bull that was used in the IVP and were subsequently placed in a brachiaria grass pasture with free access to water and mineral salt. Among the progeny of those heifers, we collected blood and semen from seven bulls (three animals from heifers of the control group and four from heifers of the low-S/Co group) to confirm the methylome data. Semen was collected by electroejaculation, and samples were stored in liquid nitrogen (−196 °C) until DNA isolation was performed. The scheme of the experimental design is shown in Fig. 1.

Blood biochemical analyses

Folic acid, vitamin B_{12}, homocysteine, insulin, glucose, and insulin growth factor 1 (IGF1) were measured in the blood plasma of all heifers (n = 20) every 15 days (n = 11 assays per animal). All animals were fasted for 8 h before blood sample collection. Analyses were performed by the Instituto Sabin, Brasília-DF, Brazil. Glucose was measured using Advia 2400 equipment following the hexokinase methodology (Siemens, Berlin, Germany). Folic acid and basal insulin were measured using ADVIA Centaur equipment according to a chemiluminescence-based methodology (Siemens, Berlin, Germany). Vitamin B_{12} was measured using ADVIA Centaur equipment according to the Centaur methodology (Siemens, Berlin,

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Table 2. Chemical composition of sugarcane and mineral premix

Item	Composition	
Nutrient composition – sugarcane (SD)		
Ca (%)	0.23(0.15)	
Crude protein (%)	5.2(1.15)	
K (%)	0.74(0.09)	
Co (%)	0.01(0.0)	
Na (ppm)	213.33(25.17)	
P (%)	0.19(0.14)	
Crude fiber (%)	22.5(5.73)	
TDN (%)	66.7(4.89)	
Mg (%)	0.14(0.06)	
Cu (ppm)	0.07(0.05)	
Humidity (%)	77.7(6.30)	
Ether extract (%)	2.1(1.67)	
Dry matter (%)	22.2(6.30)	
S (%)	0.08(0.01)	
F (g/kg)	0.001(0.0)	
Zn (ppm)	3.33(2.08)	
Mineral premix	complete[1]	-sulfur -cobalt carbonate
Na	21.01	20.77
Ca	10.15	11.15
P	8.30	8.55
S	1.76	0.28
Zn	0.28	0.23
Mg	0.18	0.24
Cu	0.07	0.07
Co	0.015	0.002

SD, standard deviation.
[1]Mineral premix (GRAMA®). Basic composition of mineral premix: dicalcium phosphate, sodium chloride, calcium iodate, ventilated sulfur, zinc oxide, copper oxide, cobalt carbonate, and sodium selenite.

Germany). The levels of IGF1 and homocysteine were measured via chemiluminescence analysis using IMMULITE XPi 2000 equipment (Siemens, Berlin, Germany).

Oocyte retrieval by ovum pickup (OPU)

OPU (n = 7/animal) was performed weekly, beginning 3 months after the experimental diets were first offered. For OPU, we used an ultrasonographic scanner (Aloka SSD 500® Japan) coupled to a probe with a microconvex sector transducer, a 7.5-MHz model UST-9125-7.5 (Aloka®, Japan), a WTA® transvaginal guide (Brazil), and a VMAR5100 Cook® vacuum pump (Australia) coupled to a system with a WTA® 18 G needle (0.9 x 70) (Brazil). Prior to follicular aspiration, caudal epidural anesthesia using 3–5 mL of 2% lidocaine (Pearson®; Eurofarma, Brazil) was administered. The oocytes were aspirated in phosphate-buffered saline medium with 5% bovine fetal serum (Sigma-Aldrich® St. Louis, MO) and 1 μL/mL sodium heparin (Liquemin® iv; Roche, Switzerland) at a constant temperature of 39°C. After aspiration, the cumulus–oocyte complexes (COCs) were sent to the laboratory to be selected[20] and subjected to IVP.

IVP

All media used for IVP were supplied by GENEAL Genetics and Animal Biotechnology S/A, Uberaba-MG, Brazil. COC grades I, II, and III, at 15–30 per treatment (control and low S/Co), were transferred to 150 μL drops of maturation medium, coated with silicone oil, and incubated for 22 h at 39°C in 5% CO_2. After maturation, the oocytes were washed and transferred to 150 μL of fertilization medium drops. Semen with known in vitro fertility from a bull that is routinely used in our laboratory was thawed at 37°C for 30 s in a water bath, and the sperm cells were selected via centrifugation on discontinuous Percoll gradient of 45–90%.[21] Sperm and COCs were coincubated for 18 h at 39°C in 5% CO_2. The day of in vitro insemination was considered day zero (D0). Following coincubation, potential zygotes derived from the fertilized oocytes were washed and transferred to drops containing 150 μL of culture medium. The zygotes were cultured at 39°C in 5% CO_2. The embryos were evaluated on D2, D7, and D8 to calculate their cleavage, blastocyst, and hatching rates, respectively.

Whole-genome bisulfite sequencing (WGBS) and identification of candidate DMRs

WGBS was performed at a facility at the Roy J. Carver Biotechnology Center, University of Illinois, as follows. One pool of grade I D8 expanded blastocysts was used for each treatment (n = 68 embryos for the control group and n = 41 embryos for the low-S/Co group) to collect the genomic DNA for WGBS. All heifers by group contributed proportionally to the embryos in each pool. As we used a single biological replicate, it was necessary to employ specific methods that can identify DMRs through two-group comparisons without biological replicates.[22,23] The WGBS experiment served as an initial screening for identifying potential genomic regions as candidate DMRs for characterization (next subsection). Genomic DNA from the embryos was isolated using the QIAamp DNA Micro kit (Qiagen®) following the manufacturer's instructions. The shotgun DNA libraries were prepared using the Library Construction kit from Kapa Biosystems® with one modification: following adaptor ligation, the libraries were treated with the EZ DNA Methylation Lightning kit (Zymo Research®). Bisulfite-treated libraries were amplified with Kapa HiFi Uracil+ DNA polymerase. The libraries were pooled in equimolar concentrations, quantified through quantitative polymerase chain reaction (PCR), and sequenced on two lanes for 166 cycles from each end of the fragments on an Illumina HiSeq2500. For quality control, the Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) program was utilized. PCR duplications and nonconverted reads were removed using MarkDuplicates.jar from the PICARD toolkit ("Picard Toolkit." 2019. Broad Institute, GitHub Repository. http://broadinstitute.github.io/picard/; Broad Institute). The Bismark package[24] was used to map bisulfite-treated sequencing reads to a *B. taurus* reference genome (UMD3.1), and methylation calls were performed. For the two libraries, the total number of reads was 375,817,934, and the mapping is summarized in Table 3. Non-CpG methylation was 0.6% for each sample, indicating high sodium bisulfite conversion efficiency (>99%). Genomic features were categorized as exon, intron, intragenic, and intergenic regions according to the information available in the UMD3.1 reference. The Bis-single nucleotide polymorphism (SNP) method was employed for SNP

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Table 3. Summary of whole genome bisulfite sequencing

Library	Reads (R1,R2)	Reads after cleaning (R1,R2)	Mapped read (R1,R2)	Uniq Mapped reads (R1,R2)	Coverage (*)	C's in CpG context analyzed
Control	94,363,495 x 2	93,269,406	64,771,159	61,543,239 (66.0%)	11.56	112,685,381
Low S/Co	93,545,472 x 2	92,527,690	71,258,496	68,162,063 (73.7%)	11.47	112,084,880
Total	375,817,934	371,594,192				224,770,261

(*)Coverage = (read count * read length)/total genome size.
Control.
93,269,406 * 2 * 165/2,660,922,743 = 11.56.
Low S/Co.
92,527,690 * 2 * 165/2,660,922,743 = 11.47

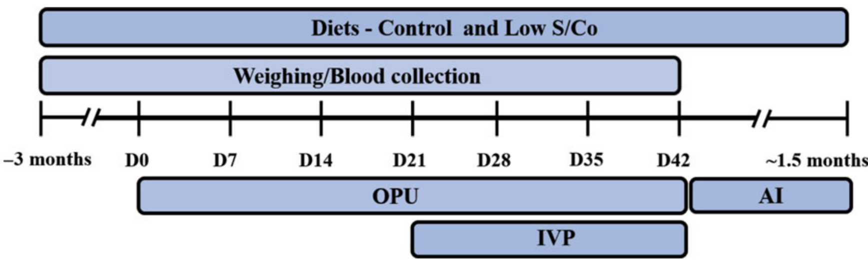

Fig. 1. Scheme of the experimental design. Heifers (n = 10 per treatment) were randomly assigned to one of two dietary treatments, control and low S/Co (S – sulfr; Co – cobalt). The diets were administered over 6 months. The animals were weighed every 15 days with 8 h of fasting to calculate the average daily gain (ADG) and for blood collection. Ovum pickup (OPU) (n = 7/animal) was performed weekly, beginning 3 months after the experimental diets were first offered. After OPU has finished, all heifers (n = 20) were inseminated (AI) with the same bull that was used in the in vitro embryo production (IVP). D0 – Day 0; D7 – Day 7; D14 – Day 14; D21 – Day 21; D28 – Day 28; D35 – Day 35; and D42 – Day 42.

calling.[25] Homemade scripts were developed to convert the output from Bis-SNPs for use in the next step, which was the detection of differential DNA methylation. To annotate differentially methylated CpGs and DMRs, the Metilene method was employed.[22] Metilene was utilized in this analysis because it provides several parameter adjustments, and this technique is one of the few methods developed to date for annotating DMRs without biological replicates. The following parameters were employed to run Metilene: a minimum of 10 CpG distant at a maximum of 50 nucleotides from one other and $P \leq 0.05$. The annotation of the Gene ID was made using an in-house script and the UMD3 annotation file. The predicted proteins from the Ensembl79_UMD3.1 genome were annotated with PFAM_33.1 version. A correspondence table between PFAM and gene ontology (GO) terms (PFAM2GO – version date: 2018/09/08) was employed to infer GO terms in each bovine protein. The hypergeometric test within the FUNC program (PMID: 17284313) was utilized to determine enriched GO terms (FDR < 0.05) in the significantly differentially methylated genes. The GO term fold-enrichment values were calculated as the ratio between the observed (methylated) and expected (genome) gene frequencies.

Characterization of candidate differentially methylated genes using bisulfite PCR (BS-PCR) in adult progeny

Among the candidate DMRs that were identified in D8 expanded blastocysts using WGBS, we first preselected 32 candidate DMRs (Table 4) that are physically linked to genes related to epigenetic mechanisms and reproduction, due to the research interest of our laboratory. Subsequently, we selected two DMRs located in the *DNMT1* and *DNMT3B* genes (in boldface in Table 4) to validate the WGBS using BS-PCR. As biological samples, we used genomic DNA from the blood and sperm cells of seven bulls (three progenies from heifers of the control group and four from the low-S/Co group). Genomic DNA was isolated from the blood using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Genomic DNA from the sperm was isolated using a salting-out procedure[26] with an additional step in which 5 mM Dithiothreitol (DTT) was added. Subsequently, genomic DNA (500 ng) was treated with sodium bisulfite using the EZ DNA Methylation-Lightning kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Sodium bisulfite-treated DNA was stored at −80°C until PCR amplification was performed. Primers for amplifying the CpG islands of the *DNMT1* and *DNMT3B* genes were designed using MethPrimer[27] and Bisulfite Primer Seeker software (http://www.zymoresearch.com/tools/bisulfite-primer-seeker). Primer sequences, GenBank access number, number of CpG sites, amplicon size, and annealing temperature are described in Table 5. The total volume of the reaction mixtures was 20 µL, comprising 1× Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U Platinum™ Taq polymerase (Invitrogen, CA, USA), 0.5 µM of each primer (forward and reverse), and 2 µL of bisulfite-treated DNA. PCR was performed with an initial denaturing step at 94°C for 3 min followed by 29 cycles at 94°C for 40 s, annealing temperature for 1 min, and 72°C for 1 min with a final extension at 72°C for 15 min. The annealing temperature for each gene is specified in Table 5. The amplicons were purified on an agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purified amplicons were cloned into the TOPO TA Cloning® vector (Invitrogen, CA, USA) and transferred into DH5α cells using a heat shock procedure. Plasmidial DNA was isolated using Pure Yield Plasmid Miniprep (Promega), and individual clones were sequenced using BigDye® cycle sequencing chemistry and an ABI3100 automated sequencer. Electropherogram quality was analyzed using Chromas®, and methylation patterns were processed using the QUantification tool for Methylation Analysis (QUMA, http://quma.cdb.riken.jp/top/index.html).[28] The DNA sequences

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Table 4. Differentially methylated regions between control and low-S/Co groups for genes related to epigenetic machinery, spermatogenesis, and one-carbon cycle metabolism in bovine embryos

chr	#CpGs	p (MWU)	% methylation Control	% methylation Low S/Co	DMR length	DMR position relative to gene	Feature	Gene id	Protein
1	15	0.000037	71.113	43.333	107	14042	Intron	ENSBTAG00000024162	Folate transporter 1
1	10	0.0011	85	25	177	−176,259	Inter gene	ENSBTAG00000000502	Deleted in azoospermia-like
2	10	0.0029	41.166	85	109	28,944	Exon	ENSBTAG00000003843	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a-like 1
2	10	0.0011	100	20	116	37,410	Exon	ENSBTAG00000013281	Spermatogenesis associated 21
3	10	0.0015	28.333	75.001	130	−57,641	Intergene	ENSBTAG00000002078	Lysine (K)-specific demethylase 4A
3	10	0.0011	60.945	90	134	−179,685	Intergene	ENSBTAG00000017764	Histone deacetylase 4
3	10	0.00021	3.333	65	45	−3475	Intergene	ENSBTAG00000017764	Histone deacetylase 4
3	12	0.000014	70.833	0	130	31,003	Exon	ENSBTAG00000017764	Histone deacetylase 4
3	13	0.0012	70.513	15.385	98	36,888	Intron	ENSBTAG00000017764	Histone deacetylase 4
3	10	0.0011	0	76.667	108	123,494	Intron	ENSBTAG00000017764	Histone deacetylase 4
4	10	0.019	28.333	70	110	43,994	Intron	ENSBTAG00000024199	Lysine (K)-specific methyltransferase 2C
5	10	0.000022	96.667	46.667	159	23,854	Intron	ENSBTAG00000014429	Lysine (K)-specific methyltransferase 2D
5	11	0.000085	4.5455	46.969	220	−26,629	Intergene	ENSBTAG00000026819	Histone deacetylase 7 (HDAC7)
5	10	0.0039	51.5	15	132	6820	Exon	ENSBTAG00000020472	Lysine-specific demethylase 5A
5	10	0.00032	13.333	86.667	174	3285	Intron	ENSBTAG00000005595	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase
7	10	0.00032	10	77	222	6584	Intron	**ENSBTAG00000002736**	**DNA (cytosine-5-)-methyltransferase 1**
7	12	0.00005	70.833	8.3333	220	87,056	Exon	ENSBTAG00000019220	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 4
7	10	0.0089	20	80	95	37,231	Exon	ENSBTAG00000009996	DOT1-like histone H3K79 methyltransferase
13	10	0.0039	46.666	90	115	20,788	Exon	**ENSBTAG00000003901**	**Bos taurus DNA (cytosine-5-)-methyltransferase 3 beta (*DNMT3B*), mRNA**
17	12	0.0000015	65.198	6.1108	222	53,399	Intron	ENSBTAG00000002328	Lysine (K)-specific demethylase 2B
17	13	0.0015	0	53.846	125	111,012	Exon	ENSBTAG00000002328	Lysine (K)-specific demethylase 2B
18	10	0.00032	30	95	73	4541	Intron	ENSBTAG00000015917	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
19	11	0.00028	46.818	90.909	65	−83,535	Intergene	ENSBTAG00000012293	Sperm-associated antigen 9
19	10	0.00049	19.666	75.001	156	25,672	Exon	ENSBTAG00000012293	Sperm-associated antigen 9
19	16	0.0000061	40.625	100	197	61,444	Exon	ENSBTAG00000004723	Testis-expressed sequence 2 protein
20	11	0.00028	90.909	40.909	160	29,712	Intron	ENSBTAG00000015466	NOP2/Sun RNA methyltransferase family, member 2
22	10	0.0011	27.047	62.5	192	−49,318	Intergene	ENSBTAG00000007208	Histone deacetylase 11 (HDAC11)
22	10	0.011	65.285	38.665	182	−48,400	Intergene	ENSBTAG00000007208	Histone deacetylase 11 (HDAC11)
24	10	0.0039	21.667	80	72	−267,493	Intergene	ENSBTAG00000037457	Methyl-CpG binding domain protein 2
26	10	0.000011	95	10	111	−580,838	Intergene	ENSBTAG00000007011	Testis-expressed 36
26	10	0.000011	15	100	103	−367,582	Intergene	ENSBTAG00000007011	Testis-expressed 36
29	10	0.000011	10	100	82	35,825	Exon	ENSBTAG00000044032	Lysine (K)-specific demethylase 2A

Differentially methylated regions in boldface were used to validate the WGBS using BS-PCR.

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Table 5. Primers for methylation analysis

Gene		Primer Sequence (5'–3')	Genomic coordinate	Genbank accession number	CpG sites	Amplicon length (bp)	Annealing (ºC)
DNMT1	F:	AGAGGGGAGTTGGTGTTTTTTTTGTAGAT	chr7: 14693613-14693640	281119	10	317	60
			chr7: 14693324-14693354				
	R:	CCCCAAATCAAAACAATTCCTAATAAACTCT					
DNMT3B	F:	TTGTTTGTGAATAGTTTTTTAGGTATTAGGG	chr13: 62175291-62175321	353354	13	312	61
			chr13: 62175567-62175602				
	R:	ACTATAAACCCAAAAAAAAAAAAAAAAAAAAAAACC					

F, forward; R, reverse; bp, base pair.

were compared with GenBank reference sequences (GenBank ID listed in Table 5), and only sequences that originated from clones with ≥ 95% identity and ≥ 97% cytosine conversion were utilized in the analysis.

Statistical analysis

For the blood biochemical parameters, to evaluate the effects of time, diet, and the interaction of time and diet on the response variables, a factorial design was employed considering two factors, namely, time and diet. A normal distribution was assigned, and a covariance analysis model, with weight serving as a covariate, was used. A Poisson distribution was assigned to the number of aspirated follicles and the number of total oocytes. We employed a generalized linear model to evaluate the effect of diet on these responses. The fit using the Poisson distribution did not adjust all the variability. Thus, we used the quasi-likelihood method for estimation of the heterogeneity factor. We also evaluated the effects of time, diet, and the interaction of time and diet on the number of total oocytes. For the embryo production data, a natural choice was the binomial distribution. The fit using the binomial distribution did not adjust all the variability. The heterogeneity present was treated using the quasi-likelihood method (quasi-binomial). All analyses were performed using the R Program "Development Core Team" (R Foundation for Statistical Computing, Vienna, Austria).[29] ADG, daily food consumption, and BS-PCR data were compared using the t test and the Mann–Whitney test for data showing normal distribution and data not showing normal distribution, respectively. The data were compared using the Prophet program Version 5.0 (BBN Technologies System; NIH, Bethesda, MD, USA, 1996). Analysis of the WGBS data is described in detail above under the heading "Whole-genome bisulfite sequencing (WGBS) and identification of candidate DMRs." Global methylation data were compared between groups and genomic features using two-tailed Fisher's exact test and two-tailed paired t test. The data were compared using GraphPad Prism software (GraphPad Software, La Jolla, California, USA; www.graphpad.com) and the R program "Development Core Team" (R Foundation for Statistical Computing, Vienna, Austria).[29]

Results

ADG and daily food consumption

Body weight measurements were performed 11 times in each heifer every 15 days. Every time we weighed the heifers, they were subjected to 8 h of fasting to calculate the ADG. No significant differences (Student's t test, $P \geq 0.05$) were found in ADG (control: 0.104 ±0.311 kg/d; low S/Co: 0.207 ± 0.208 kg/d) or average daily food consumption (Table 1) between the control and low-S/Co groups.

Plasma biochemical profile

Analysis of covariance showed no significant interaction of diet and time for any plasma metabolites between treatments, including homocysteine ($P = 0.126$), folic acid ($P = 0.99$), B12 ($P = 0.541$), IGF-I ($P = 0.95$), insulin ($P = 1.00$), and glucose ($P = 0.256$). Time significantly affected the folic acid ($P < 0.001$), vitamin B12 ($P < 0.001$), IGF-I ($P < 0.001$), insulin ($P < 0.001$), and glucose ($P < 0.001$) concentrations. The profile of the low-S/Co group was significantly different from that of the control group with respect to the following metabolites: homocysteine ($P < 0.001$), folic acid ($P = 0.011$), vitamin B_{12} ($P < 0.001$), IGF1 ($P = 0.034$), and glucose ($P = 0.003$). The low-S/Co group exhibited higher levels of homocysteine, folic acid, and glucose but lower levels of vitamin B_{12} and IGF1 than did the control group (Fig. 2 and Table 6).

Oocyte retrieval and embryo production

The diets significantly affected the total numbers of oocytes. No effects of time or the interaction of diet and time were observed on the total numbers of oocytes. The low-S/Co group produced fewer oocytes than the control group (ANOVA, $P = 0.0438$) (Fig. 3 and Table 7). The diets did not influence any of the embryo production parameters (Table 8). Heifers from the low-S/Co group produced an average of 8.66 D8 embryos by OPU, whereas heifers from the control produced an average of 17.33 D8 embryos by OPU.

Identification of candidate DMRs in embryos

We characterized the global methylation profile of F1 embryos using WGBS.

The mean CpG methylation values were 23.42% and 26.21% for the control and low-S/Co embryo groups, respectively. Independent of the treatments, the mean value for global CpG methylation of the embryos was 24.19%. Figure 4a presents the mean CpG methylation values for each chromosome. Although both groups exhibited a similar methylation profile for all chromosomes, the low-S/Co group exhibited a globally higher level of methylation than the control (Fisher's exact test, $P = 4.32E-27$). We additionally evaluated the CpG methylation percentage for different genomic regions (exon, intron, and intergenic regions); independent of the treatment,

Table 6. Effects of the low-S/Co diet on metabolites of one-carbon cycle, IGF1, insulin, and glucose levels in the peripheral circulation

	Diet				
	Low S/Co		Control		
	Mean	± SD	Mean	± SD	*P* value
Homocysteine (micromol/L)	13.70[a]	3.52	10.64[b]	3.19	< 0.001
Folic acid (ng/mL)	29.58[a]	10.47	25.98[b]	10.52	0.011
B12 (pg/mL)	143.84[a]	46.48	165.59[b]	48.73	< 0.001
IGF1 (ng/mL)	375.30[a]	99.17	410.24[b]	121.00	0.034
Insulin (μUI/mL)	4.53[a]	6.12	3.33[a]	3.23	1.00
Glucose (mg/dL)	82.16[a]	18.47	76.18[b]	13.88	0.003

[a,b] Different letters indicate different means at a 5% significance level.

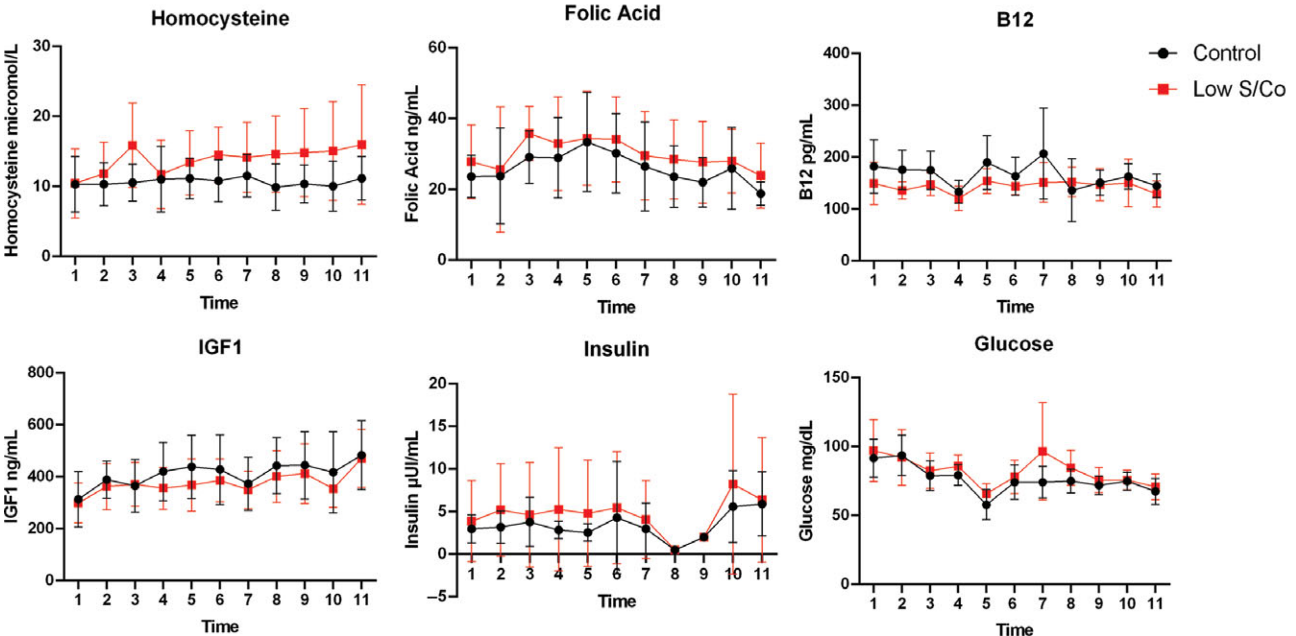

Fig. 2. Distribution of plasmatic levels of the biochemical metabolites measured in the control and low-S/Co groups over time. Time represents blood sample collection every 15 days with animals with 8 h of fasting. No significant interactions of diet and time were observed for any plasma metabolites. Time significantly affected the folic acid, B12, IGF1, insulin, and glucose concentrations ($P < 0.05$). The low-S/Co group exhibited higher levels of homocysteine, folic acid, and glucose but lower levels of vitamin B_{12} and IGF1 than did the control group ($P < 0.05$). Data are represented by means ± SD. S, sulfur; Co, cobalt; IGF1, insulin growth factor 1.

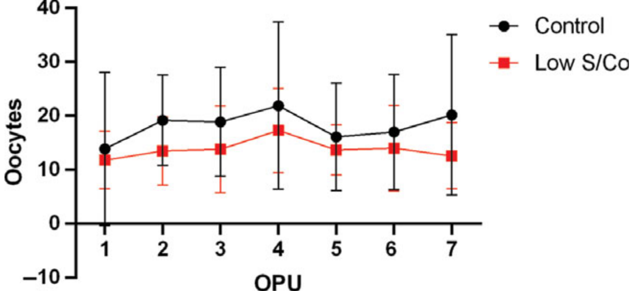

Fig. 3. Number of oocytes recovered by ovum pickup (OPU). Each OPU represents the average number of oocytes recovered from 10 heifers of each treatment, control and low S/Co. No effects of time or the interaction of diet and time were observed on the total number of oocytes. The low-S/Co group produced fewer oocytes than the control group ($P < 0.05$). Data are represented by means ± SD.

the intergenic regions exhibited less methylation than did the other regions (Student's *t* test, $P < 2E\text{-}16$) (Fig. 4b). Finally, we identified potential candidate DMRs distributed according to different genomic features (Fig. 4c). Overall, we identified 2320 candidate DMRs between the low-S/Co and control groups (Fig. 4d and Supplementary Table S1). For all genomic features analyzed, the low-S/Co group showed higher methylation for most candidate DMRs compared to the control group [1322 (56.98%) and 998 (43.02%), respectively] (Fig. 4d). In addition, the exons exhibited fewer DMRs between the control and low-S/Co groups [388; ~16.7% (Fig. 4c)]. Table 4 presents the candidate DMRs identified between the groups for genes with functions related to the epigenetic machinery, spermatogenesis, and one-carbon cycle metabolism. With respect to GO analysis, no specific terms or pathways were enriched among the 2320 candidate differentially methylated genes (Fig. 5).

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Table 7. Effect of the diets on the total number of oocytes per animal recovered by ovum pickup

Diet	Oocyte mean	± SD	*p*-value
Control	17.79[a]	11.63	
Low S/Co	13.81[b]	6.60	0.0438

[a,b] Different letters indicate different means at a 5% significance level.

Characterization of candidate DMRs in adult progeny by BS-PCR

We also evaluated the DNA methylation patterns of candidate DMRs in blood and sperm cells of adult animals. To characterize the WGBS data in adult progeny, we selected two DMRs identified in the *DNMT1* and *DNMT3B* genes (Fig. 6b and Table 4). Overall, we sequenced 406 clones to characterize the DNA methylation patterns of those two DMRs in the blood and sperm cells of the seven progeny bulls from heifers of both groups. The DNA methylation profile of each animal and tissue is presented in Fig. 6c and 6d, indicating a hypermethylated pattern in both tissues and genes for all animals. WGBS for embryos exhibited 10% and 77% methylation in the control and low-S/Co groups for *DNMT1*, respectively, and 46.66% and 90% methylation in the control and low-S/Co groups for *DNMT3B*, respectively (Fig. 6a and Table 4). Therefore, the blood cells and sperm of the adult progeny exhibited a similar result to the embryos from the low-S/Co group and a hypermethylated pattern compared to the embryos in the control group for both genes.

For both genes, the blood cells exhibited similar DNA methylation for both groups (Fig. 6c and 6d). In contrast, for *DNMT1*, the low-S/Co group exhibited a slightly higher methylation than the control in the sperm (Mann–Whitney test, $P = 0.0356$) (Fig. 6c), thereby corroborating the WGBS results, which showed that the control exhibited less methylation in the blastocysts than the low-S/Co group (Table 4).

In addition, we compared the methylation status for each CpG. We identified two specific CpGs (3 and 6) and one CpG (6) that were differentially methylated between the control and low-S/Co groups in the blood cells and sperm cells, respectively, for *DNMT1* (Fig. 6c). No individual CpG sites were observed to be differentially methylated for *DNMT3B* (Fig. 6d).

Discussion

During the prenatal period, epigenetic reprogramming can be susceptible to environmental factors, such as maternal nutrition.[1,12,30,31] SAM, which is produced by the one-carbon cycle, is the substrate that methylates genomic DNA and histones.[32] Therefore, determining the role played by specific compounds of this cycle in embryo and fetal epigenetic programming is essential to elucidate the effects of nutrition in this window of development.

Mammalian folliculogenesis and oogenesis are constant and continuous processes in the ovaries that begin during fetal life with primordial follicle formation and end with ovulation.[33] However, the entire duration of these processes in cattle is still widely discussed in the literature.[34–36] When a primordial follicle is recruited for growth, the enclosed oocyte immediately starts its molecular activity, initiating its transcriptional and epigenetic programming. In this study, we subjected the animals to experimental diets for 3 months before initiating OPU, and the diets were administered until the OPU cycles were completed. This strategy ensured that all the aspirated oocytes were under the effect of the diets during complete period of oogenesis.

We first sought to evaluate the influence of low levels of S and Co in the diet on the blood plasma levels of certain metabolites related to the methionine cycle. We observed significant differences between the low-S/Co and control groups with respect to all metabolites, except insulin (Fig. 2 and Table 6). We suggest that the synthesis of S amino acids and B vitamins by ruminal microbiota could be decreased in the low-S/Co group, thereby impairing the one-carbon cycle. In addition to the lower levels of S and Co in this diet, a substantial amount of nitrogen in the diet was supplied by urea, a nonprotein source of nitrogen, which could contribute to decreased S amino acid synthesis, considering that in this situation, the supply of an extra source of S is essential. We observed that the low-S/Co diet decreased vitamin B_{12} levels and increased homocysteine and folic acid levels (Fig. 2 and Table 6). Our results are supported by reports from the literature, which demonstrated that vitamin B_{12} deficiency decreased methionine synthesis through 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), thereby inducing hyperhomocysteinemia and folate accumulation as 5-methyl-THF,[37,38] which is the most common form detected in bovine plasma.[39] Thus, the low-S/Co diet may lead to an accumulation of homocysteine because of the lack of vitamin B_{12}, a cofactor of the MTR enzyme, which leads to decreased activity of the remethylation pathway of homocysteine, which uses 5-methyl-THF as a substrate. Instead, this diet may induce the use of an alternative to the transsulfuration or choline/betaine pathway through the BHMT enzyme. However, in the 1980s, pioneering studies in ruminants demonstrated that the folate pathway, which uses 5-methyl-THF as the substrate, is more important than the choline/betaine pathway in these animals,[40,41] possibly explaining the accumulation of homocysteine in the low-S/Co group.

In our study, the low-S/Co group exhibited higher glucose and lower IGF1 concentrations than the control group (Fig. 2 and Table 6). IGF1 is a mitogenic factor[42] that participates in glucose regulation through homeostasis. The increased glucose level may have contributed to a decrease in IGF1 secretion, thereby preventing further increases in glucose and causing an even greater decrease in plasmatic IGF1 levels. Studies with mice[43] showed that gluconeogenesis was induced in response to an increase in homocysteine, which contributed to insulin resistance. A study in sheep that evaluated the progeny of animals subjected to a folate- and B_{12}-deficient diet demonstrated similar results with respect to insulin resistance.[10] Reports from the literature additionally support the association between nutrition and ovarian function, and the glucose–insulin–IGF system may be one of the major mechanisms underlying this association.[44,45] Most circulating IGF1 is derived from liver synthesis and regulated by growth hormone, insulin, and nutritional intake.[46] IGFs are observed to be important in the early stages of folliculogenesis, and their deregulation may cause severe impairment in preantral and antral follicular development.[36] In the present study, we found that animals from the low-S/Co group produced fewer oocytes and showed lower levels of IGF1 than the control group (Figs. 2 and 3 and Tables 6 and 7), which may be explained by the importance of the IGF system for ovarian function, as mentioned above. Considering our hypothesis that the synthesis of S amino acids and B vitamins by ruminal microbiota could be decreased in the low-S/Co group, we suggest that this decrease is also a cause for the lower levels of IGF1 observed in this group. We also

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Table 8. Percentage of blastocysts produced in vitro on day 7 (D7) and day 8 (D8) of development with respect to the number of oocytes, and D8 hatched blastocyst percentage with respect to the number of D8 blastocysts

		D2		D7		D8			
	Oocytes	Cleavage		Blastocyst		Blastocyst		Hatched Blastocysts	
Diet	n	n	(%)	n	(%)	n	(%)	n	(%)
Control	229	211	92.14	146	63.76	156	68.12	73	46.79
Low S/Co	129	123	95.35	73	56.59	78	60.47	36	46.15

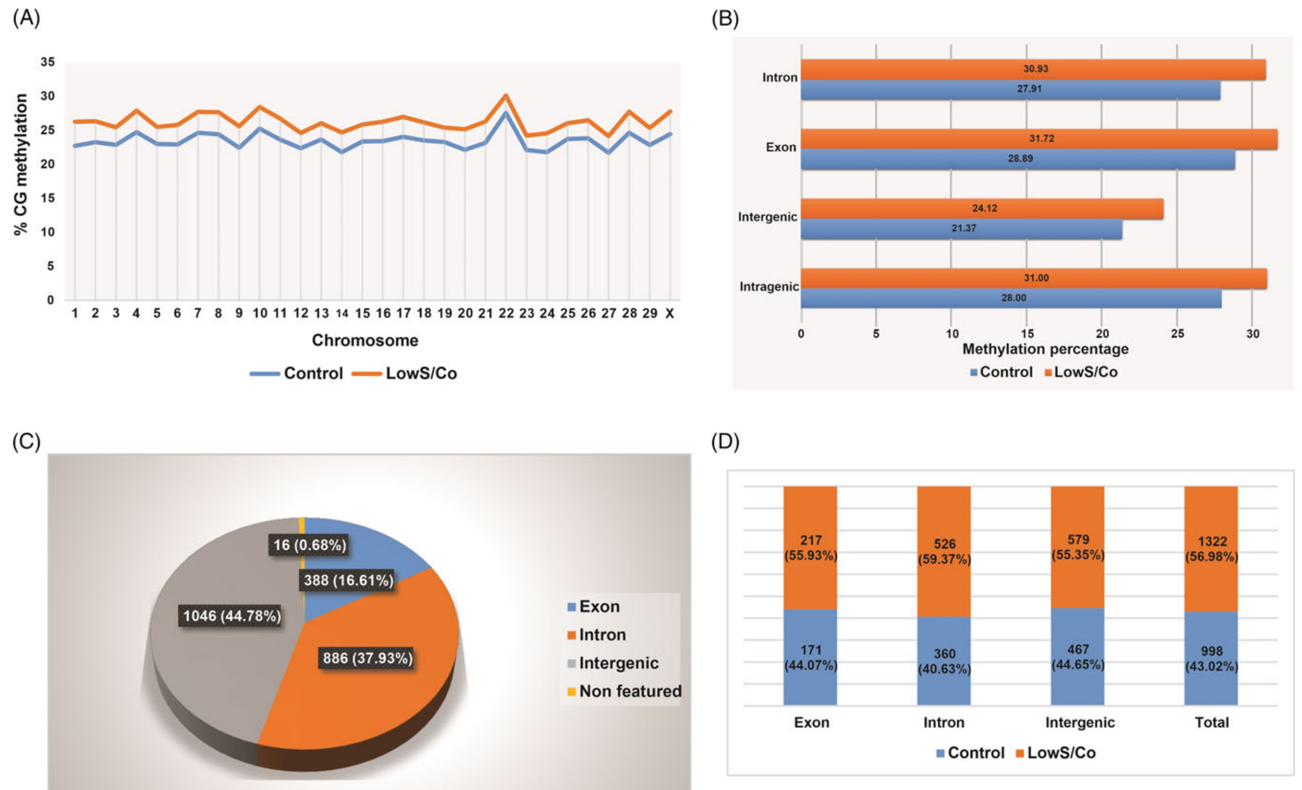

Fig. 4. Whole genome bisulfite sequencing (WGBS) of preimplantation bovine embryos. A - Percentage of global CpG methylation by chromosome generated by WGBS of embryos produced using oocytes aspirated from Nellore heifers that were offered diets with different levels of sulfur and cobalt. Control – control group; low S/Co – diet without any source of sulfur and cobalt. B – Percentage of methylated cytosines (CpGs) by different genomic features (intron, exon, intergenic, and intragenic genomic regions). C – Distribution, in percentage, of the differentially methylated regions (DMRs) between the low S/Co and control groups by different genomic features (exon, intron, and intergenic regions). D – Numbers and percentages of DMRs that were more methylated in each group by each genomic feature.

investigated the effect of time on the total number of oocytes, and no significant effect was found (Fig. 3). This result may be observed because OPUs started 3 months after the animals were receiving the experimental diets. Figure 3 shows that since the first OPUs, heifers from the control group were already producing more oocytes. Despite the significant effect of the diet on the total number of oocytes (Table 7), the treatments produced no effects on the embryo production rate (Table 8). However, whether this diet could affect the quality of the produced embryos has not been determined to date. The pregnancy rate is one of the best parameters to test embryo quality; however, it was not possible to evaluate this aspect in this study. Instead, based on our initial hypothesis, we performed preliminary molecular analyses in F1 embryos and in the progeny in adulthood. Considering the relevance of nutrition for normal epigenetic reprogramming in the initial development and its influence on the health of the progeny in childhood and adulthood,[1] we investigated whether the manipulation of specific compounds of the one-carbon cycle in the diet offered to the oocyte donors during the pre- and periconceptional periods could alter the genome-wide DNA methylation patterns of F1 in vitro-produced embryos and, if so, whether these altered methylation patterns are maintained in the tissues in adult animals.

Despite the low availability of S and Co in the diet, F1 embryos produced from the low-S/Co group showed higher levels of global methylation than did the F1 embryos from the control: 26.21% and 23.42%, respectively. This result is in contrast to the observation of Acosta *et al.* (2016) who showed that embryos from cows supplemented with methionine were less heavily methylated.[12] However, Acosta *et al.* measured global methylation through immunofluorescence, and we evaluated embryo methylomes through sequencing at single-base-pair resolution. In contrast, Mattocks *et al.* (2017) observed increased hepatic global methylation when

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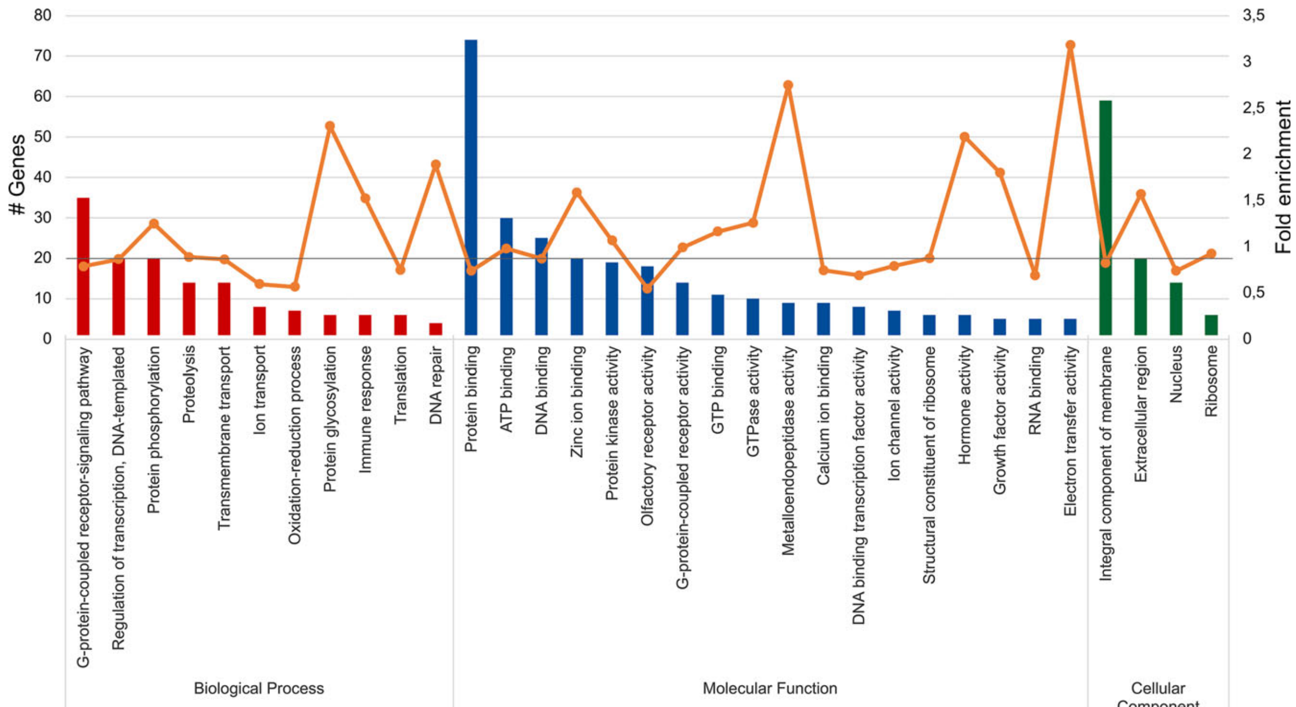

Fig. 5. Gene ontology annotation of significantly differentially methylated genes. Bars represent the number of genes annotated in each GO category. The orange dotted line represents the fold enrichment of each term. Values above 1 suggest a possible term enrichment, but no statistical support was found. Red bars = biological process. Blue bars = molecular function. Green bars = cellular component.

a methionine-restricted diet was administered to mice and suggested that this result could be observed because of a protective effect of the methionine-restricted diet on hepatic DNA hypomethylation.[47] Although we had not measured the levels of methionine, it is possible that animals from the low S/Co group had less methionine due to the low availability of S in their diet. In this regard, as Mattocks *et al.* (2017) suggested, our results may be the consequence of a protective effect on the oocytes in the low-S/Co group, in which the DNA methylation reprogramming of the oocytes was altered, consequently inducing a protective effect against the loss of DNA methylation in the embryos. Animals from the low-S/Co group exhibited differences with respect to all blood biochemical parameters, except insulin, compared to the control animals (Fig. 2 and Table 6). We hypothesize that the disturbance in the plasma metabolite profile of the one-carbon cycle compounds in the low-S/Co animals may serve as an indicator of changes in metabolism which, in turn, would interfere with the DNA methylome[32] of F1 embryos. Irrespective of the groups, we observed that the average global CpG methylation of the embryos was 24.19%, which was very similar to that in other studies, including a study indicating that human embryos show 25.7% global methylation.[48] These results are in keeping with the findings of other studies, which showed that embryonic cells exhibit less DNA methylation than spermatozoa[48,49] or adult somatic tissues,[50] considering that embryonic cells are in the initial process of de novo DNA methylation.[51] In any case, global methylation data must be interpreted with caution because even if global methylation patterns have increased or decreased, specific regions of the genome can behave in the opposite manner. Therefore, we believe that global methylation values alone are not informative and relevant.

We additionally evaluated the CpG methylation percentage according to individual chromosomes. Embryos from both groups showed similar methylation profiles; however, the low-S/Co group exhibited a globally higher level of methylation than the control when the data from all chromosomes were examined (Fig. 4a). This result suggests that the low-S/Co diet affected the methylation profile of the embryos in a nonspecific manner in the genome.

We observed that for all genomic regions, the embryos produced from the low-S/Co group exhibited a higher CpG methylation percentage than did the embryos from the control (Fig. 4b). However, the intergenic regions showed less methylation than the intragenic regions (gene body). This result is in keeping with the findings of other studies, including those performed in mice[52] and chickens,[53] that showed gene bodies with higher methylation.

Although the global CpG methylation levels of the embryos did not change markedly with respect to the diets (23.42% and 26.21% for the control and low-S/Co groups, respectively), we identified DMRs across the genome that were distributed among the different genomic features (Fig. 4c and Fig. 4d), which is in keeping with the findings described in other reports from the literature.[54] For all genomic features analyzed, that is, exons, introns, and intergenic regions, the low-S/Co group exhibited higher methylation for most DMRs compared to the control (Fig. 4d). In addition, the exons were less differentially methylated between the low-S/Co and the control groups (Fig. 4c and 4d). In contrast, intergenic regions, in addition to being less methylated (Fig. 4b), were more differentially methylated between the low-S/Co and control groups (Fig. 4c and 4d); this result was similar to that observed by Masser *et al.* [54] These results may suggest that the effects of environmental stimuli on DNA methylation patterns in the genome may depend on the genomic region. We identified DMRs related to a wide variety of different biological functions (Supplementary Table S1). We performed a GO analysis, and no specific terms or pathways were enriched among the 2320 candidate differentially methylated genes (Fig. 5). This result suggests that the possible disturbance of the

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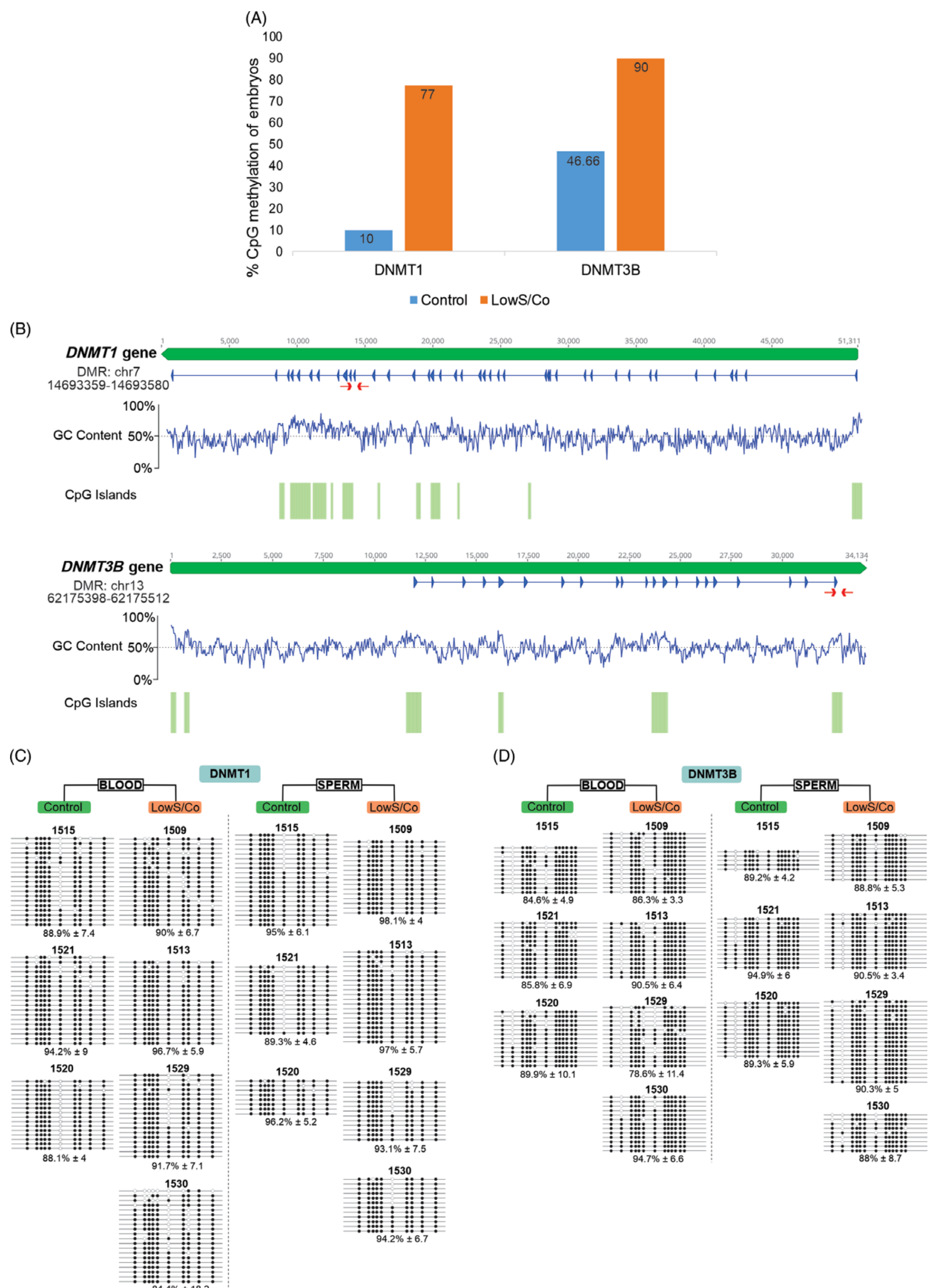

Fig. 6. Representation of *DNMT1* and *DNMT3B* gene structures, GC content, CpG island prediction, and DNA methylation profiles. (A) Percentage of CpG methylation of *DNMT1* and *DNMT3B* genes on embryos obtained by whole genome bisulfite sequencing. (B) The green bars represent the input sequences; below, blue lines represent introns, blue arrows represent exons and red arrows represent the PCR primer positions. For each gene, *DNMT1* and *DNMT3B*, the GC percentage, CpG island positions, and DMR genomic coordinates are shown. The figure was generated by using Geneious v2020.0.5 (Biomatters, New Zealand). (C) and (D) DNA methylation profiles of *DNMT1* (10 CpGs) and *DNMT3B* genes (13 CpGs), respectively, in blood cells and sperm. Each line represents an individual DNA clone, and each circle represents a CpG dinucleotide. Black circles represent methylated cytosines, and white circles represent unmethylated cytosines. The percentage of methylation of each animal (1515, 1521, 1509, 1513, 1520, 1529, and 1530) is represented by the mean ± standard deviation of the mean.

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methionine cycle (Table 6), which is the universal supplier of methyl groups in the cell, impairs the global genome methylation in a nonspecific manner. In Table 4, we present certain DMRs for genes involved in the epigenetic machinery, spermatogenesis, and one-carbon cycle metabolism, which are of special interest to our laboratory. Therefore, these DMRs are candidates to be investigated in the future studies focusing on the influence of nutrition on embryonic and/or fetal epigenetic reprogramming and fertility.

Despite the several differences that we observed in the DNA methylome of embryos between the two groups, our data require further characterization for two major reasons. First, we performed WGBS using only one biological sample. The second and primary reason is that blastocysts undergo extensive epigenetic reprogramming at this stage of embryo development, and it is possible that several DMRs that we identified in the embryos could be correctly reprogrammed later in development, with no inter- or transgenerational effects being detected.[55] Therefore, WGBS was performed on the embryos, and we tested its results using tissues from the progeny in adulthood. We believe that it is more relevant to identify whether certain candidate DMRs in groups of embryos maintain differences in methylation patterns in progeny in adulthood. We selected two candidate DMRs for characterization using BS-PCR and performed a consistent experiment using seven progenies in adulthood and sequenced 406 plasmidial DNA clones from blood and sperm cells to confirm the DNA methylation profile of the two candidate DMRs. Although we characterize only two DMRs, we are providing a supplementary file that lists all candidate DMRs. The BS-PCR results showed that both candidate DMRs, *DNMT1* and *DNMT3B*, showed a hypermethylated pattern in both tissues for all animals; this result is in contrast to the WGBS data for the embryos of the control group, which showed a hypomethylated pattern (Fig. 6 and Table 4). These results suggest that even blastocysts of both groups showed different patterns of methylation in *DNMT1* and *DNMT3B*, throughout development, the initial embryo cells from both groups of embryos were reprogrammed similarly in both blood and sperm progenitor cells. Thus, our results suggest that DMRs identified in embryos have a high probability of losing their differentially methylated pattern, owing to the extensive epigenetic reprogramming that occurs during development.[56] Therefore, we strongly believe that any embryo methylome data must be confirmed in adult individuals and in different tissues.

Finally, we emphasize that we made a considerable effort to evaluate the effects of specific compounds of the methionine cycle in the diet offered over the entire peri- and preconceptional periods on the DNA methylome of embryos and adult progeny, which was a highly expensive and time-consuming experiment. Notably, most studies in cattle only evaluate the final stages of oogenesis; and in this study, we evaluate the effects of nutrition on the entire period of oogenesis in which epigenetic reprogramming occurs in oocytes. Therefore, our results may improve our understanding of the impact of maternal diet on epigenetic reprogramming in cattle and may contribute to the development of specific diets that can be supplied to oocyte donors during specific phases of the reproductive cycle for IVP systems. Although considerable research remains regarding livestock, this information opens new avenues of study for investigations of the impact of altered epigenetic reprogramming in early life on the growth and development of the fetus and the health and production capacity of the offspring in adulthood. Importantly, as we evaluated the effect of maternal nutrition on the epigenetic reprogramming of in vitro embryos and in adult progeny, our results may help to establish a foundation for studies on the developmental origins of health and disease in humans.

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Author' Contributions. ARF, RS, RGJ, DBA, RAF, MAND, and MMF, conceived and designed the project, performed the experiments, and analyzed and interpreted the data. ARF, LNV, RCT, MMCC, PG, ASM, NRK, IP, BDMS, JFWS, LOL, JP, and ARC performed the experiments. In addition, all authors made substantial contributions to the composition or critical revision of the manuscript and supplied important intellectual content. ARF, LNV, RCT, and MMF wrote the manuscript.

Conflict of Interest. The authors have no conflicts of interest to declare.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174421000222

References

1. Grandjean P, Barouki R, Bellinger DC, *et al.* Life-long implications of developmental exposure to environmental stressors: new perspectives. *Endocrinology*. 2015; 156(10), 3408–3415.
2. Lillycrop K, Burdge GC. Maternal diet as a modifier of offspring epigenetics. *J Dev Orig Health Dis*. 2015; 6(2), 88–95.
3. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature*. 2007; 447(7143), 425–432.
4. Watson AJ, De Sousa P, Caveney A, *et al.* Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biology of Reproduction*. 2000; 62(2), 355–364.
5. Jorritsma R, Cesar ML, Hermans JT, Kruitwagen CL, Vos PL, Kruip TA. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci*. 2004; 81(3–4), 225–235.
6. Kadokawa H, Tameoka N, Uchiza M, Kimura Y, Yonai M. Short communication: a field study on the relationship between body condition and embryo production in superovulated Holstein yearling heifers. *J Dairy Sci*. 2008; 91(3), 1087–1091.
7. Leroy JL, Opsomer G, Van Soom A, Goovaerts IG, Bols PE. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part I. The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows. *Reprod Domest Anim*. 2008; 43(5), 612–622.
8. Mann MR, Lee SS, Doherty AS, *et al.* Selective loss of imprinting in the placenta following preimplantation development in culture. *Development*. 2004; 131(15), 3727–3735.
9. Young LE, Fernandes K, McEvoy TG, *et al.* Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet*. 2001; 27(2), 153–154.
10. Sinclair KD, Allegrucci C, Singh R, *et al.* 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*. 2007; 104(49), 19351–19356.
11. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003; 33, 245–254.

https://doi.org/10.1017/S2040174421000222 Published online by Cambridge University Press

12. Acosta DAV, Denicol AC, Tribulo P, *et al.* Effects of rumen-protected methionine and choline supplementation on the preimplantation embryo in Holstein cows. *Theriogenology*. 2016; 85(9), 1669–1679.
13. Olthof MR, van Vliet T, Boelsma E, Verhoef P. Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women. *J Nutr*. 2003; 133(12), 4135–4138.
14. Saw SM, Yuan JM, Ong CN, *et al.* Genetic, dietary, and other lifestyle determinants of plasma homocysteine concentrations in middle-aged and older Chinese men and women in Singapore. *Am J Clin Nutr*. 2001; 73(2), 232–239.
15. Bechdel SI, Honeywell HE, Dutcher RA, Kunutsen MH. Synthesis of vitamin B in the rumen of cow. *J Biol Chem*. 1928; 80, 231–238.
16. Inoue-Choi M, Nelson HH, Robien K, *et al.* One-carbon metabolism nutrient status and plasma S-adenosylmethionine concentrations in middle-aged and older Chinese in Singapore. *Int J Mol Epidemiol Genet*. 2012; 3(2), 160–173.
17. Ikeda S, Namekawa T, Sugimoto M, Kume S. Expression of methylation pathway enzymes in bovine oocytes and preimplantation embryos. *J Exp Zool A Ecol Genet Physiol*. 2010; 313(3), 129–136.
18. Kwong WY, Adamiak SJ, Gwynn A, Singh R, Sinclair KD. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. *Reproduction*. 2010; 139(4), 705–715.
19. Johnson SA, Javurek AB, Painter MS, *et al.* Effects of a maternal high-fat diet on offspring behavioral and metabolic parameters in a rodent model. *J Dev Orig Health Dis*. 2017; 8(1), 75–88.
20. Caixeta ES, Ripamonte P, Franco MM, Junior JB, Dode MA. Effect of follicle size on mRNA expression in cumulus cells and oocytes of Bos indicus: an approach to identify marker genes for developmental competence. *Reprod Fertil Dev*. 2009; 21(5), 655–664.
21. Machado GM, Carvalho JO, Filho ES, *et al.* Effect of Percoll volume, duration and force of centrifugation, on in vitro production and sex ratio of bovine embryos. *Theriogenology*. 2009; 71(8), 1289–1297.
22. Juhling F, Kretzmer H, Bernhart SH, Otto C, Stadler PF, Hoffmann S. Metilene: fast and sensitive calling of differentially methylated regions from bisulfite sequencing data. *Genome Res*. 2016; 26(2), 256–262.
23. Wu H, Xu T, Feng H, *et al.* Detection of differentially methylated regions from whole-genome bisulfite sequencing data without replicates. *Nucleic Acids Res*. 2015; 43(21), e141.
24. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*. 2011; 27(11), 1571–1572.
25. Liu Y, Siegmund KD, Laird PW, Berman BP. Bis-SNP: combined DNA methylation and SNP calling for Bisulfite-seq data. *Genome Biol*. 2012; 13(7), R61.
26. Carvalho JO, Michalczechen-Lacerda VA, Sartori R, *et al.* The methylation patterns of the IGF2 and IGF2R genes in bovine spermatozoa are not affected by flow-cytometric sex sorting. *Mol Reprod Dev*. 2012; 79(2), 77–84.
27. Li L-C, Dahiya RJB. MethPrimer: designing primers for methylation PCRs. *Bioinformatics*. 2002; 18(11), 1427–1431.
28. Kumaki Y, Oda M, Okano MJNar. QUMA: quantification tool for methylation analysis. *Nucleic Acids Res*. 2008; 36(suppl_2), W170–W175.
29. R Foundation for Statistical Computing V, Austria. R: A language and environment for statistical computing. 2011.
30. Laanpere M, Altmae S, Stavreus-Evers A, Nilsson TK, Yngve A, Salumets A. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. *Nutr Rev*. 2010; 68(2), 99–113.
31. Young LE, Rees WD, Sinclair KD. Programming in the pre-implantation embryo. In *Fetal Nutrition and Adult Disease: Programming of Chronic Disease through Fetal Exposure to Undernutrition* (ed. S.C. Langley-Evans). 2004; pp. 333–351. CAB International, Oxford.
32. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem*. 2000; 275(38), 29318–29323.
33. van den Hurk R, Zhao J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology*. 2005; 63(6), 1717–1751.
34. Lussier JG, Matton P, Dufour JJ. Growth rates of follicles in the ovary of the cow. *J Reprod Fertil*. 1987;81(2), 301–307.
35. Russe I. Oogenesis in cattle and sheep. *Bibl Anat*. 1983; 24, 77–92.
36. Webb R, Garnsworthy PC, Gong JG, Armstrong DG. Control of follicular growth: local interactions and nutritional influences. *J Anim Sci*. 2004; 82, E63–E74.
37. Scott JM. Folate and vitamin B12. *Proc Nutr Soc*. 1999;58(2), 441–448.
38. Stangl GI, Schwarz FJ, Muller H, Kirchgessner M. Evaluation of the cobalt requirement of beef cattle based on vitamin B12, folate, homocysteine and methylmalonic acid. *Br J Nutr*. 2000; 84(5), 645–653.
39. Interspecies differences in folate metabolism. *Nutr Rev*. 1992; 50(4 (Pt 1)), 116–118.
40. Xue GP, Snoswell AM. Comparative studies on the methionine synthesis in sheep and rat tissues. *Comp Biochem Physiol B*. 1985; 80(3), 489–494.
41. Xue GP, Snoswell AM. Developmental changes in the activities of enzymes related to methyl group metabolism in sheep tissues. *Comp Biochem Physiol B*. 1986; 83(1), 115–120.
42. Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. *Genes Dev*. 2006; 20(3), 267–275.
43. Wang Y, Yang L, Wang W, *et al.* Effect of homocysteine on gluconeogenesis in mice. *Nan Fang Yi Ke Da Xue Xue Bao*. 2013; 33(4), 507–510.
44. Armstrong DG, McEvoy TG, Baxter G, *et al.* Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biology of Reproduction*. 2001; 64(6), 1624–1632.
45. Scaramuzzi RJ, Campbell BK, Downing JA, *et al.* A review of the effects of supplementary nutrition in the ewe on the concentrations of reproductive and metabolic hormones and the mechanisms that regulate folliculogenesis and ovulation rate. *Reprod Nutr Dev*. 2006; 46(4), 339–354.
46. Yakar S, Setser J, Zhao H, *et al.* Inhibition of growth hormone action improves insulin sensitivity in liver IGF-1-deficient mice. *J Clin Invest*. 2004; 113(1), 96–105.
47. Mattocks DA, Mentch SJ, Shneyder J, *et al.* Short term methionine restriction increases hepatic global DNA methylation in adult but not young male C57BL/6J mice. *Exp Gerontol*. 2017; 88, 1–8.
48. Okae H, Chiba H, Hiura H, *et al.* Genome-wide analysis of DNA methylation dynamics during early human development. *PLoS Genet*. 2014; 10(12), e1004868.
49. MacDonald WA, Mann MR. Epigenetic regulation of genomic imprinting from germ line to preimplantation. *Mol Reprod Dev*. 2014; 81(2), 126–140.
50. Zhou Y, Xu L, Bickhart DM, *et al.* Reduced representation bisulphite sequencing of ten bovine somatic tissues reveals DNA methylation patterns and their impacts on gene expression. *BMC Genomics*. 2016; 17(1), 779.
51. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science*. 2001; 293(5532), 1089–1093.
52. Shirane K, Toh H, Kobayashi H, *et al.* Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. *PLoS Genet*. 2013; 9(4), e1003439.
53. Li J, Li R, Wang Y, *et al.* Genome-wide DNA methylome variation in two genetically distinct chicken lines using MethylC-seq. *BMC Genomics*. 2015; 16, 851.
54. Masser DR, Hadad N, Porter HL, *et al.* Sexually divergent DNA methylation patterns with hippocampal aging. *Aging Cell*. 2017; 16(6), 1342–1352.
55. Jenkins TG, James ER, Aston KI, *et al.* Age-associated sperm DNA methylation patterns do not directly persist trans-generationally. *Epigenetics Chromatin*. 2019; 12(1), 1–8.
56. Reik W, Dean W, Walter JJS. Epigenetic reprogramming in mammalian development. *Science*. 2001; 293(5532), 1089–1093.

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