

1 **Molecular responses of chicken embryos to maternal heat stress through DNA**  
2 **methylation and gene expression**

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24 **Abstract**

25 Climate change, with its repercussions on agriculture, is one of the most important adaptation  
26 challenges for livestock production. Poultry production is a major source of proteins for human  
27 consumption all over the world. With a growing human population, improving poultry's adaptation  
28 to environmental constraints becomes critical. Extensive evidence highlights the influence of  
29 environmental variations on epigenetic modifications. The aim of this paper is therefore to explore  
30 chickens' molecular response to maternal heat stress. We employed Reduced Representation  
31 Bisulfite Sequencing (RRBS) to generate genome-wide single-base resolution DNA methylation  
32 profiling and RNA sequencing (RNA-seq) to profile the transcriptome of the brains of embryos  
33 hatched from dams reared under either heat stress (32 °C) or thermoneutrality (22°C). We detected  
34 289 significant differentially methylated CpG sites (DMCs) and one differentially methylated  
35 region (DMR) between heat stressed and control groups. These DMCs were associated with 357  
36 genes involved in processes such as cellular response to stimulus, developmental processes and  
37 immune function. In addition, we identified 11 genes differentially expressed between the two  
38 groups of embryos, and identified ATP9A as a target gene of maternal heat stress on offspring.  
39 This study provides a body of fundamental knowledge on adaptive mechanisms concerning heat  
40 tolerance in chickens.

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42 **Keywords** *heat stress, epigenetics, DNA methylation, chicken, embryos*

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## 46 Introduction

47 Climate change and its direct and indirect consequences represent one of the most important  
48 adaptation challenges for livestock production, as unpredictable and rapid environmental changes  
49 are a source of stress. Chicken meat and eggs are major sources of proteins for human food  
50 worldwide, but their production is affected by global warming. Rising temperatures have adverse  
51 effects on poultry growth, production and survival. It has been shown that heat stress causes a  
52 decrease in productivity in many species<sup>1-3</sup>. Heat stress in chickens, as in other species, leads to  
53 reduced feed consumption, resulting in decreased energy and nutrient intake. This ultimately leads  
54 to compromised growth and reduced quality of broiler products, as well as decreased egg quantity  
55 and quality in layers<sup>4-9</sup>. The increased demand for animal products worldwide combined with a  
56 growing human population urges the need to improve the ability of animals to respond to heat  
57 stress<sup>10</sup>. Research has demonstrated that the environment exerts influence on gene expression in  
58 both plants and animals, resulting in phenotypic plasticity; this phenomenon leads to the  
59 emergence of different phenotypes from the same genotype in response to different environmental  
60 conditions, and can even affects the phenotype of future generations through transgenerational  
61 plasticity<sup>11-13</sup>. Some of these effects are mediated by epigenetics phenomena: in response to the  
62 environment, epigenetic mechanisms can induce changes in gene expression, linking  
63 environmental changes to the physiology and health of animals<sup>14,15</sup>. These mechanisms may act as  
64 catalysts and trigger the adaptation of organisms to their environment.

65 Epigenetics covers all mechanisms that modify gene expression in a reversible and transmissible  
66 way through mitosis or meiosis, without modifying the DNA sequence<sup>16</sup>. These phenomena  
67 include DNA methylation, histone modification, remodeling of chromatin, and regulation of gene

68 expression by non-coding RNAs (ncRNAs). Numerous studies, particularly in humans and  
69 mammals, showed that maternal stress can lead to epigenetic alterations in offspring, which  
70 ultimately may affect their phenotype<sup>17,18</sup>.

71 In avian species, Tzschenk and Basta (2002) reported that, in ducks, prenatal temperature  
72 experience has a clear influence on postnatal neural hypothalamic thermosensitivity and could be  
73 the result of epigenetic temperature adaptation<sup>19</sup>. In chickens, research focused on the effect of  
74 thermal manipulations during embryogenesis on post-hatch heat tolerance and showed an  
75 increased heat tolerance in broilers within the first 5 weeks of life, when exposed to an acute heat  
76 stress<sup>20,21</sup>. In Japanese quails, a study by Vitorino Carvalho et al. (2020) reported that thermal  
77 manipulation during embryogenesis significantly reduced the hatching rate and increased mortality  
78 during the first four weeks of life<sup>22</sup>. Subsequent research (Vitorino Carvalho et al., 2021) reported  
79 that thermal manipulation during embryogenesis had little to no effect on gene expression  
80 regulation in the hypothalamus of 35-day-old quails<sup>23</sup>. On the contrary, exposure to a heat  
81 challenge before this sampling resulted in an increase in the number of differentially expressed  
82 genes, reinforcing the hypothesis that embryonic thermal conditioning has a beneficial effect and  
83 increases thermotolerance later in life<sup>10,21,24</sup>.

84 The response to heat stress can also be triggered by heat exposure in the previous generation. For  
85 example, Ahmed et al., (2017) reported that maternal heat stress during late gestation increased  
86 acute thermal tolerance of the calf at maturity<sup>25</sup>. In birds several studies have also tried to elucidate  
87 the effect of the environmental experience of mothers on their offspring. In Japanese quails, it has  
88 been reported that maternal stress may affect and prepare future generations to cope with later  
89 environmental difficulties<sup>26,27</sup>. Santana et al. (2021) reported that maternal stress led to lower  
90 laying rate, egg mass and higher chick mortality rate at the 1–15 days of age. They observed that

91 the performance and oxidative metabolism of offspring raised in thermoneutral conditions were  
92 unaffected by maternal heat stress, while offspring subjected to heat stress during growth showed  
93 increased levels of protein oxidation<sup>18</sup>. In a recent study<sup>27</sup>, it was shown that thermal manipulation  
94 repeated during 4 generations in Japanese quail had a transgenerational effect on body weight and  
95 egg weight, suggesting non-genetic inheritance mechanisms. The hypothesis made to justify the  
96 improved resistance was that heat stress-induced epigenetic modifications were occurring as a  
97 consequence of the embryonic thermal manipulation, leading to increased thermal tolerance and  
98 adaptability in adults. A recent study confirmed the epigenetic nature of the transmission of heat-  
99 induced effects between generations through epigenetic mechanisms in chicken<sup>28</sup>.

100 Unlike mammals, birds have not been extensively studied for the effect of maternal heat stress on  
101 offspring heat tolerance. In this study, we explored this aspect by analyzing the genome-wide  
102 methylation and transcriptomic profiling of embryos whose mothers were reared under high  
103 ambient temperatures or under thermoneutral conditions. The underlying hypothesis is that  
104 maternal heat stress induces changes in DNA methylation in chicken embryos leading to changes  
105 in gene expression.

## 106 **Results**

107 In order to assess the epigenomic response to maternal heat stress on the DNA methylation levels  
108 in 13-day-old embryos, 22 embryos (10 controls and 12 stressed) were analysed. The results  
109 showed that heat stress of hens can mediate changes in the methylation patterns and also  
110 differential expression of some genes in offspring.

## 111 **DNA methylation changes**

112 Some general statistics of RRBS sequencing results are summarized in Table S2. An average of  
113 20 million reads per sample were obtained.. The average mapping efficiency was 64.84%, in  
114 accordance with what is expected from this type of data<sup>29</sup>. We have assessed 1,075,291 CpG sites  
115 (after preprocessing; Fig 2A) with an average depth of 18.34. The distribution of methylation level  
116 around the transcription start site (TSS) showed a decreased value in this region (Fig 2B). Among  
117 the analysed CpGs, we detected a total of 289 DMCs between HS and CT groups, of which 138  
118 were hypermethylated and 151 were hypomethylated in the HS group (Fig 3). The DMCs were  
119 present along most chromosomes (Fig 4 and Fig S2). Their distribution was not constant along the  
120 genome and some regions had a high density of DMCs. Notably, one region on chromosome 4  
121 (Chr4:2858109,2858165) was identified as a DMR. This region harbored two lncRNA genes  
122 (LOC121110553, LOC121110554) with unknown functions. As shown in Fig 5 these two genes  
123 have contrasted expression patterns across 47 tissues<sup>30</sup>, and only LOC121110553 was expressed  
124 in embryo.

## 125 **Annotation of differentially methylated cytosine**

126 DMCs were annotated according to gene features. From the detected DMCs, 28.85% were located  
127 in promoter regions, 40.28% in introns and 18.42% in exons (Fig 6). Chi2 test showed that these  
128 distributions among CpGs and DMCs (p-value < 2.2e-16) and among hyper and hypo DMCs (p-  
129 value < 2.2e-16) were significantly different. The fraction of the DMCs located in the promoter  
130 region was more frequently hypermethylated (37.25%) than hypomethylated (19.98%), while  
131 hypomethylation was more frequent in exons and introns.

## 132 **Gene ontology functional analysis**

133 Based on the DMCs location, we identified 357 differentially methylated genes (DMGs) that  
134 harbored at least one DMC in one of the gene features considered (Table S3) out of 35,995 genes  
135 with at least one CpG. The functional analysis of these genes has enabled us to identify as enriched  
136 several biological processes (BP) linked to the development stage. The gene ontology ViSEAGO  
137 output showed also the significance of embryo development, metabolic process, cellular response  
138 to stimulus, immune function (Fig 7).

139 **Gene expression analysis**

140 RNA sequencing analysis was performed to investigate the impact of heat stress on embryo gene  
141 expression. Among the 17,939 genes identified as expressed in embryos, eleven DEGs were  
142 detected between HS and CT groups as listed in Table 1, all being protein coding genes. Among  
143 these, four genes were upregulated and seven genes were down regulated. ATP9A (ATPase  
144 phospholipid transporting 9A), one of the upregulated genes in the HS embryos, was also in the  
145 list of DMGs, with 4 DMCs in the introns and exon regions, all of them being hypermethylated  
146 (Fig 8).

147 **Pyromark validation**

148 Pyrosequencing validation of seven DMCs with PyroMark confirmed all the positions as DMCs.  
149 Fig 9 shows the methylation level obtained with RRBS and PyroMark.

150 **Discussion**

151 The livestock industry faces a growing number of challenges due to climate change and global  
152 warming, which have a direct impact on animal growth, reproduction, health, and welfare. The  
153 exposure of animals to climate changes and other associated stressors has both short- and long-

154 term effects over the course of the animal's life. There is growing evidence that epigenetics, in  
155 interaction with the environment, may also contribute to the phenotypic diversity of animals<sup>31</sup>. In  
156 addition, these effects can be passed across generations with multigenerational inheritance and  
157 perhaps provide the ability to adapt to climate change for the subsequent generations<sup>32,33</sup>.

158 Our study aimed to elucidate the effect of maternal heat exposure on DNA methylation and gene  
159 expression in chicken embryos. The results revealed a slight influence of maternal heat stress on  
160 embryo transcriptomic levels, with eleven differentially expressed genes. We detected a total of  
161 289 DMCs between HS and CT groups, consistent with findings from previous studies in  
162 chicken<sup>28</sup>, cow<sup>34</sup> or guinea pig<sup>35</sup>, which have demonstrated changes in DNA methylation linked to  
163 parental heat exposure.

164 We observed that promoter DMCs were more frequently hypermethylated than hypomethylated in  
165 contrast with what was observed in exon and intron regions. This suggests that the promoter region  
166 may be more prone to hypermethylation in response to the mother heat stress than the other parts  
167 of the genes. A slight similar trend was observed in rainbow trout sperm after heat exposure of  
168 males during spermatogenesis<sup>36</sup>.

169 We identified 357 DMGs containing at least one DMC in various gene features, with a number of  
170 6 DMCs per gene on average. In contrast, only 11 genes exhibited significant differential  
171 expression. This highlighted the observation that the majority of differential methylation sites are  
172 not simultaneously associated with changes in gene expression. Such finding is consistent with the  
173 well-established knowledge that gene expression is highly context dependent, presenting a very  
174 fine tissue and stage specificity<sup>37</sup>. The lack of association at this developmental stage does not  
175 exclude a potential functional impact of methylation marks on gene expression later in life, which

176 could facilitate responses to heat stress exposures. It is indeed expected that during embryogenesis,  
177 some epigenetic marks are programmed and largely maintained throughout development,  
178 contributing to better cope with environmental stressors later in life<sup>38</sup> (Skinner, 2011).

179 Among the identified DMGs, ERBB4 (Erb-B2 Receptor Tyrosine Kinase 4), NFATC2 (Nuclear  
180 Factor Of Activated T-Cells 2) and ATP9A (ATPase Phospholipid Transporting 9A) have been  
181 linked to GWAS signals associated with thermotolerance in pigs, as reported by Kim et al.,  
182 (2018)<sup>39</sup>. Another study by Ramírez-Ayala et al., (2021) linked the ATP9A gene to thermogenesis  
183 in cattle<sup>40</sup>. Interestingly, in our study, ATP9A emerged as both DMG and DEG, and harbored  
184 numerous DMCs in both its intronic and exonic regions. This observation suggests the existence  
185 of temperature regulation pathways potentially shared between mammals and birds.

186 The DMR on chromosome 4 is associated with two long non-coding RNAs whose function has  
187 yet to be characterized: LOC121110553 is weakly expressed but not differentially expressed  
188 between the two groups, while LOC121110554 does not appear to be expressed.

189 The gene ontology analysis of DMGs identified important biological processes including cellular  
190 response to stimulus, embryo development, and telencephalon development. Cellular response to  
191 stimulus encompasses any process that alters the state or activity of a cell, such as movement,  
192 secretion, enzyme production, or gene expression. Indeed, cellular reaction to stress is diverse,  
193 ranging from activation of pathways involved in survival strategies to programmed cell death,  
194 which eliminate damaged cells<sup>41</sup>. Cellular apoptosis was reported as upregulated after a longer  
195 period of heat stress in highland and lowland chicken<sup>10</sup>. The cell's initial reaction to a stressful  
196 stimulus tends to support its defense and recover from injury. However, if distressing stimuli  
197 persist without resolution, cells activate signaling pathways leading to programmed cell death<sup>41</sup>.

198 Adaptive immune response is another pathway that was associated with DMGs. Heat stress in  
199 commercial laying hens has been shown to reduce production performance and inhibit immune  
200 function leading to an increase in mortality<sup>42</sup>. Similarly, a study showed that HS causes immune  
201 abnormalities in broiler chickens by impairing T and B cell development and maturation in primary  
202 and secondary lymphoid tissues<sup>43</sup>. In another study, transcriptome analysis revealed the genes and  
203 pathways involved in bursal responses to heat stress and lipopolysaccharide, showing that the  
204 combined treatments had the greatest effect<sup>44</sup>. The negative link between heat stress and immune  
205 function was also observed in cattle. For example, Dahl et al., (2020) reported that lactating cows  
206 often exhibit higher disease incidence in summer (metritis, mastitis, respiratory disease), possibly  
207 linked to compromised immune cell activity due to heat stress<sup>45</sup>. Additionally, calves born to  
208 mothers experiencing heat stress and dry period during the late gestation had lower weight at birth  
209 and through puberty<sup>46-48</sup>.

210 Epigenetics has the capability of conveying information to next generations without DNA  
211 sequence alteration. Epigenetic marks may represent the signature of environment stresses and  
212 specific physiological states acquired by the parental generation that could enhance adaptability of  
213 next generations to new situations. The outcome of the current study illustrates that maternal  
214 exposure to heat stress has an effect on the DNA methylation pattern of offspring. However, even  
215 with the exclusion of observed SNPs at CpG sites, we cannot rule out the hypothesis that some of  
216 the identified DMCs may be caused by genetic polymorphisms. Although these methylome  
217 changes were not associated with extensive transcriptional changes at the embryonic level, the  
218 affected genes and pathways identified from differentially methylated genes suggest a potential  
219 foundation for adaptive responses in progeny. This aligns with the studies of McGuigan et al.  
220 (2021) and Weyrich et al. (2016), indicating that under conditions of climate change and stressful

221 environments, epigenetic factors, through intergenerational and transgenerational effects, play a  
222 role in promoting adaptability of exposed populations<sup>33,35</sup>. This has been observed in chicken, with  
223 an intergenerational inheritance of heat resilience after fathers' embryonic heat conditioning,  
224 associated with DNA methylation changes in anterior preoptic hypothalamus<sup>28</sup>.

225 This study shows that maternal exposure to heat stress can induce hundreds of changes in  
226 methylation level and minor changes in transcriptome level in offspring. These DNA methylation  
227 modifications during the embryonic development as a consequence of their mother's heat stress  
228 may provide the capability of an adaptive response to subsequent heat stress exposure.

## 229 **Materials and Methods**

### 230 **Sample preparation and experimental design**

231 A total of 4 hens (2 controls and 2 heat-stressed) from an experimental layer population (R-) issued  
232 from selection for feed efficiency<sup>49</sup> were used. All birds were reared under standard conditions  
233 (22°C, *ad libitum* feeding) at the INRAE UE 1295 PEAT Poultry Experimental Unit (Nouzilly).  
234 In the heat stress group (Fig 1), hens were reared at 22°C until 28 weeks of age. Between 28 and  
235 32 weeks of age, the hens were kept at 32°C (increasing by 2°C per hour for 5 hours). The four  
236 hens were inseminated by the same male at week 30. Their eggs were collected between 31 and  
237 32 weeks and incubated for 13 days.

238 The experiments were carried out at the PEAT experimental unit under license number C37-175-  
239 1 for animal experimentation, in compliance with European Union legislation, and were approved  
240 by the local ethics committee for animal experimentation (Val de Loire) and by the French

241 Ministries of Higher Education and Scientific Research, and Agriculture and Fisheries (n°2873-  
242 2015112512076871), complying with the ARRIVE guidelines.

243 **DNA and RNA extraction**

244 DNA and RNA from brain of 13-day-old embryos were extracted, according to the manufacturer's  
245 instructions, with AllPrep DNA/RNA Mini Kit (Qiagen catalog No. / ID: 80204). Total RNA and  
246 DNA were quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The  
247 dsDNA concentration was measured using the Quant-iT PicoGreen dsDNA (Invitrogen) assay  
248 according to the manufacturer instructions. The fluorometric measurements were performed using  
249 ABI7900HT (Applied Biosystem).

250 The RNA quality was controlled using an Agilent 2100 bioanalyzer (Agilent Technologies France)  
251 with the Eukaryote Total RNA Nano Assay. Results were analysed with the 2100 Expert Software.  
252 RNA integrity (RIN) was 9.9 on average.

253 **Reduced representation bisulfite sequencing**

254 We obtained Reduced Representation Bisulfite Sequencing (RRBS) data from whole brains of 22  
255 embryos of unknown sex (10 controls and 12 stressed) at 13 days of age, derived from R- hens  
256 with or without heat stress. RRBS libraries were prepared using the Premium RRBS Kit  
257 (Diagenode, #C02030033), according to the manufacturer's instructions. Briefly, the protocol  
258 consisted in the digestion of 100 ng of genomic DNA by the *Msp*I enzyme followed by fragment  
259 end repair, and addition of adaptors. A size selection step was performed with AMPure XP Beads  
260 (Beckman Coulter). Next, samples were quantified by qPCR and the Ct values were used to pool  
261 samples by equimolarity. Then the bisulfite conversion was realized on the pool and the final

262 libraries were amplified using MethylTaq Plus Master Mix (Diagenode kit). After a clean-up with  
263 AMPure XP Beads, the RRBS library pools were analysed with the Qubit dsDNA HS Assay Kit  
264 (Thermo Fisher Scientific), and the profile of the pools was verified using the High Sensitivity  
265 DNA chip for 2100 Bioanalyzer (Agilent) or DNF-474 NGS fragment kit on a Fragment Analyzer  
266 (Agilent). Libraries were sequenced in single-end mode of 50 bp on an Illumina HiSeq 4000 on  
267 the GenomEast platform ([https://www.igbmc.fr/en/platforms-and-  
268 services/platforms/genomeast](https://www.igbmc.fr/en/platforms-and-services/platforms/genomeast)).

## 269 **Bioinformatics analyses**

270 The nf-core/methylseq pipeline<sup>50</sup> version 2.1.0 was used for analysing methylation bisulfite  
271 sequencing data. Bismark version 0.24.2 with Bowtie2 as an alignment tool was used for mapping  
272 on the *Gallus gallus* genome GRCg7b obtained from Ensembl ([bGalGal1.mat.broiler.GRCg7b,  
273 https://ftp.ensembl.org/pub/release-  
274 109/fasta/gallus\\_gallus/dna/Gallus\\_gallus.bGalGal1.mat.broiler.GRCg7b.dna.toplevel.fa.gz](https://ftp.ensembl.org/pub/release-109/fasta/gallus_gallus/dna/Gallus_gallus.bGalGal1.mat.broiler.GRCg7b.dna.toplevel.fa.gz)).

275 Pipeline's default parameters were used, with the option --clip\_r1 3 for adapter trimming  
276 (trimming 3 bases from the 5' end of each read).

## 277 **Differential methylation analyses**

278 The Bioconductor package edgeR v3.28.1<sup>51</sup> was used to detect differentially methylated CpGs  
279 sites (DMCs), The callDMR function from the DSS package v2.38.0<sup>52</sup> was used to call DMRs  
280 (differentially methylated regions) from the edgeR outputs. A DMR was defined as a region with  
281 a minimum number of 3 CpGs and a percentage of CpG sites with significant p-values (less than  
282 0.05) greater than 50% between Heat Stress (HS) and Control (CT) groups. Here a two-step

283 process has been implemented: preprocessing and differential methylation analysis. During the  
284 preprocessing step, CpGs that overlapped with C-T single nucleotide polymorphisms (SNPs) were  
285 filtered out to avoid erroneous identification of C-T polymorphisms as methylation changes. SNPs  
286 were detected by gemBS v4.053 with option “bs\_call”, CpGs were further filtered using other  
287 criteria (maximum Coverage: 200, minimum coverage: 5 and minimum fraction of samples present  
288 per position: 0.8). Differential methylation analysis was performed with edgeR using a multifactor  
289 model (HS/CT and Sex) with False Discovery Rate (FDR)  $\leq 0.05$ . Identification of the sex of  
290 embryos was performed through the average of read mapped on sex chromosomes (Fig S1).

291 Genomic features annotation was done with the GenomeFeatures package version 1.3  
292 (<https://forgemia.inra.fr/aurelien.brionne/GenomeFeatures>) with default defined promoter region  
293 upstream:3000 bp and downstream:500 bp. An in-house enriched annotation file was used in this  
294 study<sup>54</sup>.

## 295 **Functional enrichment analysis**

296 We analyzed all the genes that had at least one DMC in their genomic features (promoter, UTR5,  
297 introns, UTR3, downstream). Functional enrichment analysis was done with the R package  
298 ViSEAGO v1.14.0<sup>55</sup>, and the full list of genes having at least one CpG in genomic features was  
299 used as background.

## 300 **RNA-seq data acquisition**

301 Paired-end sequencing was performed using an Illumina HiSeq3000 (Illumina, California, USA)  
302 system, with  $2 \times 150$  bp, as in Jehl et al, 2019<sup>56</sup>. FASTQ files were mapped on the GRCg7b  
303 reference genome (GCF\_016699485.2) and the nf-co.re/rnaseq<sup>50</sup> pipeline version 3.8.1 was used

304 for providing raw count and transcript per kilobase million (TPM) normalized expression per gene  
305 and sample.

306 **RNA-seq analysis**

307 The normalized expression level was obtained using the trimmed mean of M-values (TMM)  
308 scaling factor method, implemented in Bioconductor package edgeR version 3.32.1, with the  
309 functions of “calcNormFactors” and “rpkm” used to scale the raw library sizes and scale of gene  
310 model size respectively. In situations where TPM and TMM normalized expressions were  $\geq 0.1$   
311 and read counts  $\geq 6$  in at least 80% of the samples, the gene was considered as expressed. For  
312 differential expression analysis we used the raw counts from the expressed genes previously  
313 selected and normalized by the TMM method. The Bioconductor package edgeR was used to  
314 perform the differential expression analysis, which is based on a generalized negative binomial  
315 model for model fitting. The method of “edgeR-Robust” was used to account for potential outliers  
316 when estimating per gene dispersion parameters. P-values were corrected for multiple testing using  
317 the Benjamini-Hochberg approach to control the false discovery rate (FDR), and  $FDR < 0.05$  was  
318 used to identify significant DEG (Differentially Expressed Gene).

319 **Pyromark validation**

320 For the DMC validation, the Pyrosequencing method was used to perform a quantitative  
321 methylation analysis of bisulfite-converted DNA for each individual. The pyrosequencing was  
322 performed using PyroMark Q24 (QIAGEN). All the primers (forward, reverse and sequencing  
323 primers) were designed with the PyroMark Assay Design software (Version 2.0.1.15, Qiagen)  
324 using the assay type "Methylation Analysis" (CpG) (Table S1).

325 The PCR reaction contained 2  $\mu$ l of bisulfite treated DNA sample (EZ DNA Methylation-Gold kit,  
326 Zymo Research), 2.5  $\mu$ l of buffer + 0.05  $\mu$ l of Taq Polymerase (PCR BIO Classic Taq, Eurobio),  
327 2.5  $\mu$ l of dNTP (2mM, Promega), 1  $\mu$ l of each primer (10  $\mu$ M), and 5.95  $\mu$ l of water. The program  
328 on the thermal cycler (Thermocycler ABI2720, Applied Biosystem) was: 95 °C for 5 min; followed  
329 by 35 cycles of: 95 °C for 30 sec, hybridization temperature for 30 sec, and 72 °C for 30 sec; and  
330 a final extension at 72 °C for 5 min.

331 Ten  $\mu$ l of PCR product were then mixed with 1  $\mu$ l of Streptavidin sepharose™ high performance  
332 (GE Healthcare) and 40  $\mu$ l of PyroMark binding buffer (Qiagen). The mix was shaken at 1400 rpm  
333 on a microplate mixer for at least 10 min. The immobilized PCR products were purified using  
334 PyroMark Q24 vacuum workstation (manufacturer instructions, QIAGEN), mixed with 1  $\mu$ l of a  
335 sequencing primer (5 $\mu$ M) and 24  $\mu$ l of Pyromark annealing buffer, and heated at 80 °C for 5 min  
336 to anneal the sequencing primer before analysis on the PyroMark Q24. Results were analysed with  
337 the PyroMark Q24 software (version 2.0.8, build 3, Qiagen). DNA methylation values obtained  
338 via pyrosequencing were compared between the HS and Control groups using a Wilcoxon test.

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479 **Acknowledgments**

480 We are grateful to the entire staff of the UE1295 PEAT (Nouzilly, France, doi:  
481 10.15454/1.5572326250887292E12) for their excellent animal care. We are grateful to the  
482 genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul,  
483 <https://doi.org/10.15454/1.5572369328961167E12>) for providing help, computing, and storage  
484 resources. RRBS sequencing was performed by the GenomEast platform, a member of the  
485 “France Génomique” consortium (ANR-10-INBS-0009). This work was funded by the French  
486 National Agency of Research (ChickStress project, ANR-13-ADAP). KK also thanks the French  
487 National Program MOPGA (Make Our Planet Great Again) for funding and support.

488

489 **Author contribution**

490 FP, TZ and SLa conceived the experimental design and secured the funding. KK, JS and SLa  
491 performed the analyses. KK, CC, GD, SF, AH and JNH participated in the bioinformatic and  
492 statistical analyses. DG performed animal breeding. SLe performed molecular experiments. KK  
493 drafted the manuscript, FP, SLa and TZ revised the manuscript draft. All authors read and  
494 approved the final version.

495 **Data availability**

496 The DNA methylation and RNA-seq datasets analyzed in the current study are available at ENA  
497 (<https://www.ebi.ac.uk/ena/browser/home>) with accession numbers PRJEB70935 and  
498 PRJEB28745, respectively.

499

500 **Competing interests**

501 The authors declare no competing interests.

502

503 **Figure legend**

504 Fig 1: Experimental design

505 Fig 2: Preprocessed data A) Number of CpGs kept after each step of the pre-processing  
506 workflow. B) Average methylation level around TSS regions.

507 Fig 3: Volcano plot of CpG methylation and DMCs between HS and CT.

508 *Hyper=hypermethylated; Hypo=hypomethylated, FDR=False Discovery Rate,*  
509 *DMC=differentially methylated CpG site*

510 Fig 4: Manhattan plot of differential methylation analysis between HS and CT groups. The above  
511 dashed line represents  $FDR \leq 0.05$  and solid line represents  $FDR \leq 0.01$ .

512 *FDR=False Discovery Rate*

513 Fig 5: Expression pattern of two lncRNA genes (LOC121110553, LOC121110554) across 47  
514 tissues (<https://gega.sigenae.org/>)

515 Fig 6: Distribution of total CpGs and DMCs (hypermethylated and hypomethylated) across the  
516 different genomic regions.

517 Fig 7 : Gene Ontology functional analysis of the genes related to DMCs. The clustering heat map  
518 plot of the functional sets of gene ontology (GO) terms was obtained using ViSEAGO. Gene  
519 Ontology functional analysis with count showing information content and a dendrogram on  
520 enriched GO terms based on BMA semantic similarity distance and Ward's clustering criterion.

521 Fig 8: Expression and methylation level of the 4 DMCs per group (CT and HS) for ATP9A.

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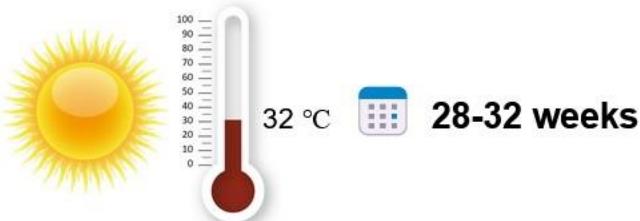
527 Table 1. Differentially expressed genes between Heat Stress and Control groups.

Gene id	Gene Name	Chr	start	end	strand	Expression*	padj	fc	lfc
LOC100858942	LOC100858942	34	2198709	2201885	+	UP	0.01	242.44	7.92
LOC112531412	LOC112531412	JAENSK010000420.1	8859	18598	-	UP	0.03	98.82	6.63
LOC396217	MBP	2	90091375	90199666	+	UP	0.02	12.92	3.69
LOC419345	ATP9A	20	13450938	13503307	+	UP	0.01	2.57	1.36
LOC107054346	LOC107054346	12	1193659	1196336	+	DOWN	0.04	0.04	-4.48
LOC121108245	LOC121108245	Z	169136	201956	-	DOWN	0.01	0.08	-3.69
LOC100857335	LOC100857335	34	1513723	1516547	-	DOWN	0.01	0.1	-3.34
LOC107057116	ZNFY4	16	1583937	1595533	+	DOWN	0.01	0	-12.35
LOC121108653	LOC121108653	MU179258.1	33562	38085	+	DOWN	0.01	0.33	-1.62
LOC100502566	TMSB15B	4	1940045	1942512	+	DOWN	0.00	0.34	-1.57
LOC417488	CLIP2	19	3258667	3318184	-	DOWN	0.00	0.41	-1.28

528 \*Up: more expressed in HS than in CT, Down: less expressed in HS than in CT. fc: fold change, lfc:  $\log_2$ (fold change)

529

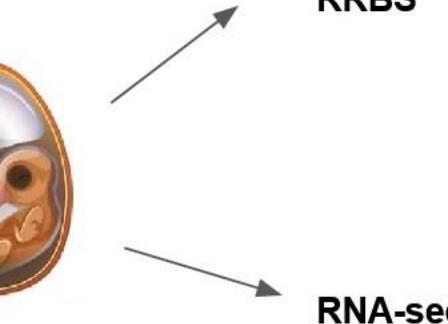
## Heat Stress



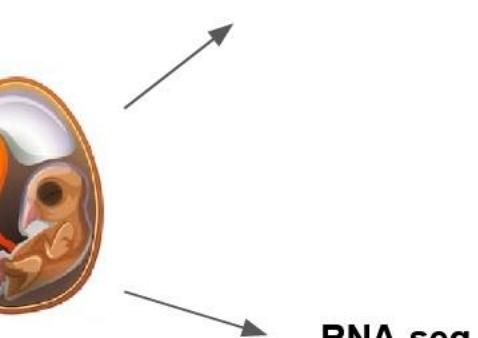
28-32 weeks



31-32 weeks



## Control



## CpG filtering

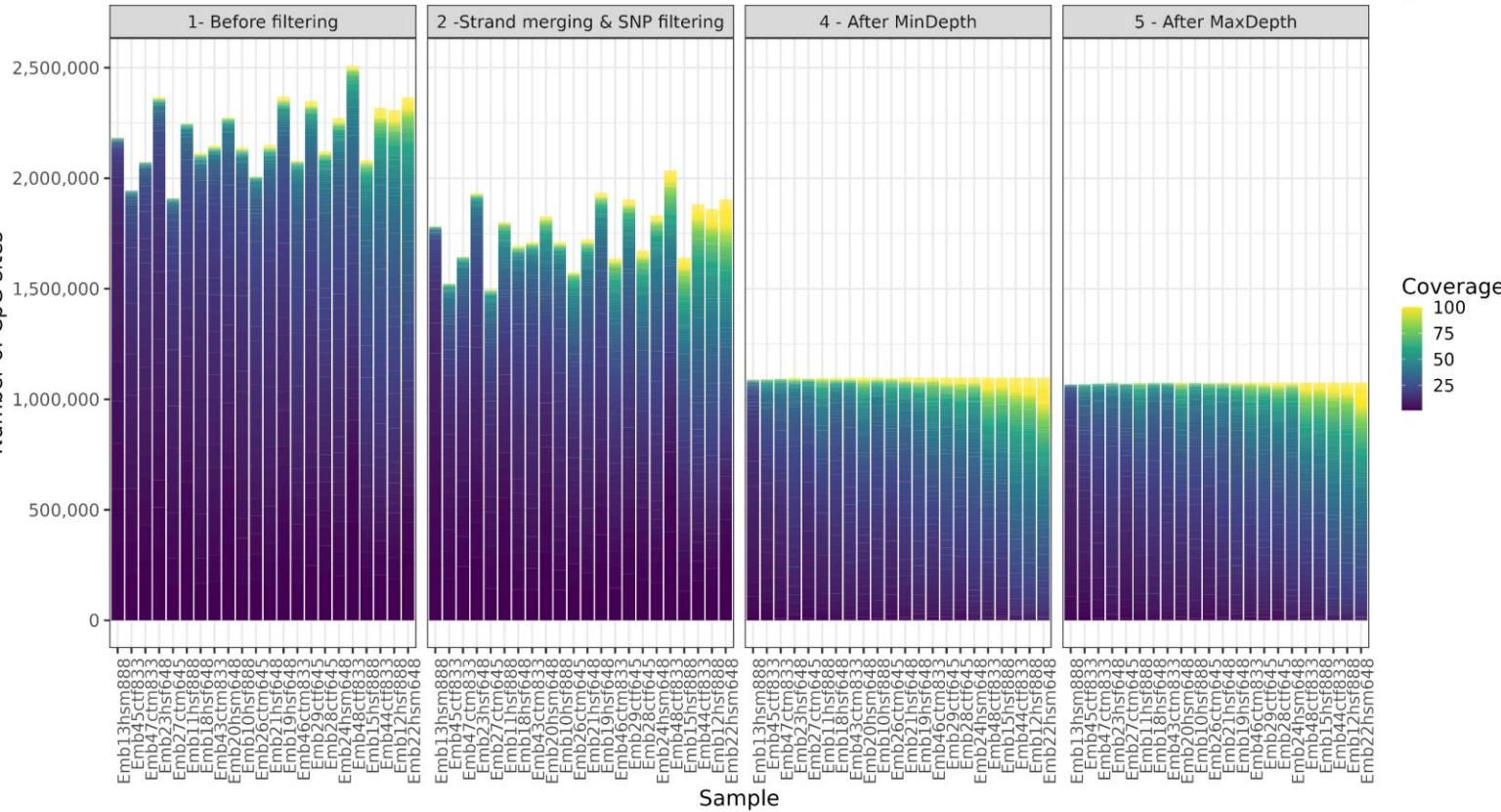


Fig 2A

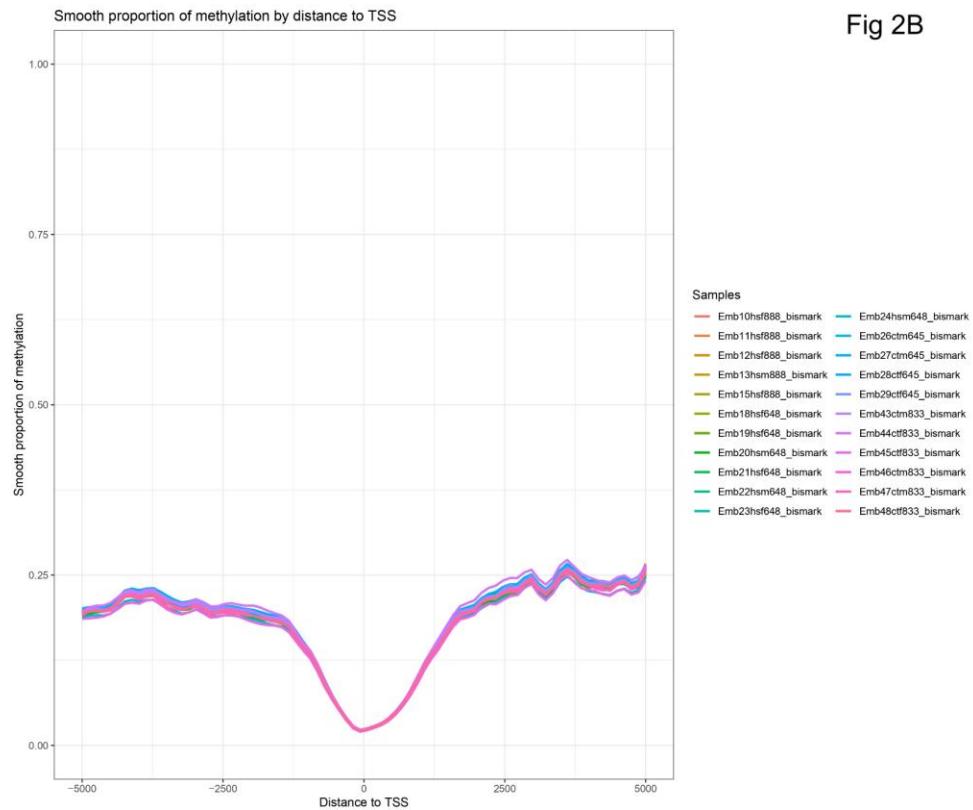
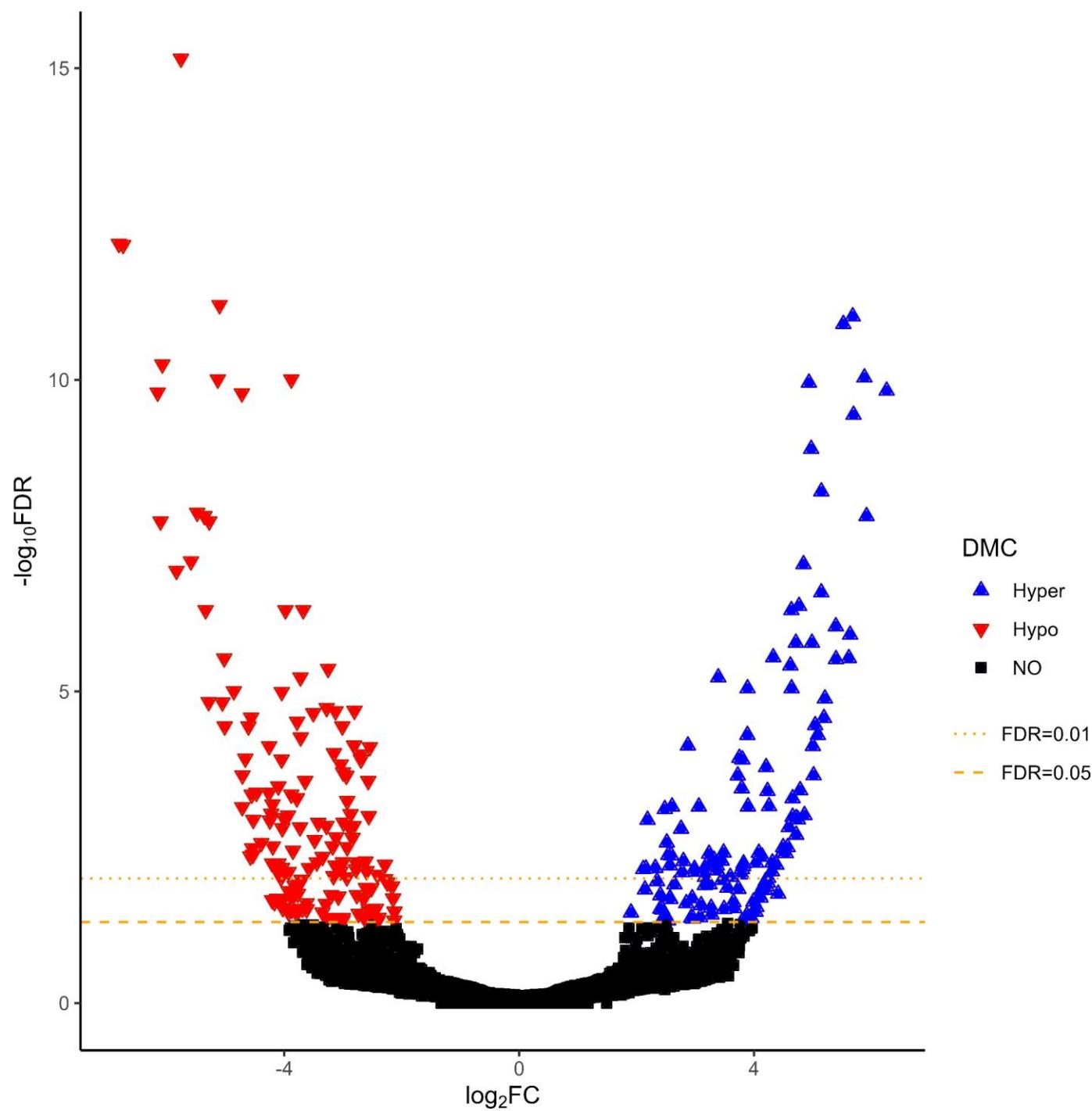
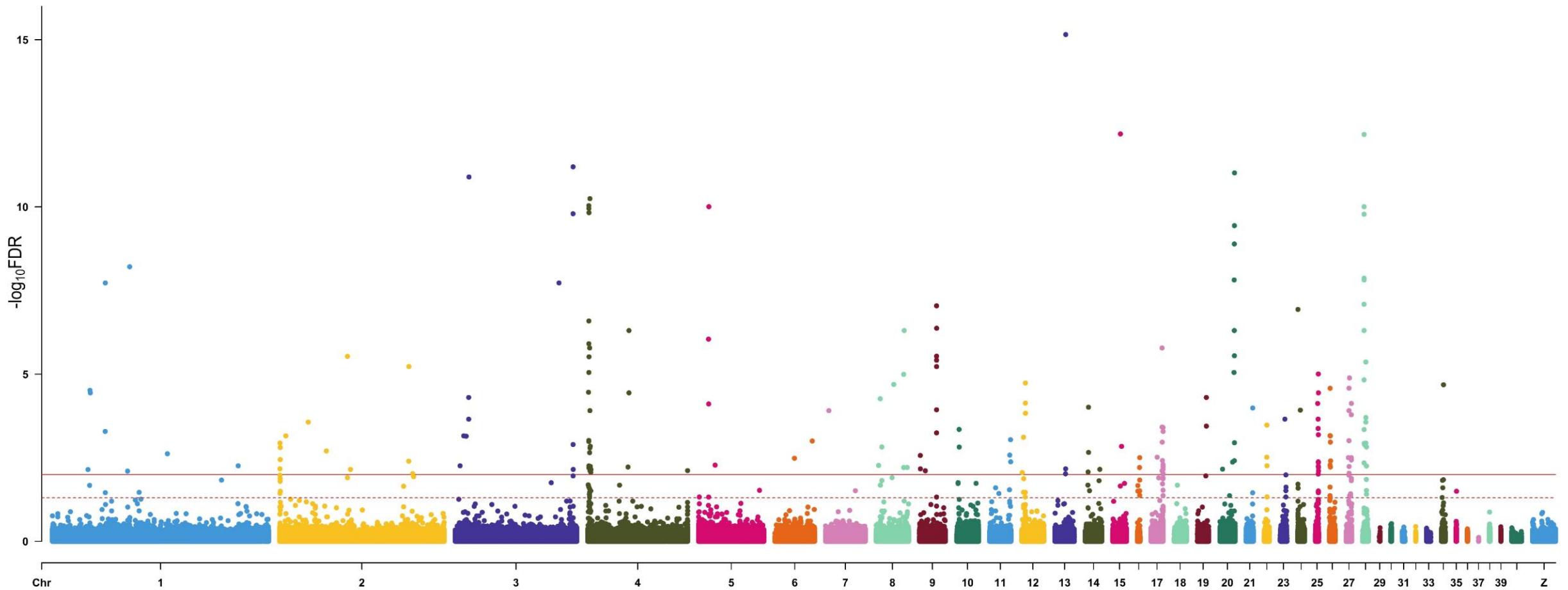
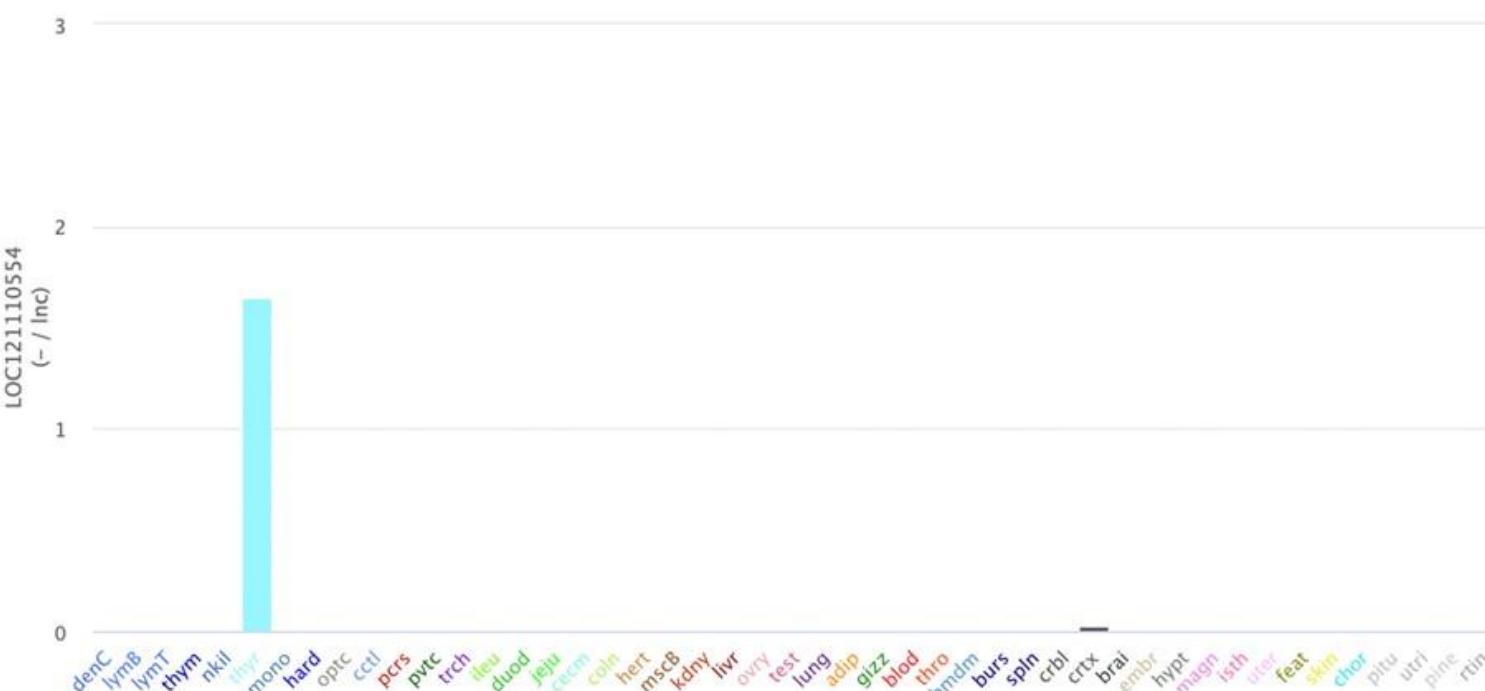
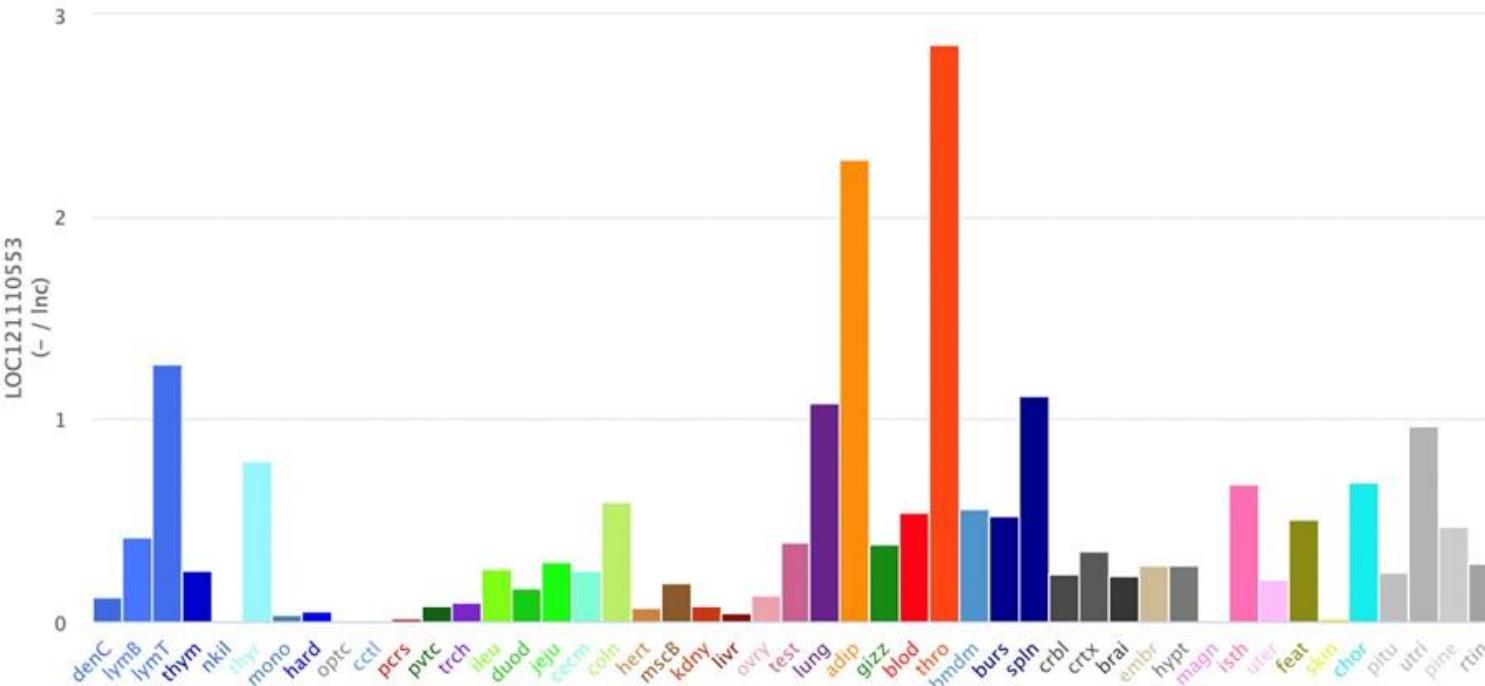


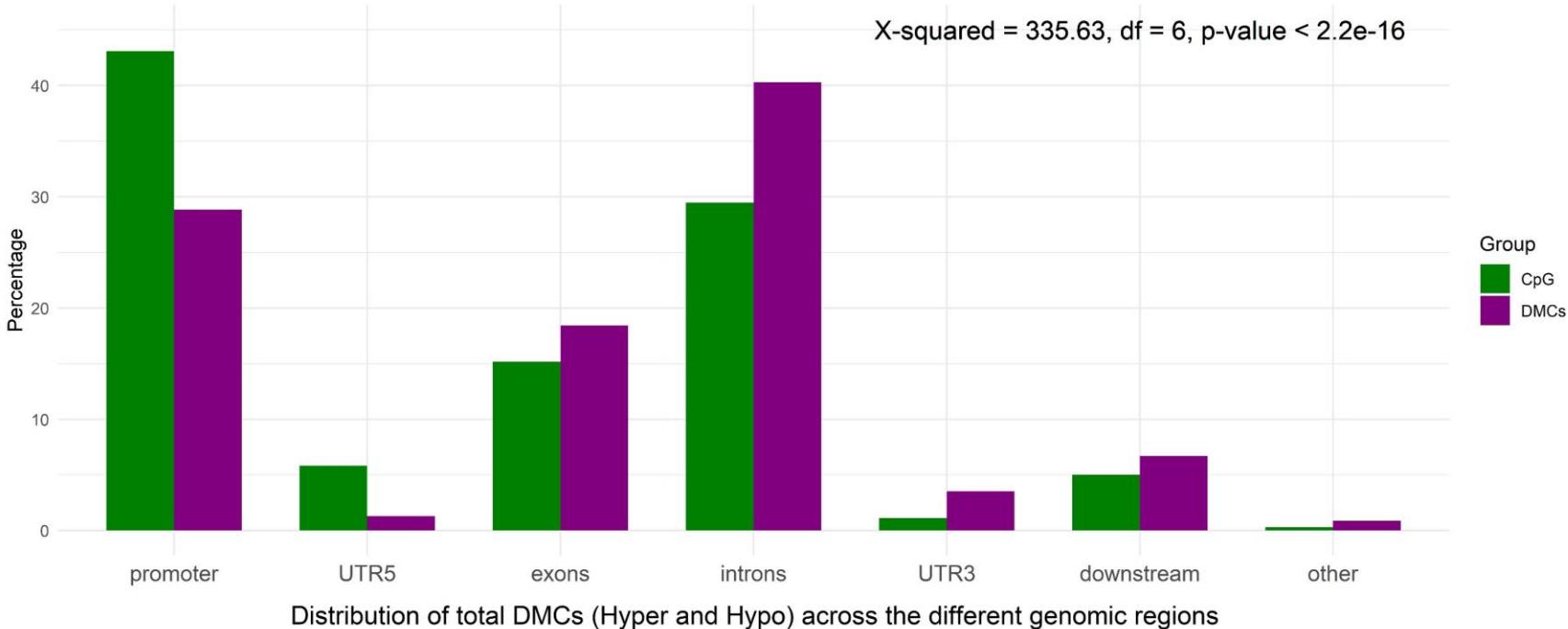
Fig 2B



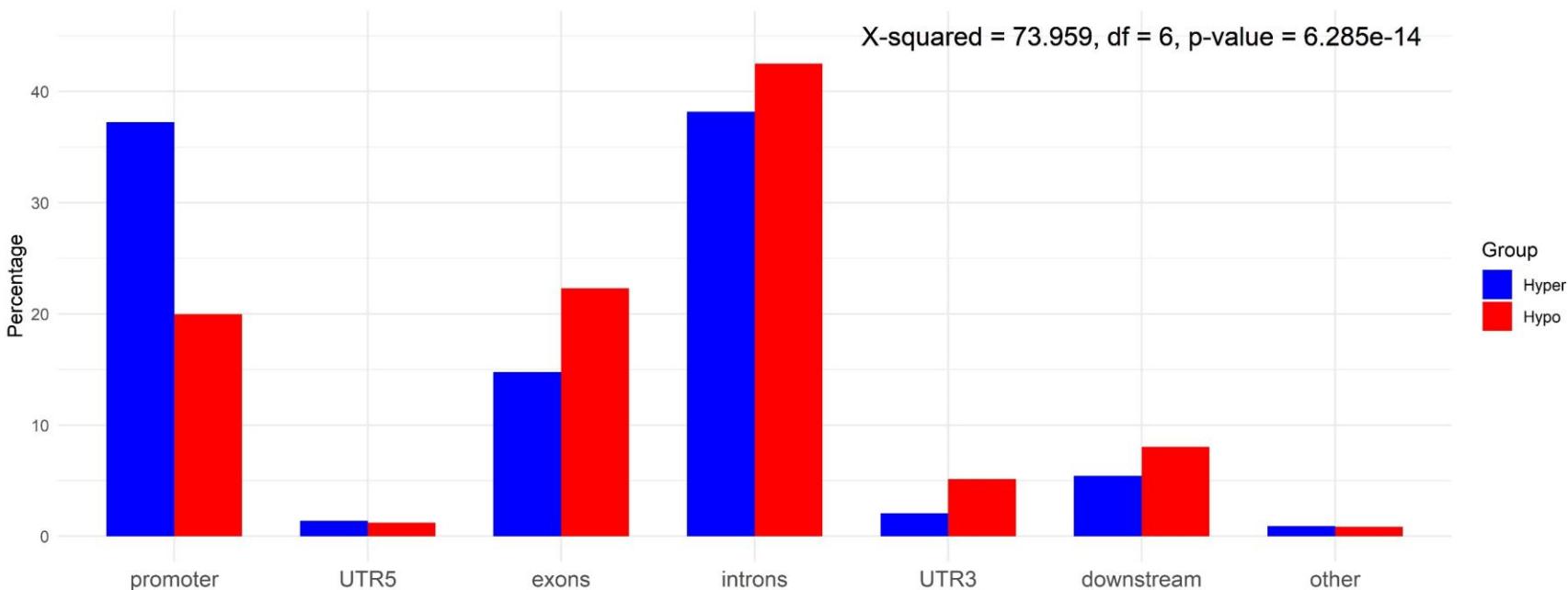




Distribution of total CpGs and DMCs across the different genomic regions



Distribution of total DMCs (Hyper and Hypo) across the different genomic regions



## BMA GOclusters distance heatmap

