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1 Sources of variation of DNA methylation in rainbow trout: combined effects of temperature and
2 genetic background

3

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26

27

28 **Abstract**

29

30 Phenotypic plasticity is a key component of the ability of organisms to respond to changing
31 environmental conditions. The role of DNA methylation in mediating environmentally induced
32 phenotypic variation has been evidenced in only a limited number of studies in fish. In this study, we
33 aimed to study the establishment of DNA methylation marks in response to an environmental stress
34 in rainbow trout and to assess whether these marks depend on the genetic background. The
35 environmental stress chosen here was temperature, a known induction factor of epigenetic marks in
36 fish. To disentangle the role of epigenetic mechanisms such as DNA methylation in generating
37 phenotypic variations, 9 rainbow trout isogenic lines with no genetic variability within a line were used.
38 For each line, half of the eggs were incubated at standard temperature (11°C) and the other half at
39 high temperature (16°C), from eyed-stage to hatching. Samples were collected at eyed-stage, 3 days
40 and 7 days after the beginning of the high temperature regime. An upregulation of *hsp47* (heat shock
41 protein 47) gene, a good molecular biomarker for thermal stress, in the 16°C batches confirmed that
42 the early temperature treatment was efficient regardless of the genetic background. In order to gain
43 a first insight into the establishment of DNA methylation marks in response to an early temperature
44 regime (control 11°C vs. heated 16°C), we have studied the expression of 8 *dnmt3* (DNA
45 methyltransferase) genes, potentially involved in *de novo* methylation, and analysed global DNA
46 methylation in the different rainbow trout isogenic lines using LUMA (LUminometric Methylation
47 Assay). Finally, finer investigation of genome-wide methylation patterns was performed using
48 EpiRADseq, a reduced-representation library approach based on the ddRADseq (Double Digest
49 Restriction Associated DNA) protocol, for 6 rainbow trout isogenic lines. Our results suggest that
50 expression of *dnmt3* genes was only moderately modulated by temperature and may be temporally
51 dynamic and dependent on the genetic background. LUMA analysis revealed no overall effect of
52 incubation temperature (11°C vs 16°C) on global DNA methylation. Differential methylation analysis
53 between the control (11°C) and high temperature (16°C) groups was performed on 51,668 to 55,607
54 EpiRADseq loci depending on the line. The great majority of observed changes in methylation differed
55 across genetic backgrounds, as there were between-lines differences in the number of differentially
56 methylated loci (ranging from 19 to 155 depending on the line) and the vast majority of differentially
57 methylated loci (375 out of 409, or 91.7%) were only detected in one line. A functional analysis, based
58 on pathway classification, performed using KEGG on 268 genes, may suggest the potential modulation
59 of genes belonging to signal transduction, metabolism, endocrine and immune systems pathways. In
60 conclusion, we have demonstrated that thermal history during embryonic development alters patterns
61 of DNA methylation, but to a greater or lesser extent depending on the genetic background.

62

63 **Keywords**

64 Temperature, EpiRADseq, DNA methylation, *dnmt3*, rainbow trout, isogenic lines

1. Introduction

Phenotypic plasticity, defined as the ability of a given genotype to vary its phenotype depending on the environment, is a key component of the ability of organisms to respond to changing environmental conditions (Pigliucci, 1996). Epigenetic mechanisms (Goldberg et al., 2007) are involved in the long-term persistence of physiological effects resulting from events that occurred earlier in the life of an animal. These epigenetic marks can be modified by environmental stimuli and hence regulate genome-wide gene expression and ultimately modulate phenotypes. The role of epigenetic processes such as DNA methylation in mediating environmentally induced phenotypic variation has been reviewed in Angers et al. (2010). To date, only a limited number of studies in fish could independently analyse genetic and epigenetic variations, using asexual fish vertebrate model systems such as the clonal fish *Chrosomus eos-neogaeus* (Cyprinidea) (Angers et al., 2020) or the naturally self-fertilizing hermaphroditic mangrove killifish *Kryptolebias marmoratus* (Ellison et al., 2015; Berbel-Filho et al., 2019). However, the aforementioned studies were limited to a single or a few genotypes.

In this study, we aimed to study the establishment of DNA methylation marks in response to an early (during embryonic development) environmental stress in rainbow trout and importantly to assess whether these marks depend on the genetic background.

A recent review highlighted the role of epigenetic marks in response to abiotic (hypoxia, temperature, salinity, nutrition, contaminants) or biotic (social interactions, pathogens) environmental factors in the modulation of physiological responses in teleost fish (Best et al., 2018). In fish, early life stages (embryonic and larval stages that occur externally) are particularly sensitive to such events.

The environmental stress chosen here was temperature, highly relevant in the context of climate change as fish are poikilothermic. Indeed, global warming is expected to impact negatively cold-water fish aquaculture, such as Salmonids. Several studies, reviewed in Jonsson and Jonsson (2019), have shown that temperature experienced during early development can impact phenotypes later in life in fish, such as thermal acclimation ability (Scott and Johnston, 2012), growth and muscle development (Johnston, 2006; Albokhadaim et al., 2007; Macqueen et al., 2008; Johnston et al., 2009; Steinbacher et al., 2011; Garcia de la serrana et al., 2012; Schnurr et al., 2014), sex differentiation (Valdivia et al., 2014) or intermediary metabolism (Seibert, 1985; Couto et al., 2008; Qiang et al., 2014). Moreover, temperature is a known induction factor of epigenetic marks in fish. For example, in response to early exposure to certain temperature regimes, through differential methylation of specific promoters (aromatase and myogenin), epigenetic mechanisms are involved in sex determination in sea bass (Navarro-Martín et al., 2011) or muscle development in Senegalese sole (Campos et al., 2013) and Atlantic salmon (Burgerhout et al., 2017). Also, Metzger and Schulte (2017) have demonstrated that temperature experienced during development has prolonged effects on DNA

100 methylation levels throughout the genome of threespine stickleback. However, rearing temperature
101 had no effect on genome-wide DNA methylation patterns during post-embryonic development in
102 turbot (Suarez-Bregua et al., 2020).

103 To disentangle genetic and environmental sources of variation of epigenetic marks, rainbow
104 trout isogenic lines, previously established at INRAE (Quillet et al., 2007), are a unique and highly
105 relevant biological model. Indeed, within each line, all fish have the same genome *i.e.* there is no
106 genetic variability. This will allow the very fine analysis of epigenetic marks established in response to
107 an environmental stress within a line (*i.e.* at constant genotype). A collection of 19 isogenic lines (*i.e.*
108 19 genotypes) is available, making it possible to study whether the establishment of these epigenetic
109 marks depend on the genetic background. These lines have also recently been characterized for their
110 response to temperature and the existence of a high between-line variability was shown (Dupont-Nivet
111 et al., 2015).

112 DNA methylation, the addition of methyl groups to cytosines, was chosen here as it is a well-
113 characterized epigenetic mark and has predominantly been studied in fish. In an organism, DNA
114 methyltransferases (*dnmt*) are the enzymes involved in DNA methylation and several types exist:
115 *dnmt1*, which is involved in the maintenance of methylation profiles during cell divisions; and *dnmt3a*
116 and *dnmt3b*, which are involved in *de novo* methylation. It has been shown that temperature during
117 embryonic development can modulate *dnmt3* expression in zebrafish (Campos et al., 2012; Dorts et
118 al., 2016). Recently, expression patterns of DNA methylation genes (including *dnmt1* and *dnmt3* genes)
119 were assessed during ontogenesis in rainbow trout (Liu et al., 2020). However, few studies have
120 analysed the expression levels of *dnmt* genes in response to temperature in fish, especially in rainbow
121 trout. Expression profiling of *dnmt* genes could help to understand the establishment of differential
122 methylation profiles during an early temperature stress. Further, in order to gain a first insight into the
123 impact of temperature during embryonic development on DNA methylation levels, Luminometric
124 Methylation Assay (LUMA) analysis (Karimi et al., 2006) is a low cost option that allows the acquisition
125 of the global percentage of DNA methylation. For finer investigation of genome-wide methylation
126 patterns established in response to an early temperature stress, we have chosen EpiRADseq, a
127 reduced-representation library approach that is scalable and provides higher resolution compared to
128 a global approach such as LUMA. It is based on the ddRADseq (Double Digest Restriction Associated
129 DNA) protocol, except that it utilizes a methylation-sensitive restriction enzyme (Schield et al., 2016).
130 It has been developed on a single clone of water fleas (*Daphnia ambigua*), *i.e.* in absence of genetic
131 diversity, but the protocol can be modified to account for it. Based on the differences in the
132 frequencies of reads obtained per locus between two conditions (control vs. treatment), this approach
133 allows the identification of differentially methylated loci in response to the treatment applied.

134 The present study reports the impact of early temperature regime (control 11°C vs. heated
135 16°C) on i) *dnmt3* genes expression; ii) global DNA methylation assessed by LUMA; and iii) genome-
136 wide patterns of DNA methylation assessed by EpiRADseq. The utilization of 9 rainbow trout isogenic
137 lines allowed us to study the interactions between genetics, epigenetics and environment.

138

139 **2. Materials and methods**

140

141 2.1. Ethical statement

142

143 All the experiments were carried out at the INRAE experimental facilities (PEIMA, Sizun, France)
144 authorized for animal experimentation under the French regulation C29-277-02. The investigation
145 reported here does not need approval by a specific ethical committee since it implies only classical
146 rearing practices and stops before the first feeding.

147

148 2.2. Biological material

149

150 Experimental fish were produced and reared at the INRAE experimental fish farm (PEIMA, Sizun,
151 France). Rainbow trout homozygous isogenic lines, previously established and maintained at INRAE
152 (Quillet et al., 2007) by single within-line pair mating, were used as breeders. The experimental design
153 is illustrated in Figure 1. Nine heterozygous isogenic lines were produced by mating several
154 homozygous females from a single isogenic line (B57) with nine homozygous sex-reversed XX males
155 from 9 other isogenic lines (A02, A03, A22, A36, AB1, AP2, G17, N38 and R23). To avoid confusion with
156 the INRAE homozygous isogenic lines, heterozygous lines will be named A02h, A03h etc. Importantly,
157 these lines have recently been characterized for their phenotypic response to acute temperature
158 challenges (Dupont-Nivet et al., 2015): resistant lines (A03h, G17h and R23h), intermediate lines (A02h,
159 A22h, AB1h and AP2h) and sensitive line (N38h). A fin clip from each doubled haploid (DH) parent was
160 taken and kept in ethanol. In order to obtain large enough numbers, eggs were collected from 49 2
161 year-old females that had spawn on the same day and had similar egg weight (ranging from 0.031 to
162 0.038 g). To avoid unexpected maternal effects, eggs were mixed and then divided into nine batches
163 (8 batches of 8,600 eggs and one of 6,060 eggs), each batch being fertilized by one of the 9 homozygous
164 males. For line R23h, fewer eggs were used because fertilization was performed with cryopreserved
165 sperm; this can explain the lower percentage of eyed eggs (37.6% compared to 59.1-76.1%). Fertilized
166 eggs were kept at 11.4°C into vertical tray incubators. Sixteen days after fertilization, eyed eggs of each
167 line were distributed into small incubators installed in two separate 200-litre tanks supplied with
168 natural spring water at either 11.4°C (control) or heated to 16°C (treatment). Several studies have

169 shown that the optimal temperature for rainbow trout egg incubation ranged between 8 to 12°C
170 (Billard, 1992; Baeverfjord, 2003; Weber et al., 2016), in particular to control mortalities and
171 malformations. Some studies showed that high incubation temperatures (16°C) from fertilization to
172 50% hatch (Velsen, 1987) or first feeding (Baeverfjord, 2003) was associated with important
173 mortalities. A similar study on brown trout also showed an upper limit for embryo development
174 between 14 and 16°C (Ojanguren and Braña, 2003). This is why we decided to incubate eggs at high
175 temperature (16°C) from eyed-stage and not straight after fertilization. From now on, the control
176 condition will be referred to as “11°C”. For 8 out of 9 lines, 3 incubators containing 500 to 625 eyed
177 eggs were used for each incubation temperature regime. For R23h, due to the lower survival at eyed-
178 stage, only 2 incubators containing 557 eyed eggs were used for each incubation temperature regime.
179 Temperature treatment lasted for 7 days. For our study, sampling was done as follows: three days after
180 the beginning of the temperature treatment (19 days post fertilization, dpf), 3 pools of 5 eggs per line
181 and per incubation temperature were collected for RNA extraction; at the end of the temperature
182 treatment (22 dpf), 6 pools of 5 eggs per line and per incubation temperature were collected, 3 pools
183 for RNA extraction (except for line R23h) and 3 pools for DNA extraction. All samples were snap frozen
184 in liquid nitrogen and kept at -80°C before processing.

185

186 2.3. Quantitative PCR (qPCR)

187

188 The expression of 9 target genes was assessed by qPCR in nine isogenic lines at two sampling dates, 19
189 and 22 dpf (Figure 1). In order to confirm that the early temperature treatment was successful, *hsp47*
190 (Heat Shock Protein 47) was chosen as it has been shown to be a good molecular biomarker for thermal
191 stress in salmonids (Akbarzadeh *et al.*, 2018). Expression profiling of 8 *dnmt3* genes, which are
192 potentially involved in *de novo* DNA methylation (*dnmt3aa*, *dnmt3ab1*, *dnmt3ab2*, *dnmt3ba1*,
193 *dnmt3ba2*, *dnmt3bba1*, *dnmt3bba2*, *dnmt3bbb*), was also performed to try to understand the
194 establishment of differential methylation profiles during an early temperature stress. Total RNA was
195 isolated from 102 pools of fertilised eggs (eyed-stage; 5 eggs per pool) using TRIzol reagent (Invitrogen)
196 (2 incubation temperatures; 3 biological replicates per line and per temperature; 8 isogenic lines at 2
197 sampling dates and 1 isogenic line (R23h) at 1 sampling date; Figure 1). RNA samples were then purified
198 using RNeasy kit (Qiagen) and quantified by Nanodrop. RNA integrity was assessed using RNA Nano
199 Chips (Agilent Technologies) in a 2100 Bioanalyzer instrument (Agilent Technologies).

200 Complementary DNA (cDNA) was synthesized from 1 µg total RNA using the SuperScript II
201 (Invitrogen, ThermoFisher Scientific). cDNA samples were then quantified using RNA Pico Chips
202 (Agilent Technologies) in a 2100 BioAnalyzer instrument (Agilent Technologies) and normalised to 20
203 pg/µl. Efficiencies were calculated using a 5-point standard curve from a 10-fold dilution series (1:1 to

204 1:10000) of a pool of all cDNA samples. Quantitative PCR was performed with AmpliTaq Gold®
205 Polymerase (Power SYBR® Green PCR MasterMix, Life Technologies) in the Applied Biosystems™
206 QuantStudio™ 12K Flex Real-Time PCR System (ThermoFisher Scientific). The qPCR conditions used
207 were 1 cycle at 95°C for 10 min then 40 cycles at 95°C for 30 s and 60°C for 1 min. At the end of each
208 PCR run, a melting curve analysis was performed to ensure the specificity of the PCR product. Two
209 technical replicates were used for each sample. No template control (NTC) was included on each plate
210 to check the absence of contaminations. The most stable reference genes (ref) were selected using the
211 BestKeeper software (Pfaffl et al., 2004). Five reference genes were selected: elongation factor 1α
212 (*ef1α*), ribosomal protein S20 (*rps20*), β-actin, RNA terminal phosphate cyclase like 1 (*rcl1*) and 60S
213 acidic ribosomal protein (*arp*). Primers sequences are shown in Table 1. The analysis software REST
214 2009 (Relative Expression Software Tool; Pfaffl, 2001; Pfaffl et al., 2002) was used to calculate fold
215 change in expression of each target gene (*hsp47* or *dnmt3*) between Control (incubation at 11°C) and
216 Treatment (incubation at 16°C) groups, using the following formula:

$$217 \quad \text{Fold change (FC)} = \frac{(E_{target})^{\Delta Ct_{target} (Control-Treatment)}}{(E_{ref})^{\Delta Ct_{ref} (Control-Treatment)}}$$

218 where *E* is the qPCR efficiency (Table 1). This software automatically includes statistical analysis based
219 on a two-sided pairwise fixed reallocation randomisation test and bootstrapping methods including
220 2000 iterations which allows to get a good estimate of the p-value (Pfaffl, 2001). These statistical
221 analyses were performed line by line. Data are presented as the geometric average ± standard error
222 (SE) of the fold change relative to Control.

223

224 2.4. DNA extraction

225

226 DNA was extracted from the fin clips collected from the DH parents using the Wizard® Genomic DNA
227 purification kit (Promega). DNA was also extracted from 54 pools of fertilised eggs (eyed-stage; 5 eggs
228 per pool), collected at the end of the temperature treatment (22 dpf) (9 isogenic lines; 2 incubation
229 temperatures; 3 biological replicates per line and per temperature; Figure 1), using the same kit but
230 with modifications. Briefly for the pools of eggs, lysis was performed in 10 ml of Nuclei Lysis Solution
231 and 292 µl of proteinase K (20 mg/µl), with overnight incubation at 55°C. RNA was digested by adding
232 100 µl of RNase Solution and incubating 1H at 37°C. Proteins precipitation was performed by adding
233 3.4 ml of Protein Precipitation solution and centrifugating 20 min at 13,000 g at room temperature.
234 Genomic DNA was then concentrated and desalted by isopropanol precipitation (volume to volume)
235 and washed with 70% ethanol. Genomic DNA was rehydrated in 200 µl of DNA Rehydration Solution.
236 30 µl of genomic DNA was then purified with Agencourt AMPure XP paramagnetic beads (Beckman

237 Coulter). Purified genomic DNA was finally quantified by NanoDrop and a Qubit 2.0 fluorometer
238 (Invitrogen), and normalised to a concentration of 100 ng/ μ l.

239

240 2.5. Global DNA methylation analysis

241

242 Global DNA methylation levels were quantified using Luminometric Methylation Assay (LUMA), as
243 previously described (Mohsen Karimi et al., 2006; Karimi et al., 2006; Robles et al., 2019) on the 54
244 pools of eggs collected at 22 dpf (Figure 1). Briefly, 500 ng of genomic DNA was cleaved using the
245 isochizomeres HpaII (methylation sensitive) and MspI (non-methylation-sensitive) in two separate
246 reactions and in the presence of EcoRI to standardize for DNA amounts (New England Biolabs). The
247 protruding ends were then used as templates for pyrosequencing with the Pyromark Q24 device and
248 Pyromark Gold Q96 reagents (Qiagen). The luminometric signals were quantified using the Pyromark
249 Q24 software (Qiagen). The level of cytosine methylation was determined in duplicate reactions by
250 comparing the ratio of HpaII to MspI cleavage, standardized using EcoRI cleavage. Statistical analyses
251 were carried out using non-parametric tests suited for small samples (permutation tests for two/K
252 independent samples with Monte-Carlo sampling; coin plug-in in RCommander) at a 95% level of
253 significance. To test for the effect of incubation temperature (11°C vs. 16°C) on global DNA
254 methylation, two-sample Fisher-Pitman permutation tests were performed on the whole dataset and
255 line by line. To test for between-lines differences in global DNA methylation at each incubation
256 temperature, K-sample Fisher-Pitman permutation tests were performed, followed by non-parametric
257 testing of Tukey-type multiple comparisons using nparcomp software (Konietzschke et al., 2015) in R.
258 Data are presented as mean percentages of global DNA methylation \pm SE.

259

260 2.6. EpiRADseq analysis

261

262 2.6.1. Sequence library preparation

263 Genome-wide patterns of DNA methylation were analysed by EpiRADseq on the same DNA extracts
264 used for LUMA, for 6 out of 9 rainbow trout isogenic lines: resistant lines (A03h, G17h and R23h),
265 intermediate lines (AB1h and AP2h) and sensitive line (N38h). EpiRADseq is a reduced-representation
266 library approach, based on ddRADseq protocol, that has been recently developed and tested on a
267 single clone of water fleas (Schield et al., 2016). The protocol was here modified to account for genetic
268 variability and allow both within and between-lines comparisons. Briefly, for 36 biological samples (6
269 isogenic lines x 2 incubation temperatures x 3 pools of 5 eggs), 200 ng of DNA from each sample was
270 digested in parallel with restriction enzymes PstI (CATCAG recognition site) and MspI or HpaII (CCGG
271 recognition site). As suggested by Schield et al. (2016), digestion by PstI/MspI (cuts at CCGG sites like

272 HpaII, but is insensitive to methylation) was used to account for differences in genotypes (e.g. to
273 identify EpiRADseq loci present in some lines but absent in others). In addition, the 7 DH parents used
274 to produce the 6 heterozygous isogenic lines (6 males and one B57 female) were digested with
275 PstI/MspI in order to confirm the presence/absence of particular EpiRADseq loci in the different lines.
276 Digested samples were purified using AMPure beads, quantified using a Qubit fluorometer and then
277 the amount of DNA was standardized. Then, each digested DNA sample was ligated with a mixture of
278 8 double-stranded sequencing adapters containing UMIs (Unique Molecular Identifiers) and a 5-bp
279 barcode. UMI are random sequences of 8 bases used to tag each molecule (fragment) prior to library
280 amplification, in order to identify PCR duplicates. Eight different 5-bp barcodes for each sample were
281 used at the ligation stage to add base heterogeneity and hence improve Illumina fluorescence reading.
282 Adapter assembling was based on Peterson et al. (2012) and using the 'ddRAD ligation molarity
283 calculator' excel spreadsheet. After adapter ligation, a 2-step PCR strategy was used to construct the
284 sequencing library, as described in Lluch et al. (2015). The 6-bp indexes, allowing for sample
285 multiplexing, were added during the second PCR step with the second Illumina adapter. The final
286 EpiRADseq library construction was as followed: P1-Illumina-adapter,UMI,barcode,PstI-cut-site-
287 overhang,DNA,MspI/HpaII-cut-site-overhang,P2-Illumina-adapter-Index-included. All libraries were pooled in
288 an equimolar fashion and the resulting pooled library was size-selected. The size selection for
289 fragments within a range 340-400 bp was done using a Blue Pippin Prep (Sage Science) with a 2%
290 agarose cartridge and purified and concentrated by 0.8X AMPure XP beads purification. All steps of
291 library construction were controlled by a fragments profile analysis with a Fragment Analyzer (Agilent).
292 The final library was quantified by qPCR and sequenced on an Illumina HiSeq 3000 in paired-end 2x150
293 bp.

294

295 2.6.2. EpiRADSeq loci definition (supplemental data epiRAD_script.zip)

296 As sequence libraries contained 8-bp UMIs, we used the 'clone_filter' program from the Stacks suite
297 version 1.48 (Catchen et al., 2013) to remove PCR duplicates (i.e. 100% identical paired sequences, also
298 known as 'optical duplicates'). Then, because each sample had 8 barcodes of 5 nucleotides before the
299 PstI restriction site, we used the Stacks 'process_radtags' function on reads pairs to remove those
300 barcodes, to check for the presence of restriction sites at the beginning of each read, and to apply
301 quality filters (i.e. remove reads with uncalled bases and with an average quality score below 20 on a
302 sliding window of 15% of the reads length). At the end of 'process_radtags' step, 8 pairs of filtered
303 reads files were obtained per sample (i.e. one for each barcode). These 8 paired files were
304 concatenated into a single pair (R1 and R2) of fastq file per sample. Sequences of each sample were
305 then aligned to the reference genome Omyk_1.0 (Genbank accession number: GCA_002163495.1)
306 using the BWA-MEM aligner version 0.7.15 (Li, 2013). Alignments were filtered with samtools version

307 1.4 (Li et al., 2009), bedtools version 2.26 (Quinlan and Hall, 2010) and an in-house script. Non properly
308 paired alignment, supplementary alignment, alignment with soft clipped positions at the restriction
309 sites extremities, and mapping with a mapping score below 20 were filtered out. EpiRADseq loci were
310 defined with bedtools for each sample separately by merging strictly identical mapping intervals,
311 calculating the coverage (i.e. the number of reads) of each defined locus and generating a .bed file. To
312 construct a full catalogue of EpiRADseq loci, the loci separately defined for each sample were merged
313 with bedtools when exhibiting strictly identical intervals. An in-house python script was used to
314 generate the final count table for each defined EpiRADseq locus, i.e. the number of reads per sample.

315 The rainbow trout genome has undergone four whole-genome duplications as in all salmonids
316 species, and it is known that large parts of the genome remain duplicated (Berthelot et al., 2014). To
317 identify duplicated loci, we performed a variant calling analysis with Stacks version 1.48 (Catchen et
318 al., 2013). Briefly, loci were identified by sample using the mapping coordinates (pstacks program),
319 each allele must have a minimum coverage of 3 reads. A full catalogue including the loci from all the
320 samples was then constructed (cstacks), individual loci were mapped back to the catalogue (sstacks),
321 and finally each sample was genotyped for each locus (populations). Since DH parents are expected to
322 be homozygous, all loci that appeared heterozygous in at least two DH parents were considered as
323 duplicated and removed from further analyses.

324 In order to further characterize the EpiRADseq loci, their location in a genomic feature was
325 determined based on the current rainbow trout genome annotation release 100 (Omyk_1.0). Several
326 genomic features were considered for this analysis, namely intergenic regions, regions 1 kb upstream
327 or 1 kb downstream of annotated genic regions, exons, introns or pseudogenes.

328

329 2.6.3. Differential methylation analysis

330 In order to define a set of usable EpiRADseq loci for each of the 6 isogenic lines, the output count table
331 (number of reads per locus and per sample) for the 6 PstI/HpaII libraries of a given line (2 incubation
332 temperatures x 3 biological replicates) was filtered to keep loci with positive counts in at least 3 of the
333 6 corresponding PstI/MspI libraries (filter called 'PM Filter'). Then, only loci with positive counts in at
334 least one of the two doubled haploid parents used to produce the given isogenic line were kept (filter
335 called 'DH Filter'). These two steps of filtering ensured the removal of potentially artefactual loci and
336 resulted in 6 sets of EpiRADseq loci, one for each isogenic line (Figure 2). The comparison of these 6
337 sets of loci was carried out using jvenn (Bardou et al., 2014) in order to calculate the total number of
338 EpiRADseq loci, the number of shared loci (i.e. present in the 6 isogenic lines), as well as the number
339 of line-specific loci. Also, the genomic distribution of EpiRADseq loci per Mb windows was assessed
340 using the 'geom_bin2d' function of ggplot2 package in R v.3.5.1. Finally, in order to identify
341 differentially methylated loci between the two incubation temperatures (11°C vs. 16°C), the analysis

342 was performed on each single line using the set of PH EpiRADseq filtered loci count tables (Figure 2).
343 Statistical analyses were performed with edgeR version 3.26.5 (Robinson et al., 2010) in R v.3.6.1.
344 EpiRADseq loci were filtered out to keep loci with CPM (counts per million) > 1 in at least 3 of the 6
345 samples considered for each line, followed by TMM normalization (Robinson and Oshlack, 2010). We
346 chose to implement quasi-likelihood (QL) F-test as it provides more robust and reliable error rate
347 control when the number of replicates is small (Lun et al., 2016). The QL dispersion estimation and
348 testing procedure were done using the functions glmQLFit() (with the ROBUST = TRUE option) and
349 glmQLFTest(). All PH EpiRADseq loci with a Benjamini-Hochberg corrected FDR value <0.05 were
350 considered to be differentially methylated between the two temperature conditions. The numbers of
351 unique and shared differentially methylated loci between the two temperature conditions (11°C vs.
352 16°C) for 6 rainbow trout isogenic lines were drawn using UpSetR package (Conway et al., 2017).

353

354 2.6.4. Functional analysis of the differentially methylated EpiRADseq loci

355 The current annotation (release 100) of the rainbow trout assembly (Omyk_1.0) describes over 55k
356 genes. However, only 11,234 (20.17%) of these genes are associated with a gene symbol. More
357 surprisingly, of the 42k protein coding genes associated with characterized proteins, only 5,619
358 (13.11%) have a gene symbol. Unfortunately, functional analyses heavily rely on gene symbols. We
359 compared the protein coding trout genes and proteins (BLASTX and BLASTP) to the newest catalogue
360 of salmon and zebrafish proteins (respectively 17,668 and 46,038 proteins with gene symbols, from
361 NCBI annotation release 100 and 106) to enrich the number of gene symbols associated with the
362 rainbow trout genes. Briefly, we defined 3 classes based on similarity and reciprocal coverage: class I
363 (similarity ≥80%, coverage ≥80%), class II (similarity ≥80%, coverage ≥50%) and class III (similarity ≥60%,
364 coverage ≥50%). If the best match for a trout gene was from class I or II, we added the corresponding
365 gene symbol as a putative gene symbol. We also added as synonyms the gene symbols of other close
366 blast matches (same blast class than the best match). These BLAST results allowed us to add 24,823
367 gene symbols (19,589 from class I matches and 5,234 from class II matches) to the 5,619 gene symbol
368 of the NCBI annotation for protein coding genes. We also used the recently released rainbow trout
369 annotation on Ensembl (v100.1 from April 2020) and used the Ensembl gene symbols of the genes
370 showing more than 50% of reciprocal coverage with NCBI genes. With the 21,379 Ensembl genes
371 associated with NCBI genes, we have added 797 gene symbols (242 and 555 for coding and non-coding
372 genes). Ultimately, 36,353 trout genes were associated with gene symbols accounting for more than
373 65% of the total trout genes up from the original 20% (Supplementary Table S1).

374 We have then extracted the gene symbols and putative gene symbols from the genes found in
375 the differentially methylated loci using the intersect tool from Bedtools suite (v2.27.1) (Quinlan and
376 Hall, 2010). Then, we used the ZFIN website to select the best gene symbol for each gene. Briefly, we

377 selected the gene symbols with the highest number of associated ZFIN phenotypes and highest
378 number of GO terms, as they were the most likely to give results for the functional analysis.

379 The gene symbols obtained from the previous step were used as an input for the downstream
380 functional analysis steps. Because of the limited number of genes available (see Results section), it was
381 not possible to perform an enrichment analysis. Therefore, in order to gain a first insight into the
382 biological functions involved in the response to temperature, a descriptive classification approach was
383 chosen instead on the whole dataset, i.e. the functional analysis was not performed line by line. First,
384 the gene symbols were checked and their names were modified whenever needed using the
385 information available from GeneCards (<https://www.genecards.org/>). Then, they were converted into
386 the corresponding KEGG Orthology (KO) codes using the 'db2db' tool ([https://biodbnet-
387 abcc.ncifcrf.gov/db/db2db.php](https://biodbnet-abcc.ncifcrf.gov/db/db2db.php)) from the bioDBnet suite, using the then up to date underlying
388 databases (Supplementary Table S2). Subsequently, the retrieved KO codes were analysed using the
389 'Reconstruct Pathway' tool (https://www.genome.jp/kegg/tool/map_pathway.html) from the KEGG
390 Mapper suite. The obtained KEGG pathways were manually edited in order to keep only the first two
391 hierarchy levels, and the first-level hierarchy 'Human diseases' was discarded. Eventually, the gene
392 counts corresponding to each second-level hierarchy were entered in an excel spreadsheet in order to
393 obtain the final bar plot.

394

395 3. Results

396

397 3.1. Effect of incubation temperature on the expression of candidate genes (*hsp47* and *dnmt3*)

398

399 At eyed-stage, the transcription of *hsp47*, a thermal stress biomarker, overall was upregulated at 16°C
400 (fold-change relative to the control 11°C: FC=3.6, p<0.001 at 19 dpf; FC=4.1, p<0.001 at 22 dpf).
401 Analyses line by line showed an increase of the expression of this gene in all lines at 16°C, with fold-
402 changes ranging between 2.2 (A03h) and 5.4 (A36h) at 19 dpf; between 2.3 (N38h) and 7.1 (A36h) at
403 22 dpf, hence confirming that the early temperature stress was efficient regardless of the genetic
404 background (Figure 3). Spearman's rank correlation tests revealed no correlation between the FC of
405 *hsp47* at 16°C and the rank (1 for sensitive; 2 for intermediate and 3 for resistant) of the known
406 phenotypic response of the lines at 19 dpf ($r=-0.391$, $p=0.338$); but a significant correlation at 22 dpf
407 ($r=0.777$, $p=0.040$) indicating that induction of *hsp47* expression was higher in resistant lines compared
408 to intermediate and sensitive lines. Also, the resistant line A03h and sensitive line N38h seemed to
409 exhibit very different patterns of *hsp47* expression at the two sampling dates (Figure 3).

410 When comparing *dnmt3* genes expression between the two incubation temperatures (11°C vs.
411 16°C) within each line, 5 lines (A03h, AP2h, G17h, N38h and R23h) exhibited up or down regulation of

412 some of the *dnmt3* genes (Table 2, Supplementary Figure S1). Results also differed between the two
413 sampling dates, with some genes up/downregulated at a single sampling date or genes shifting
414 direction between sampling dates (downregulated at 19 dpf but upregulated at 22 dpf; e.g. genes
415 *dnmt3ab1* and *dnmt3ba2* for line N38h) (Table 2, Supplementary Figure S1).

416

417 3.2. Effect of incubation temperature on global DNA methylation analysis

418

419 At eyed-stage just before hatching (22 dpf), analysis of global DNA methylation with LUMA
420 (LUminometric Methylation Assay) revealed no overall effect of incubation temperature ($z = 0.56222$,
421 $p=0.578$) but significant differences between lines at 11°C ($\chi^2 = 16.603$, $p=0.007$) and 16°C ($\chi^2 = 14.828$,
422 $p=0.028$) (Figure 4). Lines A22h and AB1h exhibited significantly lower DNA methylation levels
423 compared to the other lines at 11°C; AB1h and AP2h at 16°C. There was also a tendency of lower DNA
424 methylation level at 11°C compared to 16°C for line A22h and at 16°C compared to 11°C for lines AB1h,
425 AP2h and N38h, although not significant due to the low number (3) of biological replicates in each
426 temperature condition. Spearman's rank correlation tests revealed no correlation between global DNA
427 methylation levels and the rank (1 for sensitive; 2 for intermediate and 3 for resistant) of the known
428 phenotypic response of the lines ($r=-0.091$, $p=0.830$ at 11°C; $r=0.469$, $p=0.240$ at 16°C).

429

430 3.3. Effect of incubation temperature on genome-wide patterns of DNA methylation

431

432 A total of 565,954,096 reads were generated. Using Stacks v1.48, 'clone_filter' identified 9.7% of reads
433 as PCR duplicates and removed them. A further 8% of reads did not pass the quality filters of
434 'process_radtags' and were removed from the analysis. After removal of PCR duplicates and quality
435 filtering, a total of 467,437,915 reads were used for the analysis. Reads aligned very well to the rainbow
436 trout reference genome (98% of reads mapped on average). After alignment filtering (removal of
437 alignments that are supplementary, not representing properly paired reads for which one of the
438 restriction site has been soft-clipped, or with mapping quality below 20), 64% of reads on average were
439 kept.

440 Overall, 284,825 EpiRADseq loci were defined on the whole dataset. Variant calling analysis
441 with Stacks identified 2,671 potentially duplicated loci that were removed from downstream analyses.
442 After two steps of filtering (Figure 2), the total number of EpiRADseq loci kept was 99,712, ranging
443 between 72,263 (line R23h) to 83,494 (line A03h) depending on the line (Table 3). Among the 99,712
444 EpiRADseq loci kept for further analysis, 96,522 were located on the 29 chromosomes and 3,190 on
445 the unassembled scaffolds. The number of EpiRADseq loci per chromosome was highly correlated with
446 the chromosome size (Spearman's rank correlation $r = 0.967$; $p<0.001$). By plotting the number of loci

447 per Mb windows, distribution of EpiRADseq loci throughout the genome seemed relatively
448 homogeneous, with a tendency of lower number of loci towards the ends of the chromosomes
449 (Supplementary Figure S2). Among the 99,712 EpiRADseq loci kept, 63,359 (63.5%) were common to
450 the 6 isogenic lines and 12,036 (12.1%) were line-specific (Figure 5). Based on the current annotation
451 of rainbow trout genome, 38.1% of EpiRADseq loci mapped to intergenic regions, 44.2% within introns,
452 12.5% within exons, 1.8% upstream genes and 1.9% downstream genes (Figure 6).

453 Differential methylation analysis between the two incubation temperatures (11°C vs 16°C) was
454 performed for each line separately and using only the 99,712 EpiRADseq loci identified in the PstI/Mspl
455 libraries, but by using the PstI/HpaII counts at these loci (Figure 2). After filtering loci with low
456 abundances of reads (counts per million > 1 in at least 3 of the 6 samples), between 65.7% and 71.5%
457 of the loci were kept (Table 3). The number of differentially methylated EpiRADseq loci between the
458 two temperature conditions (11°C vs. 16°C, adjusted p-value < 0.05) ranged from 19 (N38h) to 155
459 (AB1h) (Figure 7). These differentially methylated loci were spread across the genome, located on 13
460 different chromosomes for N38h, 16 for A03h, 27 for AP2h and G17h, 28 for R23h and 29 for AB1h
461 without defining hot spots. Interestingly, the vast majority of differentially methylated loci (375) were
462 detected in only one of the six lines, while 34 loci were detected in at least 2 lines (24 in 2 lines, 6 in 3
463 lines, 2 in 4 lines and only 2 loci detected in 5 lines) (Figure 7). Analysis of the location of differentially
464 methylated EpiRADseq loci revealed that 33.6% mapped to intergenic regions, 37.6% within introns,
465 15.4% within exons, 8.4% upstream genes and 2.5% downstream genes (Figure 6). The proportions of
466 these EpiRADseq loci among the annotation features categories were significantly different from the
467 ones obtained with all EpiRADseq loci (Figure 6; $\chi^2 = 110.1$; $p < 0.001$), notably with a shift in the
468 proportion of loci located upstream genes (8.4% vs. 1.8%), suggesting that the distribution of
469 differentially methylated EpiRADseq loci might not be random.

470 Out of the 385 differentially methylated loci identified among the 6 rainbow trout isogenic
471 lines and located on the 29 rainbow trout chromosomes, 251 loci overlapped with one or several
472 genes. A total of 268 genes overlapped with these loci and 204 of them were associated with gene
473 symbols (Supplementary Table S3). The KEGG Ontology (KO) codes were retrieved for 138 of these
474 gene symbols (Supplementary Table S3) and were used as an input for a pathway classification
475 functional analysis using KEGG. By a general point of view, it was possible to observe that the most
476 represented second-level KEGG pathways were 'signal transduction' (36 genes), 'global and overview
477 maps' (presenting global and overall pictures of metabolism, 17 genes), 'cellular community' (14
478 genes), and 'endocrine and immune systems' (14 and 12 genes respectively) (Figure 8). The list of gene
479 symbols in each KEGG pathway is provided in Supplementary Table S3, as well as the information for
480 each of the 6 isogenic lines.

481

482 4. Discussion

483

484 Fish inbred/isogenic lines constitute powerful biological models to investigate the role of DNA
485 methylation in mediating environmentally induced phenotypic variation

486 Model systems like naturally clonal vertebrates (Laskowski et al., 2019) or experimental isogenic lines
487 like in this study, where genetic and epigenetic variation can be studied independently, constitute very
488 powerful tools to investigate the role of epigenetic mechanisms in generating phenotypic variation.
489 Indeed, within each line, all fish have the same genome, *i.e.* there is no genetic variability. Here, we
490 aimed to investigate how different genotypes can modulate, through epigenetic mechanisms, to a
491 greater or lesser extent, their phenotype in response to environmental stimuli. Specifically, we have
492 looked at the impact of early temperature regime (control 11°C vs. heated 16°C) on the expression of
493 *dnmt3* genes and global DNA methylation in nine rainbow trout isogenic lines, as well as finer genome-
494 wide patterns of DNA methylation in six of the lines. This allowed us to compare DNA methylation
495 patterns within a line (*i.e.* at constant genotype) between two early temperature regimes. Also,
496 performing these analyses on 6-9 rainbow trout isogenic lines made it possible to study whether the
497 establishment of these methylation marks depend on the genetic background.

498 There has been empirical evidence of the important role of epigenetic processes in generating
499 ecologically relevant phenotypic variation, using naturally inbred lines of plants such as *Arabidopsis*
500 (e.g. Bossdorf et al., 2010), clonal invertebrates such as *Daphnia* (e.g. Schield et al., 2016) or clonal
501 replicates of corals via fragmentation of coral colonies (Dixon et al., 2018). To date however, there has
502 been only a limited number of studies in fish because it is difficult to disentangle the different effects
503 due to genetic variation among individuals. In this context, the use of asexual fish vertebrate models,
504 such as the clonal fish *Chrosomus eos-neogaeus* (Cyprinidea), that reproduces asexually through
505 gynogenesis, has been pioneering as it allowed to have biological replicates without genetic variation
506 (reviewed in Angers et al., 2020). Several studies using this biological model in natural populations
507 have revealed that i) levels of DNA methylation variation were higher than genetic variation
508 (Massicotte et al., 2011); ii) both environmentally induced and stochastic modifications of DNA
509 methylation were sources of epigenetic variation (Massicotte and Angers, 2012; Leung et al., 2016); iii)
510 the relative abundance of environmentally induced and randomly established epigenetic marks was
511 correlated to the predictability of environmental conditions, both in natural sites and common garden
512 experiments (Leung et al., 2016). Another interesting biological model is the naturally self-fertilizing
513 hermaphroditic mangrove killifish *Kryptolebias marmoratus*. Two recent studies have used two highly
514 inbred lines of mangrove killifish to study the relative contributions to DNA methylation plasticity of
515 the genetic background and environment, *i.e.* temperature during embryonic development (Ellison et
516 al., 2015) or environmental enrichment (Berbel-Filho et al., 2019). Both studies have identified

517 significant methylation differences among genotypes and environments but showed that the effect of
518 the genotypes on DNA methylation plasticity is greater than that caused by environment.

519 The aforementioned studies on clonal or inbred fish have demonstrated the role of DNA
520 methylation in mediating environmentally induced phenotypic variation. However, they were
521 restricted to a single genotype (Massicotte et al., 2011) or a few ones (2 in Ellison et al., 2015 and
522 Berbel-Filho et al., 2019 or 5 in Leung et al., 2016). Therefore, our investigation using nine different
523 rainbow trout isogenic lines is powerful to study whether the establishment of the methylation marks
524 in response to an environmental stimulus depends on the genetic background. Also, working with
525 experimental populations in controlled rearing conditions allowed us to investigate clearly the role of
526 a single environmental stimulus (temperature here) on genome-wide patterns of DNA methylation.

527

528 Upregulation of *hsp47* validates exposure to a thermal stress

529 We have shown here that *hsp47* (Heat Shock Protein 47) gene expression was upregulated in response
530 to high incubation temperature (16°C) applied during embryonic development (from eyed-stage to just
531 before hatching) whatever the genetic background at the two sampling points. This is in agreement
532 with previous articles which reported that *hsp47* was induced by an increase in temperature in various
533 experimental situations (Akbarzadeh *et al.*, 2018; Rebl et al., 2013; Verleih et al., 2015; Wang et al.,
534 2016). This demonstrates that eggs were actually exposed to a thermal stress. Interestingly, our results
535 suggested that induction of *hsp47* expression was higher in resistant lines compared to intermediate
536 and sensitive lines at 22 dpf. This should be confirmed by analysing more samples per line, more lines
537 with contrasted phenotypic response to temperature and more time points in order to study the
538 dynamics of *hsp47* induction.

539

540 Modulation of *dnmt3* genes expression by high incubation temperature

541 Expression of all *dnmt3* genes has been detected which is in agreement with the expression pattern of
542 these genes during ontogenesis in rainbow trout, from oocyte to hatching (Liu et al., 2020).

543 We have investigated whether incubation temperature modulated *dnmt3* genes expression.
544 When comparing *dnmt3* genes expression between the two incubation temperatures line by line,
545 results differed between the two sampling dates, with some genes up/downregulated at a single
546 sampling date or genes shifting direction between sampling dates (downregulated at 19 dpf but
547 upregulated at 22 dpf) (Table 2, Supplementary Figure S1). This would suggest that the expression
548 patterns of *dnmt3* genes are temporally dynamic. Overall, the expression of *dnmt3* genes was only
549 moderately modulated by temperature. To our knowledge, our study is the first to look at modulation
550 of *dnmt3* genes expression by temperature in rainbow trout. Extending the analysis to other fish
551 species can allow gaining interesting insights. Campos et al. (2013) showed the impact of rearing

552 temperature on *dnmt* genes expression in Senegalese sole larvae undergoing metamorphosis, with a
553 downregulation of *dnmt1* and *dnm3b* genes at 21°C compared to 15°C but no impact on *dnmt3a* genes
554 expression. Two other studies have been performed in zebrafish which showed that *dnmt3* (2 *dnmt3a*
555 and 4 *dnmt3b*) genes expression was modified after exposure to a thermal stress (Campos et al., 2012;
556 Dorts et al., 2016). It is however difficult to compare with our work since exposure timing, duration
557 and sampling stages are different. Nevertheless, they revealed dynamic and differential changes
558 between *dnmt3* genes after exposure to a thermal stress. Specifically, Campos et al. (2012) showed
559 that expression of *dnmt3b* paralogues was more dynamic than *dnmt3a* paralogues and that *dnmt3a*
560 and *dnmt3b* paralogues exhibited a different response to temperature. This fits with results obtained
561 in Senegalese sole (Campos et al., 2013), and we could therefore expect different patterns and
562 dynamics of gene expression in response to temperature between *dnmt3a* and *dnm3b* paralogues.
563 Some of our results indeed pointed towards a partially different response to high incubation
564 temperature of *dnmt3a* and *dnm3b* paralogues: *dnmt3aa* was the only *dnmt3* gene not being impacted
565 by incubation temperature whatever the line or the sampling date. However, our experimental design
566 with only three biological replicates per condition lacked statistical power to be able to conclude about
567 a differential modulation of *dnmt3a* vs. *dnmt3b* paralogues by temperature. It would be interesting in
568 the future to finely study the dynamics of *dnmt3* genes expression in response to temperature, with
569 more biological replicates per sampling point and more time points. It would also be interesting to
570 decipher the specific roles of the different *dnmt3a* and *dnmt3b* paralogues in establishing *de novo*
571 methylation patterns in response to environmental stimuli.

572 Another interesting question is whether modulation of *dnmt3* genes expression by high
573 temperature depends on the genetic background and whether this relates to the known phenotypic
574 response of the lines to acute temperature challenges. In our study, 4 intermediate lines (A02h, A22h,
575 A36h and AB1h) did not show any modulation of *dnmt3* genes expression at any of the sampling dates;
576 the 3 resistant lines (A03h, G17h and R23h) showed some modulation only at 19 dpf; 2 lines
577 (intermediate AP2h and sensitive N38h) showed some modulation at the two sampling dates. This
578 would suggest that expression of *dnmt3* genes in response to an environmental stimulus (here
579 temperature during embryonic development) may indeed depend on the genetic background.
580 Recently, Burgerhout et al. (2017) have showed in Atlantic salmon that both genetic background and
581 embryonic temperature seemed to influence to some extent *dnmt* (*dnmt1*, *dnmt3a* and *dnmt3b*) genes
582 expression. However, only two different genetic backgrounds were compared and expression patterns
583 of a limited number of *dnmt* genes were assessed, preventing to draw general conclusions. Our
584 experimental design investigating the impact of high incubation temperature on 8 *dnmt3* (3 *dnmt3a*
585 and 5 *dnmt3b* paralogues) genes expression in 9 rainbow trout isogenic lines (i.e. 9 different
586 genotypes) is therefore quite unique, despite the low statistical power due to the low number of

587 biological replicates. Once again, in order to investigate more finely the potential role of the genetic
588 background on the modulation of *dnmt3* genes expression by temperature and the link with the known
589 contrasted phenotypic response of the lines, more samples should be analysed (e.g. more biological
590 replicates per isogenic line, individual eggs instead of pooled samples, more isogenic lines or more
591 time points).

592

593 Impact of high incubation temperature on global and genome-wide patterns of DNA methylation

594 We have chosen to analyse the impact of high incubation temperature on global and genome-wide
595 patterns of DNA methylation on the same DNA extracts at a single sampling date, 22 dpf, *i.e.* at the
596 end of the thermal treatment. When designing the experiment, we hypothesized that the impact of
597 high temperature on DNA methylation would be strongest at the end of the thermal treatment.

598 Analysis of global DNA methylation with LUMA (LUminometric Methylation Assay) revealed no
599 overall effect of incubation temperature (11°C vs 16°C) at eyed-stage. In a recent study performed on
600 rainbow trout juveniles, Defo et al. (2019) also found no impact of high temperature (23°C compared
601 to 15°C during 28 days) on global DNA methylation assessed by LUMA in brain and liver tissues. The
602 fact that there is no overall effect of temperature on global DNA methylation levels does not mean
603 that the treatment did not have any impact on DNA methylation patterns. This is in line with several
604 recent studies in zebrafish that have reported no changes in global DNA methylation levels but
605 differences in DNA methylation levels of specific gene promoters in response to high temperature
606 (Dorts et al., 2016) or environmental contaminants (e.g. Aluru et al., 2015; Bouwmeester et al., 2016).

607 However, we revealed between-lines differences in global DNA methylation, with lines A22h
608 and AB1h exhibiting lower DNA methylation levels compared to the other lines at 11°C; and AB1h and
609 AP2h at 16°C (Figure 4). Sampling of eyed-eggs was done on the same day (*i.e.* at the same number of
610 degree-days) for all the lines. Hence, we cannot rule out that the between-lines differences in global
611 DNA methylation levels could be due to the fact that the lines were not at the exact same
612 developmental stage because of differences in developmental rates. To limit this, all females spawned
613 on the same day. Differences in their genomes could also lead to different numbers of CpG between
614 the lines. It would be interesting to further investigate this point using whole genome resequencing
615 data of the different isogenic lines. Interestingly, there was also a tendency of lower DNA methylation
616 level at 16°C compared to 11°C for lines AB1h, AP2h (2 intermediate lines) and N38h (sensitive line),
617 although not significant due the low number (3) of biological replicates in each temperature condition.
618 This could suggest that different genetic backgrounds might react differently to temperature, and
619 modulate differently their epigenome.

620 Our study, which investigated the establishment of genome-wide patterns of DNA methylation
621 in 6 rainbow trout isogenic lines in response to an early temperature treatment by EpiRADseq, is novel.

622 Indeed, to our knowledge, very few papers have looked at the role of DNA methylation in mediating
623 plastic responses to environmental temperature changes. In a recent study, Metzger and Schulte
624 (2017) have reared threespine stickleback at three temperatures (cold 12°C, control 18°C or warm
625 24°C) during their embryonic development then at 18°C after hatching. After nine months of
626 development, adult stickleback from the control group were acclimated to three different
627 temperatures (5°C, 18°C or 25°C). By comparing DNA methylation patterns in muscle between the
628 experimental groups, they showed that both developmental temperature and adult acclimation
629 temperature altered DNA methylation patterns. Importantly, there was a common core response of
630 the methylome to thermal change with 50 differentially methylated regions common to all
631 experimental groups. Therefore, they concluded that epigenetic mechanisms (DNA methylation) are a
632 component of both persistent and plastic responses to environmental change. It is important to note
633 that experimental fish were composed of six families but that the effect of the genetic background was
634 not taken into account. The strength of our experimental design is that it can account for this effect on
635 the establishment of DNA methylation marks in response to temperature during embryonic
636 development. Also, as most isogenic lines were previously tested for their response to acute thermal
637 challenges (Dupont-Nivet et al., 2015), we could analyse the variations of DNA methylation patterns in
638 light of the known phenotypic response (resistance or sensitivity) of the lines to acute temperature
639 challenges.

640 Our study identified 19 to 155 differentially methylated loci depending on the line between
641 the control (11°C) and high temperature (16°C) groups, thus demonstrating that the thermal history
642 during embryonic development can alter patterns of DNA methylation. It would be interesting to test
643 the impact of a longer exposure or exposure to higher temperatures during embryonic development
644 on the establishment of DNA methylation patterns. Also here, the analysis was performed at the end
645 of the thermal treatment. As a future development, it would be interesting to analyse DNA methylation
646 patterns a few months after the end of the thermal treatment, in order to investigate the persistence
647 in time of the observed changes and the impact on the response to acute temperature challenges. For
648 comparison purposes, Metzger and Schulte (2017) identified 480 differentially methylated cytosines
649 in 10-month old sticklebacks that developed at 24°C then were reared at 18°C after hatching,
650 compared to the control group (18°C).

651

652 Effect of genetic background in the patterns of DNA methylation established in response to high 653 incubation temperature

654 Another interesting result is that the great majority of the observed changes in methylation seemed
655 to be dependent on the genetic background. This was exemplified by between-lines differences in the
656 number of differentially methylated loci, ranging from 19 (N38h) to 155 (AB1h) (Figure 7). As for the

657 LUMA results, we cannot rule out that these between-lines differences could be due to the fact that
658 the lines were not at the exact same developmental stage because of differences in developmental
659 rates. No clear link could be established between the observed plasticity of DNA methylation and the
660 known phenotypic response of the lines to acute temperature challenge. Indeed, N38h is the most
661 sensitive line to temperature challenge, A03h, G17h and R23h being resistant, AB1h and AP2h being
662 intermediate (Dupont-Nivet et al., 2015). The fact that N38h, the most sensitive line, exhibited the
663 lowest number (19) of differentially methylated loci could suggest that DNA methylation plasticity
664 plays a role in the phenotypic response to temperature. However, the most resistant line A03h only
665 showed a marginally higher number of differentially methylated loci (29) compared to N38h, while the
666 other two resistant lines G17h and R23h as well as the two intermediate lines exhibited a much higher
667 number of differentially methylated loci (Figure 7). Once again, it would be interesting to analyse DNA
668 methylation patterns at the juvenile stage (on 4-5 month old fish), i.e. at the time acute thermal
669 challenges are usually performed, in order to investigate more finely the role of DNA methylation in
670 the contrasted phenotypic response to temperature of the different isogenic lines.

671 Furthermore, the vast majority of differentially methylated loci (375 out of 409, or 91.7%) were
672 unique to one of the six lines, while 34 loci were shared between at least two lines (Figure 7). These
673 results were consistent with recent studies performed on two inbred lines of mangrove killifish (Ellison
674 et al., 2015; Berbel-Filho et al., 2019). Interestingly, Berbel-Filho et al. (2019) also classified their
675 differentially methylated cytosines or regions into the three classes of epigenetic variation defined by
676 Richards (2006) based on the degree of autonomy from the underlying genotype: obligatory epialleles
677 (completely dependent), facilitated (partially dependent) or pure epialleles (independent). They found
678 only a few differentially methylated cytosines that could be considered facilitated or pure epialleles,
679 suggesting a strong influence of the genotype on DNA methylation variation in response to
680 environmental change. Similarly to Berbel-Filho et al. (2019), we have classified the 34 differentially
681 methylated loci shared between at least 2 lines as facilitated (*i.e.* displaying different directions of
682 variation across the lines) for 8 of them, or pure (*i.e.* displaying the same direction of variation) for 26
683 of them (Supplementary Table S4).

684

685 Functional analysis of the differentially methylated EpiRADseq loci: potential KEGG pathways involved 686 in the response to high incubation temperature

687 Due to the limited number of genes found in the differentially methylated loci, functional analysis was
688 restricted to a descriptive analysis and was not performed line by line. Indeed, the 385 differentially
689 methylated loci located on the 29 rainbow trout chromosomes overlapped with 268 genes. In order to
690 investigate the functions of these genes, pathway classification functional analysis was performed
691 using KEGG. It might be more insightful to perform functional analysis line by line as several lines could

692 respond differently to temperature; however this was prevented by the limited number of impacted
693 genes (13 to 102 depending on the line; Supplementary Table S3). This global analysis revealed that
694 the most represented second-level pathways were 'environmental information processing' (signal
695 transduction), 'metabolism' (global and overview maps), 'cellular processes' (cellular community), and
696 'organismal systems' (endocrine and immune systems) (Figure 8). As there is no previous study on the
697 response of the methylome to high temperature during embryonic development in rainbow trout, it
698 seemed relevant to compare the identified pathways with those highlighted by transcriptomic studies.
699 Indeed, a limitation of this study is that we have focused on changes in DNA methylation patterns
700 without investigating concomitantly the changes in gene expression on the same samples. The higher
701 proportion of differentially methylated loci identified upstream genes (8.4% compared to 1.8%; Figure
702 6) suggest that these changes in DNA methylation might occur in promoters and hence regulate
703 expression of these genes. This would have given interesting insights into the correlated responses of
704 the transcriptome and methylome. To date, several studies have revealed transcriptomic changes in
705 response to thermal stress in several tissues of rainbow trout. In these studies, the thermal stress was
706 not applied during embryonic development but on juvenile or adult fish. It also varied greatly both in
707 duration (from 30 min to 4 weeks) and temperature range (from 18°C to 26°C compared to control
708 temperature of 10 to 18°C). The different experimental designs between these transcriptomic studies
709 and our study, in terms of timing and duration of the thermal stress applied, as well as in the type of
710 samples (embryos vs tissues) analysed, make these comparisons tentative. However, KEGG pathway
711 analysis and/or GO enrichment of transcriptomic data (microarray or RNAseq) revealed several
712 pathways influenced by heat stress in rainbow trout: stress response with heat shock proteins in all
713 studies, immune response (Lewis et al., 2010; Rebl et al., 2013; Verleih et al., 2015; Li et al., 2017;
714 Huang et al., 2018; Defo et al., 2019), apoptosis (Lewis et al., 2010; Verleih et al., 2015; Defo et al.,
715 2019), metabolism (Vornanen et al., 2005; Verleih et al., 2015; Li et al., 2017; Defo et al., 2019), cell
716 structure (Lewis et al., 2010; Rebl et al., 2013; Verleih et al., 2015), cell transport (Verleih et al., 2015),
717 protein processing (Lewis et al., 2010; Li et al., 2017; Huang et al., 2018), or post-transcriptional
718 regulation of spliceosome (Huang et al., 2018). Endocrine, metabolic and immunological pathways
719 were also shown to be regulated by high temperature in another salmonid species, maraena whitefish
720 (Rebl et al., 2018). Therefore, it is possible to say that our results suggesting the potential modulation
721 of genes belonging to metabolism, endocrine and immune systems pathways were consistent with
722 transcriptomic studies.

723

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735

736 **6. Disclosure of interest**

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738 The authors report no conflict of interest.

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740 **7. References**

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987 **Table 1.** Primers used for gene expression analysis of *hsp47* and *dnmt3* by qPCR. E: qPCR efficiency.

Gene	Accession		Primers (5'-3')	Size (bp)	E (%)	Reference
<i>hsp47</i>	NM_001124234.1	F	CAGTCAACAGACGAGCGAAC	70	106	Ojima et al., 2005
		R	CCAGGAGGCACAGAACTACA			
<i>dnmt3aa</i>	XM_021614345.1	F	AATTTGAGGCAGCCAGGTTG	177	102	Liu et al., 2020
		R	CGATCCCCACGGTGATAGAA			
<i>dnmt3ab1</i>	XM_021585300.1	F	TGGCACCAGAAGAGAAATCCT	200	107	Liu et al., 2020
		R	CATGACTCCATTCTGCACCTG			
<i>dnmt3ab2</i>	XM_021573815.1	F	GTGTGCGAGGACTCCGTC	168	111	Liu et al., 2020
		R	CCTAGCCGGTTGACAATAGAG			
<i>dnmt3ba1</i>	XM_021566506.1	F	CAAGGGTTTTGGCATTGGAGA	156	124	Liu et al., 2020
		R	ACCTCAGAGAACTTGCCATCA			
<i>dnmt3ba2</i>	XM_021615717.1	F	ACAAGGGTTTTGGTATTGGGGA	164	105	Liu et al., 2020
		R	AGCAGACACCTCAGAGAACTT			
<i>dnmt3bba1</i>	XM_021567210.1	F	GGCGATGGAACCTTTGACAAG	185	112	Liu et al., 2020
		R	TGGGCAGGAATGGAGGGATA			
<i>dnmt3bba2</i>	XM_021616487.1	F	GGCGATGGAACCTTTAAGCAA	145	115	Liu et al., 2020
		R	CGAGGGCACTGTTGTTGATG			
<i>dnmt3bbb</i>	XM_021566510.1	F	AGGACCATCACCACCAACC	166	118	Liu et al., 2020
		R	TCTGCTGGCGATTCATGTTCT			
<i>ef1α</i>	AF498320.1	F	GGCAAGAACTTGAGGATGC	149	114	Makesh et al., 2015
		R	ACAGTCTGCCTCATGTCACG			
<i>rps20</i>	NM_001124364.1	F	AGCCGCAATGTCAAGTCTCT	93	111	Pasquier et al., 2016
		R	CATACGGACTGGACCCTTCA			
<i>rcl1</i>	NM_001160621.1	F	GAACGGGACGTTCTTAGTG	96	106	Pasquier et al., 2016
		R	AGCTTGGCACAGTTTCTTCC			
<i>β-actin</i>	AJ438158.1	F	GGTGGTACGGCCAGAGGC	101	107	Johnson et al., 2004
		R	GGGAGAAGATGACCCAGATCATG			
<i>arp</i>	XM_021568901.1	F	CTCTGTCCCTCACACCATCA	196	111	Pasquier et al., 2016
		R	CTCCTCCTTGGCCTTCTT			

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991 **Table 2.** Gene expression of 8 *dnmt3* genes at 16°C, expressed as fold-change (FC) relative to the
 992 control 11°C, performed line by line. Status of the lines: R for resistant, I for intermediate and S for
 993 sensitive; dpf: days post fertilization; UP: gene being upregulated at 16°C compared to 11°C; DOWN:
 994 gene being downregulated at 16°C compared to 11°C. Pairwise fixed reallocation randomisation test
 995 was carried out: n.s. not significant; * p<0.05; ** p<0.01; *** p<0.001; n/a: not available for line R23h
 996 at 22 dpf due to technical problems during RNA extraction, meaning that the samples were lost. See
 997 Supplementary Figure S1 for visualisation of fold changes for each *dnmt3* gene.

Line	19 dpf		22 dpf	
	Gene UP	Gene DOWN	Gene UP	Gene DOWN
A02h (I)	n.s.	n.s.	n.s.	n.s.
A03h (R)	n.s.	<i>dnmt3bba1</i> (FC=0.681*)	n.s.	n.s.
A22h (I)	n.s.	n.s.	n.s.	n.s.
A36h	n.s.	n.s.	n.s.	n.s.
AB1h (I)	n.s.	n.s.	n.s.	n.s.
AP2h (I)	<i>dnmt3ab1</i> (FC=1.154*)	<i>dnmt3bba1</i> (FC=0.793*)	n.s.	<i>dnmt3ab2</i> (FC=0.621***) <i>dnmt3ba2</i> (FC=0.638***) <i>dnmt3bba2</i> (FC=0.735***)
G17h (R)	n.s.	<i>dnmt3bbb</i> (FC=0.688*)	n.s.	n.s.
N38h (S)	n.s.	<i>dnmt3ab1</i> (FC=0.507*) <i>dnmt3ba2</i> (FC=0.773*) <i>dnmt3bba2</i> (FC=0.808*)	<i>dnmt3ab1</i> (FC=6.551*) <i>dnmt3ab2</i> (FC=5.532***) <i>dnmt3ba2</i> (FC=1.876*) <i>dnmt3bbb</i> (FC=2.688***)	n.s.
R23h (R)	<i>dnmt3ba1</i> (FC=4.344*)	<i>dnmt3ba2</i> (FC=0.680*) <i>dnmt3bba1</i> (FC=0.674*) <i>dnmt3bbb</i> (FC=0.498*)	n/a	n/a

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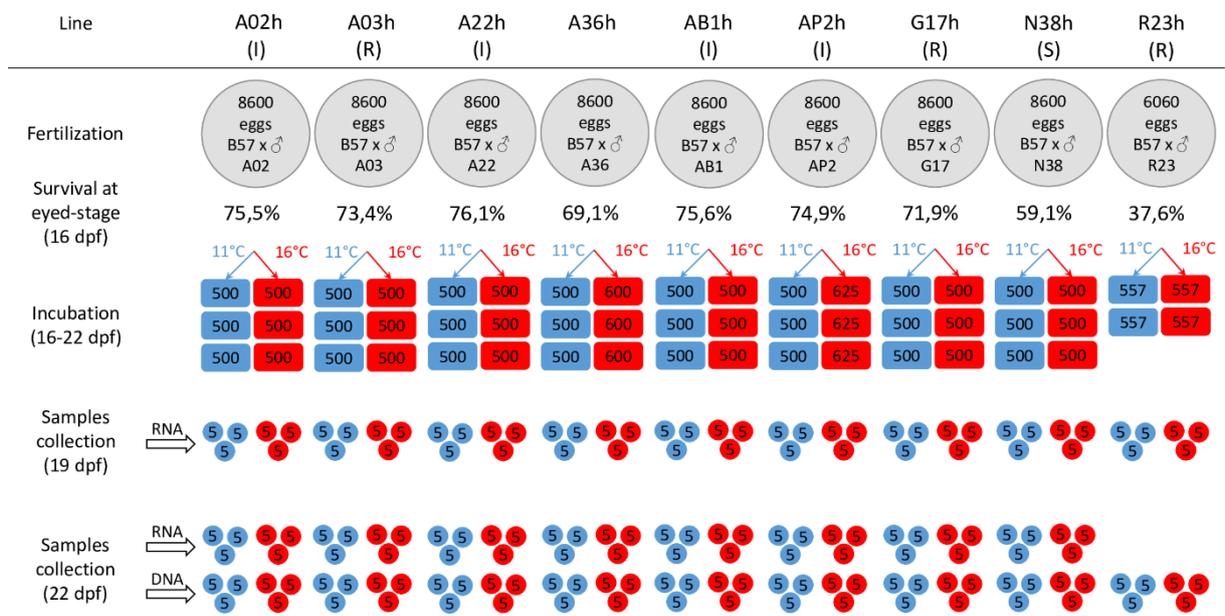
1000 **Table 3.** Number of filtered EpiRADseq loci per line. ¹Number of EpiRADseq loci kept after PM and PH
1001 filters; see Figure 2 for the overall EpiRADseq analysis strategy. ²Number of EpiRADseq loci kept after
1002 filtering on read abundance (counts per million > 1 in at least 3 of 6 samples considered).

Line	¹ No. after PM and PH filters	² No. after filtering on read abundance
A03h	83,494	55,607 (66.6%)
AB1h	78,593	53,987 (68.7%)
AP2h	82,566	54,213 (65.7%)
G17h	79,479	53,609 (67.4%)
N38h	78,256	52,577 (67.2%)
R23h	72,263	51,668 (71.5%)

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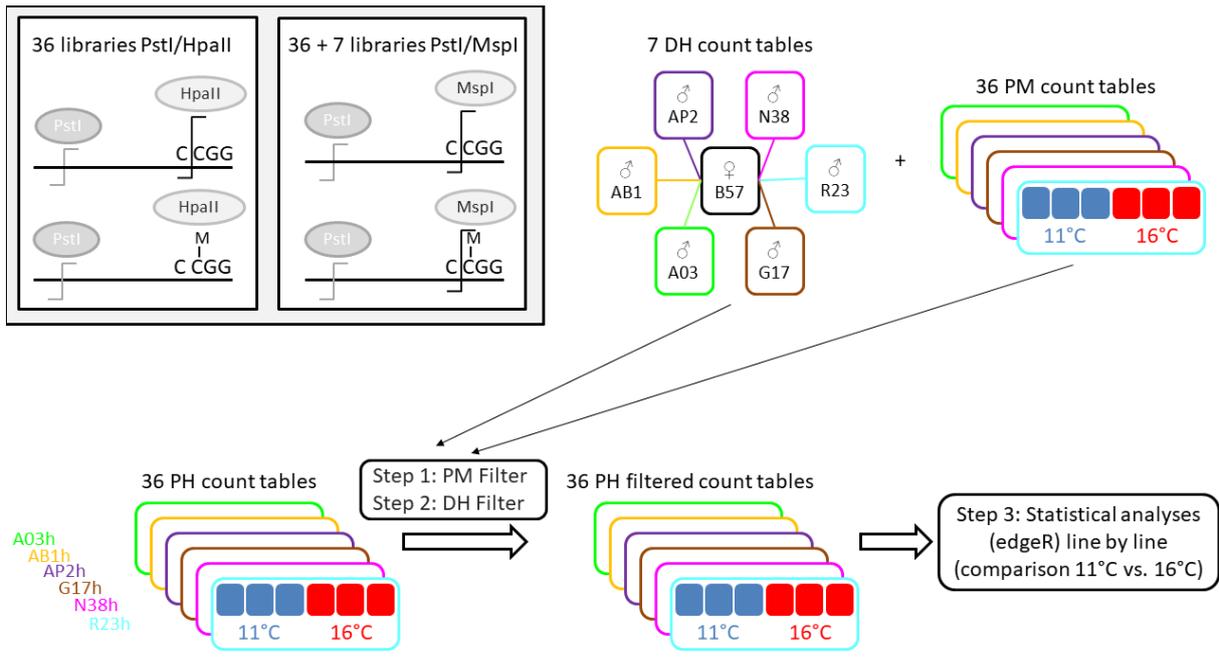
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Figure 1



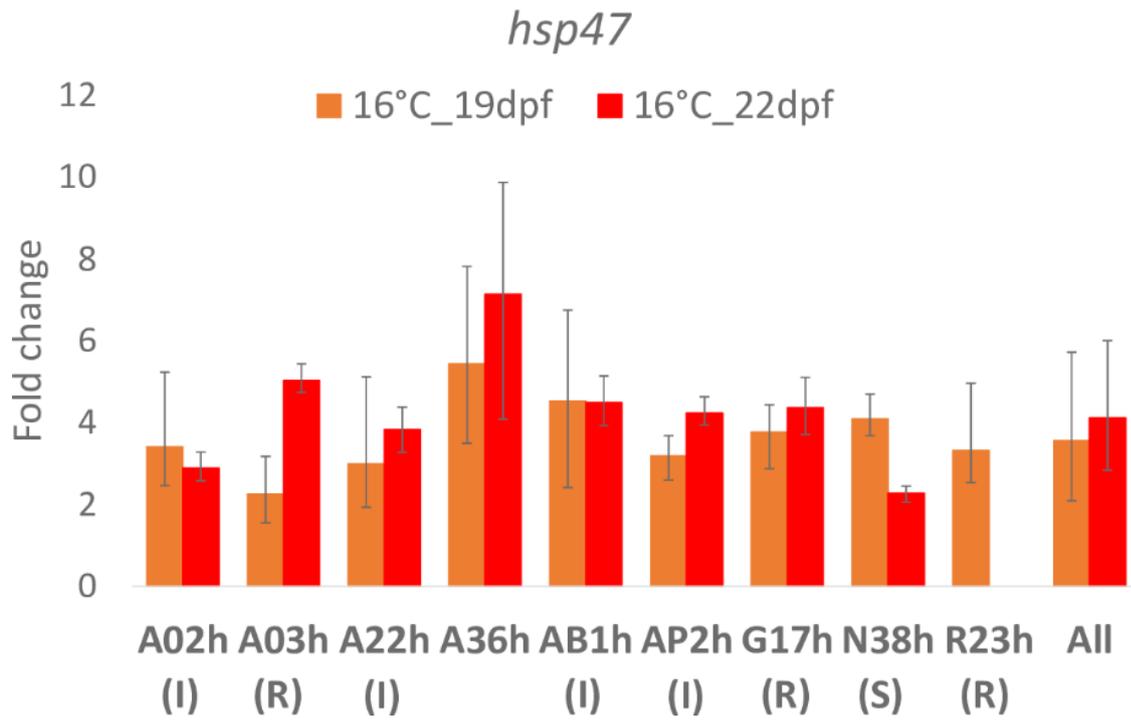
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Figure 2

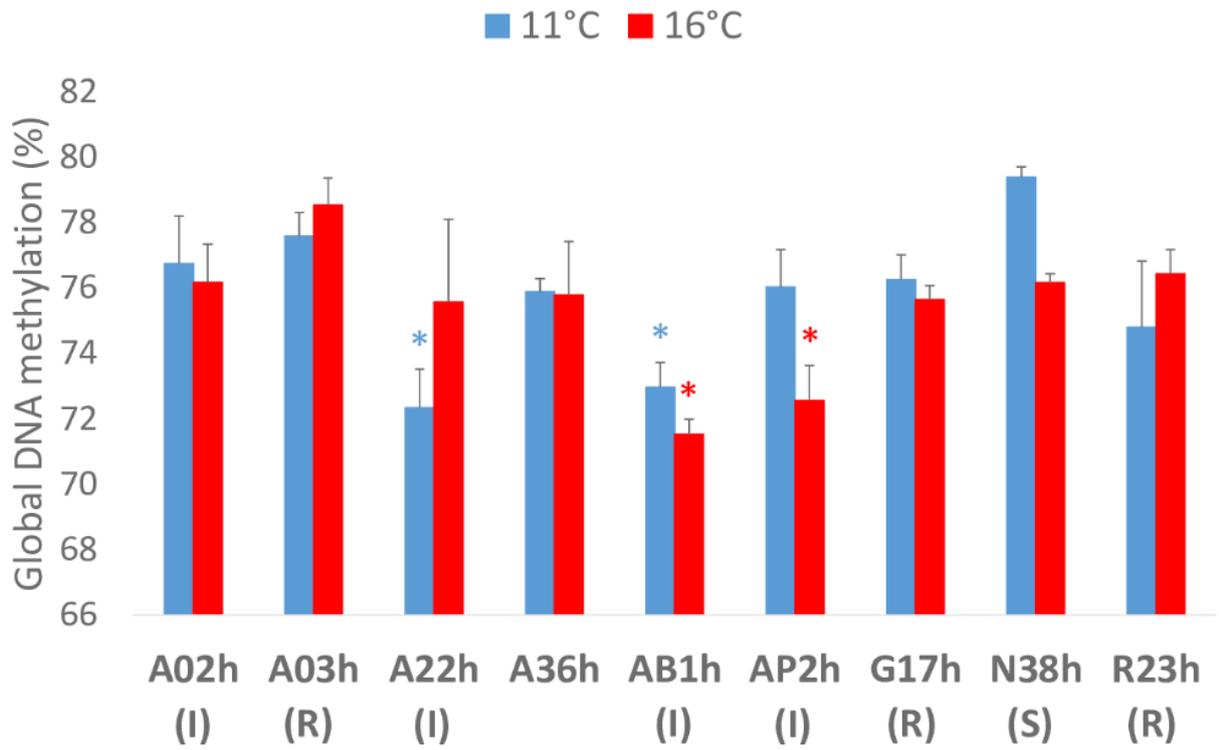


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Figure 3



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Figure 4

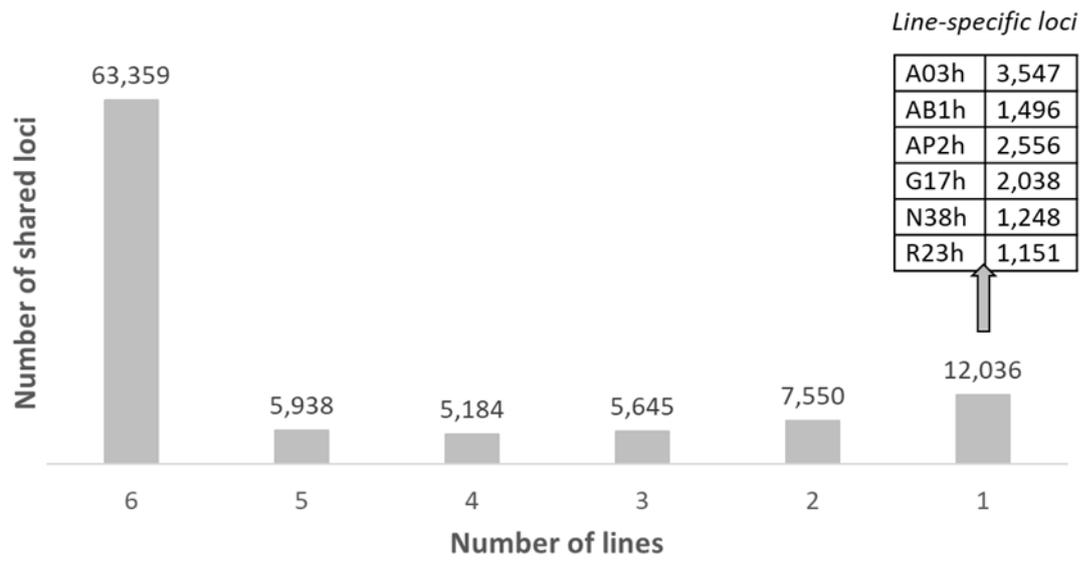
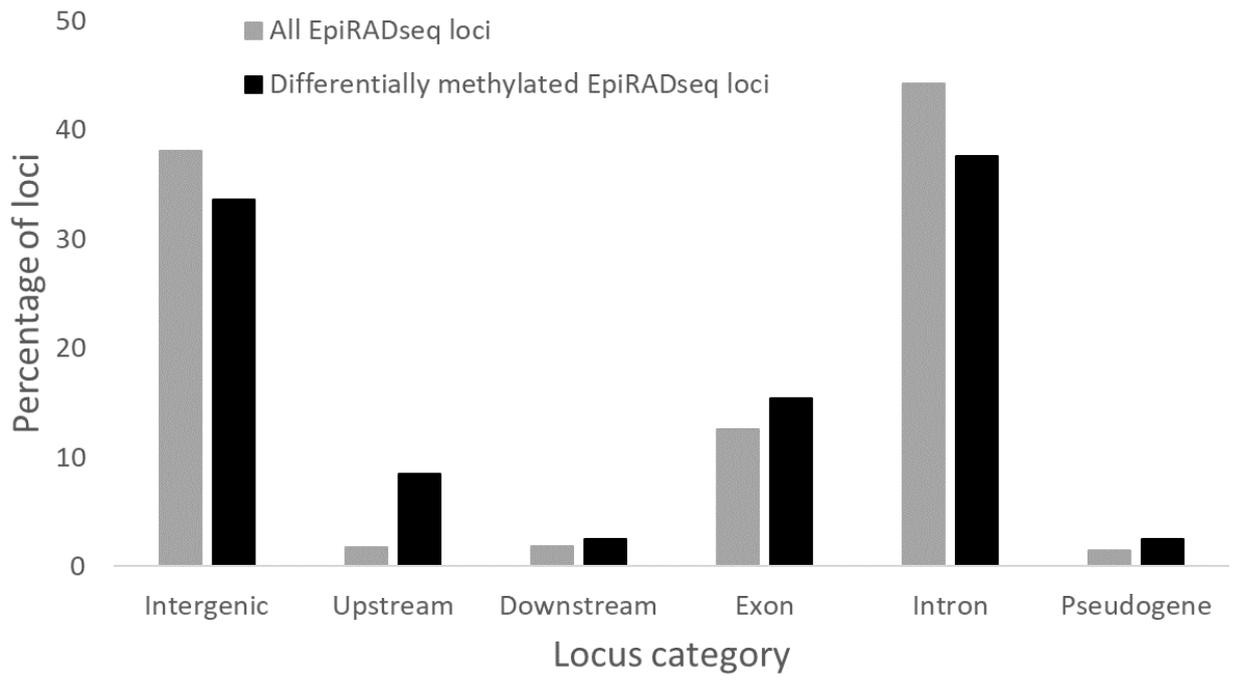


Figure 5

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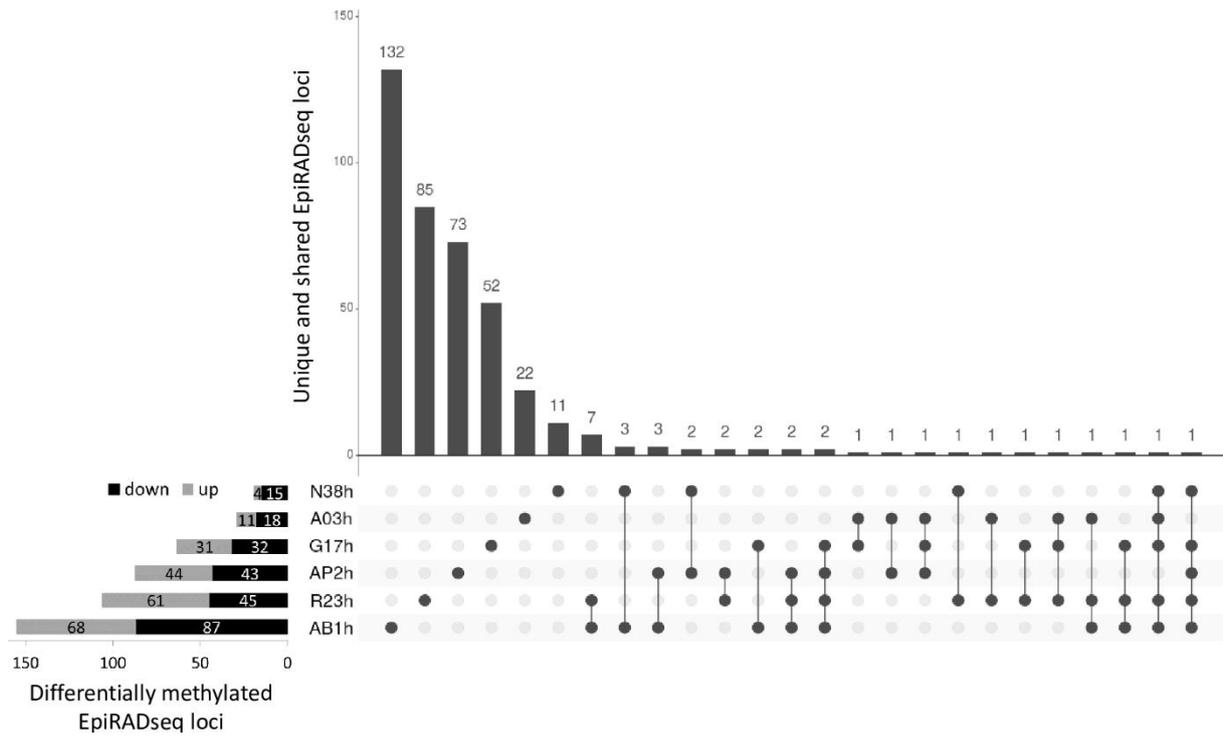


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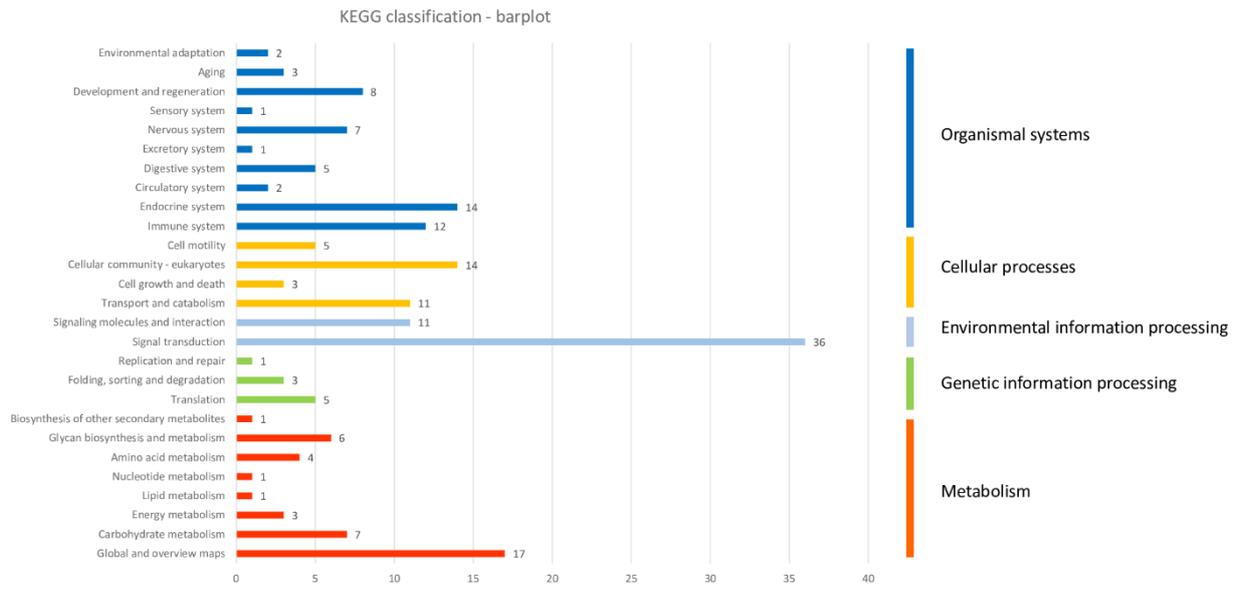
Figure 6



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Figure 7

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Figure 8

Figure captions

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1041 Figure 1. Experimental design and samples collection. With the exception of A36h, all lines have been
1042 previously tested for their response to acute thermal challenges (Dupont-Nivet et al., 2015) and their
1043 status is mentioned: R for resistant, I for intermediate and S for sensitive. dpf: days post fertilization.

1044

1045 Figure 2. Analysis strategy of EpiRADseq data. DH and PM count tables are the results of PstI/MspI
1046 sequence libraries, PH count tables are the results of the PstI/HpaII sequence libraries. Filters were
1047 applied per line on PstI/HpaII EpiRADseq loci count tables. PM filter: loci with positive counts for at
1048 least 3 out of 6 PstI/MspI libraries were kept. DH filter: loci with positive counts in at least one of the
1049 two doubled haploid parents PM libraries.

1050

1051 Figure 3. Comparison of *hsp47* gene transcription at 16°C in 9 rainbow trout isogenic lines, as
1052 expressed as fold-change relative to control 11°C and expressed as mean \pm SE. Status of the lines: R
1053 for resistant, I for intermediate and S for sensitive. 19 dpf (days post fertilization): three days after
1054 the beginning of the temperature treatment; 22 dpf: at the end of the temperature treatment. Data
1055 are not available for line R23h at 22 dpf due to technical problems during RNA extraction, meaning
1056 that the samples were lost.

1057

1058 Figure 4. Global DNA methylation assessed at eyed-stage (22 dpf) by LUMA (LUminometric
1059 Methylation Assay) in 9 rainbow trout isogenic lines at two incubation temperatures, 11°C vs. 16°C,
1060 expressed as mean \pm SE. Status of the lines: R for resistant, I for intermediate and S for sensitive. Two-
1061 sample Fisher-Pitman permutation tests performed line by line revealed no effect of incubation
1062 temperature on global DNA methylation levels. For each incubation temperature, K-sample Fisher-
1063 Pitman permutation tests were performed, followed by non-parametric testing of Tukey-type multiple
1064 comparisons: * indicate the lines with significantly ($p < 0.05$) lower global DNA methylation level
1065 compared to the other lines.

1066

1067 Figure 5. Number of EpiRADseq loci shared between 2 to 6 rainbow trout isogenic lines, and number
1068 of line-specific EpiRADseq loci.

1069

1070 Figure 6. Percentages of EpiRADseq loci that mapped to various categories of annotated genomic
1071 regions of the rainbow trout genome (Omyk_1.0). Upstream: within 1 kb upstream of annotated genic
1072 regions. Downstream: within 1 kb downstream of annotated genic regions.

1073

1074 Figure 7. Number of unique and shared differentially methylated loci between the two temperature
1075 conditions (11°C vs. 16°C) for 6 rainbow trout isogenic lines, using UpSetR package (Conway et al.,
1076 2017). Down: number of loci that were less methylated at 16°C compared to 11°C; Up: number of loci
1077 that were more methylated at 16°C compared to 11°C.

1078

1079 Figure 8. Descriptive KEGG pathway classification bar plot obtained using the genes found in the 385
1080 differentially methylated EpiRADseq loci. The horizontal bars represent the absolute number of genes
1081 found in second-level KEGG pathways, grouped in first-level KEGG pathways using a colour code. The
1082 vertical bars on the right indicate the names of first-level pathways.

1083

Supplemental material

1084

1085

1086 **Supplementary Figure S1.** Comparison of *dnmt3* gene transcription at 16°C in 9 rainbow trout isogenic
1087 lines, as expressed as fold-change relative to control 11°C and expressed as mean \pm SE. 19 dpf (days
1088 post fertilization): three days after the beginning of the temperature treatment; 22 dpf: at the end of
1089 the temperature treatment. Data are not available for line R23h at 22 dpf due to technical problems
1090 during RNA extraction, meaning that the samples were lost. Black stars indicate upregulation at 16°C
1091 compared to 11°C; grey stars indicate downregulation.

1092

1093 **Supplementary Figure S2.** Distribution of EpiRADseq loci, by Mb windows, throughout the rainbow
1094 trout genome.

1095

1096 **Supplementary Table S1.** Rainbow trout genes and their associated gene symbols.

1097

1098 **Supplementary Table S2.** Version of databases used for functional analysis.

1099

1100 **Supplementary Table S3.** Genomic locations of 385 differentially methylated EpiRADseq loci and their
1101 associated gene symbols, KO codes and KEGG pathways. KO codes were retrieved after modifying gene
1102 symbols whenever needed using the information available from GeneCards
1103 (<https://www.genecards.org/>) and after conversion using the 'db2db' tool from the bioDBnet suite.

1104

1105 **Supplementary Table S4.** Genomic locations of 34 EpiRADseq loci that are differentially methylated in
1106 a least two rainbow trout isogenic lines. Classification into pure or facilitated epiallele is given
1107 according to Richards (2006).

1108