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

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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/ $\mu$ 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](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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725

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735

## 736 **6. Disclosure of interest**

737

738 The authors report no conflict of interest.

739

## 740 **7. References**

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742 Akbarzadeh, A., Günther, O.P., Houde, A.L., Li, S., Ming, T.J., Jeffries, K.M., Hinch, S.G., Miller, K.M.,  
743 2018. Developing specific molecular biomarkers for thermal stress in salmonids. BMC Genomics  
744 19, 749. <https://doi.org/10.1186/s12864-018-5108-9>

745 Albokhadaim, I., Hammond, C.L., Ashton, C., Simbi, B.H., Bayol, S., Farrington, S., Stickland, N., 2007.  
746 Larval programming of post-hatch muscle growth and activity in Atlantic salmon (*Salmo salar*). J.  
747 Exp. Biol. 210, 1735–1741. <https://doi.org/10.1242/jeb.003194>

748 Aluru, N., Kuo, E., Helfrich, L.W., Karchner, S.I., Linney, E.A., Pais, J.E., Franks, D.G., 2015.  
749 Developmental exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin alters DNA methyltransferase  
750 (dnmt) expression in zebrafish (*Danio rerio*). Toxicol. Appl. Pharmacol. 284, 142–151.  
751 <https://doi.org/10.1016/j.taap.2015.02.016>

752 Angers, B., Castonguay, E., Massicotte, R., 2010. Environmentally induced phenotypes and DNA  
753 methylation: how to deal with unpredictable conditions until the next generation and after.  
754 Mol. Ecol. 19, 1283–1295. <https://doi.org/10.1111/j.1365-294X.2010.04580.x>

755 Angers, B., Perez, M., Menicucci, T., Leung, C., 2020. Sources of epigenetic variation and their  
756 applications in natural populations. Evol. Appl. eia.12946. <https://doi.org/10.1111/eva.12946>

757 Baeverfjord, G., 2003. Optimal incubation temperatures for rainbow trout (*Onchorhynchus mykiss*)  
758 eggs. Beyond monoculture, Abstr. Aquac. Eur. 2003, Trondheim 33, 119–120.

759 Bardou, P., Mariette, J., Escudié, F., Djemiel, C., Klopp, C., 2014. jvenn: an interactive Venn diagram  
760 viewer. BMC Bioinformatics 15, 293. <https://doi.org/10.1186/1471-2105-15-293>

761 Berbel-Filho, W.M., Rodríguez-Barreto, D., Berry, N., Garcia De Leaniz, C., Consuegra, S., 2019.  
762 Contrasting DNA methylation responses of inbred fish lines to different rearing environments.  
763 *Epigenetics* 14, 939–948. <https://doi.org/10.1080/15592294.2019.1625674>

764 Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C.,  
765 Labadie, K., Alberti, A., Aury, J.-M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C.,  
766 Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M., Quillet, E., Guyomard, R., Galiana, D.,  
767 Bobe, J., Volff, J.-N., Genêt, C., Wincker, P., Jaillon, O., Roest Crolius, H., Guiguen, Y., 2014. The  
768 rainbow trout genome provides novel insights into evolution after whole-genome duplication in  
769 vertebrates. *Nat. Commun.* 5, 3657. <https://doi.org/10.1038/ncomms4657>

770 Best, C., Ikert, H., Kostyniuk, D.J., Craig, P.M., Navarro-Martin, L., Marandel, L., Mennigen, J.A., 2018.  
771 *Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. Comp.*  
772 *Biochem. Physiol. Part B Biochem. Mol. Biol.* 224, 210–244.  
773 <https://doi.org/10.1016/j.cbpb.2018.01.006>

774 Billard, R., 1992. Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis,  
775 biology and preservation of gametes. *Aquaculture* 100, 263–298. [https://doi.org/10.1016/0044-](https://doi.org/10.1016/0044-8486(92)90385-X)  
776 [8486\(92\)90385-X](https://doi.org/10.1016/0044-8486(92)90385-X)

777 Bossdorf, O., Arcuri, D., Richards, C.L., Pigliucci, M., 2010. Experimental alteration of DNA  
778 methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis*  
779 *thaliana*. *Evol. Ecol.* 24, 541–553. <https://doi.org/10.1007/s10682-010-9372-7>

780 Bouwmeester, M.C., Ruiter, S., Lommelaars, T., Sippel, J., Hodemaekers, H.M., van den Brandhof, E.J.,  
781 Pennings, J.L.A., Kamstra, J.H., Jelinek, J., Issa, J.P.J., Legler, J., van der Ven, L.T.M., 2016.  
782 Zebrafish embryos as a screen for DNA methylation modifications after compound exposure.  
783 *Toxicol. Appl. Pharmacol.* 291, 84–96. <https://doi.org/10.1016/j.taap.2015.12.012>

784 Burgerhout, E., Mommens, M., Johnsen, H., Aunsmo, A., Santi, N., Andersen, O., 2017. Genetic  
785 background and embryonic temperature affect DNA methylation and expression of myogenin  
786 and muscle development in Atlantic salmon (*Salmo salar*). *PLoS One* 12, e0179918.  
787 <https://doi.org/10.1371/journal.pone.0179918>

788 Campos, C., Valente, L.M.P., Conceição, L.E.C., Engrola, S., Fernandes, J.M.O., 2013. Temperature  
789 affects methylation of the myogenin putative promoter, its expression and muscle cellularity in  
790 Senegalese sole larvae. *Epigenetics* 8, 389–397. <https://doi.org/10.4161/epi.24178>

791 Campos, C., Valente, L.M.P., Fernandes, J.M.O., 2012. Molecular evolution of zebrafish *dnmt3* genes  
792 and thermal plasticity of their expression during embryonic development. *Gene* 500, 93–100.  
793 <https://doi.org/10.1016/j.gene.2012.03.041>

794 Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A., Cresko, W.A., 2013. Stacks: An analysis tool set  
795 for population genomics. *Mol. Ecol.* 22, 3124–3140. <https://doi.org/10.1111/mec.12354>

796 Conway, J.R., Lex, A., Gehlenborg, N., 2017. UpSetR: An R package for the visualization of intersecting  
797 sets and their properties. *Bioinformatics* 33, 2938–2940.  
798 <https://doi.org/10.1093/bioinformatics/btx364>

799 Couto, A., Enes, P., Peres, H., Oliva-Teles, A., 2008. Effect of water temperature and dietary starch on  
800 growth and metabolic utilization of diets in gilthead sea bream (*Sparus aurata*) juveniles. *Comp.*  
801 *Biochem. Physiol. Part A* 151, 45–50. <https://doi.org/10.1016/j.cbpa.2008.05.013>

802 Defo, M.A., Gendron, A.D., Head, J., Pilote, M., Turcotte, P., Marcogliese, D.J., Houde, M., 2019.  
803 Cumulative effects of cadmium and natural stressors (temperature and parasite infection) on  
804 molecular and biochemical responses of juvenile rainbow trout. *Aquat. Toxicol.* 217, 105347.  
805 <https://doi.org/10.1016/j.aquatox.2019.105347>

806 Dixon, G., Liao, Y., Bay, L.K., Matz, M. V., 2018. Role of gene body methylation in acclimatization and  
807 adaptation in a basal metazoan. *Proc. Natl. Acad. Sci. U. S. A.* 115, 13342–13346.  
808 <https://doi.org/10.1073/pnas.1813749115>

809 Dorts, J., Falisse, E., Schoofs, E., Flamion, E., Kestemont, P., Silvestre, F., 2016. DNA  
810 methyltransferases and stress-related genes expression in zebrafish larvae after exposure to  
811 heat and copper during reprogramming of DNA methylation. *Sci. Rep.* 6, 34254.  
812 <https://doi.org/10.1038/srep34254>

813 Dupont-Nivet, M., Crusot, M., Rigaudeau, D., Labbé, L., Quillet, E., 2015. Genetics of thermotolerance  
814 in rainbow trout, *Oncorhynchus mykiss*, in: 12th International Symposium for Genetics in  
815 Aquaculture, Santiago de Compostela, Spain, 22-27th June 2015.

816 Ellison, A., Rodríguez López, C.M., Moran, P., Breen, J., Swain, M., Megias, M., Hegarty, M.,  
817 Wilkinson, M., Pawluk, R., Consuegra, S., 2015. Epigenetic regulation of sex ratios may explain  
818 natural variation in self-fertilization rates. *Proc. R. Soc. B Biol. Sci.* 282, 20151900.  
819 <https://doi.org/10.1098/rspb.2015.1900>

820 Garcia de la serrana, D., Vieira, V.L.A., Andree, K.B., Darias, M., Estévez, A., Gisbert, E., Johnston, I.A.,  
821 2012. Development Temperature Has Persistent Effects on Muscle Growth Responses in  
822 Gilthead Sea Bream. *PLoS One* 7, e51884. <https://doi.org/10.1371/journal.pone.0051884>

823 Goldberg, A.D., Allis, C.D., Bernstein, E., 2007. Epigenetics: A Landscape Takes Shape. *Cell* 128, 635–  
824 638. <https://doi.org/10.1016/j.cell.2007.02.006>

825 Huang, J., Li, Y., Liu, Z., Kang, Y., Wang, J., 2018. Transcriptomic responses to heat stress in rainbow  
826 trout *Oncorhynchus mykiss* head kidney. *Fish Shellfish Immunol.* 82, 32–40.  
827 <https://doi.org/10.1016/j.fsi.2018.08.002>

828 Johnson, M.C., Sangrador-Vegas, A., Smith, T.J., Cairns, M.T., 2004. Molecular cloning and expression  
829 analysis of rainbow trout (*Oncorhynchus mykiss*) matrix metalloproteinase-9. *Fish Shellfish*  
830 *Immunol.* 17, 499–503. <https://doi.org/10.1016/j.fsi.2004.05.005>

831 Johnston, I.A., 2006. Environment and plasticity of myogenesis in teleost fish. *J. Exp. Biol.* 209, 2249–  
832 2264. <https://doi.org/10.1242/jeb.02153>

833 Johnston, I.A., Lee, H.-T., Macqueen, D.J., Paranthaman, K., Kawashima, C., Anwar, A., Kinghorn, J.R.,  
834 Dalmay, T., 2009. Embryonic temperature affects muscle fibre recruitment in adult zebrafish:  
835 genome-wide changes in gene and microRNA expression associated with the transition from  
836 hyperplastic to hypertrophic growth phenotypes. *J. Exp. Biol.* 212, 1781–1793.  
837 <https://doi.org/10.1242/jeb.029918>

838 Jonsson, B., Jonsson, N., 2019. Phenotypic plasticity and epigenetics of fish: Embryo temperature  
839 affects later-developing life-history traits. *Aquat. Biol.* 28, 21–32.  
840 <https://doi.org/10.3354/ab00707>

841 Karimi, M., Johansson, S., Ekström, T.J., 2006. Using LUMA: a Luminometric-based assay for global  
842 DNA-methylation. *Epigenetics* 1, 45–48. <https://doi.org/10.4161/epi.1.1.2587>

843 Karimi, M., Johansson, S., Stach, D., Corcoran, M., Grandér, D., Schalling, M., Bakalkin, G., Lyko, F.,  
844 Larsson, C., Ekström, T.J., 2006. LUMA (LUMinometric Methylation Assay)--a high throughput  
845 method to the analysis of genomic DNA methylation. *Exp. Cell Res.* 312, 1989–1995.  
846 <https://doi.org/10.1016/j.yexcr.2006.03.006>

847 Konietzke, F., Placzek, M., Schaarschmidt, F., Hothorn, L.A., 2015. nparcomp : An R Software  
848 Package for Nonparametric Multiple Comparisons and Simultaneous Confidence Intervals. *J.*  
849 *Stat. Softw.* 64. <https://doi.org/10.18637/jss.v064.i09>

850 Laskowski, K.L., Doran, C., Bierbach, D., Krause, J., Wolf, M., 2019. Naturally clonal vertebrates are an  
851 untapped resource in ecology and evolution research. *Nat. Ecol. Evol.* 3, 161–169.  
852 <https://doi.org/10.1038/s41559-018-0775-0>

853 Leung, C., Breton, S., Angers, B., 2016. Facing environmental predictability with different sources of  
854 epigenetic variation. *Ecol. Evol.* 6, 5234–5245. <https://doi.org/10.1002/ece3.2283>

855 Lewis, J.M., Hori, T.S., Rise, M.L., Walsh, P.J., Currie, S., 2010. Transcriptome responses to heat stress  
856 in the nucleated red blood cells of the rainbow trout (*Oncorhynchus mykiss*). *Physiol. Genomics*  
857 42, 361–373. <https://doi.org/10.1152/physiolgenomics.00067.2010>

858 Li, H., 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM  
859 [arXiv:1303.3997v2](https://arxiv.org/abs/1303.3997v2) [q-bio.GN]. <https://doi.org/10.1303.3997> [q-bio.GN]

860 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R.,  
861 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079.  
862 <https://doi.org/10.1093/bioinformatics/btp352>

863 Li, Y., Huang, J., Liu, Z., Zhou, Y., Xia, B., Wang, Y., Kang, Y., Wang, J., 2017. Transcriptome analysis  
864 provides insights into hepatic responses to moderate heat stress in the rainbow trout (  
865 *Oncorhynchus mykiss* ). *Gene* 619, 1–9. <https://doi.org/10.1016/j.gene.2017.03.041>

866 Liu, J., Hu, H., Panserat, S., Marandel, L., 2020. Evolutionary history of DNA methylation related genes  
867 in chordates: new insights from multiple whole genome duplications. *Sci. Rep.* 10, 970.  
868 <https://doi.org/10.1038/s41598-020-57753-w>

869 Lluch, J., Servant, F., Païssé, S., Valle, C., Valière, S., Kuchly, C., Vilchez, G., Donnadieu, C., Courtney,  
870 M., Burcelin, R., Amar, J., Bouchez, O., Lelouvier, B., 2015. The Characterization of Novel Tissue  
871 Microbiota Using an Optimized 16S Metagenomic Sequencing Pipeline. *PLoS One* 10, e0142334.  
872 <https://doi.org/10.1371/journal.pone.0142334>

873 Lun, A.T.L., Chen, Y., Smyth, G.K., 2016. It's DE-licious: A Recipe for Differential Expression Analyses of  
874 RNA-seq Experiments Using Quasi-Likelihood Methods in edgeR, in: Mathé, E., Davis, S. (Eds.),  
875 *Statistical Genomics. Methods in Molecular Biology* Volume1418. NY, pp. 391–416.

876 Macqueen, D.J., Robb, D.H.F., Olsen, T., Melstveit, L., Paxton, C.G.M., Johnston, I.A., 2008.  
877 Temperature until the “eyed stage” of embryogenesis programmes the growth trajectory and  
878 muscle phenotype of adult Atlantic salmon. *Biol. Lett.* 4, 294–298.  
879 <https://doi.org/10.1098/rsbl.2007.0620>

880 Makesh, M., Sudheesh, P.S., Cain, K.D., 2015. Systemic and mucosal immune response of rainbow  
881 trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by  
882 different routes. *Fish Shellfish Immunol.* 44, 156–163. <https://doi.org/10.1016/j.fsi.2015.02.003>

883 Massicotte, R., Angers, B., 2012. General-purpose genotype or how epigenetics extend the flexibility  
884 of a genotype. *Genet. Res. Int.* Article ID 317175. <https://doi.org/10.1155/2012/317175>

885 Massicotte, R., Whitelaw, E., Angers, B., 2011. DNA methylation: A source of random variation in  
886 natural populations. *Epigenetics* 6, 421–427. <https://doi.org/10.4161/epi.6.4.14532>

887 Metzger, D.C.H., Schulte, P.M., 2017. Persistent and plastic effects of temperature on DNA  
888 methylation across the genome of threespine stickleback ( *Gasterosteus aculeatus* ). *Proc. R.*  
889 *Soc. B Biol. Sci.* 284, 20171667. <https://doi.org/10.1098/rspb.2017.1667>

890 Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., Piferrer, F., 2011. DNA  
891 Methylation of the Gonadal Aromatase (*cyp19a*) Promoter Is Involved in Temperature-  
892 Dependent Sex Ratio Shifts in the European Sea Bass. *PLoS Genet.* 7, e1002447.  
893 <https://doi.org/10.1371/journal.pgen.1002447>

894 Ojanguren, A.F., Braña, F., 2003. Thermal dependence of embryonic growth and development in  
895 brown trout. *J. Fish Biol.* 62, 580–590. <https://doi.org/10.1046/j.1095-8649.2003.00049.x>

896 Ojima, N., Yamashita, M., Watabe, S., 2005. Quantitative mRNA expression profiling of heat-shock  
897 protein families in rainbow trout cells. *Biochem. Biophys. Res. Commun.* 329, 51–57.  
898 <https://doi.org/10.1016/j.bbrc.2005.01.097>

899 Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P.,  
900 Klopp, C., Postlethwait, J.H., Guiguen, Y., Bobe, J., 2016. Gene evolution and gene expression

901 after whole genome duplication in fish: The PhyloFish database. *BMC Genomics* 17, 368.  
902 <https://doi.org/10.1186/s12864-016-2709-z>

903 Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., Hoekstra, H.E., 2012. Double Digest RADseq: An  
904 Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model  
905 Species. *PLoS One* 7, e37135. <https://doi.org/10.1371/journal.pone.0037135>

906 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic  
907 Acids Res.* 29, e45. <https://doi.org/10.1093/nar/29.9.e45>

908 Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-  
909 wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic  
910 Acids Res.* 30, e36. <https://doi.org/10.1093/nar/30.9.e36>

911 Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping  
912 genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool  
913 using pair-wise correlations. *Biotechnol. Lett.* 26, 509–515.  
914 <https://doi.org/10.1023/B:BILE.0000019559.84305.47>

915 Pigliucci, M., 1996. How organisms respond to environmental changes: from phenotypes to  
916 molecules (and vice versa). *Trends Ecol. Evol.* 11, 168–173. [https://doi.org/10.1016/0169-  
5347\(96\)10008-2](https://doi.org/10.1016/0169-<br/>917 5347(96)10008-2)

918 Qiang, J., He, J., Yang, H., Wang, H., Kpundeh, M.D., Xu, P., Zhu, Z.X., 2014. Temperature modulates  
919 hepatic carbohydrate metabolic enzyme activity and gene expression in juvenile GIFT tilapia  
920 (*Oreochromis niloticus*) fed a carbohydrate-enriched diet. *J. Therm. Biol.* 40, 25–31.  
921 <https://doi.org/10.1016/j.jtherbio.2013.12.003>

922 Quillet, E., Dorson, M., Le Guillou, S., Benmansour, A., Boudinot, P., 2007. Wide range of  
923 susceptibility to rhabdoviruses in homozygous clones of rainbow trout. *Fish Shellfish Immunol.*  
924 22, 510–519. <https://doi.org/http://dx.doi.org/10.1016/j.fsi.2006.07.002>

925 Quinlan, A.R., Hall, I.M., 2010. BEDTools: A flexible suite of utilities for comparing genomic features.  
926 *Bioinformatics* 26, 841–842. <https://doi.org/10.1093/bioinformatics/btq033>

927 Rebl, A., Verleih, M., Köbis, J.M., Kühn, C., Wimmers, K., Köllner, B., Goldammer, T., 2013.  
928 Transcriptome Profiling of Gill Tissue in Regionally Bred and Globally Farmed Rainbow Trout  
929 Strains Reveals Different Strategies for Coping with Thermal Stress. *Mar. Biotechnol.* 15, 445–  
930 460. <https://doi.org/10.1007/s10126-013-9501-8>

931 Rebl, A., Verleih, M., Nipkow, M., Altmann, S., Bochert, R., Goldammer, T., 2018. Gradual and Acute  
932 Temperature Rise Induces Crossing Endocrine, Metabolic, and Immunological Pathways in  
933 Maraena Whitefish (*Coregonus maraena*). *Front. Genet.* 9, 241.  
934 <https://doi.org/10.3389/fgene.2018.00241>

935 Richards, E.J., 2006. Inherited epigenetic variation — revisiting soft inheritance. *Nat. Rev. Genet.* 7,

936 395–401. <https://doi.org/10.1038/nrg1834>

937 Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential  
938 expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140.  
939 <https://doi.org/10.1093/bioinformatics/btp616>

940 Robinson, M.D., Oshlack, A., 2010. A scaling normalization method for differential expression analysis  
941 of RNA-seq data. *Genome Biol.* 11, R25. <https://doi.org/10.1186/gb-2010-11-3-r25>

942 Robles, M., Couturier-Tarrade, A., Derisoud, E., Geeverding, A., Dubois, C., Dahirel, M., Aioun, J.,  
943 Prezelin, A., Calvez, J., Richard, C., Wimel, L., Chavatte-Palmer, P., 2019. Effects of dietary  
944 arginine supplementation in pregnant mares on maternal metabolism, placental structure and  
945 function and foal growth. *Sci. Rep.* 9, 6461. <https://doi.org/10.1038/s41598-019-42941-0>

946 Schield, D.R., Walsh, M.R., Card, D.C., Andrew, A.L., Adams, R.H., Castoe, T.A., 2016. EpiRADseq:  
947 scalable analysis of genomewide patterns of methylation using next-generation sequencing.  
948 *Methods Ecol. Evol.* 7, 60–69. <https://doi.org/10.1111/2041-210X.12435>

949 Schnurr, M.E., Yin, Y., Scott, G.R., 2014. Temperature during embryonic development has persistent  
950 effects on metabolic enzymes in the muscle of zebrafish. *J. Exp. Biol.* 217, 1370–1380.  
951 <https://doi.org/10.1242/jeb.094037>

952 Scott, G.R., Johnston, I.A., 2012. Temperature during embryonic development has persistent effects  
953 on thermal acclimation capacity in zebrafish. *Proc. Natl. Acad. Sci. U. S. A.* 109, 14247–52.  
954 <https://doi.org/10.1073/pnas.1205012109>

955 Seibert, H., 1985. Effects of temperature on glucose release and glycogen metabolism in isolated  
956 hepatocytes from rainbow trout (*Salmo gairdneri*). *Comp Biochem Physiol* 81B, 877–883.  
957 [https://doi.org/10.1016/0305-0491\(85\)90082-3](https://doi.org/10.1016/0305-0491(85)90082-3)

958 Steinbacher, P., Marschallinger, J., Obermayer, A., Neuhofer, A., Sanger, A.M., Stoiber, W., 2011.  
959 Temperature-dependent modification of muscle precursor cell behaviour is an underlying  
960 reason for lasting effects on muscle cellularity and body growth of teleost fish. *J. Exp. Biol.* 214,  
961 1791–1801. <https://doi.org/10.1242/jeb.050096>

962 Suarez-Bregua, P., Pérez-Figueroa, A., Hernández-Urcera, J., Morán, P., Rotllant, J., 2020.  
963 Temperature-independent genome-wide DNA methylation profile in turbot post-embryonic  
964 development. *J. Therm. Biol.* 88, 102483. <https://doi.org/10.1016/j.jtherbio.2019.102483>

965 Valdivia, K., Jouanno, E., Volff, J.-N., Galiana-Arnoux, D., Guyomard, R., Helary, L., Mourot, B., Fostier,  
966 A., Quillet, E., Guiguen, Y., 2014. High Temperature Increases the Masculinization Rate of the  
967 All-Female (XX) Rainbow Trout “Ma” Population. *PLoS One* 9, e113355.  
968 <https://doi.org/10.1371/journal.pone.0113355>

969 Velsen, F.P.J., 1987. Temperature and incubation in Pacific salmon and rainbow trout: compilation of  
970 data on median hatching time, mortality and embryonic staging. *Can. Data Rep. Fish. Aquat. Sci.*

971 626, 67 pp.

972 Verleih, M., Borchel, A., Krasnov, A., Rebl, A., Korytář, T., Kühn, C., Goldammer, T., 2015. Impact of  
973 Thermal Stress on Kidney-Specific Gene Expression in Farmed Regional and Imported Rainbow  
974 Trout. *Mar. Biotechnol.* 17, 576–592. <https://doi.org/10.1007/s10126-015-9640-1>

975 Vornanen, M., Hassinen, M., Koskinen, H., Krasnov, A., 2005. Steady-state effects of temperature  
976 acclimation on the transcriptome of the rainbow trout heart. *Am. J. Physiol. - Regul. Integr.*  
977 *Comp. Physiol.* 289, 1177–1184. <https://doi.org/10.1152/ajpregu.00157.2005>

978 Wang, Y., Liu, Z., Li, Z., Shi, H., Kang, Y., Wang, J., Huang, J., Jiang, L., 2016. Effects of heat stress on  
979 respiratory burst, oxidative damage and SERPINH1 (HSP47) mRNA expression in rainbow trout  
980 *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* 42, 701–710. [https://doi.org/10.1007/s10695-](https://doi.org/10.1007/s10695-015-0170-6)  
981 [015-0170-6](https://doi.org/10.1007/s10695-015-0170-6)

982 Weber, G.M., Martin, K., Kretzer, J., Ma, H., Dixon, D., 2016. Effects of incubation temperatures on  
983 embryonic and larval survival in rainbow trout, *Oncorhynchus mykiss*. *J. Appl. Aquac.* 28, 285–  
984 297. <https://doi.org/10.1080/10454438.2016.1212447>

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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	CTCCTCCTTGGCCTCTTCTT			

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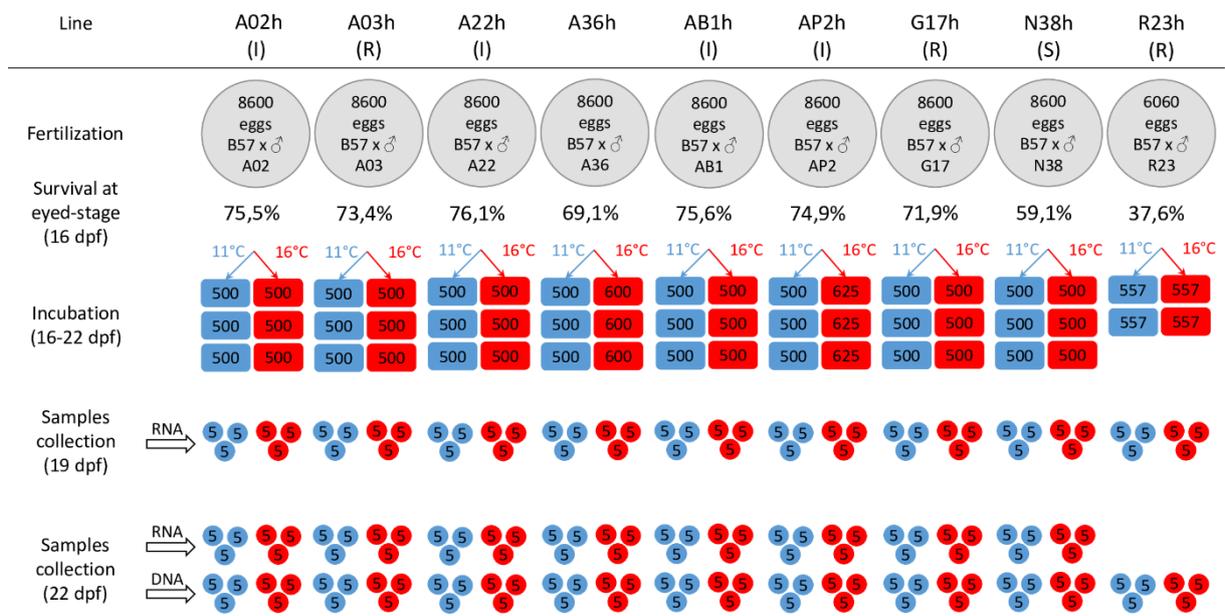
1000 **Table 3.** Number of filtered EpiRADseq loci per line. <sup>1</sup>Number of EpiRADseq loci kept after PM and PH  
1001 filters; see Figure 2 for the overall EpiRADseq analysis strategy. <sup>2</sup>Number of EpiRADseq loci kept after  
1002 filtering on read abundance (counts per million > 1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

Line	<sup>1</sup> No. after PM and PH filters	<sup>2</sup> 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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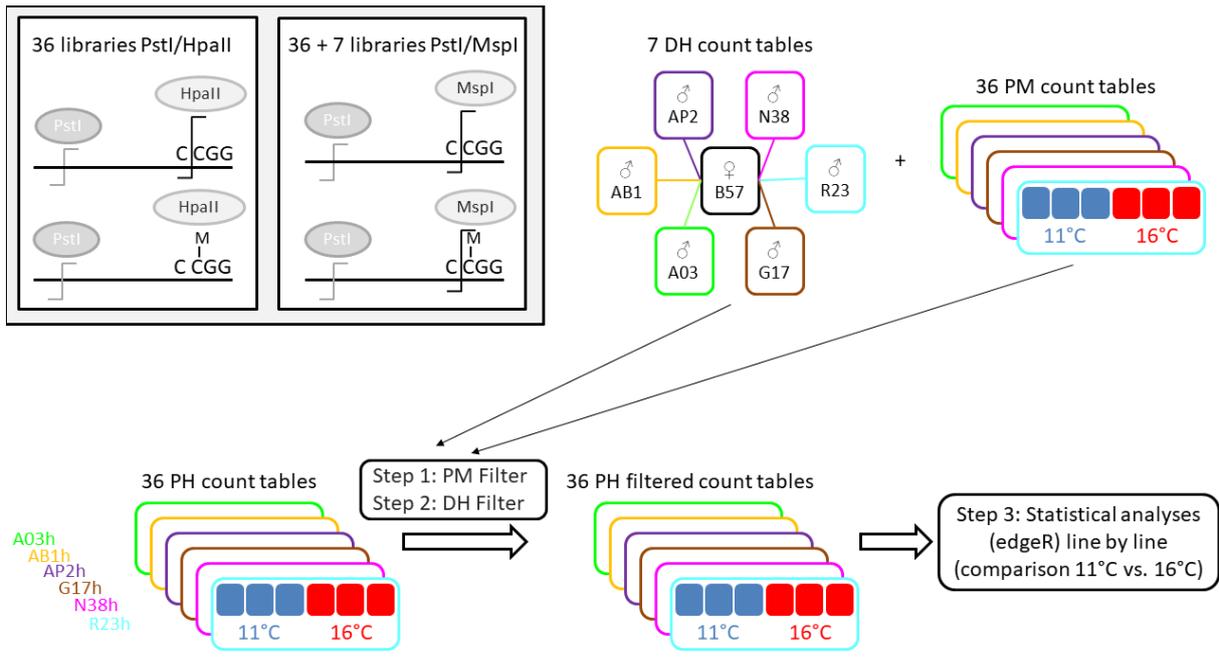
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Figure 1



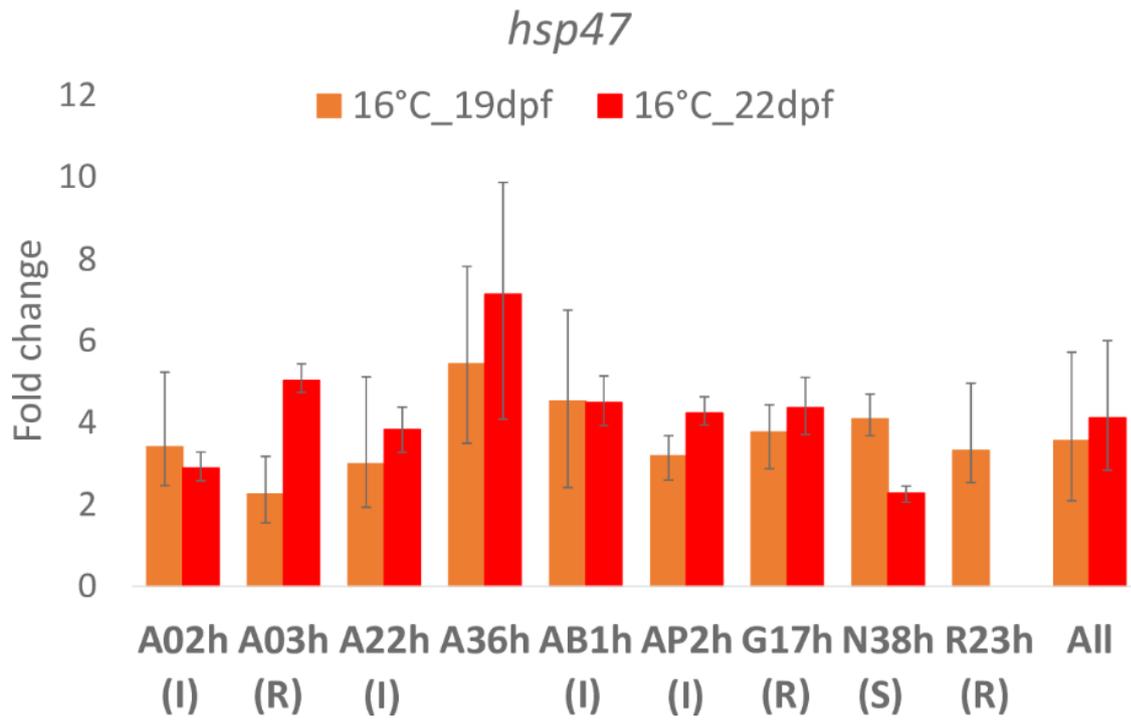
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Figure 2

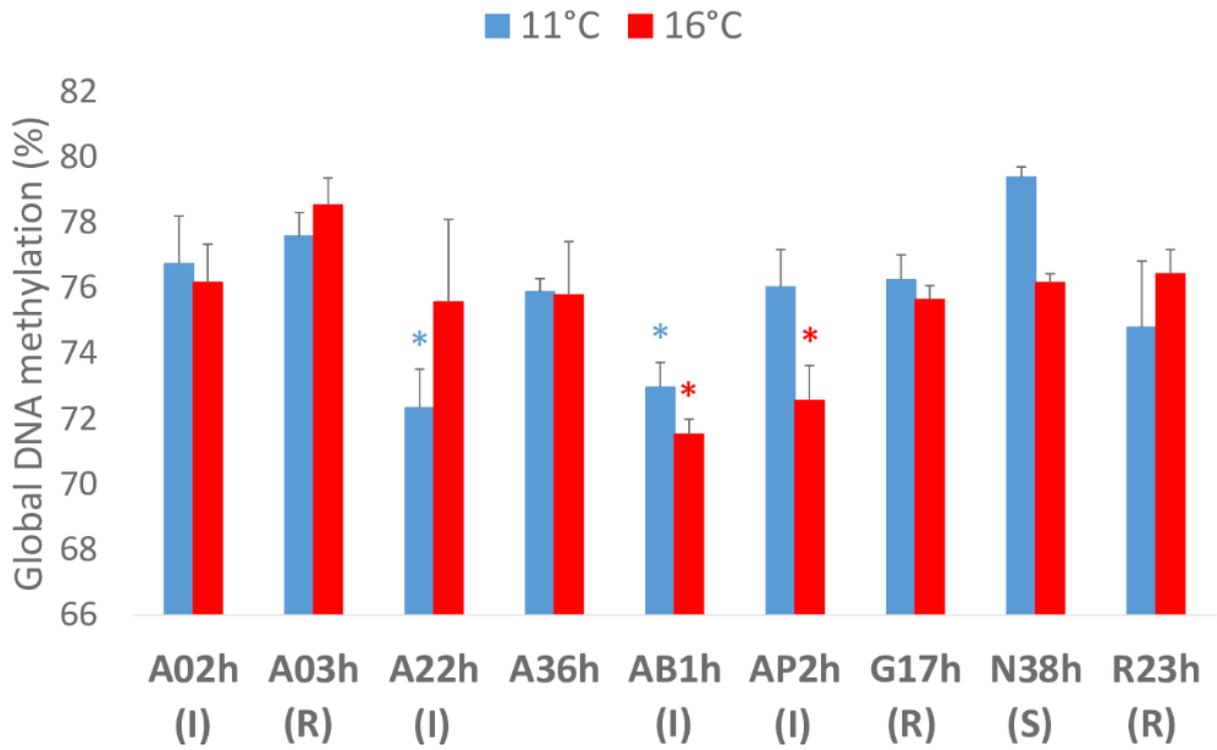


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**Figure 3**



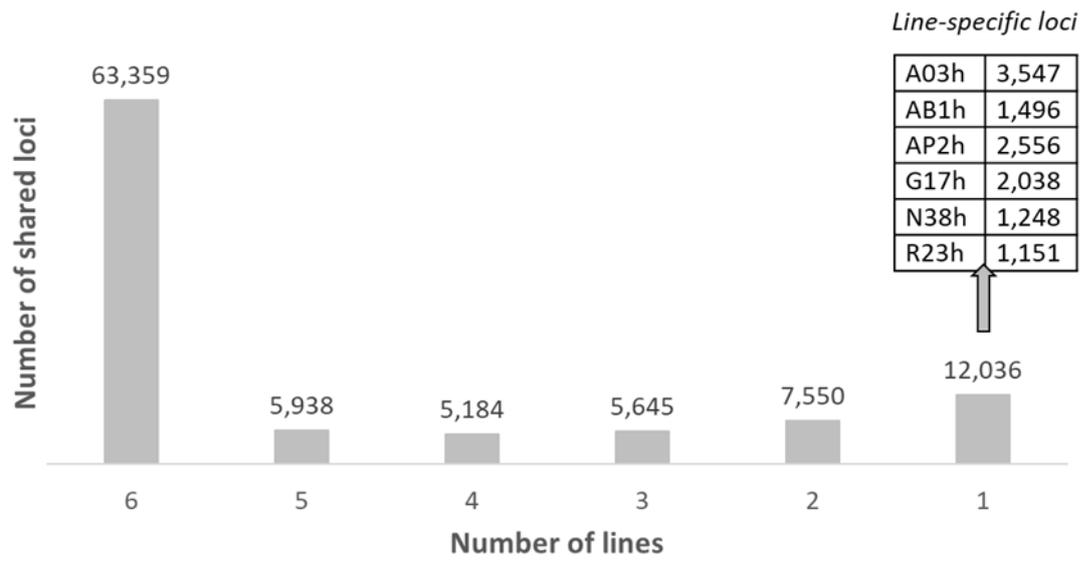
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Figure 4

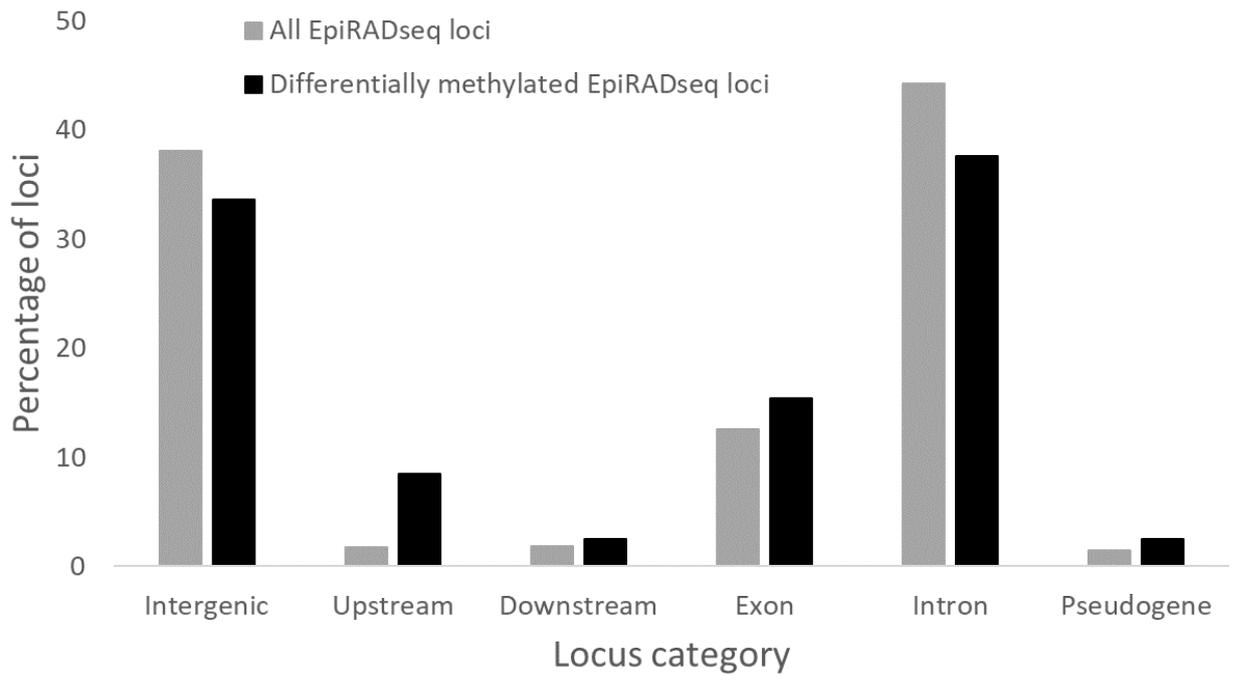


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Figure 5



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**Figure 6**

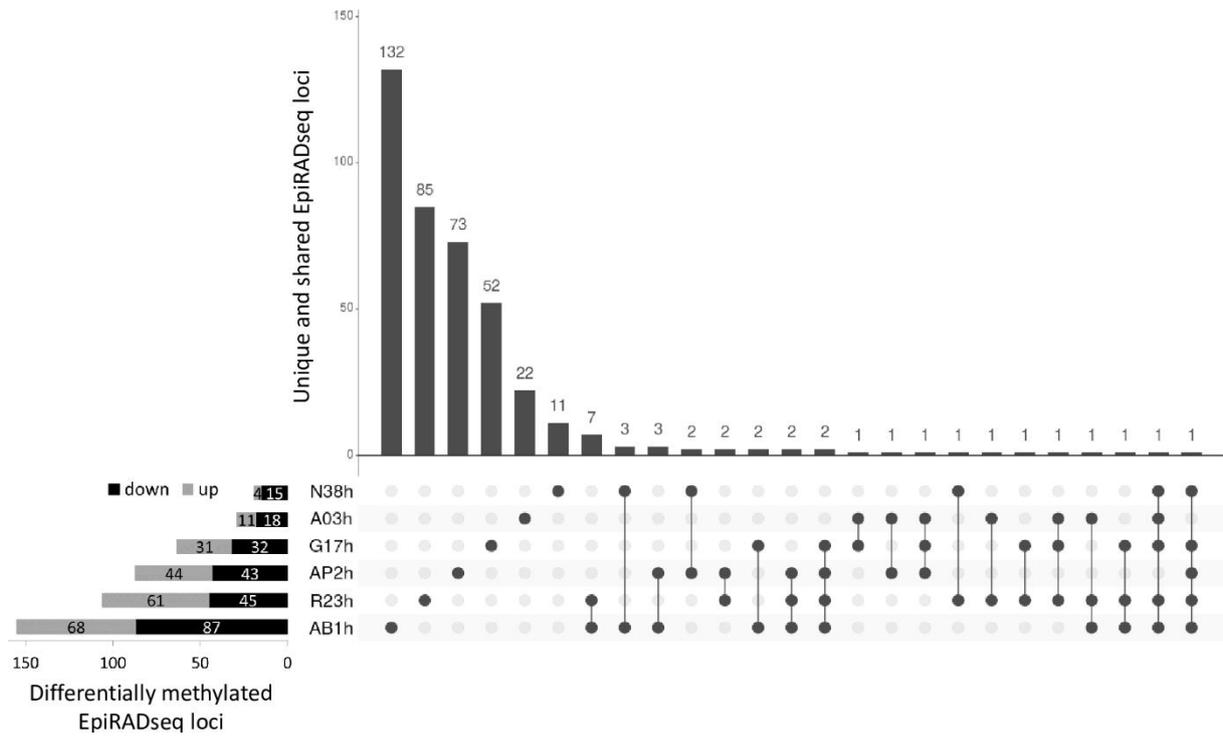
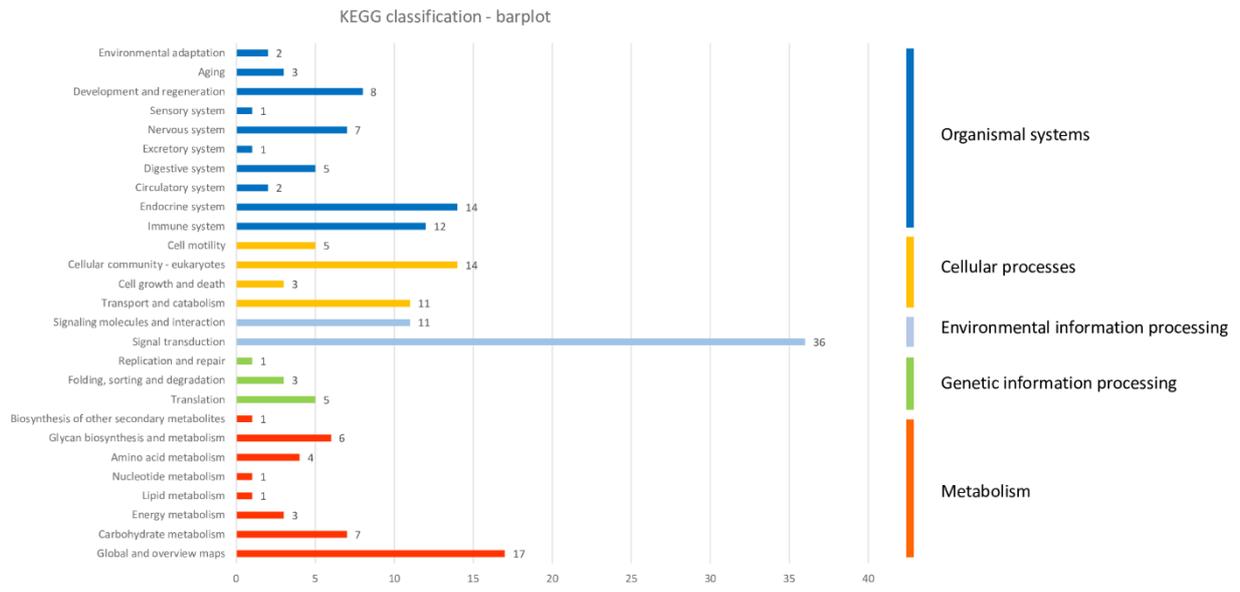


Figure 7

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Figure 8

## Figure captions

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1040

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

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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