



# Prepubertal nutritional modulation in the bull and its impact on sperm DNA methylation

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## Abstract

Enhanced pre-pubertal nutrition in Holstein bulls increased reproductive hormone production and sperm production potential with no negative effects on sperm quality. However, recent trends in human epigenetic research have identified pre-pubertal period to be critical for epigenetic reprogramming in males. Our objective was to evaluate the methylation changes in sperm of bulls exposed to different pre-pubertal diets. One-week-old Holstein bull calves ( $n=9$ ), randomly allocated to 3 groups, were fed either a high, medium or low diet (20%, 17% or 12.2% crude protein and 67.9%, 66% or 62.9% total digestible nutrients, respectively) from 2 to 32 weeks of age, followed by medium nutrition. Semen collected from bulls at two specific time points, i.e. 55–59 and 69–71 weeks, was diluted, cryopreserved and used for reduced representation bisulfite sequencing. Differential methylation was detected for dietary treatment, but minimal differences were detected with age. The gene ontology term, “regulation of Rho protein signal transduction”, implicated in sperm motility and acrosome reaction, was enriched in both low-vs-high and low-vs-medium datasets. Furthermore, several genes implicated in early embryo and foetal development showed differential methylation for diet. Our results therefore suggest that sperm epigenome keeps the memory of diet during pre-pubertal period in genes important for spermatogenesis, sperm function and early embryo development.

**Keywords** Pre-pubertal diet · Bull sperm · DNA methylation · RRBS · Epigenetics

## Introduction

The increasing demand for animal proteins over the years have compelled for an increased efficiency of global food production (Thundathil et al. 2016). Improving reproductive efficiency, especially bull fertility, is particularly critical,

as one bull can breed thousands of females by artificial insemination (AI) (Thundathil et al. 2016). Recent adoption of bovine genotyping arrays has facilitated early-life, genotype-based selection of candidate bulls. Consequently, semen from these young bulls can be marketed as soon as they reach puberty. However, there is considerable variation

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among bulls regarding age at puberty (Brito et al. 2012); furthermore, semen quality and fertility improve markedly for ~3 months after puberty (Brito et al. 2007b). Therefore, early onset of puberty and production of freezable semen have substantial financial implications (Dance et al. 2016). Although genetic selection affects those parameters, the environment (e.g. nutrition) has a profound impact on performance of animals and their progeny (Dance et al. 2016, 2015). In addition, recent studies have documented profound benefits of strategic supplemental nutrition on reproductive performance of bulls (Brito et al. 2007a; Barth et al. 2008; Dance et al. 2015, 2016; Johnson et al. 2019). We reported that feeding beef and dairy bulls supplemental energy and protein during calthood (2–30 weeks) increased serum luteinizing hormone concentrations before 20 weeks of age, hastened puberty (~1 month) and produced mature bulls with larger testes and greater sperm production (~20–25% for each) compared to bulls fed less than recommended amounts of energy and protein with no apparent effect on sperm function (Dance et al. 2016, 2015). Although nutrition modulates reproductive potential of bulls, its impact on post-pubertal sperm epigenome remains largely unknown (Wennmann et al. 2014).

The epigenome represents a group of molecular processes targeting the chromatin that influences gene expression without altering the DNA sequence (Goldberg et al. 2007). Among these molecular processes, modifications to DNA (DNA methylation; Holliday and Pugh 1975) or its associated proteins (histone modifications; Allfrey et al. 1964) act in combination to drive the transcriptomic profiles related to cell identity and function. Furthermore, alterations of these epigenetic marks could be influenced by environmental conditions (e.g. nutrition; Burton and Lillycrop 2019). In human, nutritional influences on epigenome were also evoked to explain inter-generational effects, opening a completely new field of research backing up to Barker's hypothesis on developmental origin of health and diseases (DOHaD) (Barker 2004, 1992; Barker and Martyn 1992; Kaati et al. 2002). More and more studies also demonstrated that paternal environment could influence the non-genetic information transmission carried by sperm cells and modify the trajectory of offspring (Donkin and Barres 2018; Champroux et al. 2018). Indeed, the differentiation of male germ cells into mature spermatozoa is based on a long and complex process including a large panel of epigenetic modifications taking place during the foetal life, the prepubertal period and after puberty during each spermatogenesis cycle (Champroux et al. 2018). All these epigenetic remodelling steps are plastic to environmental influences. Specifically, it has been shown that pre-pubertal nutrition has effects on gonad development through the proliferation of Sertoli cells, spermiogenesis supporting cells (Gaysinskaya et al. 2018; Perrier et al. 2018). In bull, we observed differential regulation of several genes in testicular tissue of post-pubertal

bulls exposed to either low-, normal- or high-nutrition diets during their pre-pubertal period (Johnson et al. 2019). One hypothesis would be that dietary modulations done on bull calves during their pre-pubertal period influence the epigenetic maturation of germ cells through alterations of Sertoli cell transcriptome and function.

Although the DNA methylation profile appears to be acquired at the stage of prespermatogonia at birth (Oakes et al. 2007), a transient DNA methylation reduction was observed at onset of meiosis suggesting potential plasticity at prepubertal period (Gaysinskaya et al. 2018). The main objective of our study is to generate preliminary data on effects of pre-pubertal dietary modulations on bull sperm DNA methylation using the reduced-representation bisulfite sequencing (RRBS) technique, which offers a cost-effective genome-wide view of DNA methylation changes at gene- and CpG-rich regions and at a base resolution level. The data generated will allow us to conduct focused studies on the effects of dietary modulations during specific stages of a bull's reproductive life on the sperm epigenome and predict consequences for his fertility and for progeny development.

## Materials and methods

### Animals and treatments

Dietary treatments have been described in detail in Dance et al. (2015). Briefly, 1-week-old Holstein bull calves ( $n=9$ ), randomly allocated to 3 groups ( $n=3$ /group), were fed either a high, medium or low diet from 2 to 32 weeks of age. From 2 to 8 weeks of age, calves were fed milk (8, 6 and 4L/day in high-, medium- and low-diet groups respectively), followed by a transition into a barley silage-based diet. All diets contained 1.6% vitamin-mineral premix (as fed). The high nutrition diet consisted of 49.7% rolled barley, 9.7% rolled corn, 7.6% canola meal and 7.6% soybean meal (20.0% crude protein (CP) and 67.9% total digestible nutrients (TDN)). The medium nutrition diet contained 4.8% rolled barley, 4.8% rolled corn, 3.8% canola meal and 3.8% soybean meal (overall, 17.0% CP and 66.0% TDN). The low-nutrition diet was barley silage (plus premix, but no concentrate) and had 12.2% CP and 62.9% TDN (note that for this and all other diets, CP and TDN are reported on a dry matter basis). The high-nutrition group was fed ad libitum and based on their intake, the same amount of feed (on an as-fed basis) was offered to the low- and medium-nutrition groups. Calves were on their respective diets until 32 weeks of age, after which they were all fed the medium-nutrition diet. Semen was collected from bulls by electroejaculation at frequent intervals from 51 to 73 weeks, but for the present study we utilised semen samples collected from two specific time points 55–59 (Y) and 69–71 (O) weeks of age. The semen samples were then chilled, diluted and cryopreserved as described previously (Dance et al. 2016).

## Genomic DNA extraction

Frozen-thawed sperm cells from two 0.5-ml straws were used for extraction from all 18 samples (3 diets, 2 time points,  $n = 3$  per condition) as described previously (Perrier et al. 2018). In brief, semen samples were washed with water followed by phosphate-buffered saline and incubated overnight with lysis buffer (10 mM Tris–HCl pH 7.5, 25 mM EDTA, 1% SDS, 75 mM NaCl, 50 mM dithiothreitol and 0.5 µg glycogen) containing 0.2 mg/ml proteinase K. Following RNase digestion (25 µg/ml RNase A for 1 h, 37 °C), genomic DNA was extracted twice using phenol:chloroform (1:1) and chloroform, then ethanol precipitated and washed. The dried pellet was re-suspended in TE buffer (10 mM Tris–HCl, pH 7.5, 2 mM EDTA). Both Nanodrop (2000/2000c; ThermoFisher Scientific) and Qubit (ThermoFisher Scientific) were used to analyse the purity and concentration of extracted DNA. The integrity of genomic DNA was confirmed by electrophoresis on agarose gel. Mitochondrial DNA elimination was not ensured as sperm tails were included in the DNA extraction procedure.

## Preparation of RRBS libraries

Library preparation for RRBS was performed as described elsewhere [64–65] from 200 ng of genomic DNA digested with MspI (Fermentas), except that magnetic bead-based size selection was performed using SPRIselect magnetic beads (Beckman-Coulter). Briefly, after ligation to 55-bp methylated Illumina adapters for paired-end sequencing, H<sub>2</sub>O was added up to 50 µl, which was followed by the addition of 35-µl magnetic beads. The larger fragments bound to the beads were removed using a magnetic rack according to the manufacturer's instructions, and 85 µl of supernatant containing the smaller fragments were transferred into a new tube. The addition of 25 µl of fresh beads next allowed to select fragments ranging from 150 to 400 bp. After washing with 85% ethanol, the DNA bound to the beads was eluted in 20 µl EB buffer (Qiagen). The DNA was then bisulfite converted twice using the EpiTect bisulfite kit (Qiagen) followed by PCR amplification of converted DNA for 14 cycles using Pfu Turbo Cx hotstart DNA polymerase (Agilent). All pipetting steps before the final amplification were carried out using a NGS STARlet liquid handling system with four channels (Hamilton). The final libraries were purified using AMPure XP beads (Beckman-Coulter). Concentration of libraries was estimated using Qubit and concentrations suitable for sequencing were confirmed in all the 18 samples. To evaluate the integrity of the final libraries, electrophoresis was performed on a pre-cast 4–20% polyacrylamide gel (Invitrogen) to reveal a smear of DNA from 150 to 400 bp (Supplementary File 1). Sequencing was performed on an

Illumina HiSeq4000 sequencer to produce 75-bp paired-end reads (Integrage SA, France).

## RRBS data analysis

RRBS sequences were analysed as described previously (Perrier et al. 2018). An integrated pipeline combining scripts developed in house in Python, R and Shell, together with external tools (<https://github.com/FAANG/faang-methylation/tree/master/RRBS-toolkit/>) was used for this purpose. In brief, RRBS libraries were subjected to FastQC quality control analysis. Additional quality check and trimming was performed with TrimGalore v0.4.0 to remove adapter sequences, unsatisfactory bases and reads, also reads shorter than 20 nucleotides (Krueger 2014). Good-quality reads were aligned using Bismark v0.14.3 with Bowtie 1 [67–68] to the bovine reference genome (UMD3.1) in which the sequence of Y chromosome has been incorporated (GenBank: CM001061.2).

Principal component analysis (PCA) and hierarchical clustering were computed using the FactoMineR R package on the matrix of CpGs covered by at least 10 reads per sample. For hierarchical clustering, the distance between samples was calculated using Pearson correlation coefficients.

MethylKit (Akalin et al. 2012) software was used to detect differentially methylated CpGs (DMCs). A minimum threshold of 10 reads was applied for CpG coverage. All CpGs fulfilling this coverage threshold in at least 4 samples within a treatment (2 groups related to age,  $n = 9$ /group; 3 groups related to diet,  $n = 6$ /group) were included in the analysis. CpGs with a  $q$ -value lower than 0.01 and methylation difference greater than 25% were considered differentially methylated. A region should have had minimum 3 DMCs with a maximum inter-DMC distance of 100 bp to be considered a differentially methylated region (DMR). Identified DMRs were further extended by the incorporation of CpGs with a  $q$ -value comprised between 0.05 and 0.01 (Perrier et al. 2018).

## Annotation and enrichment analysis

All analysed CpGs, DMCs and DMRs were annotated relative to gene features, CpG density and repetitive elements using an in house pipeline (<https://github.com/FAANG/faang-methylation/tree/master/RRBS-toolkit/Annotation>) as described previously (Perrier et al. 2018). The reference files were downloaded at the following sites: [ftp://ftp.ensembl.org/pub/release-94/gtf/bos\\_taurus/Bos\\_taurus.UMD3.1.94.gtf.gz](ftp://ftp.ensembl.org/pub/release-94/gtf/bos_taurus/Bos_taurus.UMD3.1.94.gtf.gz), <http://oct2018.archive.ensembl.org/biomart/martview/2c4d22063af3b241ef9322512fae0c> (Ensembl Genes 94, Cow genes (UMD3.1)), <http://hgdownload.cse.ucsc.edu/goldenPath/bosTau6/database/cpgIslandExt.txt.gz> and <http://hgdownload.cse.ucsc.edu/goldenPath/bosTau6/database/rmsk.txt.gz>. The following

criteria were applied for annotation: promoter-TSS, –2000 to +100 bp relative to the transcription start site (TSS); TTS, –100 to +100 bp relative to the transcription termination site (TTS); shore, up to 2000 bp from a CpG island (CGI); and shelf up to 2000 bp from a shore. A site/fragment was considered to belong to a CGI (respective shore and shelf) if an overlap of at least 75% was observed between the site/fragment and the CGI (respective shore and shelf). A site/fragment was considered being overlapped by a repetitive element whatever the extent of this overlapping.

CpGs from all analysis covered a total of 17,257 Ensembl gene IDs and this was used as background for enrichment analysis. The Ensembl gene IDs covered by DMCs and DMRs from low vs high, low vs medium and medium vs high comparisons were uploaded to Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) to check enrichment for Gene Ontology (GO) terms (Huang et al. 2009) and KEGG pathway analysis (Kanehisa and Goto 2000). The functional annotation and clustering option were used for most analysis. All significant GO terms and pathways with an adjusted  $P$  value  $\leq 0.01$  (Benjamini-Hochberg) and gene count  $\geq 2$  have been reported.

### Bisulfite pyrosequencing

The DMRs associated with *IGF2*, *INS*, *WWC2* and *WNT1* were subjected to bisulfite pyrosequencing (Tost and Gut 2007). Bisulfite conversion was performed either using the Epiect bisulfite kit (Qiagen) or as described previously (Dupont et al. 2004) using 1  $\mu$ g of genomic DNA. In brief, genomic DNA was denatured followed by treatment with sodium bisulfite (5 M) and hydroquinone (130 mM) for 4 h at 55 °C followed by purification using a pressure activated aspirator system and the Wizard DNA cleanup system (Promega). After purification, DNA was eluted with water, following incubation with 0.3 N NaOH and addition of ammonium acetate (6 M), glycogen and 100%

ethanol. After overnight incubation, the DNA pellet was ethanol precipitated and resuspension in water.

PCR cycle conditions used for amplification of bisulfite converted DNA were as follows: 15 min at 95 °C followed by 45 cycles of 30 s at 94 °C (denaturation), 30 s at 56 °C (annealing), 30 s at 72 °C (extension) and 10 min at 72 °C. The primers for all genes were designed using Pyromark assay design software (Qiagen) and sequences are provided in Table 1. The reverse primers were 5' biotinylated for all genes evaluated in our study. Amplifications were performed using the Pyromark PCR kit (Qiagen) according to the manufacturer's instructions with varying concentration of  $MgCl_2$  (Table 1). Electrophoresis on 1% agarose gels confirmed the appropriate size and homogenous amounts of the PCR products among samples (data not shown).

Pyrosequencing was used to validate quantitative DNA methylation data (Tost and Gut 2007). PCR products (15  $\mu$ L) were added to a mixture containing 2  $\mu$ L Streptavidin Sepharose HP™ (Amersham Biosciences), 38  $\mu$ L binding buffer (Qiagen), 25  $\mu$ L MilliQ  $H_2O$  and mixed at 1400 rpm for 10 min at room temperature. PyroMark Q24 Vacuum Workstation (Qiagen) was used to prepare the single-stranded PCR products according to the manufacturer's instructions. The sepharose beads with the single stranded templates attached were released into a Pyromark Q24 plate (Qiagen) containing 0.3  $\mu$ M of the sequencing primer (Table 2) diluted in annealing buffer (Qiagen). Pyrosequencing reactions were performed in duplicate with the PyroMark Q24 instrument using PyroMark Gold Q24 reagents, according to the manufacturer's instructions (Qiagen). Methylation analysis of CpG sites was performed using PyroMark Q24 Software (Qiagen), and the average methylation values obtained from duplicated assays were considered for statistical analysis.

All statistical analysis was performed using R software (v 3.5.3; Team 2008). Diet groups were compared at each CpG using non-parametric tests suited to small samples (permutation tests for two independent samples, with Monte-Carlo

**Table 1** Details of primers used for PCR amplification. Reverse primers are biotinylated

Region	Chromosome	Strand	Primer sequence	Amplicon size (bp)	$MgCl_2$ Conc (mM)	Hybridisation temperature (C)
<i>WWC2</i>	27	Forward	GTTTGGAGAATTTAATGGGTAGAG	323	2	56
		Reverse	AAATAAAACAAAACACAAACCACTC			
<i>IGF2</i>	29	Forward	TTTTTTAGGGGTGTTTGTGAG	237	2.5	56
		Reverse	AATTCCAAAACAATCAAAAAAATCTTCCT			
<i>INSR</i>	7	Forward	GGTGGTTTAGTGTAAGAATTTGTT	154	2	56
		Reverse	ATTTCCCCTATTACCAAACAC			
<i>WNT1</i>	5	Forward	GGTTGATTTGTGATTTTGTAGATG	158	2.5	56
		Reverse	ACTTCCAAACCCACCTCTT			

**Table 2** Details of primers used for pyrosequencing including their oligonucleotide sequence and CpG sites included

Gene	Pyrosequencing primer	Sequence	CpG sites included
<i>WWC2</i>	Pyro 1	AGGTAGATAGTAATTAGTTTT	6–10
	Pyro 2	ATGAGTAGGTTTAGTGAGGT	11–16
	Pyro 3	GGAGGAAGTAGTGTTATGGAGA	17–18
<i>IGF2</i>	Pyro 1	GGTGTGTTGTGAGTAGG	1–7
<i>INSR</i>	Pyro 1	AGAATTTGTTTGTAAATGTAGTAT	1–3
<i>WNT1</i>	Pyro 1	AGGTGATTTAGAGTTTTTTTTGGTTT	1–14

sampling of 100,000 permutations and with correction for the stratification of the population according to the age). Significance was declared at  $P < 0.05$ .

## Results and discussion

### Results

#### RRBS library characterisation

An average of 28 million read pairs were obtained from all the 18 libraries included in our study (Supplementary Dataset 1). Average mapping rate was 88.9%, with a unique mapping rate of 36.4%. Taking account of the high representation of repeat sequences in the bovine genome, this unique mapping rate was expected and in accordance with the rate observed in a recent RRBS study in cattle (Perrier et al. 2018). CpGs were covered by 21.4 reads on average, with 55.1% of CpGs covered by at least 10 reads (CpG<sub>10</sub>) displaying an average methylation of 49.6%. Bisulfite conversion rate was on average 98.7% with a standard deviation of 0.2 suggestive of low technical variability. Further details on the individual libraries are provided in Supplementary Dataset 1.

#### Descriptive analysis

The matrix of methylation percentages for every CpG<sub>10</sub> and every sample was used to perform correlation clustering and PCA. With correlation clustering, the samples from the same bull (young and older) were grouped together irrespective of dietary treatments (Fig. 1a). No specific clustering was observed in the PCA plot (Fig. 1b). The results suggest that inter-individual variation unrelated to either age or dietary treatment represents the major variation in our methylation data.

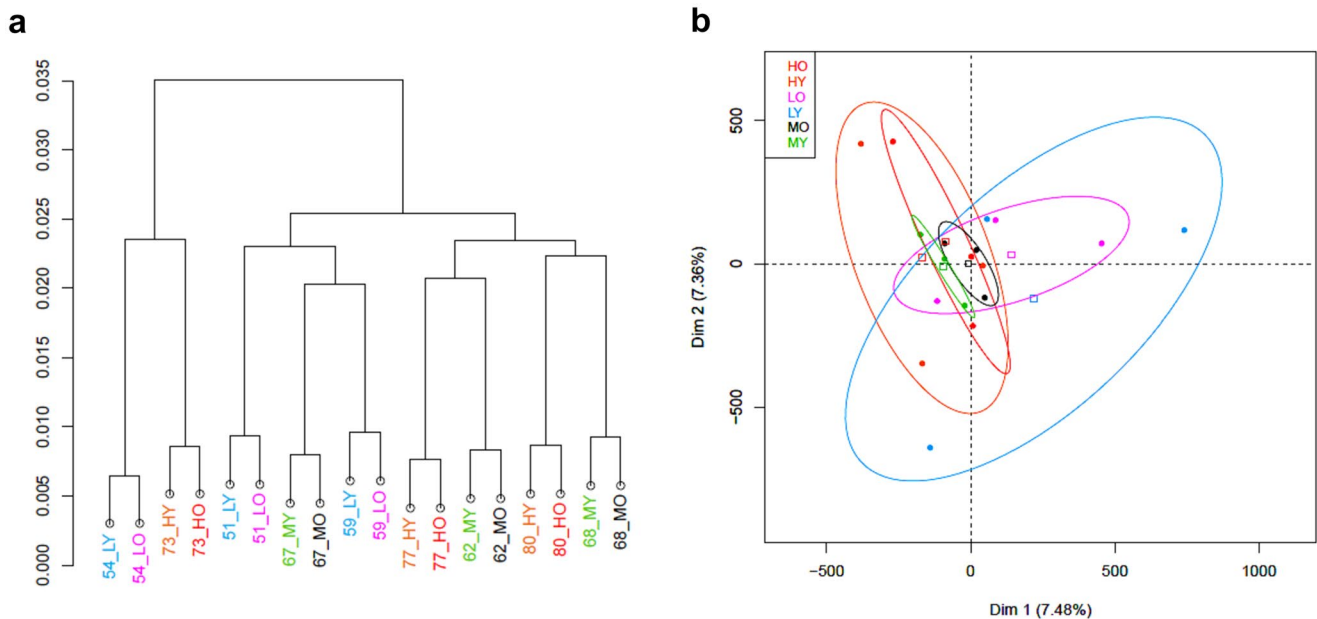
#### Determination of age or diet effects by differential analysis

Two independent factors were considered for analysis of differential methylation: diet and age. The young vs older

comparison for age ( $n = 9/\text{group}$ ) revealed differential methylation in 39 CpG sites with no DMRs, supporting the findings from our descriptive analysis of lack of intra-individual age differences. Accordingly, paired samples collected from the same bull were grouped to run the dietary comparisons. Since in methylKit, reads obtained under the same condition are aggregated regardless of the intra-condition inter-individual variability; this approach allows increasing the coverage at each tested CpG. Pairwise comparisons were therefore conducted on sperm samples from bulls fed low vs high diets, low vs medium and medium vs high diets ( $n = 6$  samples from 3 bulls per diet group). A total of 7931 CpGs were differentially methylated in the low- vs high-diet comparison (5678 hypermethylated and 2253 hypomethylated in the low diet as compared to the high diet), 6144 in low vs medium (4073 hypermethylated and 2071 hypomethylated in the low diet as compared to the medium diet) and 5244 in medium- vs high-diet (2762 hypermethylated and 2482 hypomethylated in the low diet as compared to the high diet) comparisons (Fig. 2a). In addition, 395 DMRs were identified in the low vs high (367 vs 28, hyper vs hypo relative to low diet), 270 in low vs medium (238 vs 32, hyper vs hypo relative to low diet) and 118 in medium- vs high-diet (70 vs 48, hyper vs hypo relative to low diet) groups (Fig. 2b). All together, these results suggest that pre-pubertal low diet led to hypermethylation of some regions of the genome compared to enriched diets. A list of DMCs and regions significantly different across treatments (low vs high, low vs medium and medium vs high) is provided in Supplementary Datasets 2, 3 and 4 (DMCs) and Supplementary Datasets 5, 6 and 7 (DMRs) respectively. The overlap between the three DMC lists was limited (Fig. 2c), since 217 DMCs were common to all the three dietary datasets without significant enrichment for any function or pathway.

#### Genomic distribution of diet-DMCs

The genomic distribution of the DMCs relative to gene features, CpG islands and repetitive elements for the three comparisons between diets was compared to the genomic distribution of the 1,254,808 CpG<sub>10</sub> analysed by RRBS



**Fig. 1** **a** Correlation clustering and **b** principal component analysis (PCA) run on the totality of CpG<sub>10</sub> covered in all 18 samples. Low and young ( $n=3$ , blue), low and older ( $n=3$ , pink), medium

and young ( $n=3$ , green), medium and older ( $n=3$ , black), high and young ( $n=3$ , orange), high and older ( $n=3$ , red)

(Background) (Fig. 3a–l). Interestingly, the genomic distribution of the DMCs was similar for the three comparisons and differed from that of the background. Concerning the gene features, the DMCs were more associated with intergenic regions and gene introns, in detriment of the promoter-TSS regions. The diet-DMCs were also more associated to CpG poor regions. Finally, diet-DMCs targeted more LINE and SINE regions and less simple repeat that was observed for the background. Together these data suggested that an important part of diet effect may concern more a general chromatin organisation than gene-specific regulatory regions.

### Gene specific enrichment

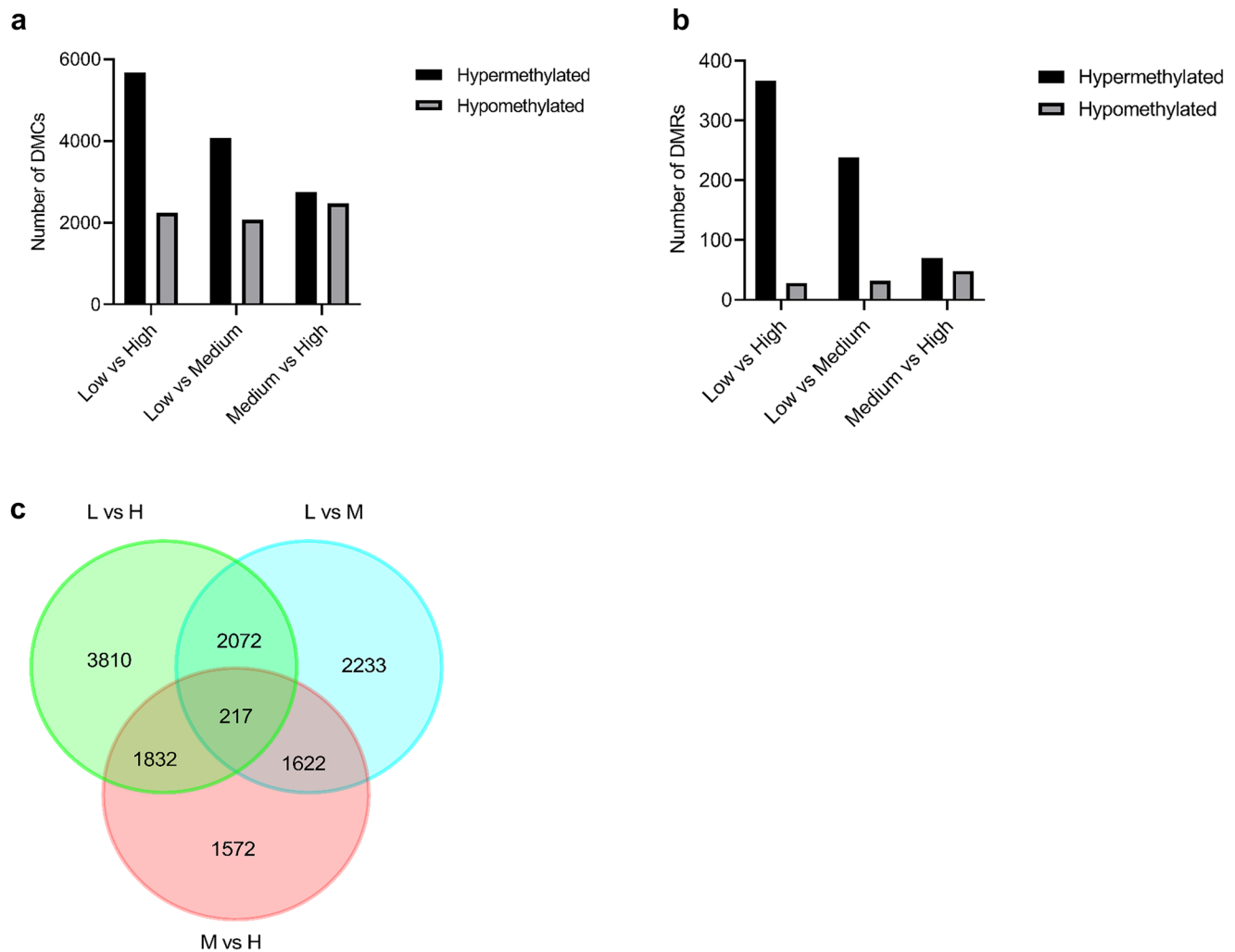
A focus was done on DMCs included in genes. It was interesting to observe that the 522 DMC-containing genes were shared by all pairwise comparisons, demonstrating that their methylation status was significantly different across all three diets. Among the 522 genes, MAPK (mitogen-activated protein kinase) signalling pathway was enriched (adjusted  $P$  value  $< 0.1$ , Benjamini–Hochberg correction, gene count-18, Fig. 4). This could point towards the vulnerability of the pathway to mild environmental influences, which in our case was pre-pubertal nutrition.

The results of enrichment analysis have been summarised in Table 3 (Adj  $P \leq 0.01$ , Benjamini–Hochberg correction). Briefly, in the low- vs high-diet dataset, a single biological process (BP), “regulation of Rho protein signal transduction”

involving genes *SPATA13* (spermatogenesis-associated protein), *ARHGEF2*, *ARHGEF7*, *ARHGEF10* and *ARHGEF19* (Rho guanine nucleotide exchange factors), was enriched (total 22 genes). The cellular component (CC) plasma membrane and proteinaceous extracellular matrix were also enriched. Rho protein signal transduction (BP, 20 genes), GTPase activity (molecular function (MF)) and Rho guanylnucleotide exchange factor activity (MF) were the GO terms enriched in the low vs medium dataset. KEGG pathways were only significantly enriched in the medium- vs high-diet comparison, and this included “MAPK signalling” (40 genes), “focal adhesion” (33 genes) and “proteoglycans in cancer” (33 genes). Major genes involved in the MAPK pathway included *AKT1*, *AKT2*, *EGFR* and *MAPK1*.

### Validation by bisulfite-pyrosequencing

Pyrosequencing was used to validate quantitative DNA methylation data (Tost and Gut 2007). Four DMRs located within (i) *WWC2*, (ii) *INSR*, (iii) *IGF2* and (iv) *WNT1* genes were selected for this. IGV browser views of these regions are provided in Fig. 5. According to our RRBS data, the DMR of *WWC2* located within the exon was hypermethylated in the low-diet group relative to both the medium- and high-diet groups. The DMRs of *WNT1* (located in an exon) and *INSR* (intron) were both hypermethylated in the low (vs high) diet and the DMR of *IGF2* (promoter) being hypomethylated in low (vs high) diet. *IGF2* is a major regulator of development and the disruption of its paternal allele reduced foetal growth in



**Fig. 2** Number of hypermethylated and hypomethylated **a** DMCs and **b** DMRs in low vs high, low vs medium and medium vs high datasets. *Y* axis—number of DMCs or DMRs; *X* axis—dataset. The black bars represent the number of DMCs hypermethylated in low

diet (low vs high and low vs medium comparisons), or in medium diet (medium vs high comparison). **c** Venn diagram showing the DMCs identified in low (L) vs high (H), low (L) vs medium (M) and medium (M) vs high (H) comparisons

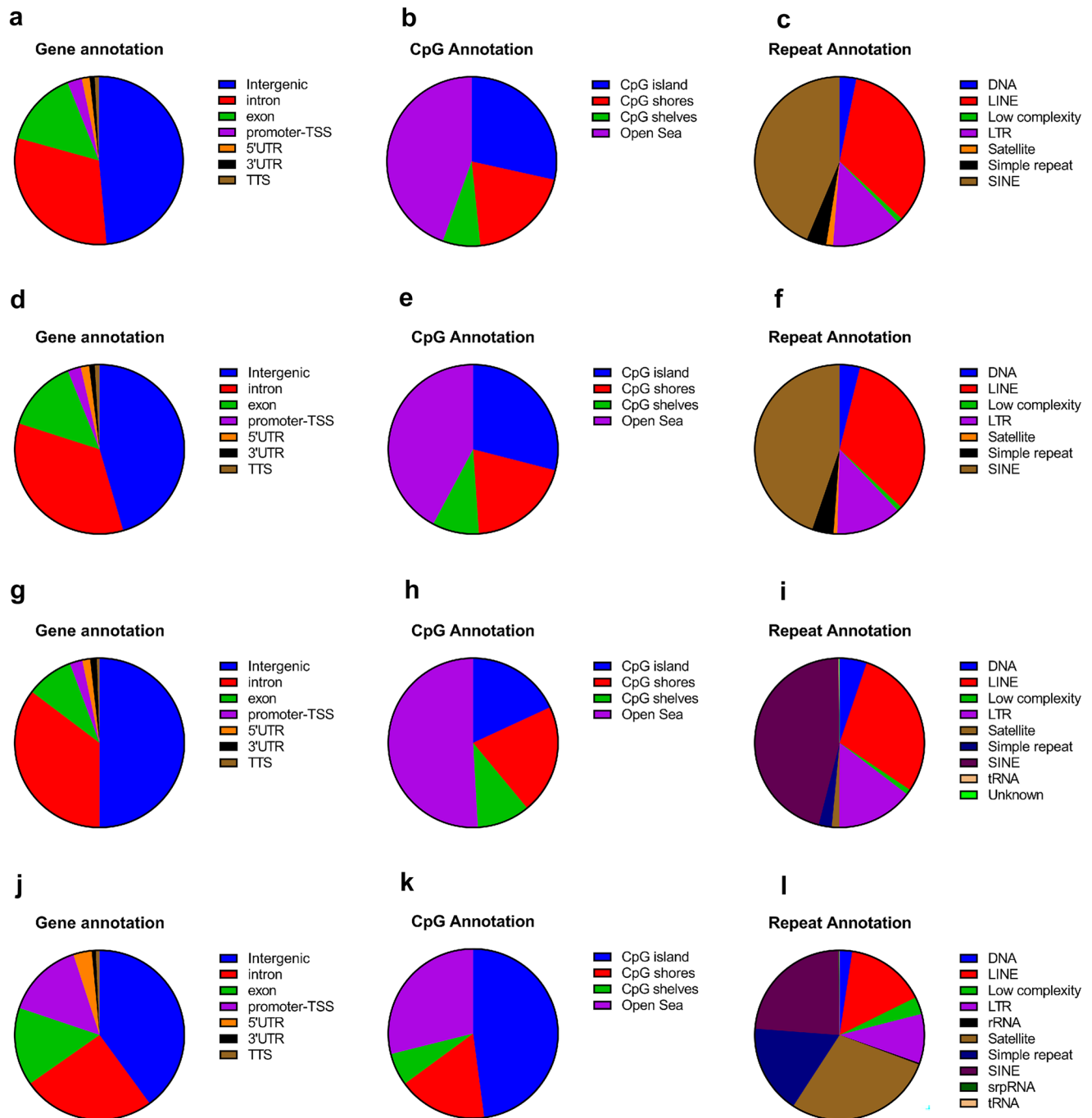
mice (Burns and Hassan 2001). Some of the growth-promoting effects of *IGF2* is mediated by the *INSR* (Louvi et al. 1997). WNT signalling (*WNT1*) is critical for mammalian spermatogenesis and is also involved in sexual differentiation (Ronfani and Bianchi 2004). *WWC2* is a regulator of hippo signalling involved in the male reproductive development and spermatogenesis in sheep (Zhang et al. 2019). Results obtained using pyrosequencing successfully validated the results of RRBS (Fig. 5).

## Discussion

Through our RRBS study, we analysed the effects of differential pre-pubertal diet and advancing age on methylation of bovine sperm DNA. Even though age did not impact the sperm methylome, probably due to the short interval

between semen collections (2–3 months), our results suggest that pre-pubertal nutrition can cause dynamic changes in the sperm epigenome.

Sexual development in the bull is a complicated process in which the testes grow comparatively slow during the first 6 months followed by a rapid phase during puberty (9–12 months, Rawlings et al. 2008). The proliferation and differentiation of germ cells occur during the rapid phase and are characterised by the increased production of testosterone. Even though a significant portion of the germ cell pattern is established prior to type A spermatogonia stage, de novo methylation and demethylation changes have been reported during spermatogenesis, specially during meiosis (Oakes et al. 2007). It is possible that in young bulls these mechanisms are not fully established and could be characterised by differences in DNA methylation. Thus, age was



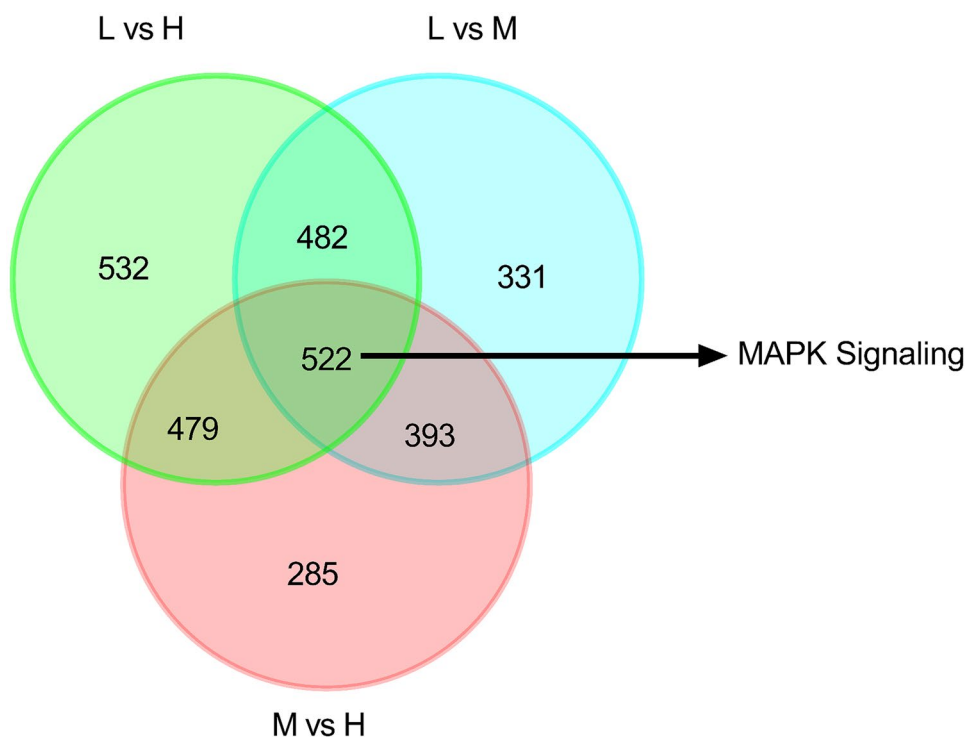
**Fig. 3** Gene, CpG and repeat annotations for DMCs across **a–c** low vs high, **d–f** low vs medium, **g–i** medium vs high and **j–l** background

included as an independent variable in our study to determine if methylation changes induced by pre-pubertal diet could be modified over time in bulls. A study investigating effects of age on sperm DNA methylation in bulls detected no DMRs when evaluating sperm at 12 (late-pubertal) and 16 months (post-pubertal) of age supporting our findings (Lambert et al. 2018). Since testes growth is maximum during puberty, most DMRs were detected across 10 (early pubertal) and 16 months

(post-pubertal) of age in the above study. Other studies have also reported age-related DMRs in bovine sperm (Takeda et al. 2017, 2019). Even though isolated CpG sites were differentially methylated in our study, no DMRs were detected, demonstrating that sperm DNA methylation is stable over an interval of 2–3 months within the post-pubertal period. Another study in bovine evaluated the effects of feeding a high vs medium nutrition in bulls until 24 weeks of age followed



**Fig. 4** Venn diagram showing the DMC-containing genes identified using RRBS in low vs high, low vs medium and medium vs high comparisons. A total of 522 genes were common across all datasets and were significantly enriched for MAPK signalling



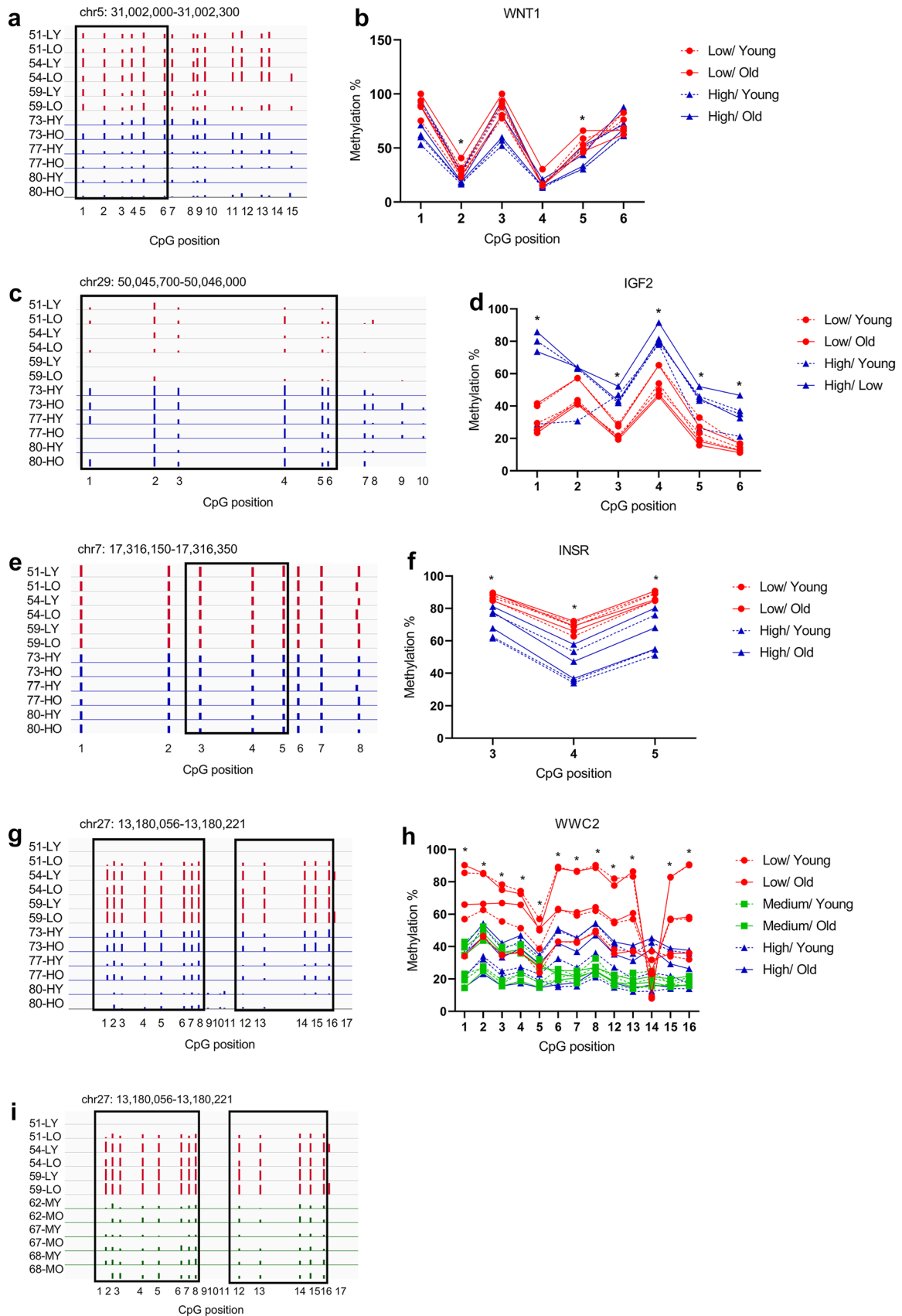
by medium nutrition until puberty at two different ages (15 vs 16 months, Perrier et al. 2020). No differential methylation was detected in sperm for age, but for diet. Based on their findings, they speculated that the changes in DNA methylation were associated with advanced sexual maturity of high-diet vs medium-diet bulls as no differences were detected when comparing medium group at 16 months vs high at 15 months as methylation changes would be counterbalanced with maturity (Perrier et al. 2020). Our lab previously reported advanced puberty, larger testes and increased sperm production in bulls fed a high vs low pre-pubertal nutrition (Dance et al. 2016; 2015). A similar speculation that the advanced puberty of high-diet bulls facilitated by their enhanced diet induced differential methylation in sperm is plausible but inconclusive.

It is also imperative to note that majority of the differences in methylation were irrespective of diet or age, but between individuals likely rely on genetic polymorphisms as described in humans (Lappalainen and Grealley 2017). Considering the random selection of bulls in our study and the vital role of genetics in addition to environment (nutrition) in epigenetic regulation (Triantaphyllopoulos et al. 2016), this is conceivable and indirectly ascertains the supremacy of genetics in epigenetic regulation.

With regard to diet, we identified several DMCs and regions (DMR). Interestingly, for all the comparisons, the major part of DMCs were more methylated in the low diet, indicating that nutritional limitation is associated with an increase of DNA methylation at specific sites. Previous

**Table 3** (a) KEGG pathways and (b) GO terms enriched in the list of DMC-containing genes identified using DAVID (Huang et al. 2009) (Adj  $P \leq 0.01$ , Count  $\geq 2$ ). *BP* biological process, *CC* cellular component

a			
KEGG Pathways	Dataset	No. of genes	Adj <i>P</i> -value
MAPK signalling pathway	Medium vs high	40	0.001
Proteoglycans in cancer	Medium vs high	33	0.003
Focal adhesion	Medium vs high	33	0.003
Choline metabolism in cancer	Medium vs high	20	0.005
b			
GO terms	Dataset	No. of genes	Adj <i>P</i> -value
Regulation of Rho protein signal transduction (BP)	Low vs high	22	0.003
Regulation of Rho protein signal transduction (BP)	Low vs medium	20	0.010
Plasma membrane (CC)	Low vs medium	219	0.006



**Fig. 5** Validation of RRBS data by bisulfite-pyrosequencing. IGV browser views of gene regions and methylation percentages of the CpGs assayed by pyrosequencing (**a, b** WNT1; **c, d** IGF2; **e, f** INSR; **g, i** WWC2) targeted for pyrosequencing. For **a, c, e, g** and **i**, the red, green and blue bar charts represent the methylation percentages at each CpG<sub>10</sub> position for low-, medium- and high-diet samples, respectively. The DMCs are enclosed in the black box. For **b, d, f** and **h**, low-, medium- and high-diet groups are represented in red, green and blue, respectively ( $n=3$  bulls/group), while the two different time points are indicated in plain and dashed lines. X-axis: CpG sites; Y-axis: Methylation %. \*Significant difference between groups (permutation test,  $P < 0.05$ )

studies investigating human infertility have associated global sperm DNA hypermethylation to lowered sperm quality (Rahiminia et al. 2018; Kumar et al. 2013). Even though there is a lack of consensus in this regard (Olszewska et al. 2017), it is worthy to note that in a previous study done in our lab, the semen from bulls fed low pre-pubertal diet also exhibited low progressive motility compared to the high-diet bulls (Johnson et al. 2020). Thus, a negative association between sperm motility and global sperm hypermethylation is plausible based on our data.

The genomic distribution of DMCs was quite similar for the three comparisons: intragenic regions and gene promoters, CpG islands and simple and low complexity repeat regions were depleted in DMCs. These data suggest nutritional effects on global architecture of spermatozoa nuclei which would have consequences on the epigenetic reprogramming of paternal nucleus after fertilisation as described for other epigenetic marks (Ost et al. 2014).

Most prominent changes in the sperm epigenome associated with pre-pubertal diet could be related to spermatogenesis, sperm function and early embryo development. We found an enrichment of MAPK signalling in the 522 genes differentially methylated in all three dietary datasets. This is important considering the involvement of MAPK pathway in numerous male reproductive processes including spermatogenesis, sperm function (maturation and activation, capacitation, acrosome reaction) and Sertoli cell function (Li et al. 2009). MAPKs have been implicated in the progression of meiosis where incubation of pachytene spermatocytes with ERK1 (MAPK downstream) inhibitor prevented chromatin condensation and progression to metaphase stages in mouse (Sette et al. 1999). In primates and rats, MAPK signalling induced germ cell apoptosis in response to mild hyperthermia and lowered testosterone levels (Johnson et al. 2008). ERK1/2 was detected in the tail of human spermatozoa and regulated sperm capacitation and hyperactivation in sperm. It is possible that Rho GTPases, and putative ERK substrates, are involved in the abovementioned regulation of sperm motility by ERK1/2 (Almog et al. 2008). MAPK signalling activated in response to cytokines lowered the steady-state protein levels of occludin, ZO-1 and cadherins, disrupting blood testes barrier integrity and spermatogenesis in adult

rat testes (Xia et al. 2006). Thus, dietary modulation in cattle could alter MAPK signalling that plays a key role in the regulation of gene expression, cellular growth and survival.

Some effects of MAPK signalling on sperm function could be activated via small GTPases as described earlier. Through analysis in DAVID, we identified the biological process, “rho protein signal transduction”, to be enriched in two datasets (low vs high, low vs medium) in our study. Rho protein signal transduction has been implicated in many cellular processes including transcription, cell cycle, cell proliferation, migration and most importantly actin reorganisation (Bustelo et al. 2007). Coming from the Ras superfamily, Rho GTPases are small (~20 kDa) signalling G proteins regulating actin-based cytoskeletal rearrangements. GTPases respond to growth factors, integrins, ion channels or G-protein-coupled receptors activated by environmental influences/external stimuli. Activation of Rho GTPase mediates its transition from the inactive GDP bound form to an active GTP bound form (Lu et al. 2009). A major regulatory protein of Rho GTPase, guanine nucleotide exchange factor, facilitates the exchange of GDP with GTP. Many downstream targets of Rho have been identified including PKN (protein kinase C-like protein) and PIP5-kinase (phosphatidylinositol 4-phosphate 5-kinase) with major regulatory effects on actin reorganisation (Mukai 2003; Tolia et al. 1995). Rho GTPases have been identified in the sperm of many mammalian species including bovine. A multi-species localisation study of Rho GTPases in the sperm revealed its presence in both the head and tail of bovine sperm and confirmed it being conserved through mammalian evolution (Ducummon and Berger 2006). Previously, in 1993, a research group performed ADP ribosylation of rho GTPases using an exoenzyme like toxin and reported lowered motility in bovine sperm (Hinsch et al. 1993). Evaluating bulls fed differential pre-pubertal diets, we previously identified greater sperm progressive motility and mitochondrial function in both the testes and sperm of high- compared to medium- and low-diet bulls (Johnson et al. 2020). It is possible that the underlying differences in DNA methylation (rho protein signal transduction) drove the differences in sperm motility observed in our previous study. Considering the association between Rho signalling and sperm motility, this is a possibility. A recent review investigated the role of Rho GTPases in spermatogenesis and supported its role in maintaining the junctional dynamics by actin organisation within the testes (Lui et al. 2003). In addition, the localisation of Rho proteins in the acrosome region of many mammals supports its role in acrosomal exostosis (Ducummon and Berger 2006). However, the association between the methylation status of genes involved in Rho signalling and its impact on sperm function is unknown.

When comparing DMCs in the medium- vs high-diet dataset, we identified focal adhesions and MAPK pathways to

be significantly enriched. Focal adhesions are multi-protein complexes forming mechanical links between the extracellular matrix and the cytoskeleton. Focal adhesion kinase has been localised in mammalian sperm and is important for signalling mechanisms required for capacitation and acrosome reaction (Roa-Espitia et al. 2016). Focal adhesion kinases can also modify the actin cytoskeletal assembly through activation of Rho GTPases for enabling cell motility (Tomar and Schlaepfer 2009).

In addition to the above, we identified several differentially methylated genes in our study with a direct impact on sperm function and embryo development. Among the many hypermethylated DMRs in the low-diet bulls, *CYP26B1* (cytochrome P450, family 26, subfamily B, polypeptide 1), a gene associated with spermatogenesis was also present. *CYP26B1*, an inhibitor of retinoic acid action, maintains the undifferentiated state of germ cells regulating their meiotic entry. In mice, mutations in *CYP26B1* gene in both germ and Sertoli cells disrupted spermatogenesis and lowered fertility in male (Hogarth et al. 2015). Hypermethylation could imply lowered gene expression in low-diet bulls (vs high and medium) causing disruption in spermatogenesis. In our previous study where we evaluated testicular gene expression of pre-pubertal bulls, a cytochrome P450 gene (*CYP51A1*) was downregulated in the low-diet (vs high diet)-fed bulls (Johnson et al. 2019). Another hypermethylated DMR in the low diet was located within the *DDX4* gene, a commonly used marker for germ cells. The gene is critical for germ cell development in many mammals including bovine (Bartholomew and Parks 2007) and was reported to have lowered expression in oligozoospermic human patients (Guo et al. 2007). Other DMRs with related functions included *SPATA13* (exonic location, spermatogenesis-associated protein 13) and *EQTN* (Equatorin; acrosome reaction). Both genes were hypermethylated in low compared to high diet, and this could be speculated as lowered gene expression in low-diet bulls. To corroborate this, our RNA-seq study on pre-pubertal testicular tissue detected differential expression of genes supporting enhanced Sertoli cell maturation in the high-diet-fed bulls (Johnson et al. 2019). Since Sertoli cells support spermatogenesis in tremendous ways, it is reasonable to speculate a positive impact of pre-pubertal feeding in bulls.

DNA methylation of sperm have a critical role in the regulation of imprinted genes and is a crucial determinant of embryo/foetal development and post natal growth (Reik et al. 2001). Compared to the somatic genome where methylation patterns are stable and heritable, gametes encounter two developmental periods (germ cell and pre-implantation embryo) where DNA methylation is reprogrammed establishing cells with vast developmental potential (Reik et al 2001). Even though a major wave of demethylation is complete by 8-cell stage in bovine embryos, a small portion (~20%) of DNA methylation is retained including the imprinted genes, which could

be critical in regulation of embryo growth and development (Jiang et al. 2018, Wu and Sirard 2020). Thus, the effects of environmental modulation on DNA methylation could be immediate in terms of sperm function and fertility or long term (progeny development). According to a recent study in sheep, a minor dietary modulation in prepubertal F0 rams altered SC and weight at puberty in F1 rams. Additionally, differential methylation was detected in genes associated with sexual development and body weight (Gross et al. 2020). In porcine, feeding methylating micronutrients vs a standard diet to F0 males lowered fat and increased shoulder muscle percentage in F2 generation which was postulated to be epigenetic inheritance via differential methylation in the liver of treatment males (Braunschweig et al. 2012).

As discussed previously, embryo development is characterised by the development of new methylation patterns during the different windows of epigenetic programming (Reik et al. 2001) which involves DNMT3a, the enzyme involved in de novo methylation and crucial for early embryonic development, parental imprinting and X chromosome inactivation (Kaneda et al. 2004; Okano et al. 1999). Interestingly within the *DNMT3a* gene, 2 CpG sites with an intronic location were found hypermethylated in low-diet bulls when compared to high-diet bulls. In human testicular germ cell tumours, intronic hypomethylation of DNMT3a was associated with upregulated expression (Ishii et al. 2007). Ablation of *DNMT3a* in mice resulted in death shortly after birth (Okano et al. 1999), and lowered expression of *DNMT3a* was correlated with increased rates of early embryonic death in humans (Gu et al. 2017). Thus, one hypothesis could be that low diet in prepubertal bulls leads to hypermethylation and lower gene expression of *DNMT3a*, resulting to lower reprogramming efficiency after fertilisation.

Several genes within the insulin/IGF family were also differentially methylated in our study. This included DMRs in *INSR*, *IGF2* and DMCs in *IGF-IR*, *IGF2R*, *IRS2* and *INSIG1* genes. A significant finding was the hypomethylation (low vs high) of a DMR (196 bp) with 7 CpGs located in the promoter region of *IGF2*, a well-recognised imprinted gene in bovine (Dindot et al. 2005) as well in mice and human (Constancia et al. 2002). *IGF2* gene is widely expressed during embryo development and encodes a critical regulator of foetal growth, nutrient delivery and placental size (Constancia et al. 2002; Fowden et al. 2009). A previous study in bulls reported hypomethylation of *IGF2* in sperm collected from peripubertal bulls (Lambert et al. 2018) and discussed the importance of age of semen collection as a critical factor. It has been shown that some of the growth-promoting effects of IGF2 during embryogenesis is mediated via the *INSR* (Louvi et al. 1997) and *IGF-IR* (Pitetti et al. 2013), another gene differentially methylated in our study. For better coordination of regulation of parental imprinting, *IGF2* shares an imprinting control region (ICR) with a non-coding gene *H19* and is located

upstream of the *H19*. In this study, diet does not affect the ICR methylation state, suggesting that *IGF2* parental imprinting is correctly operating. We found a diet-induced alteration of methylation in *IGF2* promoter. A previous study reported a correlation between hypermethylation of *IGF2* promoter and repressed expression (Zheng et al. 2018). As previously indicated, an abnormal methylation of *IGF2* promoter could be reprogrammed with lower efficiency after fertilisation, affecting *IGF2* gene expression and embryo development.

Moreover, aberrant methylation in the imprinted genes was associated with spermatogenic failure in a human case control study comparing idiopathic and normal sperm (Tang et al. 2018). *IGF2R* located on chromosome 9 is also a verified imprinted (maternal) gene in bovine (Killian et al. 2001). A single DMC located within an intron was hypermethylated in the medium-diet bulls compared to the high diet. No clear hypothesis could be established concerning the impact of this DNA methylation alteration.

In bovine species, it has been proven that nutrition impacts fertility but the transgenerational effects of the same through sperm DNA methylation specially in the progeny have not been clear. When comparing embryos generated from sperm of peripubertal (10–12 months) vs post-pubertal bulls (16 months), differential methylation and expression were detected for genes associated with energy metabolism (Wu and Sirard 2020). In a different study, DMRs in sperm and its correlation with gestation length, days to first breeding after calving, somatic cell score, body type, milk production and other traits were reported (Fang et al. 2019) suggestive of transgenerational effects via epigenetic programming. Even though we detected DMRs correlated with sperm function and embryo development in our study, we lack gene expression data which could have been correlated with sperm DNA methylation. Therefore, a future direction would be the evaluation of gene expression in earlier spermatogenic cell types and/relevant somatic cells of the offspring derived from the sperm. To accomplish this, evaluation of gene expression of embryos derived from sperm of bulls fed differential pre-pubertal diet is important which could then be correlated with our DNA methylation data to expand our understanding about the implications of our dietary modulation on the next generation.

Another limitation of our study was the inability to characterise mitochondrial DNA methylation albeit their inclusion via sperm tails. Differential mitochondrial DNA methylation in response to diet has been reported previously in human trials (Corsi et al. 2020). In our previous studies, we reported enhanced mitochondrial function and motility of sperm from bulls fed high levels of nutrition (Johnson et al. 2020). Thus, it would also be interesting to evaluate its impact on sperm mitochondrial DNA methylation and its correlation with sperm function in future studies.

## Conclusion

In brief, our results suggest that pre-pubertal diet can alter DNA methylation in the mature sperm of bulls with potential impacts on genes implicated in spermatogenesis, sperm function and early embryo development, appealing for further investigations about the programming of reproductive development in bulls during early calfhood. The question as to the impacts on embryogenesis or phenotype of the next generation is still vague in bovine and therefore will deserve further attention.

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**Author contribution** CJ performed the pyrosequencing experiment, participated in the RRBS experiments and data analysis and drafted the manuscript. HK and LJ performed the bioinformatics and statistical analyses of RRBS data. ACT constructed the RRBS libraries. HK, ES, AD, JK, JT and HJ participated in the conception of the study and editing of the manuscript.

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**Availability of data and materials** The datasets generated and/or analysed during the current study are available in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB35854 (<https://www.ebi.ac.uk/ena/data/view/PRJEB35854>).

## Declarations

**Ethics approval and consent to participate** This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and was reviewed and approved by the Lethbridge Research Centre Institutional Animal Care Committee.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

## References

- Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE (2012) MethylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol* 13:R87
- Allfrey VG, Faulkner R, Mirsky AE (1964) Acetylation and methylation of histones and their possible role in the regulation of rna synthesis. *Proc Natl Acad Sci USA* 51(5):786–794
- Almog T, Lazar S, Reiss N, Etkovitz N, Milch E, Rahamim N, Bekma D-B, Rotem R, Kalina M, Ramon J, Raziell A, Breitbart H, Seger R, Naor Z (2008) Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality. *J Biol Chem* 283:14479–14489

- Barker DJ (1992) Fetal and infant origins of adult disease. *BMJ*
- Barker DJ, Martyn CN (1992) The maternal and fetal origins of cardiovascular disease. *J Epidemiology Community Health* 46:8–11
- Barker DJ (2004) The developmental origins of adult disease. *J Am Coll Nutr* 23:588S–595S
- Barth AD, Brito LFC, Kastelic JP (2008) The effect of nutrition on sexual development of bulls. *Theriogenology* 70:485–494
- Bartholomew RA, Parks JE (2007) Identification, localization, and sequencing of fetal bovine VASA homolog. *Anim Reprod Sci* 101:241–251
- Braunschweig M, Jagannathan V, Gutzwiller A, Bee G (2012) Investigations on transgenerational epigenetic response down the male line in F2 pigs. *PLoS ONE* 7(2):e30583
- Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DH, Mir PS, Kastelic JP (2007a) Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. *Domest Anim Endocrinol* 33:460–469
- Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DH, Mir PS, Kastelic JP (2007b) Circulating metabolic hormones during the peripubertal period and their association with testicular development in bulls. *Reprod Domest Anim* 42:502–508
- Brito LFC, Barth AD, Wilde RE, Kastelic JP (2012) Effect of growth rate from 6 to 16 months of age on sexual development and reproductive function in beef bulls. *Theriogenology* 77:1398–1405
- Burns JL, Hassan AB (2001) Cell survival and proliferation are modified by insulin-like growth factor 2 between days 9 and 10 of mouse gestation. *Development* 128:3819–3830
- Burton MA, Lillycrop KA (2019) Nutritional modulation of the epigenome and its implication for future health. *Proc Nutr Soc* 78:305–312
- Bustelo XR, Sauzeau V, Berenjeno IM (2007) GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *BioEssays* 29:356–370
- Champroux A, Cocquet J, Henry-Berger J, Drevet JR, Kocer A (2018) A decade of exploring the mammalian sperm epigenome: paternal epigenetic and transgenerational inheritance. *Front Cell Dev Biol* 6:50
- Corsi S, Iodice S, Shannon O, Siervo M, Mathers J, Bollati V, Byun H (2020) Mitochondrial DNA methylation is associated with Mediterranean diet adherence in a population of older adults with overweight and obesity. *Proceedings of the Nutrition Society* 79:E95
- Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C and Reik W (2002) Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 417:945–948
- Dance A, Thundathil J, Blondin P, Kastelic J (2016) Enhanced early-life nutrition of Holstein bulls increases sperm production potential without decreasing postpubertal semen quality. *Theriogenology* 86:687–694
- Dance A, Thundathil J, Wilde R, Blondin P, Kastelic J (2015) Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. *J Dairy Sci* 98:987–998
- Dindot S, Kent K, Evers B, Loskutoff N, Womack J, Piedrahita J (2005) Conservation of genomic imprinting at the XIST, IGF2, and GTL2 loci in the bovine. *Mamm Genome* 15:966–974
- Donkin I, Barres R (2018) Sperm epigenetics and influence of environmental factors. *Mol Metab* 14:1–11
- Ducummon C, Berger T (2006) Localization of the Rho GTPases and some Rho effector proteins in the sperm of several mammalian species. *Zygote* 14:249–257
- Dupont JM, Tost J, Jammes H, Gut IG (2004) De novo quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem* 333:119–127
- Fang L, Jiang J, Li B, Zhou Y, Freebern E, Vanraden PM, Cole JB, Liu GE, Ma L (2019) Genetic and epigenetic architecture of paternal origin contribute to gestation length in cattle. *Commun Biol* 14:2
- Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ (2009) Placental efficiency and adaptation: endocrine regulation. *J Physiol* 587:3459–3472
- Gaysinskaya V, Miller B, Luca C, van der Heijden GW, Hansen KD, Bortvin A, (2018) Transient reduction of DNA methylation at the onset of meiosis in male mice. *Epigenetics Chromatin* 11:15
- Goldberg AD, Allis CD, Bernstein E (2007) Epigenetics: a landscape takes shape. *Cell* 128:635–638
- Gross N, Taylor T, Crenshaw T, Khatib H (2020) The intergenerational impacts of paternal diet on DNA methylation and offspring phenotypes in sheep. *Front Genet* 11:597943
- Gu H, Gao J, Guo W, Zhou Y, Kong Q (2017) The expression of DNA methyltransferases 3A is specifically downregulated in chorionic villi of early embryo growth arrest cases. *Mol Med Rep* 16:591–596
- Guo X, Gui YT, Tang AF, Lu LH, Gao X, Cai ZM (2007) Differential expression of VASA gene in ejaculated spermatozoa from normozoospermic men and patients with oligozoospermia. *Asian J Androl* 9:339–344
- Hinsch KD, Habermann B, Just I, Hinsch E, Pfisterer S, Schill WB, Aktories K (1993) ADP-Ribosylation of Rho proteins inhibits sperm motility. *FEBS Lett* 334
- Hogarth CA, Evans E, Onken J, Kent T, Mitchell D, Petkovich M, Griswold MD (2015) CYP26 enzymes are necessary within the post-natal seminiferous epithelium for normal murine spermatogenesis. *Biol Reprod* 93:19
- Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. *Science* 187:226–232
- Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4
- Ishii T, Kohu K, Yamada S, Ishidoya S, Kanto S, Fuji H, Moriya T, Satake M, Arai Y (2007) Up-regulation of DNA-methyltransferase 3A expression is associated with hypomethylation of intron 25 in human testicular germ cell tumors. *Tohoku J Exp Med* 212:177–190
- Jiang Z, Lin J, Dong H, Zheng X, Marjani SL, Duan J, Ouyang Z, Chen J, Tian XC (2018) DNA methylomes of bovine gametes and in vivo produced preimplantation embryos. *Biol Reprod* 1:949–959
- Johnson C, Jia Y, Wang C, Lue YH, Swerdloff RS, Zhang XS, Hu Z, Li YC, Liu YX, Hikim AP (2008) Role of caspase 2 in apoptotic signaling in primate and murine germ cells. *Biol Reprod* 79:806–814
- Johnson C, Dance A, Kovalchuk I, Kastelic J, Thundathil J (2019) Enhanced early-life nutrition upregulates cholesterol biosynthetic gene expression and Sertoli cell maturation in testes of pre-pubertal Holstein bulls. *Sci Rep* 9:6448
- Johnson C, Dance A, Kovalchuk I, Kastelic J, Thundathil J (2020) Enhanced pre-pubertal nutrition upregulates mitochondrial function in testes and sperm of post-pubertal Holstein bulls. *Sci Rep* 10:2235
- Kaati G, Bygren LO, Edvinsson S (2002) Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *Eur J Hum Genet* 10:682–688
- Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429:900–903
- Kanehisa M, Goto S (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 28:27–30
- Killian JK, Nolan CM, Wylie AA, Li T, Vu TH, Hoffman AR, Jirtle RL (2001) Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. *Hum Mol Genet* 10:1721–1728

- Krueger F (2014) Trim galore. [http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Accessed 21 July 2019
- Kumar D, Salian SR, Kalthur G, Uppangala S, Kumari S, Kumar CS, Chandraguthi SG, Krishnamurthy H, Jain N, Kumar P, Adiga SK (2013) Semen abnormalities, sperm DNA damage and global hypermethylation in health workers occupationally exposed to ionizing radiation. *PLoS ONE* 8:e69927–e69927
- Lambert S, Blondin P, Vigneault C, Labrecque R, Dufort I, Sirard MA (2018) Spermatozoa DNA methylation patterns differ due to peripubertal age in bulls. *Theriogenology* 106:21–29
- Lappalainen T, Grealis JM (2017) Associating cellular epigenetic models with human phenotypes. *Nat Rev Genet* 18:441–451
- Li MWM, Mruk DD, Cheng CY (2009) Mitogen-activated protein kinases in male reproductive function. *Trends Mol Med* 15:159–168
- Louvi A, Accili D, Efstratiadis A (1997) Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Develop Biol* 189:33–48
- Lu Q, Longo FM, Zhou H, Massa SM, Chen YH (2009) Signaling through Rho GTPase pathway as viable drug target. *Curr Med Chem* 16:1355–1365
- Lui WY, Lee WM, Cheng CY (2003) Rho GTPases and spermatogenesis. *Biochimica et Biophysica Acta (BBA) - Mol Cell Res* 1593:121–129
- Mukai H (2003) The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *J Biochem* 133:17–27
- Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM (2007) Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. *Dev Biol* 307:368–379
- Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99:247–257
- Olszewska M, Barciszewska MZ, Fraczek M, Huleyuk N, Chernykh VB, Zastavna D, Barciszewski J, Kurpisz M (2017) Global methylation status of sperm DNA in carriers of chromosome structural aberrations. *Asian J Androl* 19:117–124
- Ost A, Lempradl A, Casas E, Weigert M, Tiko T, Deniz M, Pantano L, Boenisch UPM, Stoeckius M, Ruf M, Rajewsky N, Reuter G, Iovino N, Ribeiro C, Alenius M, Heyne S, Vavouri T, Pospisilik JA (2014) Paternal diet defines offspring chromatin state and intergenerational obesity. *Cell* 159:1352–1364
- Perrier JP, Kenny DA, Chaulot-Talmon A, Byrne CJ, Sellem E, Jouneau L, Aubert-Frambourg A, Schibler L, Jammes H, Lonergan P, Fair S, Kiefer H (2020) Accelerating onset of puberty through modification of early life nutrition induces modest but persistent changes in bull sperm DNA methylation profiles post-puberty. *Front Genet* 26:945
- Perrier JP, Sellem E, Prezelin A, Gasselien M, Jouneau L, Perrier PF, JAI Adhami H, Weber M, Fritz S, Boichard D, Le Danvic C, Schibler L, Jammes H and Kiefer H, (2018) A multi-scale analysis of bull sperm methylome revealed both species peculiarities and conserved tissue-specific features. *BMC Genom* 19:404
- Pitetti JL, Calvel P, Zimmermann C, Conne B, Papaioannou MD, Aubrey F, Cederroth CR, Urner F, Fumel B, Crausaz M, Docquier M, Herrera PL, Pralong F, Germond M, Guillou F, Jégou B, Nef S (2013) An essential role for insulin and IGF1 receptors in regulating sertoli cell proliferation, testis size, and FSH action in mice. *Mol Endocrinol* 27:814–827
- Rahiminia T, Yazd EF, Fesahat F, Moein MR, Mirjalili AM, Talebi AR (2018) Sperm chromatin and DNA integrity, methyltransferase mRNA levels, and global DNA methylation in oligoasthenoterozoospermia. *Clin Exp Reprod Med* 45:17–24
- Rawlings N, Evans AC, Chandolia RK, Bagu ET (2008) Sexual maturation in the bull. *Reprod Domest Anim* 43:295–301
- Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. *Science* 293:1089–1093
- Roa-Espitia AL, Hernández-Rendón ER, Baltiérrez-Hoyos R, Muñoz-Gotera RJ, Cote-Vélez A, Jiménez I, González-Márquez H, Hernández-González EO (2016) Focal adhesion kinase is required for actin polymerization and remodeling of the cytoskeleton during sperm capacitation. *Biol Open* 5:1189–1199
- Ronfani L, Bianchi ME (2004) Molecular mechanisms in male determination and germ cell differentiation. *Cell Mol Life Sci* 61:1907–1925
- Sette C, Barchi M, Bianchini A, Conti M, Rossi P, Geremia R (1999) Activation of the mitogen-activated protein kinase ERK1 during meiotic progression of mouse pachytene spermatocytes. *J Biol Chem* 274:33571–33579
- Takeda K, Kobayashi E, Akagi S, Nishino K, Kaneda M, Watanabe S (2017) Differentially methylated CpG sites in bull spermatozoa revealed by human DNA methylation arrays and bisulfite analysis. *J Reprod Dev* 63:279–287
- Takeda K, Kobayashi E, Nishino K, Imai A, Adachi H, Hoshino Y, Iwao K, Akagi S, Kaneda M, Watanabe S (2019) Age-related changes in DNA methylation levels at CpG sites in bull spermatozoa and in vitro fertilization-derived blastocyst-stage embryos revealed by combined bisulfite restriction analysis. *J Reprod Dev* 65:305–312
- Tang Q, Pan F, Yang J, Fu Z, Lu Y, Wu X, Han X, Chen M, Lu C, Xia Y, Wang X, Wu W (2018) Idiopathic male infertility is strongly associated with aberrant DNA methylation of imprinted loci in sperm: a case-control study. *Clin Epigenetics* 10:134
- Team RDC (2008) R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing
- Thundathil J, Dance A, Kastelic J (2016) Fertility management of bulls to improve beef cattle productivity. *Theriogenology* 86:397–405
- Tolias KF, Cantley LC, Carpenter CL (1995) Rho family GTPases bind to phosphoinositide kinases. *J Biol Chem* 270:17656–17659
- Tomar A, Schlaepfer DD (2009) Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr Opin Cell Biol* 21:676–683
- Tost J, Gut IG (2007) DNA methylation analysis by pyrosequencing. *Nat Protoc* 2:2265
- Triantaphyllopoulos KA, Ikononopoulos I, Bannister AJ (2016) Epigenetics and inheritance of phenotype variation in livestock. *Epigenetics Chromatin* 9:31
- Wennmann DO, Schmitz J, Wehr MC, Krahn MP, Koschmal N, Gromnitsa S, Schulze U, Weide T, Chekuri A, Skryabin BV, Gerke V, Pavenstädt H, Duning K, Kremerskothen J (2014) Evolutionary and molecular facts link the WWC protein family to Hippo signaling. *Mol Biol Evol* 31:1710–1723
- Wu C, Sirard MA (2020) Parental effects on epigenetic programming in gametes and embryos of dairy cows. *Front Genet* 14:11
- Xia W, Mruk DD, Lee WM, Cheng CY (2006) Differential interactions between transforming growth factor-beta3/TbetaR1, TAB1, and CD2AP disrupt blood-testis barrier and Sertoli-germ cell adhesion. *J Biol Chem* 281:16799–16813
- Zhang GM, Zhang TT, An SY, El-Samahy MA, Yang H, Wan YJ, Meng FX, Xiao SH, Wang F, Lei ZH (2019) Expression of Hippo signaling pathway components in Hu sheep male reproductive tract and spermatozoa. *Theriogenology* 126:239–248
- Zheng QF, Xu B, Wang H, Ding L, Liu JY, Zhu LY, Qui H, Zhang L, Ni GY, Ye J, Gao SH, Jin GH (2018) Epigenetic alterations contribute to promoter activity of imprinting gene IGF2. *Biochimica et Biophysica Acta. Gene Regul Mech* 1861:117–124