Genome scale epigenomic analysis of response to acute stress in the European sea bass (*Dicentrarchus labrax*, L.)

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Running title: Epigenomics of stress in sea bass

Keywords: cytosine methylation, epiGBS, red blood cells, challenge test, coping styles, aquaculture

Abstract

Understanding the molecular basis of stress is of long standing interest in biology and fish science. We tackled this question by modifying the epiGBS (epiGenotyping By sequencing) technique to screen for cytosine methylation and explore the genome-wide epigenomic response to a repeated acute stress challenge in the European sea bass (*Dicentrarchus labrax*). Following a minimally invasive sampling using nucleated red blood cells (RBCs), our modified epiGBS protocol retrieved 501,108,033 sequencing reads after trimming, with a mean mapping efficiency of 73.0% (unique best hits). Sequencing reads mapped across all linkage groups (LGs). A total of 47,983 CpG coordinates with a minimum 30X read depth was retained for differential methylation analysis between pre- and post-stress fish. A family effect was demonstrated, and 57 distinct differentially methylated cytosines (DMCs) distributed on 17 of 24 LGs were found between RBCs of pre- and post-stress individuals and located close to 51 distinct stress-related genes. Thirty-eight of these genes were previously reported as differentially expressed in the brain of zebrafish, most of them involved in stress coping differences. Some DMC-related genes appear as good candidates to study the stress response, especially a set of them associated to the Brain Derived Neurotrophic Factor (BDNF), a protein that favors stress adaptation and fear memory. Limits to our study and future directions are presented, including the use of RBCs as a surrogate to other target tissues, to provide with classical physiological measurements a more complete picture of the stress response in fish.

45 # 1 - Introduction

46 The study of stress is of large interest in biological sciences. Stress affects cells as well as
47 ecosystems and influences processes that span from seconds to evolutionary time scales
48 (Balasch and Tort 2019; Petitjean et al. 2019). Reaching a common definition is quite complex
49 (Levine 1985; Schreck and Tort 2016; Del Giudice et al. 2018; Balasch and Tort 2019), but stress
50 often refers to the non-specific, total response of one organism to answer to internal and/or
51 external stimuli acting as stressors. Accordingly, the stress response represents the repertoire
52 of physiological, metabolic and behavioural changes developed during and observed after a
53 threat to counteract the effect of stressors and recover or approach the homeostatic
54 equilibrium. In teleost fish – the largest group of vertebrates - an integrative understanding of
55 the stress response is still expected (Balasch and Tort 2019; Petitjean et al. 2019). This concerns
56 natural populations that develop(ed) plastic and adaptive response to stressors (Schulte 2014;
57 Petitjean et al. 2019), but also cultured species in order to increase fish health and welfare in
58 stressful farming environments (Ashley 2007; Ellis et al. 2012; Sneddon et al. 2016).

59 The study of stress is complex as it may differ in nature, intensity and/or duration, plus
60 number of stressors. This poses many challenges that remain to be overcome (Romero et al.
61 2015) and stress studies have to integrate physiological, immunological, neurobiological and
62 behavioural components that altogether shape the stress response in fish (Pottinger 2008; Ellis
63 et al. 2012; Nardocci et al. 2014; Castanheira et al. 2017). The stress response implies, among
64 others, a primary neuroendocrine and a secondary immune response, with interacting roles of
65 neuropeptides and cytokines in each respective system (Wendelaar-Bonga 1997; Yada and
66 Nakanishi 2002; Geven and Klaren 2017). The Hypothalamus-Pituitary-Interrenal (HPI) axis plays

67 a crucial role in the manifestation and regulation of the stress response through the release of
68 neuropeptides, catecholamines and glucocorticoids. In teleosts, cortisol is the primary
69 circulating glucocorticoid and species-specific differences in the timing, magnitude and peak
70 duration of post-stress plasma cortisol concentrations have been reported (Fanouraki et al.
71 2011). Glucose - another classical biomarker of stress - is also under control of hypothalamus,
72 and its blood level depends on glycogen metabolism as well as gluconeogenesis, themselves
73 triggered by interrenal glands and catecholamines (Gamperl et al. 1994; Mommsen et al. 1999).
74 Beyond the classical HPI axis, several mediators of allostasis, organs and systems (e.g. serotonin
75 and the serotonergic system; osteocalcin and the parasympathetic nervous system) are involved
76 in stress and anxiety (Backström and Winberg 2017; Foster et al. 2017; Berger et al. 2019). A
77 proper regulation of the stress response requires the consideration of a complex regulatory
78 network of non-linear actions to integrate the whole organism response to stressors (McEwen
79 2008; Gray et al. 2017).

80 In the past few years, the stress response largely inspired genome-wide epigenetic
81 studies in model organisms (Dirven et al. 2017; Burns et al. 2018; Floriou-Servou et al. 2018;
82 Denzel et al. 2019; Perrone and Martinelli 2020). For animal models, it was established that
83 stress can especially reshape the stress axis/network through epigenetic modification of genes
84 in the hippocampus, hypothalamus and other stress-responsive brain regions (Reul et al. 2015;
85 Gray et al. 2017; Zhou and Fang 2018). In human stress studies, it has been shown that blood
86 cells responded to DNA methylation in the brain (Auta et al. 2013; Blaze et al. 2018; Vinkers et
87 al. 2019), and more generally that blood is useful whenever target tissues cannot be easily
88 sampled or biopsied, or because blood collections are available for large-scale screening of

89 methylation profiles (Houseman et al. 2015). As blood cells are also mobilized as part of the
90 stress response in fish (Tort 2011; Burgos-Aceves et al. 2019), it might be appealing to
91 investigate if an epigenomic signature of stress is present in their nucleated red blood cells
92 (RBCs). Indeed, the use of RBCs in fish transcriptomics or proteomics has received attention
93 over the last decade (Morera et al. 2011; Puente-Marin et al. 2018). However, fish epigenetics
94 has not embarked on this path yet and genome-wide epigenetic studies using RBCs are quite
95 rare in fish (Gavery et al 2018). This contrasts with the over-increasing interest to investigate
96 epigenomic variation in teleosts (e.g. zebrafish: Chatterjee et al. 2016; Olsvik et al. 2019;
97 medaka: Wang and Bhandari 2019; tongue sole: Shao et al. 2014; killifish: Berbel-Filho et al.
98 2020; salmonids: Le Luyer et al. 2017; Boltana et al. 2018; Gavery et al. 2018, 2019; stickleback:
99 Metzger and Schulte 2017, 2018; Heckwolf et al. 2020; tilapias: Wan et al. 2016; Hilliard et al.
100 2019; carp: Zhang et al. 2019; guppy: Hu et al. 2018; striped bass: Woods et al. 2018; wrasse:
101 Todd et al. 2019; goby: Sommerville et al. 2019). Furthermore, documenting more directly the
102 epigenomics of the stress axis/network also remains scarce (Uren-Webster et al. 2018), while Li
103 and Leatherland (2013) or Faught et al. (2016) asked for greater attention on this topic. Only few
104 epigenomic studies dealt explicitly with a specific component of the stress response (e.g.
105 immune function: Hu et al. 2018, Uren-Webster et al. 2018), while others are more elusive (e.g.
106 'early-life stress': Moghadam et al. 2017).

107 After salmonids, the European sea bass (*Dicentrarchus labrax*) is certainly the most
108 investigated marine fish species in Europe using molecular tools. It has been extensively studied
109 over the last three decades (reviewed in Vandeputte et al. 2019). This includes the sequencing
110 of its genome (Tine et al. 2014) and an increasing number of epigenetic studies (e.g. Navarro-

111 Martín et al. 2011; Anastasiadi et al. 2018; Anastasiadi and Piferrer 2019). The stress response of
112 European sea bass remains evaluated using blood parameters that have been shown to reflect
113 multiple components of its stress response (Vazzana et al. 2002; Fanouraki et al. 2011), despite
114 authors have proposed alternatives (e.g. Tognoli et al. 2010; Cœurdacier et al. 2011). How the
115 European sea bass methylome may capture such components is actually missing.

116 In this study, we adapted the epiGenotyping By sequencing (epiGBS) protocol originally
117 proposed by Van Gurp et al. (2016) to assess the genome-wide epigenomic variation in the RBCs
118 of *D. labrax* submitted to periods of acute stress. EpiGBS targets variation in cytosine
119 methylation – the covalent addition of a methyl group to cytosine nucleotides – that has long
120 been accepted as an important epigenetic modification in many organisms (Schübeler 2015;
121 Allis and Jenuwein 2016). Our aims were to explore the epigenomic landscape of the stress
122 response in the European sea bass using this protocol, and to investigate if nucleated RBCs from
123 minimally invasive blood samples could provide relevant information on this issue.

124

125

126 ## 2 - Materials and Methods

127

128 **2 - 1 : Rearing, stress challenge, and blood sampling**

129 Four hundred European sea bass were initially used in this study. They resulted of a single
130 crossing experiment (12 dams, 20 sires) that took place in January 2018. During the larval
131 phase, 20 different families labelled from A to T were raised. Each family was reared separately
132 in open circulation tanks at Nireus S.A. research facilities (Greece). To avoid environmental

133 effects, fish were tagged at ~280 days post-hatch (July 10-13th 2018) and distributed in 20 tanks;
134 each tank receiving 1 fish from each family (i.e., 20 fish per tank). Fish were fed twice a day, for
135 6 days a week, using a commercial diet (Blue Line 45:20 3.5 mm, Feedus S.A., Greece).
136 Throughout the experimental period, the photoperiod was set at 12L:12D, the water
137 temperature and the salinity held constant (18.1 ± 0.2°C and 28 ppt, respectively). Fish weights
138 (mean ± SD) were 48.1 ± 12.8g and 86.2 ± 24.1g in pre- and post-stress individuals,
139 respectively.

140 Fish were submitted to one acute challenge test per month for three consecutive
141 months, from July to October 2018. During this challenge, fish were exposed to high density
142 stress by lowering water levels in the tank to 1⁄3 of the original volume, followed by chasing of
143 the fish with a net for five minutes and a 30 min waiting period before sampling. These stressors
144 are classical in sea bass studies regarding response to acute stress (e.g. Fanouraki et al. 2011;
145 Samaras et al. 2016). This protocol took place in each tank then for each fish individual entering
146 the experiment. It was repeated for 3 consecutive times at 20-21 day intervals (period long
147 enough for fish to recover).

148 An initial blood sampling occurred two weeks prior to the implementation of the stress
149 challenge (hereafter T0, July 19th, 2018, pre-stress/control group) then at the end of the
150 challenge test (hereafter T4, October 5th, 2018). Blood from the caudal vessel was collected.
151 Plasma and RBCs were separated by centrifugation at 2,000g for 10 min. Careful separation of
152 the plasma and RBCs was performed using 200 µl pipettes. RBC extracts were heparinized
153 (heparin sodium; Sigma-Aldrich), transferred in microtubes and conserved at -20°C in 1 ml of
154 RNA later. DNA was extracted using the Macherey Nagel Nucleo Spin Tissue DNA kit and

155 quantified using a Qubit fluorometer (Qubit dsDNA BR Assay Kit, Q32853, Invitrogen). Thirty-
156 seven blood samples at T0 (pre-stress) and thirty-seven additional samples at T4 (post-stress)
157 have been randomly selected for the downstream epigenomic analysis.
158
159 **2 - 2 : Library preparation and sequencing**
160 We followed the epiGBS protocol published by van Gurp et al (2016). As the method was
161 developed for plants (with methylation occurring in CpG, CHH, and CHG context; H being any
162 nucleotide but a cytosine) and used a single digestion approach, the protocol was modified to
163 make it more suitable and straightforward for our vertebrate system. Particularly, a double
164 digest instead of the single digest approach was implemented. We chose the restriction enzyme
165 *MspI*, a standard choice in reduced representation bisulfite sequencing-like (RRBS) studies, as its
166 recognition site targets CpG rich regions (Gu et al. 2011). A second enzyme (*SfbI*) with a
167 recognition site length of 8 bp was used to reduce fragment numbers, and thus to increase read
168 depth per fragment. The choice of this enzyme was guided by *in silico* digestion of the European
169 sea bass genome (Tine et al. 2014) using *simRAD* (Lepais and Weir 2014). This genome is
170 available at: https://www.ensembl.org/Dicentrarchus_labrax/Info/Index.
171 One single library was prepared for a set of 74 samples. For this library, 200 ng of DNA of
172 each sample were digested in a 40 µl reaction, using 0.25 µl *MspI* (NEB, 20,000 U/ml R0106S),
173 0.25 µl S*bfI*-HiFi (NEB 20,000 U/ml R3642L) and 4 µl of 10X cutsmart buffer. The reaction was
174 run overnight at 37°C. Unique forward and reverse adapter combinations allow multiplexing
175 samples in the library. We added forward and reverse adapters in unique combinations (1 µl of
176 adapter, 2.5 µM), 0.5 µl T4 Ligase (NEB, 400,000 U/ml m0202L), 6 µl of 10X T4 ligase buffer and

177 11.5µl water were added directly to the digested DNA. Sequences of adapters are provided as
178 Suppl. Mat. Table S1. Adapters were ligated for 3 h at 23°C followed by 10 min of enzyme
179 inactivation at 65°C. After ligation, all samples were pooled and one third of the total volume
180 was used in the following step. The mixture volume was reduced using a Qiaquick PCR
181 purification kit (28104 Qiagen). The resulting product was cleaned a second time to ensure the
182 removal of small fragments and adapter remnants using CleanPCR paramagnetic beads
183 (Proteigene, CPCR-0050) with a ratio of 0.8X [sample : beads].

184 Since oligonucleotides were not phosphorylated (see Suppl. Mat. Table S1), a nick
185 translation was performed to repair the nick of the DNA at the restriction site and to fully
186 methylate the hemi-methylated adapters. To do so, 19.25 µl of the concentrated and purified
187 ligation pool were used in a 25 µl reaction, including 0.75 µl DNA Polymerase I (*E. coli*, 10,000
188 U/ml M0209S), 2.5 µl of 10 mM 5-Methylcytosine dNTP mix (Zymo Research, D1030), and
189 incubated at 15 °C for one hour. The library was then treated with sodium bisulfite using the EZ
190 DNA Methylation Gold Kit (D5005, Zymo Research) following manufacturer instructions to
191 convert unmethylated cytosines to uracil, paying attention to the optimal DNA amount per
192 reaction. After bisulfite conversion, 14 cycles of PCR (95°C for 1 min, 91°C for 10s, 65°C for 15s,
193 72°C for 10s and a final elongation step at 72°C for 5 min) were performed, followed by a
194 paramagnetic bead clean up with a 0.8X ratio. The library quality (fragment size distribution, no
195 adapters left, no primers left; fragment size range from 300 to 800 bp, Suppl. Mat. Fig. S1) was
196 verified on an Agilent 5300 Fragment Analyzer (Santa Clara, USA). It was sequenced (paired-end,
197 150 bp) on one lane of a SP flow cell on an ILLUMINA™ NovaSeq 6000 at the MGX sequencing
198 facility in Montpellier, France.

199 **2 - 3 : Bioinformatics analysis**

200 Raw sequencing data were trimmed of low quality reads and adapter residues using *Trim*
201 *Galore!* (v.0.6.4; available at
202 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The trimmed reads were
203 then demultiplexed using the `process_radtags` command of STACKS (Catchen et al 2013).
204 The option `disable_rad_check` was applied to avoid reads with bisulfite-modified
205 restriction sites to be discarded. The demultiplexed reads were trimmed a second time on both
206 5' and 3' ends with the options `--clip_R1 --clip_R2` and `--three_prime_clip_R1`
207 `--three_prime_clip_R2` to remove introduced methylated cytosines during adapter
208 ligation. Using the `bismark_genome_preparation` function of the program *bismark*
209 (Krueger & Andrews 2011), a bisulfite converted version of the European sea bass genome was
210 prepared against which the demultiplexed and trimmed reads were mapped.

211

212 **2 - 4 : Methylation analysis**

213 The R package MethylKit (Akalin et al 2012) was used to determine differential methylation
214 between pre- and post-stress fish. CpGs with less than 30 read depth and with coverage >99.9%
215 of the distribution of read counts were filtered out to account for PCR bias. Coverage was
216 normalized across samples, and only CpGs present in at least 20 out of 37 samples per group
217 (56%) were kept for further analysis. Differentially methylated CpG sites were determined
218 between pre- and post-stress individuals using logistic regression (`calculateDiffMeth`
219 function). Pre-stress individuals were considered as the baseline. Cytosines were considered as
220 differentially methylated when presenting at least 15% methylation difference (as in Anastasiadi

221 and Piferrer 2019 for epigenomic variation in *D. labrax*), and a nominal *q*-value < 0.001 between
222 pre- and post-stress fish. Reads presenting differentially methylated cytosines (DMCs) were
223 extracted using GENEIOUS (v.11.0; available at https://www.geneious.com/) and mapped along
224 LGs of the European sea bass reference genome (Tine et al. 2014;
225 http://seabass.mpipz.mpg.de/) to produce a primary annotation of potential candidate genes.
226 As the annotation of the European sea bass and the sea bream (*Sparus aurata*) reference
227 genomes have been recently released on ENSEMBL (http://www.ensembl.org/index.html), gene
228 names and models have been controlled, eventually including a TBLASTN search in case of
229 discrepancy.
230
231 **2 - 5 : Literature surveys and protein-protein association**
232 A first literature survey was performed in order to decipher if genes found associated to
233 European sea bass DMCs were candidates related to stress in vertebrates. We searched PUBMED
234 (https://pubmed.ncbi.nlm.nih.gov/) for studies in which they could have been mentioned as
235 responding to stress. This approach was preferred to, e.g. GO-terms or enrichment analyses that
236 are useful summaries of functional knowledge, but less suitable when searching how one or a
237 specific set of genes might be involved more precisely in the stress response, if they are known
238 to be associated to each other and/or how they were found related to stress (e.g. in which
239 organism, context, study, organ or tissues). As relatively few DMCs were detected (see Results),
240 this detailed approach was amenable.
241 The data mining considered a DMC relevant to the stress response when associated to a
242 gene related to a stress phenotype (typically issued from genome-wide association studies,

243 knock-out experiments or empirical evidence of impairment and dysfunction when facing
244 stressors). Eight stress response themes were identified and used to structure the mining:
245 '*blood*' (including issues on red blood cells, blood homeostasis, blood volume, hemato- and
246 erythropoiesis, platelets, hypo- and hypertension, angiogenesis), '*stress*' (including stress-
247 related genes, physiological disorders, HPI/HPA axis, adrenals, head kidney, stress
248 resistance/response), '*immunity*' (including issues on all types of leukocytes and immune
249 response, cytokines, cytotoxicity and response to pathogens), '*hormones*' (including issues
250 related to catecholamines, corticosteroids, glucocorticoids, neuro- and peptide transmitters,
251 other hormones related to the HPI axis in fish), '*brain*' (including issues on hypothalamus and
252 pituitary themselves, forebrain, limbic brain, neuronal development and plasticity, signaling
253 pathways in the brain, serotonergic/dopaminergic activities), '*glucose*' (including issues on
254 glucose metabolism [systemic and brain], lactate, glycolysis, gluconeogenesis, [pro]insulin,
255 diverse receptors, pancreatic β-cells), and '*behaviour*' as stress-induced behavioural changes
256 and not only physiological changes. Diverse health disorders related to stress often well-studied
257 in mammalian models were screened, as well as some genetic disorders that affect behaviour.
258 This may include changes in mood, emotional and affective states (e.g. fear, anxiety), cognitive
259 traits (e.g. memory, learning abilities), or psychiatric disorders and neurodegenerative diseases
260 (e.g. bipolar disorders, autism, schizophrenia). One additional category ('*others*') was included
261 to report significant items known to be important for, e.g., epigenetic regulation that could not
262 be directly addressed with previous themes. Cellular issues of stress such like, e.g., oxidative,
263 chromatin or endoplasmic reticulum stress where not covered.

264 A second literature survey focused on the literature dealing with stress, stress response
265 and high-throughput transcriptomics or epigenomics in fish. We searched PubMed for relevant
266 articles over the period 2010-2020 (June) and, when available, their associated supplementary
267 materials, and searching for difference in gene expression or methylation. Our goal was to
268 evaluate if DMC-related genes detected here in RBCs are relevant to brain and/or processes
269 specific to brain or if they are commonly detected whatever the target tissues and the nature of
270 stressors. For this survey, we first mined gene expression studies that used blood within fish
271 species in order to verify whether sea bass DMC-related genes were not commonly
272 overexpressed in this tissue. In a second time, we focused on zebrafish for which functional
273 annotation is well established. We searched for transcriptomics (RNA sequencing) studies
274 dealing with stress in various tissues (liver, kidney, gill, brain, gonad, muscle, eye and skin). The
275 following search string was used: '*zebrafish*' AND '*RNA seq**' AND '*stress*' AND ['*tissue name*'].
276 As mRNA studies often consider several tissues, results were manually curated. Studies whose
277 title referred to single gene families (e.g. heat shock proteins) or microRNAs were discarded.
278 Finally, fish epigenomic studies were also analyzed. About this issue, we first analyzed gene
279 transcripts and differentially methylated genes in the single genome-wide epigenomic study
280 performed so far in sea bass (Anastasiadi and Piferrer 2019). As genome-wide methylation
281 studies remain relatively few in fish, this was extended to other species in a second time.
282 Transcriptomics and methylation data sets that did not provide for gene names and/or gene
283 symbols were discarded. Reported gene names and symbols were considered to be exact.
284 Finally, in order to complete these manually curated surveys, we used String
285 (https://string-db.org/) to investigate if DMC-related genes encoded for proteins are known to

interact together. We used our gene list as input and the zebrafish genome as a reference for annotation. This search was done without reference to the thematic stress categories mentioned above, and when one interaction was provided we specifically explored the literature for confirmation and relevant experimental evidence.

2 – 6 : Data analysis

A principal component analysis (PCA) on the methylation profiles of individual samples at DMCs was performed to analyse the potential grouping structures within our data set. Differences in the distribution of mean individual loading scores for pre- and post-stress fish were tested along each principal components axis using a Student *t*-test. Additionally, a hierarchical clustering was performed using Ward's linkage method on Euclidean distances in order to explore whether sea bass families structure the data set.

3 - Results

Nineteen out of 20 families were represented by at least one individual among the 74 samples analyzed in this study. Fish number per family ranged from one (families A, D, N) to nine (family R) individuals. Except for the families with a single representative and family M with post-stress fish only (four), both pre-stress and post-stress individuals were present in the 15 remaining families. Four individuals from four distinct families were retained twice by chance, and were

307 thus analyzed for both pre- and post-challenge stress conditions. A total of 70 distinct fish has
308 been analyzed in this study.
309
310 **3 - 1 : epiGBS library construction and sequencing**
311 We obtained 504,271,331 total sequencing reads of which 99.4% (501,108,033) were retrieved
312 after trimming. After demultiplexing, read numbers per sample ranged from 2,284,915 to
313 16,314,759, with an average of 5,212,596 reads per sample (see Suppl. Mat. Table S2).
314 Demultiplexed samples were mapped against the *D. labrax* reference genome with a mean
315 mapping efficiency of 74.5% (73.0% for unique best hits; Suppl. Mat. Table S2). Sequencing
316 reads mapped across all linkage groups (Fig. 1). The mean per base pair read depth was 250X.
317
318 **3 - 2 : Methylation analysis**
319 Out of the 10,368,945 CG dinucleotides (0.46% of the whole) present in the *MspI-SbfI* reduced-
320 representation of *D. labrax* genome we obtained, 47,983 CpG coordinates were extracted with a
321 minimum of 30X read depth and presence in at least 20 individuals. They were filtered out using
322 a 15% methylation difference threshold and a nominal cut-off value of $q < 0.001$. With these
323 parameters, only a total of 57 cytosines in CpG context were defined as DMCs between pre- and
324 post-stress sea bass (Table 1). Methylation differences ranged from -27.5% to -15.0% for
325 hypomethylated cytosines, and 15.0% to 46.4% for hypermethylated cytosines. Hyper-
326 methylation was more frequently detected than hypomethylation (11 [19.30%] hypo- *vs* 46
327 [80.70%] hypermethylated DMCs) in post-stress sea bass. DMCs were distributed on 17 out of

328 24 LG groups and in or close to 51 distinct genes. Further information is provided in Suppl. Mat.
329 Table S3 (e.g. gene annotations, CpG context).

330 Most identified DMCs were found located within identified gene bodies (44 out of 57,
331 77.19%), one in the 3'UTR regions of the Solute Carrier family 22 Member 2 (*SLC22A2*) gene on
332 LG17, and one in a repeated region (a non LTR Retrotransposon Element on LG20). In the
333 remaining cases (*n* = 11), DMCs are intergenic and located in a window ranging from 0.9kb to
334 51kb to the closest gene (respectively: *SASH1A* on LG12 and *TRMT11* on LG16; Table 1). Two
335 pairs of overlapping, but inversely oriented genes share on their sense vs. antisense strand an
336 identical DMC: *PLG* and *SLC22A2* on LG17, and *SART3* and *FICD* on LG20 (Table 1)

337 When located on the same LG, DMCs were usually distant by at least 30kb from each
338 other. In only four instances, DMCs occurred as differentially methylated regions (DMRs) that
339 grouped 2-3 DMCs separated by only few hundreds of base pairs (Table 1). This includes three
340 hypo-methylated cytosines grouped in a DMR located ~1500 bp downstream of the predicted
341 Kelch Repeat and BDB domain 13 (*KBTBD13*) gene with at most 88 bp between the cytosines.
342 Another DMR includes three hypomethylated cytosines (>20%) on LG1A in the second intron of
343 the forkhead box J3 (*FOXJ3*) gene. Two other groups of two cytosines were found either in the
344 same exon (distant by 3 bp) of the *BTR30* gene or in two different introns (distant by 3.8Kb) of a
345 predicted CREB regulated transcription coactivator 2 (*CRTC2* or *TORC2*: Transducer Of Regulated
346 cAMP Response Element-Binding Protein [CREB] 2) (Table 1). In these two latter cases, one
347 cytosine was found hypomethylated while the other is hypermethylated. A single DMC was
348 associated to a repeat region and two DMCs were found to refer to the same gene (homologous
349 to the *Gasterosteus aculeatus* paralogue of *COL4A5*, a collagen gene of type IV mostly

350 implicated in the protein network of the basement membrane) (Table 1). For this gene, one
351 DMC is located in the first intron while the second is 16.5kb upstream of the start codon. They
352 were not considered as a DMR.
353
354 **3 - 3 : Clustering**
355 Hierarchical clustering showed a strong family effect in methylation patterns (i.e. individuals
356 within family clustered together; Fig. 3). It is interesting to note that the four individuals that
357 were caught twice displayed after-stress methylation pattern very similar to their before-stress
358 pattern, and clustered together by pairs in all four cases. These individuals have the lowest
359 levels of dissimilarity in hierarchical clustering, suggesting very few differences in their
360 methylation profiles in pre- *vs* post-stress condition.
361 Despite this strong family effect and clues of low impact of the stress on methylation,
362 pre- (T0) and post-stress (T4) groups can be distinguished based on their DMC profile in PCA.
363 Mean loading scores of individuals were found significant among T0 and T4 for PC1 that
364 explained 7.2% of total variance (Student *t*-test; $p < 0.005$, Fig. 4). No significant difference was
365 found for loading scores along PC2 (3.0% of total variation; $P = 0.404$).
366
367 **3 - 4 : Literature surveys**
368 *Thematic survey* - Our first literature survey showed that DMC-related genes detected in this
369 study were found involved in one of the eight themes we retained to describe the stress
370 response in fish (Fig. 2 and Suppl. Mat. Table S4 for a more extensive description of gene
371 involvement in the stress response, including references). Most of them were previously

372 analysed or detected in human or mice stress studies, in few cases in other model organisms
373 (e.g. *Drosophila*) (Suppl. Mat. Table S4). Additionally, the *GLI2* gene encoding for a zinc-finger
374 protein and the *BTR30* (bloodthirsty-related gene family member 30, also known as E3
375 ubiquitin-protein ligase *TRIM39*-like) gene (Table 1) were found differentially regulated in sea
376 bass cultured-cells submitted to viral infection and involved in the immune response (Chaves-
377 Pozo et al. 2019) (Fig. 2).
378
379 *Transcriptomics survey in zebrafish* - In a second time, we searched for differential expression of
380 sea bass DMC-related genes in studies using high-throughput transcriptomics in fish blood. Only
381 four studies were retained (Morera et al. 2011; Dahle et al. 2015; Martos-Sitcha et al. 2017;
382 Puente-Marin et al. 2018), and none of our DMC-related genes was found to be differentially
383 expressed in these studies.
384 After curation of PubMed lists and addition of few undetected studies, we surveyed 22
385 zebrafish studies analyzing the transcriptomics of the stress response in six tissues: brain (*n* =
386 10), liver (*n* = 7), muscle (*n* = 2), gonads (*n* = 1), intestine (*n* = 1), skin (*n* = 1) (no high-throughput
387 transcriptomics study in gill, kidney, one in muscles of eye that was accounted for 'muscle'; see
388 Suppl. Mat. Text S1). The main outcome of this survey was to document 38 out of 51 RBC's
389 DMC-related genes (74.51%) in stress-related studies. Strikingly, these 38 DMCs were reported
390 mainly from few neurotranscriptomics zebrafish studies that dealt either with reactive-proactive
391 behavioural response to stress (Rey et al. 2013; Wong et al. 2015) or with changes in social
392 regulation that may promote stressful behaviour among congeners (Greenwood and Peichel
393 2015; Oliveira et al. 2016) (Fig. 2 and Suppl. Mat. Table S4). Some genes are shared among these

394 studies. A recent studies recorded five of them as also differentially expressed in Siamese
395 fighting fish (*Betta splendens*) brain during male contests (Vu et al. 2020) (Suppl. Mat. Table S4),
396 *FBOX33* was not formerly reported in zebrafish, suggesting that 39 DMCs found in sea bass RBCs
397 could be associated to stress.

398 Only four of these DMC-related genes were reported in the former RNA sequencing
399 study of Anastasiadi and Piferrer (2019) in sea bass. This included *CILP1* and *PTGFRN* in muscle,
400 *PRKCQ* in testis, and *RRM2* in liver (blood and brain not investigated) (Fig.2). In comparison, the
401 twelve other zebrafish studies investigating other organs than brain retrieved only 15 of the sea
402 bass DMC-related genes found in RBCs (details in Suppl. Mat. Text S1).

403

404 *Epigenomic survey* - We finally looked at former studies dealing with epigenomics in fish. The
405 studies retained (*n* = 31) are presented in Suppl. Mat. Table S5. Twenty studies reported
406 differential methylation at genes also found differentially methylated in sea bass RBCs (Suppl.
407 mat. Table S5). Data sets were highly heterogeneous for different reasons (e.g. experimental set-
408 ups, nature of stressors if any, thresholds considered to define a DMC/DMR). However, main
409 observations might be summarized as follows: all articles considering brain tissue and with all
410 gene annotations made available (*n* = 6) reported at least one match with DMC-related genes
411 found in the present study, and more specifically data of the former sea bass epigenomic study
412 by Anastasiadi and Piferrer (2019) reported five genes of the present study as differentially
413 methylated in the brain (*GLG1a, GFRA2, CELF2, PRKCQ, ROBO3*), three in muscle (*CILP1, FURIN,*
414 *KBTBD13*), one in testis (*GLG1a*), and any in liver (total: eight distinct genes). This indicated
415 slightly more matches with brain. However, the single previous study analyzing RBC methylome

416 in fish so far (Gavery et al. 2018) shared no DMC-related gene with the present study. Finally,
417 the study that reported the largest number of genes found as DMC-related genes was Gavery et
418 al. (2019) (*n* = 15) in the liver of hatchery *vs* wild rainbow trout during a so-called 'temporal
419 analysis' (Suppl. Mat. Table S5). Another study by Olvsik et al. (2019) on zebrafish reported 11
420 genes in common with the present study, but was not organ specific (Suppl. Mat. Table S5).
421
422 **3 - 5 : Protein-protein interactions**
423 Further database mining screening for specific protein interactions on the STRING server revealed
424 few other possible pairs of associations (*n* = 8): *ROBO3-CHN1*, *ROBO3-PRKCQ*, *ROBO3-LRRC3*,
425 *DLG1-NCBP2*, *FURIN-PLG*, *PLG-MMRN2a*, *CELF-RRM2*, and *CRTC2-DENND4B* (Suppl. Mat. Fig.
426 S2). The *FURIN-PLG* and *DLG1-NCBP2* associations were also detected in our literature survey.
427 The first one will be presented in the 'Discussion' (see Suppl. Mat. Table S4 for the second one).
428 However, most of these associations retrieved only poor information relative to the stress
429 response. *ROBO3* and *CHN1* have been shown to interact in the brain with poorly-understood
430 implications of *CHN1* in neurological disorders (Walsh and Engle 2010; *CHN1* may have other
431 stress-related functions, see Suppl. Mat. Table S4). *CELF2* (CUGBP Elav-like family member 2)
432 and *RRM2* (Ribonucleotide reductase M2 polypeptide) are both known to participate to
433 messenger RMA (mRNA) metabolism (Vlasova-StLouis et al. 2013). *CELF2* acts to post-
434 transcriptionally stabilize mRNAs by relocating them to stress granules in the cytosol. *CELF2*
435 interferes with *RRM2* that modulates its splicing activity. As post-transcriptional activities are at
436 the core of methylation studies, the detection of this association is relevant to our study. To
437 illustrate some limits of these associations: *CRTC2* and *DENN4B* were detected apparently

438 because they are located close to the sex determining region of *Onchorhyncus mykiss* (Phillips et
439 al. 2013). No relationship with stress is suspected. The relationship between multimerin
440 (*MMRN2*) and plasminogen (*PLG*) via its co-activator remains poorly investigated and supported
441 (Øynebråten et al. 2005). This automatic search using STRING did not really enrich our findings.
442
443

444 4 - Discussion

445

446 In this study, we showed that a modified epiGBS protocol originally proposed by Van Gurp et al.
447 (2016) was applicable to further analyze patterns of cytosine methylation at a genome-wide
448 scale in *D. labrax*. This is the first use of epiGBS in fish and the second in an animal species
449 (Canadian lynx; Meröndun et al. 2019). The addition of a second restriction enzyme illustrates
450 the flexibility of the epiGBS and more generally RRBS protocols to improve data acquisition and
451 impact (Paun et al. 2019). This is however not the first RRBS protocol dedicated to the addition
452 of a second restriction enzyme (Wang et al. 2013), but this is hereby proposed in a context of
453 improved multiplexing of samples and increased sequencing capacities, thus offering more
454 opportunities to explore important biological processes at a genome-wide scale, such as the
455 stress response. Data allowed us to explore how RBC's DNA methylation may respond to
456 stressors in the European sea bass and - albeit some limitations that will be discussed below -
457 was shown to complement both the traditional evaluation of the stress response based on
458 analyses of blood serum samples (e.g. Vezzana et al. 2002; Seponka et al. 2016) or on gene
459 expression variation, often using invasive brain samples (e.g. Aluru and Vijayan 2009; Rey et al.

460 2013; Martos-Sitcha et al. 2014; Ghalambor et al. 2015. Wong et al. 2015; Metzger and Schulte
461 2016; Gerlai et al. 2017; Nyman et al. 2020).
462
463 **4 - 1 : Mining the epigenome…**
464 The information provided in this study is based on the analysis of 47,983 distinct methylated
465 sites distributed over all sea bass LGs. The mapping efficiency was high (74.5%) when compared
466 to early values retrieved in human (~65%; Krueger and Andrews 2011), or in some recent fish
467 studies implementing genome-wide methylation studies (e.g. 55-60% in Wan et al. 2016; 40% in
468 Gavery et al. 2018). Other studies reported similar mapping efficiencies, but reported
469 percentages of mapping for unique best hits that were generally lower. For example, in
470 *Kryptolebias marmoratus*, Berbel-Filho et al. (2020) reported a mean mapping efficiency of
471 74.2% but 61.1% unique best hits while, in this study, this latter percentage reached 73.0%. This
472 reflects a more robust mapping of the DMCs we detected and significantly enlarge the breadth
473 of the sites that can confidently exploit to retrieve functional information. Taking advantage of
474 the epiGBS protocol that allow to process more samples (Van Gurp et al. 2016), the number of
475 individuals considered in this study is rather high (*n* = 70 distinct individuals), when most
476 epigenomic studies in fish dealt with less than 30 individuals (range: *n* = 3 in Wang et al. 2019; *n*
477 = 106 in Heckwolf et al. 2020 for a population study). In sea bass, Anastasiadi and Piferrer (2019)
478 previously reported a study that used 27 samples and as many libraries to be sequenced while
479 our data were obtained from a unique library preparation. Our modified epiGBS protocol
480 provides a considerable amount of information, certainly at a reasonable cost, to decipher
481 methylation landscapes of sea bass or other species.

482 The operational and statistical thresholds used in the successive steps of this study are
483 large, resulting in the discovery of a rather low total number of methylated sites. For example a
484 threshold of 30X and nominal cut-off value of 0.001 are quite conservative, when some studies
485 might consider a threshold of 5X or 10X for a CpG to be analysed and associated cut-off values
486 of 0.05 or 0.01 (e.g. Wan et al. 2016; Hu et al. 2018; Anastasiadi and Piferrer 2019). Relaxing
487 thresholds would enable to retrieve more DMCs, but elevated thresholds should normally
488 ensure that relevant information is retrieved. Thus, only 57 DMCs have been found in RBCs of
489 pre- and post-stress European sea bass, and only few were grouped as DMRs. Considering DMRs
490 rather than DMCs is common in genome-wide methylation studies, including sea bass
491 (Anastasiadi and Piferrer 2019). Methylation changes grouped in clusters (i.e. DMRs) are more
492 likely to influence transcriptional activity at nearby loci than single cytosines (e.g. Richards et al.
493 2017). However, other experimental issues associated to the flexibility of RRBS protocols (e.g.
494 single- *vs* paired-end sequencing, likelihood of annotation, selection and size of fragments) can
495 made the advantages of considering DMRs over DMCs perhaps more disputable (Paun et al.
496 2019). In this study, DMCs were found mostly hypermethylated in post-stress individuals
497 compared to the pre-stress individuals, and mostly located in gene bodies of fifty-one different
498 genes (44 out of 57; 77.2%). It has been shown that such location of differential methylation
499 may regulate splicing and/or act as alternative promoters to reshape gene expression
500 (Maunakea et al. 2010; Ehrlich and Lacey 2013; Lev Maor et al. 2015). Thus, despite a low
501 number of DMRs in this study, a large fraction of DMCs points to the transcriptionally active
502 portion of the genome and to important stress-related genes (see below).

503 In addition to DMCs located in gene bodies, a dozen of DMCs were found in intergenic
504 regions (21.0%). Intergenic cytosine methylation has been frequently described, including in
505 stress studies (Hing et al. 2018), but its role remains poorly understood (Suzuki et al. 2011;
506 Rajarajan et al. 2016). While numbers of genic *vs* intergenic DMCs may greatly vary, a ratio of
507 ~80% of DMCs located in gene bodies and ~20% located in other genomic regions has been
508 reported in other fish studies (e.g. Hu et al. 2018). Sometimes proportions are approximately
509 reversed (~80% intergenic *vs* ~20% in gene bodies; e.g. Metzger and Schulte 2017). While
510 under-represented in our data, the role for differentially methylated transposable elements is
511 also poorly understood (Lerat et al. 2019; Jönnson et al. 2020), while certainly crucial in the
512 stress response (Hunter 2020).
513
514 **4 - 2 : … but mining the unknown**
515 Studies looking at the epigenomic landscape of RBCs in fish are scarce. Gavery et al. (2018)
516 reported 85 DMRs between hatchery and wild fish in *O. mykiss*. This study suggested that blood
517 DNA methylation patterns are indicative of the stressors experimented in the environment, but
518 we are still currently mining the unknown to gain supporting information.
519 We nevertheless demonstrate that fish RBCs are relevant candidates to investigate the
520 stress response at the epigenomic level in the European sea bass. Numerous DMC-related genes
521 reported here have been previously shown to respond to stress or implicated in variation of the
522 stress response in animal models. Overall, our comparative surveys showed that the DMC-
523 related genes we detected in sea bass using RBCs have more connections with results found in
524 the brain than in other tissues. Five DMC-related genes were previously found differentially

525 methylated in sea bass brain by Anastasiadi and Piferrer (2019) and reports from other studies
526 suggest that RBC methylome might integrate epigenetic components of the stress response
527 observed in the brain. This relationship is however weak at the epigenomic level and about one
528 third of our DMC-related genes have also been detected in liver of trout (Gavery et al. 2019).
529 Liver is classically used in transcriptomic and epigenomic studies, may certainly integrate
530 information because of its pivotal role in metabolism, but generally necessitates sacrificing fish.
531 Nevertheless, the relationship is far more pronounced when genome-wide gene expression
532 difference is considered. Indeed, genome-wide neurotranscriptomics studies dealing with
533 personality and stress coping styles (Rey et al. 2013; Wong et al. 2015), or social regulation
534 (Greenwood and Peichel 2015) collectively reported thirty-eight of our 51 DMC-related genes as
535 expressed and potentially associated to the stress response in zebrafish. As differential
536 methylation is classically interpreted as leading to differential gene expression (but see, e.g.,
537 Jones 2012; Lea et al. 2018 for a more nuanced consideration), this observation is one
538 interesting signal indicating that RBCs might monitor events occurring in the brain. However, it
539 remains unclear why most of them are related to coping and personality, and to studies where
540 fish were under stress for few minutes (Rey et al. 2013; Wong et al. 2015), hours or days
541 (Greenwood and Peichel 2015). How distinct stress contexts and challenges may apparently rely
542 on similar stress circuitry has to be investigated further.
543
544 **4 - 3 : A Brain-derived neutrophic factor (BDNF) network**
545 The roles of the DMC-related genes have to be exposed more extensively to illustrate their
546 possible involvement in the stress response of sea bass. This is not place to fully expand on all

547 DMC-related genes (see Suppl. Mat. Table S4 for details), but one single selected example can
548 be developed. The best representative is a set of DMC-related genes from the BDNF network
549 whose role in the stress response is commonly reported. BDNF is a protein synthesized in the
550 brain that offers resistance to neurodegenerative diseases and favors stress adaptation and
551 resilience (Taliaz et al. 2011; Notaras and van den Buuse 2020), but also energy homeostasis
552 (Marosi and Mattson 2013). BDNF has emerged as one of the most important molecule in
553 memory (Beckinschtein et al. 2014; Gonzalez et al. 2019). It consolidates both the within- and
554 between-generation fear memory owing to epigenetic regulation (Lubin et al. 2008; Coley et al.
555 2019). Its activity is strongly linked to glucocorticoid stress to imprint neurogenesis (Numakawa
556 et al. 2017) and it acts as both a regulator and a target of stress hormone signaling (Notaras and
557 van den Buuse 2020). In fish, cortisol binds the glucocorticoid receptor (*gr*) and controls BDNF
558 expression in the brain. With *gr* and relatively few other genes, BDNF has thus emerged as one
559 of the target genes of interest in stress studies, as shown for zebrafish (e.g. Pavlidis et al. 2015),
560 sea bream (Vindas et al. 2018) and sea bass (Tognoli et al. 2010; Sadoul et al. 2018; Samaras et
561 al. 2018; Alfonso et al. 2019). BDNF is also involved in other aspects of the stress response in
562 fish (e.g. regulation of the microbiota-gut-brain axis; Borrelli et al. 2016). Variation in BDNF
563 methylation status could not be investigated in this study as no *SbfI* restriction site is present
564 within or close to this gene. Nevertheless, a methylation signature of stress associated to BDNF
565 is likely present in our data and illustrates how blood methylation may provide a snapshot of
566 events studied in the brain.

567 Indeed, several DMC-related gees are involved in the maturation of proBDNF to mature
568 BDNF or the regulation of its activities (*ABLIM2, ADCY1b, CRTC2, FURIN, NPAS3, PLG,* and

569 possibly *SLC22A2*). Most of these DMC-related genes were already found differentially
570 expressed in the zebrafish brain (Rey et al. 2013; Greenwood and Peichel 2015; Wong et al.
571 2015), but an outline of their interaction was not presented. The adenyl(ate) cyclase (AC,
572 *ADCY1b* gene) is a brain-specific signaling enzyme that synthesizes the cyclic AMP (Sassone Corsi
573 2012). In the brain, AC is activated by PACAP (Protein Adenylate Cyclase Activating Protein) and
574 stimulates memory and long term potentiation (Wang H. et al. 2004). This inducible signaling
575 pathway participates to the synthesis of the active form of BDNF (proBDNF to mature BDNF; Lee
576 and Seo 2014). ProBDNF is processed by furin and the plasminogen system (Marosi and Mattson
577 2013), including processing steps that necessitate actions of actin-binding LIM kinases (ABLIM;
578 Bennett and Lagopoulos 2014). Furin - encoded by *FURIN* - is a subtilisin-like protein
579 proconvertase ubiquitously expressed in fish (Murata and Kinoshita 2015) that presents an
580 intracellular cleaving activity of numerous biologically relevant molecules in the trans-Golgi
581 network. This includes BDNF in the brain, notably in pre-synaptic axons. In mice, furin has been
582 shown to participate to dendrite morphogenesis and modulate learning abilities and memory
583 (Zhu et al. 2018). Stress imprinting at *FURIN* is likely since it has recently been shown that
584 transgenerational epigenetic effects of furin activity were active in brain of mice (Buck et al.
585 2019). ProBDNF cleavage by furin depends on brain AC and CREB (cAMP Response Element-
586 binding protein) signaling (Marcinkiewicz et al. 1996; Chen et al. 2015) and plasminogen (*PLG*;
587 Yeh et al. 2015). This activity itself is modulated by stress hormones (corticosteroids) and is
588 essential to brain hippocampal plasticity in mammals (Pang et al. 2004; McEwen 2008). One
589 interesting supplementary observation is that CREB signaling necessary to furin is associated to
590 *CRTC2* - a CREB co-activator – found as one of the four DMRs reported in this study. In mice,

591 *CRTC2* is known to act as a switch for BDNF and glucocorticoids to direct the expression of
592 corticotropin-releasing hormone (CRH) in the hypothalamus (Jeanneteau et al. 2012).

593 In the brain, plasminogen encoded by *PLG* is converted to plasmin that cleaves BDNF in
594 the extracellular synaptic domain (Gray and Ellis 2008; Marosi and Mattson 2013). *PLG* has also
595 been shown to regulate pro-opiomelanocortin (POMC) in the hypothalamic-pituitary axis, then
596 the production of peptides hormones such as the adrenocorticotropic hormone (ACTH; Wang N.
597 et al. 2004).

598 In relation to BDNF, two other DMC-related genes should be mentioned: *NPAS3*
599 (neuronal PAS domain containing protein 3) and *SLC22A2*. *SLC22A2* - also known as *OCT2*
600 (organic cation transporter 2) – associated to the unique 3'UTR DMC found in this study is
601 implied in numerous transmembrane transports (Liu 2019), including at the blood-brain barrier
602 (Strazielle and Ghersi-Egea 2015). It was found involved in memory in mice (Couroussé and
603 Gautron 2015; Couroussé et al. 2015) or *Drosophila* (Gai et al. 2016), and associated itself to
604 neurodegenerative diseases (Jedlitschky et al. 2014). Substrates of *SLC22A2* include
605 neurotransmitters such as norepinephrine, but also dopamine, serotonin and a wide-variety of
606 internal or exogenous compounds (Bacq et al. 2012; Volk 2014). Functional links of *SLC22A2*
607 with BDNF remain however poorly documented (Kendall et al. 1995), while they have been
608 found co-expressed and co-regulated in the brain during drug administration experiments (e.g.
609 methamphetamine, mimicking the action of catecholamines; Thomas et al. 2004). In this study,
610 *PLG* and *SLC22A2* are associated to the same DMC; the functional significance of this situation
611 should be investigated further. Finally, *NPAS3* has a well-established action in memory (e.g.
612 Erbel-Sieler et al. 2004; Luoma et al. 2018). It participates to neurogenesis in the hippocampus

613 within a network that also includes BDNF (Yang et al. 2016), but is also known associated to the
614 glial cell line-derived neurotrophic factor (GDNF) receptor-alpha2 gene (*GFRA2*) detected in this
615 study and related to stress and anxiety (Lavedan et al. 2009). The *DLG1* (Disk-large homolog 1)
616 gene could be also associated to BDNF, but this will be not develop further (Suppl. Mat. Table
617 S4).

618 Results suggest that even in absence of BDNF sequencing reads, some DMCs/DMRs with
619 a potential impact in fear memory and anxiety response as expected in fish after an acute stress
620 challenge certainly did not occur only by chance in this study. They have also a role in processes
621 that regulate the HPI axis and hormones. The measurement of methylation difference now used
622 to monitor the BDNF activity and its relationship to stress (Uwaya et al. 2016; Coley et al. 2019)
623 could thus potentially depend on the methylation status observed at or close to *ADCY1b,*
624 *ABLIM2, FURIN, CRTC2*, *PLG* and other genes such as *SLC22A2* and *NPAS3*. This should be
625 investigated further to improve our understanding of changes in the directionality of multigenic
626 epigenetic modifications and inter-individual variation in stress coping. Candidate genes coming
627 from other fish studies with role in memory consolidation could be added (see, e.g. Nyman et
628 al. 2020; Yang et al. 2020 for proposals).

629 As at least *ADCY1b, FURIN, CRTC2*, *PLG* and *SLC22A2* have other well-recognized
630 relationships with stress and the stress response (especially the glucose metabolism and the
631 immune function; see Suppl. Mat. Table S4), this example illustrates the complex non-linear
632 network modulating the stress response in fish with a pivotal role in the brain associated to
633 BDNF and secondary additional outcomes in other tissues and organs.

634

635 **4 - 4 : Family effect, development and few individuals**

636 While the PCA showed that pre- and post-stress individuals have in average distinct methylation
637 profiles that may represent stress imprinting during the challenge test, a strong family effect
638 was also detected. Family effects were already reported in fish (e.g. Metzger and Schulte 2018;
639 Xiu et al. 2019; Sagonas et al. 2020), and sea bass families certainly do not have the same
640 sensitivity to stress. This may suggest that (*i*) their genomic backgrounds alter how family will
641 develop an epigenetic stress response over the period covered by the challenge test, (*ii*) that the
642 developmental period during which families were distributed over different tanks was a "critical
643 time window" (e.g. Safi-Stibler and Gabory 2020) sufficient to imprint individuals and lead them
644 to develop a familial response to stress during the challenge, or that (*iii*) some degree of
645 transgenerational inheritance in methylation profiles is present. These are not mutually
646 exclusive. Transgenerational inheritance has gained interest over the last years (e.g. Perez and
647 Lehner 2019), notably in marine species (e.g. Beemelmanns and Roth 2017; Metzger and
648 Schulte 2017; Fallet et al. 2020), also including sea bass (Anastasiadi and Piferrer 2019). Parents
649 and/or the germline were unfortunately not sampled in this study for further analysis. As
650 Gavery et al. (2018) reported that *BTR30*, *DLG1*, *MMRN2*, and *NOL4B* - also found in this study -
651 were differentially methylated in the sperm of *O. mykiss*, these differentially methylated genes
652 could be interesting candidates for transgenerational inheritance study of the stress response.
653 Other candidates are possible. For example, Ghalambor et al. (2015) reported rapid evolution in
654 brain gene expression of the Trinidadian guppy (*Poecilia reticulata*) for the *TNKS* and *NCBP2*
655 genes, found as DMC-related genes in this study. It would thus be interesting to investigate how

656 gene expression and cytosine methylation at these genes may co-vary within and between
657 species over generations.

658 Anastasiadi and Piferrer (2019) showed that the domestication process in *D. labrax*
659 resulted notably in genome-wide methylation differences for genes involved in the nervous
660 system and neural crest cell differentiation. In our study, *GLI2* (e.g. Cerrizuala et al. 2018), *PBX1*
661 (Welsh et al. 2018), *DLG1* (Mostowska et al. 2018) and *FOXJ3* (Landgren and Carson 2004) are
662 DMC-related genes involved in neural crest cell proliferation and differentiation. *ROBO3* is
663 involved in the immune response, but also implied in early neurogenesis (Friocourt and
664 Chédotal 2017), as well as *NPAS3* (Kamm et al. 2013). With *BMP3* (Nie 2005), *FURIN* (Walker et
665 al. 2006); *NOL4B* (Kumari et al. 2018), and *Myf5* (Gustaffson et al. 2002; Lin et al. 2013), most of
666 these genes are engaged in the development of the anterior region and/or the craniofacial
667 skeleton which is modified during sea bass farming (Anastasiadi and Piferrer 2019). This
668 suggests that methylation patterns observed in sea bass could also potentially rely on the
669 ontogenetic regulation of a particular phenotype between pre- and post-stress fish, rather than
670 being directly related to the stress challenge. Variation in gene expression or methylation
671 accompanying ontogeny are reported in fish (Baumgart et al. 2014; Gavery et al. 2019) and, as
672 for other organisms, cytosine methylation is known to vary with age in sea bass (Anastasiadi and
673 Piferrer 2020). Collateral effects of stress – a process sometimes coined as 'epigenetic drift'
674 (Vaiserman 2018) - might thus be present in our data as suggested by the fact that fifteen genes
675 we found differentially methylated in RBCs were reported as also differentially methylated in
676 the liver of the rainbow trout by Gavery et al. (2019).

677 On another hand, the epigenetic profiles of four individuals were analyzed in the pre-
678 and post-stress situations and clustered very closely from each other in each case. While based
679 on few observations, this suggests that neither the stress challenge itself, nor development have
680 a strong impact on the cytosine methylation landscape in sea bass. How a transgenerational and
681 within-generation stress-imprinting events influenced by ontogeny may interact to shape both
682 the plastic and the heritable component of the stress response in relation to environmental
683 stimuli require in depth evaluation (Burggren 2015; Trollope et al. 2017). Nature and strength of
684 family-based epigenomic variation are of considerable importance for selection breeding
685 improvements in a cultured fish like sea bass (Houston et al. 2020), including also issues about
686 health and welfare (Triantaphyllopoulos et al. 2016).
687
688 **4 - 5 : Blood and RBCs : extending the perspective**
689 As fish engaged in aquaculture programs as sea bass are costly and based on breeders that
690 cannot be easily sacrificed, epiGBS using blood samples may offer a genome-wide assessment of
691 stress-induced epigenetic marks over a significant number of individuals and at reasonable cost.
692 Blood is widely used in human epigenomics (e.g. Houseman et al. 2015), but not in fish despite
693 the presence of nucleated cells and minimally invasive sampling that allows for multiple
694 measurements. Using RNA-sequencing, Morera et al. (2011) or Dahle et al. (2015) already
695 suggested that fish erythrocytes would be useful to provide insights on the innate immunity or
696 response to pathogens. Our study suggests that blood could effectively offer an in depth insight
697 for the evaluation of the stress response from a novel perspective, accompanied by classical

698 physiological measurements (e.g. cortisol, glucose, lactate) and may provide a more complete
699 picture of the stress response in fish than currently performed.

700 The link between brain and blood epigenomics remains however to be explored more
701 deeply in fish and requires careful evaluation and validation to correct for tissue specificity, as
702 requested in human (Lauschke et al. 2017). Our attempts to survey fish studies undoubtedly
703 showed that considerably more knowledge has to be accumulated in fish epigenomics before to
704 reach this issue. In human, the lack of access to brain tissues have been shown to impede some
705 epigenomic studies and naturally raised ethical issues certainly also valid for fish (Barr and
706 Misener 2016; Bunnik et al. 2020). *In situ* hybridization techniques in brain have been shown to
707 be especially important to link neurobiological activities and stress coping in fish (Vindas et al.
708 2017, 2018). Their use and thus sacrifice of individuals cannot be ruled out. Actually,
709 methylation differences among full brain or brain areas have been rarely studied in fish, mostly
710 in zebrafish (e.g. Zhang et al. 2016), but recently in a tilapia (Hilliard et al. 2019), a goby
711 (Sommerville et al. 2019), and a killifish (Berbel-Filho et al. 2020).

712

713

714 5 - Conclusion and future directions

715

716 With the works of Anastasiadi and co-workers (2017, 2018, 2019, 2020) and others (Navarro-
717 Martín et al. 2011; Geay et al. 2012; Terova et al. 2016), the European sea bass has become one
718 of the most studied species in fish epigenetics. Because of the availability of its genome and the
719 development of selection programs, efforts should be dedicated to further explore its

720 epigenomic landscape regarding the stress response, especially in an aquaculture context. This
721 should be done over a multigenerational scale to identify pathways structuring the stress
722 response at key life stages, on different tissues to better delineate stress-responsive cellular
723 networks, and investigating the effects of the nature, the duration, and the number of stressors.
724 Studies in epigenomics including phenotypic information (e.g. Birney et al. 2016; Lin et al. 2016)
725 could increase the panel of diagnostic tools for breeding selection in aquaculture if blood could
726 be – at least partly - substituted to brain or other tissues when studying the stress response.
727 Screening for genome-wide epigenomic variation in natural populations as already done for
728 genomic variation (Duranton et al. 2018) is now also conceivable in this species. Our modified
729 version of the original epiGBS protocol seems to be a powerful and affordable method to screen
730 a significant number of individuals with sufficient depth and coverage to reach meaningful
731 conclusions. While it should be compared to others (e.g. Wang et al. 2013; Suzuki et al. 2018),
732 our protocol is close to the double digestion restriction site DNA sequencing protocol used in
733 genomics. Their parallel use certainly deserves attention to design more integrated epigenomic-
734 genomic studies (e.g. Prudencio et al. 2018; Crotti et al. 2020), and more generally multi-omics
735 investigations of stress, health and welfare (Suravajhala et al. 2016; Schwartz 2020).
736
737

738 **Acknowledgements**

739 This work was funded by the EU ERA-NET FP7 programme for the Cooperation in Fisheries,
740 Aquaculture and Seafood Processing (COFASP) and the Agence Nationale pour la Recherche
741 (ANR-16-0001-COFA). Data used in this work were partly produced through the GenSeq

742 technical facilities at ISEM and benefited from the Montpellier Bioinformatics Biodiversity
743 (MBB) platform supported by the LabEx CeMEB, both supported by ANR “Investissements
744 d’Avenir" program (ANR-10-LABX-04-01). MGX acknowledges financial support from the France
745 Génomique National infrastructure, also funded as part of “Investissement d’Avenir” program
746 managed by ANR (ANR-10-INBS-09). We thank N. Wagemaker at the Radboud University
747 (Nijmegen, The Netherlands) for his support when implementing and developing the lab work,
748 and Prof. C. Grunau at the University of Perpignan Via Domitia (France) for hosting M.V.K at
749 various steps of this work. S. Ben Chehida greatly helped during the last stages of manuscript
750 preparation.

751

752

753 **Conflicts of interest**

754 None declared

755

756 **Ethics statements**

757 All experiments were performed in accordance with relevant guidelines and regulations. Nireus
758 S.A. research facilities are certified and have obtained the codes for the rearing and use of fish
759 for scientific purposes (EL04-BIOexp-01). All procedures on fish used in this study were
760 approved by the Departmental Animal Care Committee following the Three Rs principle, in
761 accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use
762 of experimental animals. All sample procedures were performed by FELASA certified researchers
763 (http://www.felasa.eu/).

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1228

1229 ## Data accessibility

1230 Individual Illumina raw reads (one round of trimming) and processed files are currently under
1231 submission to NCBI as a GEO project submission and a sequence read archive (SRA). Deposits
1232 will be completed during the submission to the journal.

1233

1234 ## Author contributions

1235 MVK, ED, BG developed the protocol, MVK and ED conducted the lab work, EG ensured tech-
1236 nical assistance and supervised sequencing. AS, AD, MP and CST established the study design of
1237 the challenge test. Experiments with live fish were conducted by AD, AS, MP, with contribution
1238 of MVK. MVK performed data analyses under supervision of ED and BG. MVK, ED, BG drafted
1239 the manuscript; others authors reviewed drafts. CST and BG coordinated this study.

Caption of the Table

Table 1: Location on the European sea bass linkage groups (LGs) of the 57 differentially methylated cytosines (DMCs) found in this study between pre- and post-stress individuals. The four differentially methylated regions (DMRs) are highlighted in grey. For each DMC, the false-discovery rate adjusted *q*-values at the nominal *q* = 0.001 cut-off threshold are reported, together with their methylation difference. Gene names and gene symbols (IDs) of DMC-related genes (*n* = 51) are reported. The location of each DMC is given (*gb*: gene body, *int.*: intergenic, 3'UTR or *rr*: repeat region). We did not arbitrarily defined promoter regions in this study. An extended version of this table reporting annotations and further useful information are offered in Suppl. Mat. Table S3.

Caption of the Table

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1264 ## Captions of the Figures

1265

1266 **Figure 1:** Coverage of the 24 linkage groups (LGs) of the sea bass genome by sequencing epiGBS
1267 reads. Ordinates represent the mean number of sequencing reads. LG numbers are those used
1268 by Tine et al. (2014). Complex IDs resulting from the merging of previously defined LGs during
1269 final assembly (e.g. LG22-25 resulted in the fusion of previous LG22 and LG25). SB-UN (sea bass
1270 – unknown; hereby SB) indicates a composite LG made from originally unassembled scaffolds
1271 (Tine et al. 2014).

1272

1273 **Figure 2:** Diagrammatic representation of the involvement of the 51 DMC-related genes found
1274 in this study and issues regarding the stress response (see main text for detailsOne additional
1275 issue was added for few genes that are involved in the regulation, the control and the dynamics
1276 of epigenetic process (*NCBP2, GTPF*), and acid-base homeostasis in kidney without formal proof
1277 of actual relation with a stress issue (*CLDN4*). DMCs located within gene bodies are indicated in
1278 black, in brown for intergenic regions including promoters. The hatched symbols refer to genes
1279 also reported in high-throughput transcriptomic studies in zebrafish (main text and Suppl. Mat.
1280 Table S4). This does not mean that our literature survey at DMC-related genes was supported
1281 only by these studies, but it illustrates their importance over results. Stars with full line indicate
1282 genes detected as differentially expressed (*BTR30, GLI2*) in one immunity-related transcriptomic
1283 study in sea bass (Chavez-Pozo et al. 2019), while stars with broken lines indicate genes with
1284 methylation differences (*PTPRB, SASH1A*) (stickleback: Sagonas *et al.* 2020; rainbow trout: Uren-
1285 Webster et al. 2018; respectively). Reports outside of the above-mentioned studies are

1286 indicated by filled symbols. On the right are indicated genes found differentially expressed (RNA)
1287 or differentially methylated (CpG) in the genome-wide study by Anastasiadi and Piferrer (2019)
1288 (*A&P 2019*) on European sea bass. Tissues in which genes were found differentially
1289 expressed/methylated are indicated (brain not investigated in transcriptomics). Association with
1290 the *LRRC3* gene is reported in grey as leucine-rich repeat (LRR) gene family is classically involved
1291 in neuroimmunity. Variations in transcription or methylation have been shown to impact
1292 behaviours after stress events within this family (see e.g. *LRRTM4L2* in this study). However,
1293 *LRRC3* is amongst the most poorly known gene within this family (Söllner and Wright 2009), and
1294 this remains presumptive. Additional support including references for these DMC-related genes
1295 is given in Supplementary Materials Table S4, as well as comparisons with other epigenomic
1296 studies.
1297
1298 **Figure 3:** Hierarchical clustering based on of the 57 differentially methylated cytosines (15%
1299 threshold) of the randomly caught 74 pre- and post-stress sea bass samples considered during
1300 this study. Nineteen out the twenty families (A to T) used in this study are present in this graph
1301 and each family is associated to one single colour. Capital letters refer to the family, while T0
1302 and T4 are for pre- and post-stress individuals, respectively.
1303
1304 **Figure 4:** PCA based on the methylation profiles of the 57 differentially methylated cytosines
1305 (15% threshold) reported in this study (Table 1). Pre- and post-stress individual sea bass (in red
1306 and blue, respectively) differ significantly along PC1 ($p < 0.001$), but not PC2. The insert illustrate

the distribution of individual scores along PC1. Ellipses represent the 95% confidence limits over PC1 and PC2.

1311 **Table 1**

D. labrax LG	Ensembl LG	*q* -value	methylation difference (%)	*D. labrax* Name	Gene ID	Gene Name	Location
LG1A	HG916837.1	0	-21,40	DLAgn_00094580	*FOXI3*	forkhead box protein I3	*gb*
LG1A	HG916837.1	0	-21,67				*gb*
LG1A	HG916837.1	0	-15,51				*gb*
LG3	HG916843.1	1,54E-95	18,14	DLAgn_00141440	*ABLIM2*	actin binding LIM protein family -- member 2	*gb*
LG4	HG916844.1	1,08E-84	16,41	DLAgn_00145290	*CELA3b*	proproteinase E-like (elastase 2)	
LG4	HG916844.1	0	21,38	DLAgn_00146200	*NCBP2*	nuclear cap-binding protein subunit 2	*int.*
LG5	HG916845.1	3,67E-265	21,91	DLAgn_00159160	*CILP1*	cartilage intermediate layer protein 1	*gb*
LG5	HG916845.1	8,93E-89	16,64	DLAgn_00159900	*GRPT1a*	growth hormone regulated TBC protein 1	*ge*
LG6	HG916846.1	6,44E-245	20,44	DLAgn_00164450	*GLG1*	golgi glycoprotein 1	*gb*
LG6	HG916846.1	0	-21,01	DLAgn_00166360	*GDPGP1*	gdp-d-glucose phosphorylase 1	*exon*
LG6	HG916846.1	0	-18,98	DLAgn_00172370	*KBTBD13*	kelch repeat and btb domain-containing protein 13-like	*int.*
LG6	HG916846.1	0	-18,18				*int.*
LG6	HG916846.1	0	-16,12				*int.*
LG8	HG916848.1	2,77E-247	22,52	DLAgn_00186250	*BTPF*	nucleosome-remodeling factor subunit bptf	*gb*
LG10	HG916827.1	2,44E-79	15,44	DLAgn_00005890	*PBX1a*	pre-B-cell leukemia homeobox 1	*gb*
LG10	HG916827.1	6,98E-125	21,55	DLAgn_00006210	*ADCY1b*	adenylate cyclase 1 (brain)	*gb*
LG11	HG916828.1	0	27,70	DLAgn_00016970	*MMNR2a*	multimerin 2a Precursor Elastin Microfibril Interface Located Elastin Microfibril Interfacer	*gb*
LG12	HG916829.1	0	22,32	DLAgn_00019700	*FBXO33*	F-box protein 33	*int.*
LG12	HG916829.1	1,23E-128	20,01	DLAgn_00025280	*SASH1A*	sam and sh3 domain-containing protein 1-like	*int.*
LG14	HG916831.1	1,48E-53	15,74	DLAgn_00037440	*COL4A5*	collagen type IV alpha 5 chain	*int.*
LG14	HG916831.1	0	42,05	DLAgn_00037440	*COL4A5*	collagen type IV alpha 5 chain	*gb*
LG14	HG916831.1	0	22,46	DLAgn_00038760	*ROBO3*	roundabout homolog 2-like	*int.*
LG14	HG916831.1	5,22E-212	17,93	DLAgn_00044110	*CLDN4*	claudin 4	*gb*
LG14	HG916831.1	1,25E-155	27,48	DLAgn_00046110	*TMEM132E*	transmembrane protein 132e	*gb*
LG16	HG916833.1	2,78E-168	26,24	DLAgn_00063590	*CSMD3a*	CUB and Sushi multiple domains 3a	*gb*
LG16	HG916833.1	9,78E-92	17,12	DLAgn_00063770	*TRMT11*	tRNA methyltransferase 11 homolog	*int.*
LG16	HG916833.1	8,81E-215	17,80	DLAgn_00064610	*FURIN*	furin-like protease kpc-1	*gb*
LG16	HG916833.1	0	17,76	DLAgn_00064820	*SPIRE1b*	protein spire homolog 1-like	*gb*
LG17	HG916834.1	1,91E-82	18,84	DLAgn_00073160 ; DLAgn_00073170	*PLG* (sense); *SLC22A2* (antisense)	Plasminogen (sense) / Solute carrier family 22 member 2-like (antisense)	*gb / 3'UTR*
LG17	HG916834.1	1,28E-231	19,86	DLAgn_00073770	*RRM2*	ribonucleotide reductase regulatory subunit M2	*gb*
LG20	HG916840.1	2,76E-207	15,01	DLAgn_00114460	*TNKSb*	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase b	*gb*
LG20	HG916840.1	1,34E-115	15,35	DLAgn_00114810	*LRRTM4L2*	leucine-rich repeat transmembrane neuronal protein 4-like	*gb*
LG20	HG916840.1	3,78E-68	15,32	DLAgn_00116160 ; DLAgn_00116150	*FICD* (sense) ; *SART3* (antisense)	Adenosine monophosphate-protein transferase ficd-like (sense) / Spliceosome associated factor 3, U4/U6 recycling protein (antisense)	*gb*
LG20	HG916840.1	1,02E-88	18,04	DLAgn_00121270		Coding region of a truncated Non LTR Retrotransposable Element (RTE) RET-1_AFC	*rr*
LG20	HG916840.1	0	35,61	DLAgn_00122640	*BMP3*	Bone morphogenetic protein 3	*gb*
LG20	HG916840.1	0	15,40	DLAgn_00124110	*ZMAT4b*	zinc finger matrin-type 4b	*gb*
LG22-25	HG916841.1	7,62E-96	16,89	DLAgn_00125750	*NOL4Lb*	nucleolar protein 4-like b	*gb*
LG22-25	HG916841.1	3,26E-99	15,05	DLAgn_00132500	*CCDC30*	coiled-coil domain containing protein 30 like	*gb*
LG24	HG916842.1	2,81E-43	-15,63	DLAgn_00136190	*GLI2b*	zinc finger protein gli2-like	*gb*
LG24	HG916842.1	2,87E-112	16,47	DLAgn_00136980	*LRRC3*	leucine rich repeat containing 3	*int.*
LG24	HG916842.1	0	30,68	DLAgn_00137190	*UNC80*	Protein unc-80 homolog isoform 2	*gb*
LG24	HG916842.1	0	15,42	DLAgn_00137560	*CHN1*	N-chimerin	*gb*
LG24	HG916842.1	9,47E-204	17,08	DLAgn_00139980	*PTGFRN*	prostaglandin f2 receptor negative regulator	*gb*
LGx	HG916850.1	4,18E-251	19,94	DLAgn_00209310	*MYF5*	myogenic factor 5	*gb*
LGx	HG916850.1	4,52E-204	23,46	DLAgn_00209760	*CELF2*	cugbp elav-like family member 2	*gb*
SB-UN	HG916851.1	1,61E-152	16,29	DLAgn_00218300	*PRKCQ*	protein kinase c theta type	*int.*
SB-UN	HG916851.1	1,09E-120	16,34	DLAgn_00220000	*PTPRB*	protein tyrosine phosphatase receptor type B	*gb*
SB-UN	HG916851.1	6,07E-154	18,95	DLAgn_00222790	*NPAS3*	neuronal PAS domain-containing protein 3-like	*gb*
SB-UN	HG916851.1	0	-21,55	DLAgn_00227120	*CRTC2*	CREB regulated transcription coactivator 2	*gb*
SB-UN	HG916851.1	6,11E-56	15,79				*gb*
SB-UN	HG916851.1	4,15E-61	17,53	DLAgn_00227130	*DENND4B*	Denn domain-containing protein 4b	*gb*
SB-UN	HG916851.1	1,51E-46	15,84	DLAgn_00236500	*GFRA2*	GDNF family receptor alpha-2	*gb*
SB-UN	HG916851.1	0	46,39	DLAgn_00238280	*DLG1*	disks large homolog 1-like	*gb*
SB-UN	HG916851.1	9,43E-157	26,15	DLAgn_00242570	*BTR30*	E3 ubiquitin-protein ligase TRIM39-like (bloodthirsty-related gene family, member 30)	*gb*
SB-UN	HG916851.1	6,99E-209	-17,71				*gb*
SB-UN	HG916851.1	2,11E-216	-27,49	DLAgn_00244380	*GMPPB*	GDP-mannose pyrophosphorylase B	*exon*
SB-UN	HG916851.1	5,09E-256	20,97	DLAgn_00245980	*MATR3*	matrin-3	*exon*

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1313 **Figure 1**

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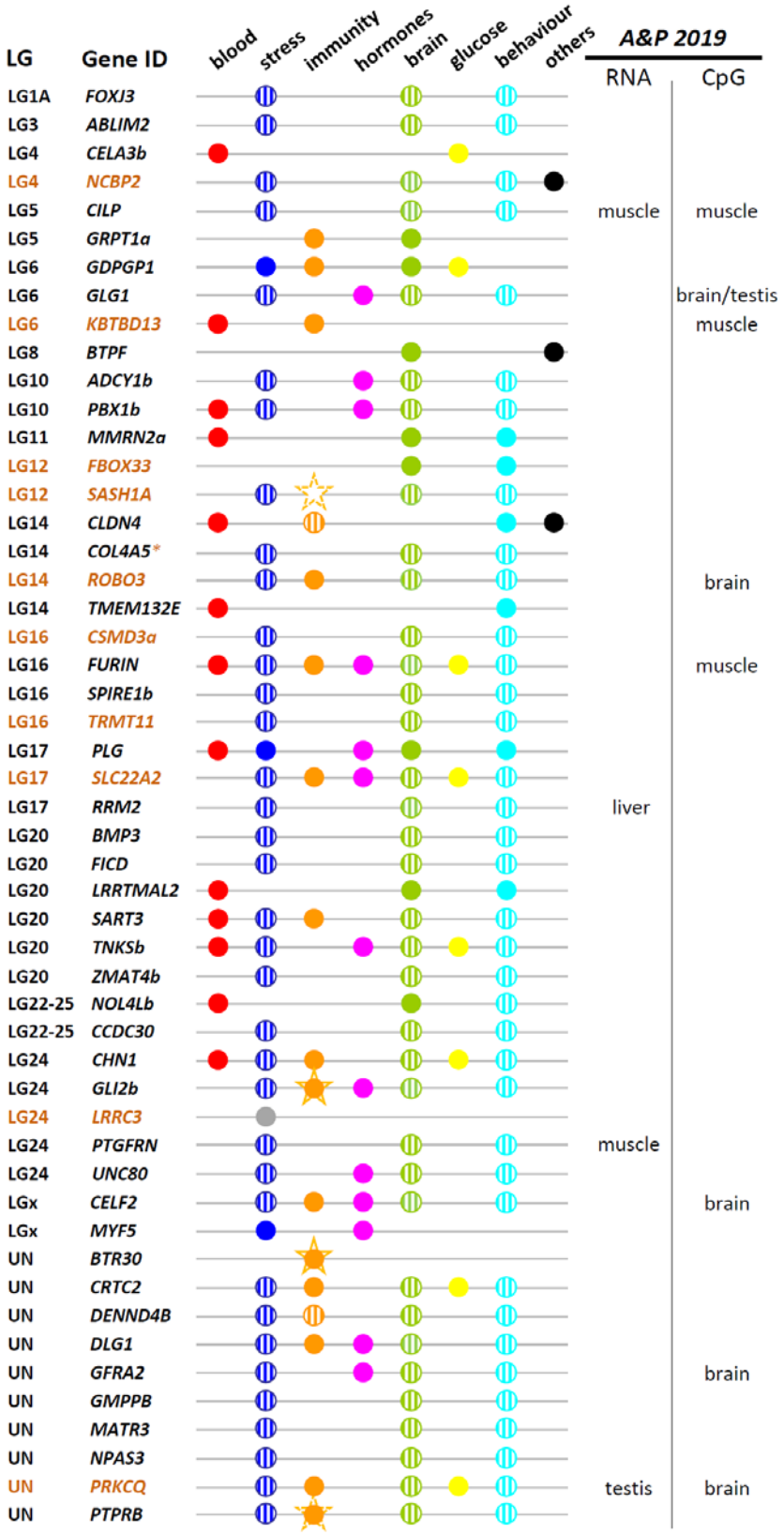
1317 **Figure 2**



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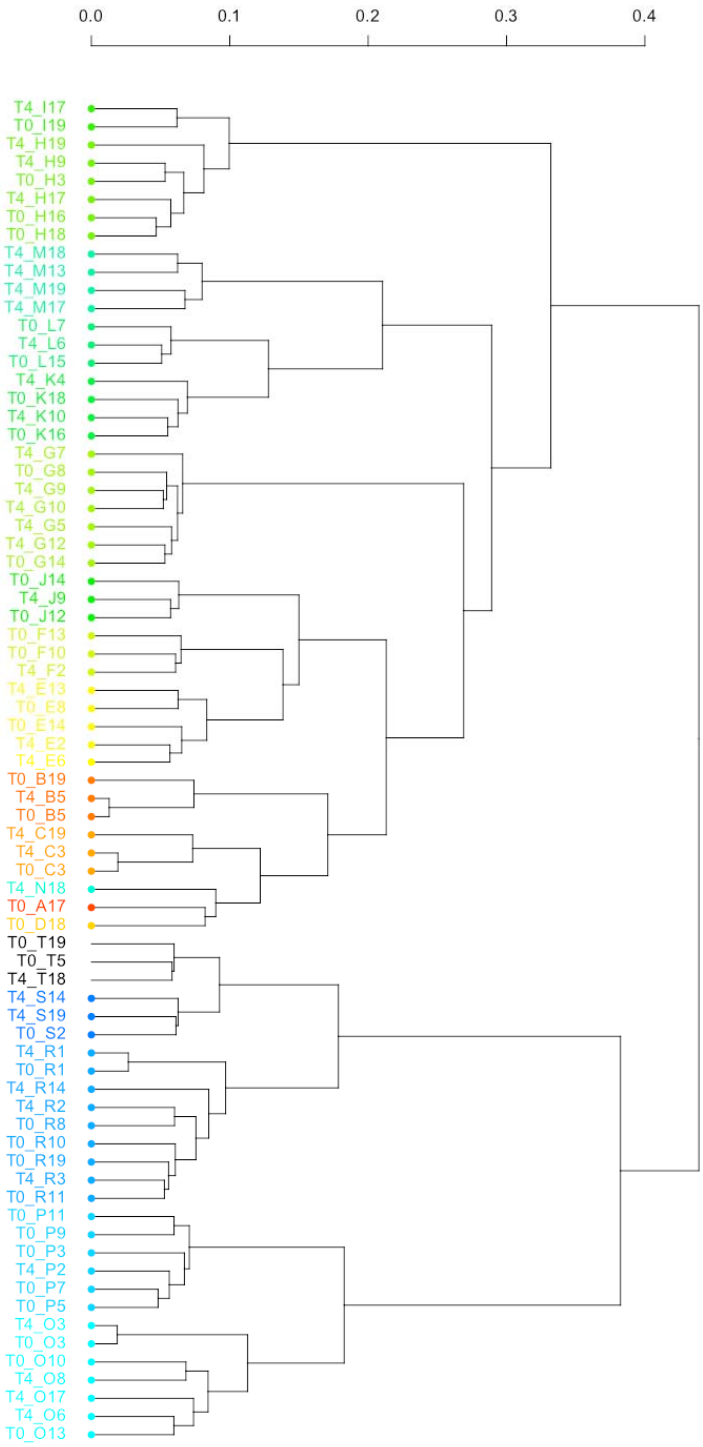
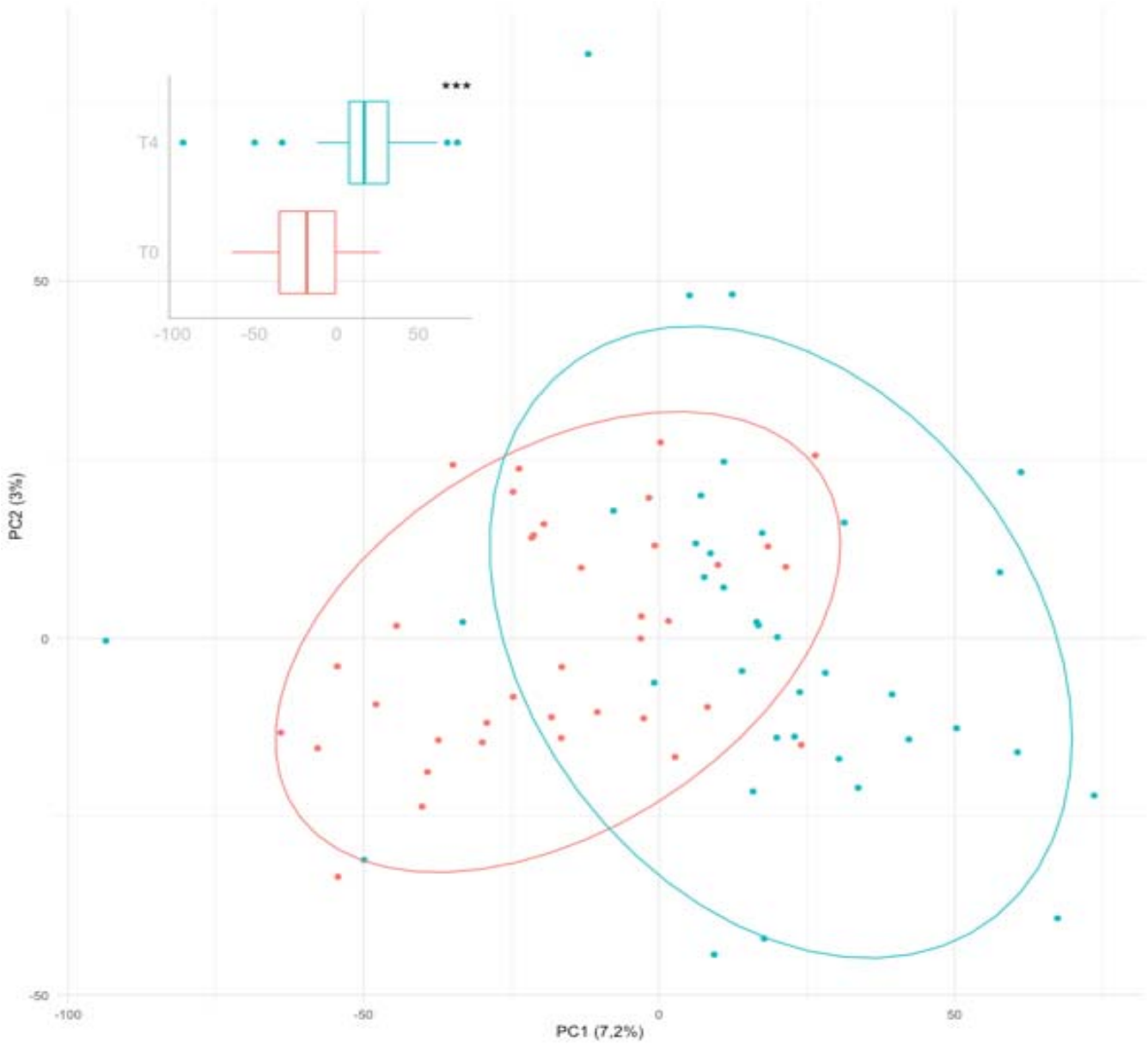
1319 **Figure 3**



1320

1321 **Figure 4**

1322



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