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

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Abstract

Understanding the molecular basis of stress is crucial in biology and of long standing interest in 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 three months 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% for unique best hits. Sequencing reads were shown to map across all linkage groups (LGs) of sea bass. 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 strong family effect was demonstrated, and fifty-seven distinct differentially methylated cytosines (DMCs) distributed on 17 of 24 LGs were found between RBCs of pre- and post-stress individuals. Twelve of them were located in intergenic regions, one in a repeated element; and forty four in gene bodies. Overall, DMCs were found inside or in the vicinity of 51 distinct genes shown previously to be related to stress. 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 in sea bass, 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, especially brain, to provide with classical physiological measurements a more complete picture of the stress response in fish.

46 # 1 - Introduction

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

60 The study of stress is complex as it may differ in nature, intensity and/or duration, plus
61 number of stressors. This poses many challenges that remain to be overcome (Romero et al.
62 2015) and stress studies have to integrate physiological, immunological, neurobiological and
63 behavioural components that altogether shape the stress response in fish (Pottinger 2008; Ellis
64 et al. 2012; Nardocci et al. 2014; Castanheira et al. 2017). The stress response implies, among
65 others, a primary neuroendocrine and a secondary immune response, with interacting roles of
66 neuropeptides and cytokines in each respective system (Wendelaar-Bonga 1997; Yada and
67 Nakanishi 2002; Tort 2011; Geven and Klaren 2017). The Hypothalamus-Pituitary-Interrenal

68 (HPI) axis plays a crucial role in the manifestation and regulation of the stress response through
69 the release of neuropeptides, catecholamines and glucocorticoids. In teleosts, cortisol is the
70 primary circulating glucocorticoid and species-specific differences in the timing, magnitude and
71 peak duration of post-stress plasma cortisol concentrations have been reported (Fanouraki et al.
72 2011). Glucose - another classical biomarker of stress - is also under control of hypothalamus,
73 and its blood level depends on glycogen metabolism as well as gluconeogenesis, themselves
74 triggered by interrenal glands and catecholamines (Gamperl et al. 1994; Mommsen et al. 1999).
75 Beyond the classical HPI axis, several mediators of allostasis, organs and systems (e.g. serotonin
76 and the serotonergic system; osteocalcin and the parasympathetic nervous system) are involved
77 in stress and anxiety (Backström and Winberg 2017; Foster et al. 2017; Berger et al. 2019). A
78 proper regulation of the stress response requires the consideration of a complex regulatory
79 network of non-linear actions to integrate the whole organism response to stressors (McEwen
80 2008; Gray et al. 2017).

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

90 (Houseman et al. 2015). As blood cells are also mobilized as part of the stress response in fish
91 (Tort 2011; Burgos-Aceves et al. 2019), it might be appealing to investigate if an epigenomic
92 signature of stress is present in their nucleated red blood cells (RBCs). Indeed, the use of RBCs
93 in fish transcriptomics or proteomics has received attention over the last decade (Morera et al.
94 2011; Puente-Marin et al. 2018). However, fish epigenetics has not embarked on this path yet
95 and genome-wide epigenetic studies using RBCs are quite rare in fish (Gavery et al 2018). This
96 contrasts with the over-increasing interest to investigate epigenomic variation in teleosts (e.g.
97 zebrafish: Chatterjee et al. 2016; Olsvik et al. 2019; medaka: Wang and Bhandari 2019; tongue
98 sole: Shao et al. 2014; killifish: Berbel-Filho et al. 2020; salmonids: Le Luyer et al. 2017; Boltana
99 et al. 2018; Gavery et al. 2018, 2019; stickleback: Metzger and Schulte 2017, 2018; Heckwolf et
100 al. 2020; tilapias: Wan et al. 2016; Hilliard et al. 2019; carp: Zhang et al. 2019; guppy: Hu et al.
101 2018; striped bass: Woods et al. 2018; wrasse: Todd et al. 2019; goby: Sommerville et al. 2019).
102 Furthermore, documenting more directly the epigenomics of the stress axis/network also
103 remains scarce (Uren-Webster et al. 2018), while Li and Leatherland (2013) or Faught et al.
104 (2016) asked for greater attention. Only few epigenomic studies dealt explicitly with a specific
105 component of the stress response (e.g. immune function: Hu et al. 2018, Uren-Webster et al.
106 2018), while others are more elusive (e.g. '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 and distributed in 20 tanks; each tank receiving 1 fish from each family

134 (i.e., 20 fish per tank). Fish were fed twice a day, for 6 days a week, using a commercial diet
135 (Blue Line 45:20 3.5 mm, Feedus S.A., Greece). Throughout the experimental period, the
136 photoperiod was set at 12L : 12D, the water temperature and the salinity held constant (18.1 ±
137 0.2°C and 28 ppt, respectively). Fish weights (mean ± SD) were 48.1 ± 12.8g and 86.2 ± 24.1g in
138 pre- and post-stress individuals, respectively.

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

147 An initial blood sampling occurred two weeks prior to the implementation of the stress
148 challenge (hereafter T0, July 19th, 2018, pre-stress/control group), then at the end of the
149 challenge test (hereafter T4, October 5th, 2018). Blood from the caudal vessel was collected.
150 Plasma and RBCs were separated by centrifugation at 2,000g for 10 min. Careful separation of
151 the plasma and RBCs was performed using 200 µl pipettes. RBC extracts were heparinized
152 (heparin sodium; Sigma-Aldrich), transferred in microtubes and conserved at -20°C in 1 ml of
153 RNA later. DNA was extracted using the Macherey Nagel Nucleo Spin Tissue DNA kit and
154 quantified using a Qubit fluorometer (Qubit dsDNA BR Assay Kit, Q32853, Invitrogen). Thirty-

155 seven blood samples at T0 (pre-stress) and thirty-seven additional samples at T4 (post-stress)
156 have been randomly selected for the downstream epigenomic analysis.
157
158 **2 - 2 : Library preparation and sequencing**
159 We followed the epiGBS protocol published by van Gurp et al (2016). As the method was
160 developed for plants (with methylation occurring in CpG, CHH, and CHG context; H being any
161 nucleotide but a cytosine) and used a single digestion approach, the protocol was modified to
162 make it more suitable and straightforward for our vertebrate system. Particularly, a double
163 digest instead of the single digest approach was implemented. We chose the restriction enzyme
164 *MspI*, a standard choice in reduced representation bisulfite sequencing-like (RRBS) studies, as its
165 recognition site targets CpG rich regions (Gu et al. 2011). A second enzyme (*SfbI*) with a
166 recognition site length of 8 bp was used to reduce fragment numbers, and thus to increase read
167 depth per fragment. The choice of this enzyme was guided by *in silico* digestion of the European
168 sea bass genome (Tine et al. 2014) using *simRAD* (Lepais and Weir 2014). This genome is
169 available at: https://www.ensembl.org/Dicentrarchus_labrax/Info/Index.
170 One single library was prepared for a set of 74 samples. For this library, 200 ng of DNA of
171 each sample were digested in a 40 µl reaction, using 0.25 µl *MspI* (NEB, 20,000 U/ml R0106S),
172 0.25 µl S*bfI*-HiFi (NEB 20,000 U/ml R3642L) and 4 µl of 10X cutsmart buffer. The reaction was
173 run overnight at 37°C. Unique forward and reverse adapter combinations allow multiplexing
174 samples in the library. We added forward and reverse adapters in unique combinations (1 µl of
175 adapter, 2.5 µM), 0.5 µl T4 Ligase (NEB, 400,000 U/ml m0202L), 6 µl of 10X T4 ligase buffer and
176 11.5µl water were added directly to the digested DNA. Sequences of adapters are provided as

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

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

198

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; Krueger 2012). The trimmed reads were then demultiplexed using the
202 `process_radtags` command of STACKS (Catchen et al 2013). The option
203 `disable_rad_check` was applied to avoid reads with bisulfite-modified restriction sites to
204 be discarded. The demultiplexed reads were trimmed a second time on both 5' and 3' ends with
205 the options `--clip_R1` `--clip_R2` and `--three_prime_clip_R1` `--`
206 `three_prime_clip_R2` to remove introduced methylated cytosines during adapter ligation.
207 Using the `bismark_genome_preparation` function of the program *bismark* (Krueger &
208 Andrews 2011), a bisulfite converted version of the European sea bass genome was prepared
209 against which the demultiplexed and trimmed reads were mapped.

210

211 **2 - 4 : Methylation analysis**

212 The R package MethylKit (Akalin et al 2012) was used to determine differential methylation
213 between pre- and post-stress fish. CpGs with less than 30 read depth and with coverage >99.9%
214 of the distribution of read counts were filtered out to account for PCR bias. Coverage was
215 normalized across samples, and only CpGs present in at least 20 out of 37 samples per group
216 (56%) were kept for further analysis. Differentially methylated CpG sites were determined
217 between pre- and post-stress individuals using logistic regression (`calculateDiffMeth`
218 function). Pre-stress individuals were considered as the baseline. Cytosines were considered as
219 differentially methylated when presenting at least 15% methylation difference (as in Anastasiadi
220 and Piferrer 2019 for epigenomic variation in *D. labrax*), and a nominal *q*-value < 0.001 (false

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

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

263 A second literature survey focused on the literature dealing with stress, stress response
264 and high-throughput transcriptomics or epigenomics in fish. We searched PubMed for relevant

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

283 Finally, in order to complete these manually curated surveys, we used STRING
284 (https://string-db.org/) to investigate if DMC-related genes encoded for proteins are known to
285 interact together. We used our gene list as input and the zebrafish genome as a reference for
286 annotation. This search was done without reference to the thematic stress categories

287 mentioned above, and when one interaction was provided we specifically explored the
288 literature for confirmation and relevant experimental evidence.
289
290 **2 – 6 : Data analysis**
291 A principal component analysis (PCA) on the methylation profiles of individual samples at DMCs
292 was performed to analyse the potential grouping structures within our data set. Differences in
293 the distribution of mean individual loading scores for pre- and post-stress fish were tested along
294 each principal components axis using a Student *t*-test. Additionally, a hierarchical clustering was
295 performed using Ward's linkage method on Euclidean distances in order to explore whether sea
296 bass families structure the data set.
297
298
299 **3 - Results**
300
301 Nineteen out of 20 families were represented by at least one individual among the 74 samples
302 analyzed in this study. Fish number per family ranged from one (families A, D, N) to nine (family
303 R) individuals. Except for the families with a single representative and family M with post-stress
304 fish only (four), both pre-stress and post-stress individuals were present in the 15 remaining
305 families. Four individuals from four distinct families were retained twice by chance, and were
306 thus analyzed for both pre- and post-challenge stress conditions. A total of 70 distinct fish has
307 been analyzed in this study.
308

309 **3 - 1 : epiGBS library construction and sequencing**

310 We obtained 504,271,331 total sequencing reads of which 99.4% (501,108,033) were retrieved
311 after trimming. After demultiplexing, read numbers per sample ranged from 2,284,915 to
312 16,314,759, with an average of 5,212,596 reads per sample (see Suppl. Mat. Table S2).
313 Demultiplexed samples were mapped against the *D. labrax* reference genome with a mean
314 mapping efficiency of 74.5% (73.0% for unique best hits; Suppl. Mat. Table S2). Sequencing
315 reads mapped across all linkage groups (Fig. 1). The mean per base pair read depth was 250X.

316

317 **3 - 2 : Methylation analysis**

318 Out of the 10,368,945 CG dinucleotides (0.46% of the whole) present in the *MspI-SbfI* reduced-
319 representation of *D. labrax* genome we obtained, 47,983 CpG coordinates were extracted with a
320 minimum of 30X read depth and presence in at least 20 individuals. They were filtered out using
321 a 15% methylation difference threshold and a nominal cut-off value of $q < 0.001$. With these
322 parameters, only a total of 57 cytosines in CpG context were defined as DMCs between pre- and
323 post-stress sea bass (Table 1). Methylation differences ranged from -27.5% to -15.0% for
324 hypomethylated cytosines and 15.0% to 46.4% for hypermethylated cytosines. Hyper-
325 methylation was more frequently detected than hypomethylation (11 [19.30%] hypo- *vs* 46
326 [80.70%] hypermethylated DMCs) in post-stress sea bass. DMCs were distributed on 17 out of
327 24 LG groups and in or close to 51 distinct genes. Further information is provided in Suppl. Mat.
328 Table S3 (e.g. gene annotations, CpG context).

329 Most identified DMCs were found located within identified gene bodies (44 out of 57,
330 77.19%), one in the 3'UTR regions of the Solute Carrier family 22 Member 2 (*SLC22A2*) gene on

331 LG17, and one in a repeated region (a non LTR Retrotransposon Element on LG20). In the
332 remaining cases (*n* = 11), DMCs are intergenic and located in a window ranging from 0.9kb to
333 51kb to the closest gene (respectively: *SASH1A* on LG12 and *TRMT11* on LG16; Table 1). Two
334 pairs of overlapping, but inversely oriented genes share on their sense vs. antisense strand an
335 identical DMC: *PLG* and *SLC22A2* on LG17, and *SART3* and *FICD* on LG20 (Table 1)

336 When located on the same LG, DMCs were usually distant by at least 30kb from each
337 other. In only four instances, DMCs occurred as differentially methylated regions (DMRs) that
338 grouped 2-3 DMCs separated by only few hundreds of base pairs (Table 1). This includes three
339 hypo-methylated cytosines grouped in a DMR located ~1500 bp downstream of the predicted
340 Kelch Repeat and BDB domain 13 (*KBTBD13*) gene with at most 88 bp between the cytosines.
341 Another DMR includes three hypomethylated cytosines (>20%) on LG1A in the second intron of
342 the forkhead box J3 (*FOXJ3*) gene. Two other groups of two cytosines were found either in the
343 same exon (distant by 3 bp) of the *BTR30* gene or in two different introns (distant by 3.8Kb) of a
344 predicted CREB regulated transcription coactivator 2 (*CRTC2* or *TORC2*: Transducer Of Regulated
345 cAMP Response Element-Binding Protein [CREB] 2) (Table 1). In these two latter cases, one
346 cytosine was found hypomethylated while the other is hypermethylated. A single DMC was
347 associated to a repeat region and two DMCs were found to refer to the same gene (homologous
348 to the *Gasterosteus aculeatus* paralogue of *COL4A5*, a collagen gene of type IV mostly
349 implicated in the protein network of the basement membrane) (Table 1). For this gene, one
350 DMC is located in the first intron while the second is 16.5kb upstream of the start codon. They
351 were not considered as a DMR.

352

353 **3 - 3 : Clustering**

354 Hierarchical clustering showed a strong family effect in methylation patterns (i.e. individuals
355 within family clustered together; Fig. 3). It is interesting to note that the four individuals that
356 were caught twice displayed after-stress methylation pattern very similar to their before-stress
357 pattern, and clustered together by pairs in all four cases. These individuals have the lowest
358 levels of dissimilarity in hierarchical clustering, suggesting very few differences in their
359 methylation profiles in pre- *vs* post-stress condition.

360 Despite this strong family effect and clues of low impact of the stress on methylation,
361 pre- (T0) and post-stress (T4) groups can be distinguished based on their DMC profile in PCA.
362 Mean loading scores of individuals were found significant among T0 and T4 for PC1 that
363 explained 7.2% of total variance (Student *t*-test; $p < 0.005$, Fig. 4). No significant difference was
364 found for loading scores along PC2 (3.0% of total variation; $P = 0.404$).

365

366 **3 - 4 : Literature surveys**

367 *Thematic survey* - Our first literature survey showed that DMC-related genes detected in this
368 study were found involved in one of the eight themes we retained to describe the stress
369 response in fish (Fig. 2 and Suppl. Mat. Table S4 for a more extensive description of gene
370 involvement in the stress response, including references). Most of them were previously
371 analysed or detected in human or mice stress studies, in few cases in other model organisms
372 (e.g. *Drosophila*) (Suppl. Mat. Table S4). Additionally, the *GLI2* gene encoding for a zinc-finger
373 protein and the *BTR30* (bloodthirsty-related gene family member 30, also known as E3
374 ubiquitin-protein ligase *TRIM39*-like) gene (Table 1) were found differentially regulated in sea

375 bass cultured-cells submitted to viral infection and involved in the immune response (Chaves-
376 Pozo et al. 2019) (Fig. 2).
377
378 *Transcriptomics survey in zebrafish* - In a second time, we searched for differential expression of
379 sea bass DMC-related genes in studies using high-throughput transcriptomics in fish blood. Only
380 four studies were retained (Morera et al. 2011; Dahle et al. 2015; Martos-Sitcha et al. 2017;
381 Puente-Marin et al. 2018), and none of our DMC-related genes was found to be differentially
382 expressed in these studies.
383 After curation of PubMed lists and addition of few undetected studies, we surveyed 20
384 zebrafish studies analyzing the transcriptomics of the stress response in six tissues: brain (*n* = 8),
385 liver (*n* = 7), muscle (*n* = 2), gonads (*n* = 1), intestine (*n* = 1), skin (*n* = 1) (no high-throughput
386 transcriptomics study in gill, kidney, one in muscles of eye that was accounted for 'muscle'; see
387 Suppl. Mat. Text S1). The main outcome of this survey was to document 38 out of 51 RBC's
388 DMC-related genes (74.51%) in stress-related studies. Strikingly, these 38 DMCs were reported
389 mainly from few neurotranscriptomics zebrafish studies that dealt either with reactive-proactive
390 behavioural response to stress (Rey et al. 2013; Wong et al. 2015) or with changes in social
391 regulation that may promote stressful behaviour among congeners (Greenwood and Peichel
392 2015) (Fig. 2 and Suppl. Mat. Table S4). Some genes are shared among studies
393 Only four of these DMC-related genes were reported in the former RNA sequencing
394 study of Anastasiadi and Piferrer (2019) in sea bass. This included *CILP1* and *PTGFRN* in muscle,
395 *PRKCQ* in testis, and *RRM2* in liver (blood and brain not investigated) (Fig.2). In comparison, the

396 twelve other zebrafish studies investigating other organs than brain retrieved only 15 of the sea
397 bass DMC-related genes found in RBCs (details in Suppl. Mat. Text S1).
398
399 *Epigenomic survey* - We finally looked at former studies dealing with epigenomics in fish. The
400 studies retained (*n* = 31) are presented in Suppl. Mat. Table S5. Twenty studies reported
401 differential methylation at genes also found differentially methylated in sea bass RBCs (Suppl.
402 mat. Table S5). Data sets were highly heterogeneous for different reasons (e.g. experimental set-
403 ups, nature of stressors if any, thresholds considered to define a DMC/DMR). However, main
404 observations might be summarized as follows: all articles considering brain tissue and with all
405 gene annotations made available (*n* = 6) reported at least one match with DMC-related genes
406 found in the present study, and more specifically data of the former sea bass epigenomic study
407 by Anastasiadi and Piferrer (2019) reported five genes of the present study as differentially
408 methylated in the brain (*GLG1a, GFRA2, CELF2, PRKCQ, ROBO3*), three in muscle (*CILP1, FURIN,*
409 *KBTBD13*), one in testis (*GLG1a*), and any in liver (total: eight distinct genes). This indicated
410 slightly more matches with brain. However, the single previous study analyzing RBC methylome
411 in fish so far (Gavery et al. 2018) shared no DMC-related gene with the present study. Finally,
412 the study that reported the largest number of genes found as DMC-related genes was Gavery et
413 al. (2019) (*n* = 15) in the liver of hatchery *vs* wild rainbow trout during a so-called 'temporal
414 analysis' (Suppl. Mat. Table S5). Another study by Olvsik et al. (2019) on zebrafish reported 11
415 genes in common with the present study, but was not organ specific (Suppl. Mat. Table S5).
416

417 **3 - 5 : Protein-protein interactions**

418 Further database mining screening for specific protein interactions on the STRING server revealed
419 few other possible pairs of associations (*n* = 8): *ROBO3-CHN1*, *ROBO3-PRKCQ*, *ROBO3-LRRC3*,
420 *DLG1-NCBP2*, *FURIN-PLG*, *PLG-MMRN2a*, *CELF-RRM2*, and *CRTC2-DENND4B* (Suppl. Mat. Fig.
421 S2). The *FURIN-PLG* and *DLG1-NCBP2* associations were also detected in our literature survey.
422 The first one will be presented in the 'Discussion' (see Suppl. Mat. Table S4 for the second one).
423 However, most of these associations retrieved only poor information relative to the stress
424 response. *ROBO3* and *CHN1* have been shown to interact in the brain with poorly-understood
425 implications of *CHN1* in neurological disorders (Walsh and Engle 2010; *CHN1* may have other
426 stress-related functions, see Suppl. Mat. Table S4). *CELF2* (CUGBP Elav-like family member 2)
427 and *RRM2* (Ribonucleotide reductase M2 polypeptide) are both known to participate to
428 messenger RMA (mRNA) metabolism (Vlasova-StLouis et al. 2013). *CELF2* acts to post-
429 transcriptionally stabilize mRNAs by relocating them to stress granules in the cytosol. *CELF2*
430 interferes with *RRM2* that modulates its splicing activity. As post-transcriptional activities are at
431 the core of methylation studies, the detection of this association is relevant to our study. To
432 illustrate some limits of these associations: *CRTC2* and *DENN4B* were detected apparently
433 because they are located close to the sex determining region of *O. mykiss* (Phillips et al. 2013).
434 No relationship with stress is suspected. The relationship between multimerin (*MMRN2*) and
435 plasminogen (*PLG*) via its co-activator remains poorly investigated and supported (Øynebråten
436 et al. 2005). This automatic search using STRING did not really enrich our findings.

437

438 # 4 - Discussion

439

440 In this study, we showed that a modified epiGBS protocol originally proposed by Van Gurp et al.
441 (2016) was applicable to further analyze patterns of cytosine methylation at a genome-wide
442 scale in *D. labrax*. This is the first use of epiGBS in fish and the second in an animal species
443 (Canadian lynx; Meröndun et al. 2019). The addition of a second restriction enzyme illustrates
444 the flexibility of the epiGBS and more generally RRBS protocols to improve data acquisition and
445 impact (Paun et al. 2019). This is however not the first RRBS protocol dedicated to the addition
446 of a second restriction enzyme (Wang et al. 2013), but this is hereby proposed in a context of
447 improved multiplexing of samples and increased sequencing capacities, thus offering more
448 opportunities to explore important biological processes at a genome-wide scale, such as the
449 stress response. Data allowed us to explore how RBC's DNA methylation may respond to
450 stressors in the European sea bass and - albeit some limitations that will be discussed below -
451 was shown to complement both the traditional evaluation of the stress response based on
452 analyses of blood serum samples (e.g. Vezzana et al. 2002; Seponka et al. 2016) or on gene
453 expression variation, often using invasive brain samples (e.g. Aluru and Vijayan 2009; Rey et al.
454 2013; Martos-Sitcha et al. 2014; Wong et al. 2015; Metzger and Schulte 2016; Nyman et al.
455 2020).

456

457 **4 - 1 : Mining the epigenome…**

458 The information provided in this study is based on the analysis of 47,983 distinct methylated
459 sites distributed over all sea bass LGs. The mapping efficiency was high (74.5%) when compared

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

476 The operational and statistical thresholds used in the successive steps of this study are
477 large, resulting in the discovery of a rather low total number of methylated sites. For example a
478 threshold of 30X and nominal cut-off value of 0.001 are quite conservative, when some studies
479 might consider a threshold of 5X or 10X for a CpG to be analysed and associated cut-off values
480 of 0.05 or 0.01 (e.g. Wan et al. 2016; Hu et al. 2018; Anastasiadi and Piferrer 2019). Relaxing
481 thresholds would enable to retrieve more DMCs, but elevated thresholds should normally

482 ensure that relevant information is retrieved. Thus, only 57 DMCs have been found in RBCs of
483 pre- and post-stress European sea bass, and only few were grouped as DMRs. Considering DMRs
484 rather than DMCs is common in genome-wide methylation studies, including sea bass
485 (Anastasiadi and Piferrer 2019). Methylation changes grouped in clusters (i.e. DMRs) are more
486 likely to influence transcriptional activity at nearby loci than single cytosines (e.g. Richards et al.
487 2017). However, other experimental issues associated to the flexibility of RRBS protocols (e.g.
488 single- *vs* paired-end sequencing, likelihood of annotation, selection and size of fragments) can
489 made the advantages of considering DMRs over DMCs perhaps more disputable (Paun et al.
490 2019). In this study, DMCs were found mostly hypermethylated in post-stress individuals
491 compared to the pre-stress individuals, and mostly located in gene bodies of fifty-one different
492 genes (44 out of 57; 77.2%). It has been shown that such location of differential methylation
493 may regulate splicing and/or act as alternative promoters to reshape gene expression
494 (Maunakea et al. 2010; Ehrlich and Lacey 2013; Lev Maor et al. 2015). Thus, despite a low
495 number of DMRs in this study, a large fraction of DMCs points to the transcriptionally active
496 portion of the genome and to important stress-related genes (see below).

497 In addition to DMCs located in gene bodies, a dozen of DMCs were found in intergenic
498 regions (21.0%). Intergenic cytosine methylation has been frequently described, including in
499 stress studies (Hing et al. 2018), but its role remains poorly understood (Suzuki et al. 2011;
500 Rajarajan et al. 2016). While numbers of genic *vs* intergenic DMCs may greatly vary, a ratio of
501 ~80% of DMCs located in gene bodies and ~20% located in other genomic regions has been
502 reported in other fish studies (e.g. Hu et al. 2018). Sometimes proportions are approximately
503 reversed (~80% intergenic *vs* ~20% in gene bodies; e.g. Metzger and Schulte 2017). While

504 under-represented in our data, the role for differentially methylated transposable elements is
505 also poorly understood (Lerat et al. 2019).
506
507 **4 - 2 : … but mining the unknown**
508 Studies looking at the epigenomic landscape of RBCs in fish are scarce. Gavery et al. (2018)
509 reported 85 DMRs between hatchery and wild fish in *O. mykiss*. This study suggested that blood
510 DNA methylation patterns are indicative of the stressors experimented in the environment, but
511 we are still currently mining the unknown to gain supporting information.
512 We nevertheless demonstrate that fish RBCs are relevant candidates to investigate the
513 stress response at the epigenomic level in the European sea bass. Numerous DMC-related genes
514 reported here have been previously shown to respond to stress or implicated in variation of the
515 stress response in animal models. Overall, our comparative surveys showed that the DMC-
516 related genes we detected in sea bass using RBCs have more connections with results found in
517 the brain than in other tissues. Five DMC-related genes were previously found differentially
518 methylated in sea bass brain by Anastasiadi and Piferrer (2019) and reports from other studies
519 suggest that RBC methylome might integrate epigenetic components of the stress response
520 observed in the brain. This relationship is however weak at the epigenomic level and about one
521 third of our DMC-related genes have also been detected in liver of trout (Gavery et al. 2019).
522 Liver is classically used in transcriptomic and epigenomic studies, may certainly integrate
523 information because of its pivotal role in metabolism, but generally necessitates sacrificing fish.
524 Nevertheless, the relationship is far more pronounced when genome-wide gene expression
525 difference is considered. Indeed, genome-wide neurotranscriptomics studies dealing with

526 personality and stress coping styles (Rey et al. 2013; Wong et al. 2015), or social regulation
527 (Greenwood and Peichel 2015) collectively reported thirty-eight of our 51 DMC-related genes as
528 expressed and potentially associated to the stress response in zebrafish. As differential
529 methylation is classically interpreted as leading to differential gene expression (but see, e.g.,
530 Jones 2012; Lea et al. 2018 for a more nuanced consideration), this observation is one
531 interesting signal indicating that RBCs might monitor events occurring in the brain. However, it
532 remains unclear why most of them are related to coping and personality, and to studies where
533 fish were under stress for a few minutes (Rey et al. 2013; Wong et al. 2015), hours or days
534 (Greenwood and Peichel 2015). How distinct stress contexts and challenges may apparently rely
535 on similar stress circuitry has to be investigated further.
536
537 **4 - 3 : A Brain-derived neutrophic factor (BDNF) network**
538 The roles of the DMC-related genes have to be exposed more extensively to illustrate their
539 possible involvement in the stress response of sea bass. This is not place to fully expand on all
540 DMC-related genes (see Suppl. Mat. Table S4 for details), but one single selected example can
541 be developed. The best representative is a set of DMC-related genes from the BDNF network
542 whose role in the stress response is commonly reported. BDNF is a protein synthesized in the
543 brain that offers resistance to neurodegenerative diseases and favors stress adaptation and
544 resilience (Taliaz et al. 2011; Gonzalez et al. 2019; Notaras and van den Buuse 2020), but also
545 energy homeostasis (Marosi and Mattson 2013). BDNF has emerged as one of the most
546 important molecule in memory (Beckinschtein et al. 2008, 2014). It consolidates both the
547 within- and between-generation fear memory owing to epigenetic regulation (Lubin et al. 2008;

548 Coley et al. 2019). Its activity is strongly linked to glucocorticoid stress to imprint neurogenesis
549 (Numakawa et al. 2017) and it acts as both a regulator and a target of stress hormone signaling
550 (Notaras and van den Buuse 2020). In fish, cortisol binds the glucocorticoid receptor (*gr*) and
551 controls BDNF expression in the brain. With *gr* and relatively few other commonly studied
552 genes, BDNF has thus emerged as one of the target genes of interest in stress studies, as shown
553 for zebrafish (e.g. Pavlidis et al. 2015), sea bream (Vindas et al. 2018) and sea bass (Tognoli et al.
554 2010; Sadoul et al. 2018; Samaras et al. 2018; Alfonso et al. 2019). BDNF is also involved in other
555 aspects of the stress response in fish (e.g. regulation of the microbiota-gut-brain axis; Borrelli et
556 al. 2016). Variation in BDNF methylation status could not be investigated in this study as no *SbfI*
557 restriction site is present within or close to this gene. Nevertheless, a methylation signature of
558 stress associated to BDNF is likely present in our data and illustrates how blood methylation may
559 provide a snapshot of events studied in the brain.

560 Indeed, several DMC-related gees are involved in the maturation of proBDNF to mature
561 BDNF or the regulation of its activities (*ABLIM2, ADCY1Bb, CRTC2, FURIN, NPAS3, PLG,* and
562 possibly *SLC22A2*). Most of these DMC-related genes were already found differentially
563 expressed in the zebrafish brain (Rey et al. 2013; Greenwood and Peichel 2015; Wong et al.
564 2015), but an outline of their interaction was not presented. The adenyl(ate) cyclase (AC,
565 *ADCY1B* gene) is a brain-specific signaling enzyme that synthesizes the cyclic AMP (Sassone Corsi
566 2012). In the brain, AC is activated by PACAP (Protein Adenylate Cyclase Activating Protein) and
567 this inducible signaling pathway that participate to the synthesis of the active form of BDNF
568 (proBDNF to mature BDNF; Lee and Seo 2014). ProBDNF is processed by furin and the
569 plasminogen system (Marosi and Mattson 2013), including processing steps that necessitate

570 actions of actin-binding LIM kinases (ABLIM; Bennett and Lagopoulos 2014). Furin - encoded by
571 *FURIN* - is a subtilisin-like protein proconvertase ubiquitously expressed in fish (Murata and
572 Kinoshita 2015) that presents an intracellular cleaving activity of numerous biologically relevant
573 molecules in the trans-Golgi network. This includes BDNF in the brain, notably in pre-synaptic
574 axons. In mice, furin has been shown to participate to dendrite morphogenesis and modulate
575 learning abilities and memory (Zhu et al. 2018). Stress imprinting at *FURIN* is likely since it has
576 recently been shown that transgenerational epigenetic effects of furin activity were active in
577 brain of mice (Buck et al. 2019). ProBDNF cleavage by furin depends on brain AC and CREB
578 (cAMP Response Element-binding protein) signaling (Marcinkiewicz et al. 1996; Chen et al.
579 2015) and plasminogen (*PLG*; Yeh et al. 2015). This activity itself is modulated by stress
580 hormones (corticosteroids) and is essential to brain hippocampal plasticity in mammals (Pang et
581 al. 2004; McEwen 2008). One interesting supplementary observation is that CREB signaling
582 necessary to furin is associated to *CRTC2* - a CREB co-activator – found as one of the four DMRs
583 reported in this study. In mice, *CRTC2* is known to act as a switch for BDNF and glucocorticoids
584 to direct the expression of corticotropin-releasing hormone (CRH) in the hypothalamus
585 (Jeanneteau et al. 2012).

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

591 In relation to BDNF, two other DMC-related genes should be mentioned: *NPAS3*
592 (neuronal PAS domain containing protein 3) and *SLC22A2*. *SLC22A2* - also known as *OCT2*
593 (organic cation transporter 2) – associated to the unique 3'UTR DMC found in this study is
594 implied in numerous transmembrane transports (Liu 2019), including at the blood-brain barrier
595 (Strazielle and Ghersi-Egea 2015). It was found involved in memory in mice (Couroussé and
596 Gautron 2015; Couroussé et al. 2015) or *Drosophila* (Gai et al. 2016) and associated itself to
597 neurodegenerative diseases (Jedlitschky et al. 2014). Substrates of *SLC22A2* include
598 neurotransmitters such as norepinephrine, but also dopamine, serotonin and a wide-variety of
599 internal or exogenous compounds (Bacq et al. 2012; Volk 2014). Functional links of *SLC22A2*
600 with BDNF remain however poorly documented (Kendall et al. 1995), while they have been
601 found co-expressed and co-regulated in the brain during drug administration experiments (e.g.
602 methamphetamine, mimicking the action of catecholamines; Thomas et al. 2004). In this study,
603 *PLG* and *SLC22A2* are associated to the same DMC; the functional significance of this situation
604 should be investigated further. Finally, *NPAS3* has a well-established action in memory (e.g.
605 Erbel-Sieler et al. 2004; Luoma et al. 2018). It participates to neurogenesis in the hippocampus
606 within a network that also includes BDNF (Yang et al. 2016), but is also known associated to the
607 glial cell line-derived neurotrophic factor (GDNF) receptor-alpha2 gene (*GFRA2*) detected in this
608 study and related to stress and anxiety (Lavedan et al. 2009). The *DLG1* (Disk-large homolog 1)
609 gene could be also associated to BDNF, but this will be not develop further (see Suppl. Mat.
610 Table S4).

611 Results suggest that even in absence of BDNF sequencing reads, some DMCs/DMRs with
612 a potential impact in fear memory and anxiety response as expected in fish after an acute stress

613 challenge certainly did not occur only by chance in this study. They have also a role in processes
614 that regulate the HPI axis and hormones. The measurement of methylation difference now used
615 to monitor the BDNF activity and its relationship to stress (Uwaya et al. 2016; Coley et al. 2019)
616 could thus potentially depend on the methylation status observed at or close to *ADCY1b,*
617 *ABLIM2, FURIN, CRTC2*, *PLG* and other genes such as *SLC22A2* and *NPAS3*. This should be
618 investigated further to improve our understanding of changes in the directionality of multigenic
619 epigenetic modifications and inter-individual variation in stress coping. Candidate genes coming
620 from other fish studies with role in memory consolidation could be added (see, e.g. Nyman et
621 al. 2020; Yang et al. 2020 for proposals).

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

627

628 **4 - 4 : Family effect, development and few individuals**

629 While the PCA showed that pre- and post-stress individuals have in average distinct methylation
630 profiles that may represent stress imprinting during the challenge test, a strong family effect
631 was also detected. Family effects were already reported in fish (e.g. Metzger and Schulte 2018;
632 Xiu et al. 2019; Sagonas et al. 2020), and sea bass families certainly do not have the same
633 sensitivity to stress. This may suggest that (*i*) their genomic backgrounds alter how family will
634 develop an epigenetic stress response over the period covered by the challenge test, (*ii*) that the

635 short developmental period during which families were distributed over different tanks was a
636 "critical time window" (e.g. Safi-Stibler and Gabory 2020) sufficient to imprint individuals and
637 lead them to develop a familial response to stress during the challenge, or that (*iii*) some degree
638 of transgenerational inheritance in methylation profiles is present. These are not mutually
639 exclusive. Transgenerational inheritance has gained interest over the last years (e.g. Perez and
640 Lehner 2019), notably in marine species (e.g. Beemelmanns and Roth 2017; Metzger and
641 Schulte 2017; Fallet et al. 2020), also including sea bass (Anastasiadi and Piferrer 2019). Parents
642 and/or the germline were unfortunately not sampled in this study for further analysis. As
643 Gavery et al. (2018) reported that *BTR30/TRIM39*, *DLG1*, *MMRN2*, and *NOL4B* - also found in
644 this study - were differentially methylated in the sperm of *O. mykiss*, these differentially
645 methylated genes found reported there could be interesting candidates for transgenerational
646 inheritance study of the stress response.

647 Anastasiadi and Piferrer (2019) showed that the domestication process in *D. labrax*
648 resulted notably in genome-wide methylation differences for genes involved in the nervous
649 system and neural crest cell differentiation. In our study, *GLI2* (e.g. Cerrizuala et al. 2018), *PBX1*
650 (Chang et al. 2008), *DLG1* (Mostowska et al. 2018) and *FOXJ3* (Landgren and Carson 2004) are
651 DMC-related genes involved in neural crest cell proliferation and differentiation. *ROBO3* is
652 involved in the immune response, but also implied in early neurogenesis (Friocourt and
653 Chédotal 2017), as well as *NPAS3* (Kamm et al. 2013). With *BMP3* (Nie 2005), *FURIN* (Walker et
654 al. 2006); *NOL4B* (Kumari et al. 2018), and *Myf5* (Gustaffson et al. 2002; Lin et al. 2013), most of
655 these genes are engaged in the development of the anterior region and/or the craniofacial
656 skeleton which is modified during sea bass farming (Anastasiadi and Piferrer 2019). This

657 suggests that methylation patterns observed in sea bass could also potentially rely on the
658 ontogenetic regulation of a particular phenotype between pre- and post-stress fish, rather than
659 being directly related to the stress challenge. Changes in gene expression or methylation
660 accompanying ontogeny are reported in fish (Baumgart et al. 2014; Gavery et al. 2019) and, as
661 for other organisms, cytosine methylation is known to vary with age in sea bass (Anastasiadi and
662 Piferrer 2020). Collateral effects of stress might thus be present in our data as suggested by the
663 fact that fifteen genes we found differentially methylated in RBCs were reported as also
664 differentially methylated in the liver of the rainbow trout by Gavery et al. (2019).

665 On another hand, the epigenetic profiles of four individuals were analyzed in the pre-
666 and post-stress situations and clustered very closely from each other in each case. While based
667 on few observations, this suggests that neither the stress challenge itself nor development have
668 a strong impact on the cytosine methylation landscape in sea bass. How a transgenerational and
669 within-generation stress-imprinting events influenced by ontogeny may interact to shape both
670 the plastic and the heritable component of the stress response in relation to environmental
671 stimuli require in depth evaluation (Burggren 2015; Trollope et al. 2017). Nature and strength of
672 family-based epigenomic variation is of considerable importance for selection breeding
673 improvements in a cultured fish like sea bass (Houston et al. 2020), including also issues about
674 health and welfare (Triantaphyllopoulos et al. 2016).

675

676 **4 - 5 : Blood and RBCs : extending the perspective**

677 As fish engaged in aquaculture programs as sea bass are costly and based on breeders that
678 cannot be easily sacrificed, epiGBS using blood samples may offer a genome-wide assessment of

679 stress-induced epigenetic marks over a significant number of individuals and at reasonable cost.
680 Blood is widely used in human epigenomics (e.g. Houseman et al. 2015), but not in fish despite
681 the presence of nucleated cells and minimally invasive sampling that allows for multiple
682 measurements. Using RNA-sequencing, Morera et al. (2011) or Dahle et al. (2015) already
683 suggested that fish erythrocytes would be useful to provide insights on the innate immunity or
684 response to pathogens. Our study suggests that blood could effectively offer an in depth insight
685 for the evaluation of the stress response from a novel perspective, accompanied by classical
686 physiological measurements (e.g. cortisol, glucose, lactate) and may provide a more complete
687 picture of the stress response in fish than currently performed.

688 The link between brain and blood epigenomics remains however to be explored more
689 deeply in fish and requires careful evaluation and validation to correct for tissue specificity, as
690 requested in human (Lauschke et al. 2017). Our attempts to survey fish studies undoubtedly
691 showed that considerably more knowledge has to be accumulated in fish epigenomics before to
692 reach this issue. In human, the lack of access to brain tissues have been shown to impede some
693 epigenomic studies and naturally raised ethical issues certainly also valid for fish (Barr and
694 Misener 2016; Bunnik et al. 2020). *In situ* hybridization techniques in brain have been shown to
695 be especially important to link neurobiological activities and stress coping in fish (Vindas et al.
696 2017, 2018). Their use and thus sacrifice of individuals cannot be ruled out. Actually,
697 methylation differences among full brain or brain areas have been rarely studied in fish, mostly
698 in zebrafish (e.g. Zhang et al. 2016), but recently in a tilapia (Hilliard et al. 2019), a goby
699 (Sommerville et al. 2019), and a killifish (Berbel-Filho et al. 2020).
700

701 **5 - Conclusion and future directions**

702

703 With the works of Anastasiadi and co-workers (2017, 2018, 2019, 2020) and others (Navarro-
704 Martín et al. 2011; Geay et al. 2012; Terova et al. 2016), the European sea bass has become one
705 of the most studied species in fish epigenetics. Because of the availability of its genome and the
706 development of selection programs, efforts should be dedicated to further explore its
707 epigenomic landscape regarding the stress response, especially in an aquaculture context. This
708 should be done over a multigenerational scale to identify pathways structuring the stress
709 response at key life stages, on different tissues to better delineate stress-responsive cellular
710 networks, and in different contexts (nature, duration, number of stressors). Studies in
711 epigenomics including phenotypic information (e.g. Birney et al. 2016; Lin et al. 2016) could
712 increase the panel of diagnostic tools for breeding selection in aquaculture if blood could be – at
713 least partly - substituted to brain or other tissues when studying the stress response. Screening
714 for genome-wide epigenomic variation in natural populations as already done for genomic
715 variation (Duranton et al. 2018) is now also conceivable in this species. Our modified version of
716 the original epiGBS protocol seems to be a powerful and affordable method to screen a
717 significant number of individuals with sufficient depth and coverage to reach meaningful
718 conclusions. While it should be compared to others (e.g. Wang et al. 2013; Suzuki et al. 2018),
719 our protocol is close to the double digestion restriction site DNA sequencing protocol used in
720 genomics. Their parallel use certainly deserves attention to design more integrated epigenomic-
721 genomic studies (e.g. Prudencio et al. 2018; Crotti et al. 2020), and more generally multi-omics
722 investigations (Suravajhala et al. 2016; Limborg et al. 2018).

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Conflicts of interest

None declared

Ethics statements

All experiments were performed in accordance with relevant guidelines and regulations. Nireus S.A. research facilities are certified and have obtained the codes for the rearing and use of fish for scientific purposes (EL04-BIOexp-01). All procedures on fish used in this study were approved by the Departmental Animal Care Committee following the Three Rs principle, in

745 accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use
746 of experimental animals. All sample procedures were performed by FELASA certified researchers
747 (http://www.felasa.eu/).
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1206 **Data accessibility**

1207 Illumina raw reads will be submitted to the NCBI sequence read archive (SRA). Additional
1208 submission of clean epigenomic data will be deposited on DRYAD. Data deposits will be
1209 completed at or during submission to a journal.

1210

1211

1212 **Author contributions**

1213 MVK, ED, BG developed the protocol, MVK and ED conducted the lab work, EG ensured techni-
1214 cal assistance and supervised sequencing. AS, AD, MP and CST established the study design of
1215 the challenge test. Experiments with live fish were conducted by AD, AS, MP, with contribution
1216 of MVK. MVK performed data analyses under supervision of ED and BG. MVK, ED, BG drafted
1217 the manuscript; others authors reviewed drafts. CST and BG coordinated this study.

1218

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.

1231 **Captions of the Figures**

1232

1233 **Figure 1:** Coverage of the 24 linkage groups (LGs) of the sea bass genome by sequencing epiGBS
1234 reads. Ordinates represent the mean number of sequencing reads. LG numbers are those used
1235 by Tine et al. (2014). Complex IDs resulting from the merging of previously defined LGs during
1236 final assembly (e.g. LG22-25 resulted in the fusion of previous LG22 and LG25). SB-UN (sea bass
1237 – unknown; hereby SB) indicates a composite LG made from originally unassembled scaffolds.

1238

1239 **Figure 2:** Diagrammatic representation of the involvement of the 51 DMC-related genes found
1240 in this study and issues regarding the stress response (see main text for detailsOne additional
1241 issue was added for few genes that are involved in the regulation, the control and the dynamics
1242 of epigenetic process (*NCBP2, GTPF*), and acid-base homeostasis in kidney without formal proof
1243 of actual relation with a stress issue (*CLDN4*). DMCs located within gene bodies are indicated in
1244 black, in brown for intergenic regions including promoters. The hatched symbols refer to genes
1245 also reported in high-throughput transcriptomic studies in zebrafish (main text and Suppl. Mat.
1246 Table S4). This does not mean that our literature survey at DMC-related genes was supported
1247 only by these studies, but it illustrates their importance over results. Stars with full line indicate
1248 genes detected as differentially expressed (*BTR30, GLI2*) in one immunity-related transcriptomic
1249 study in sea bass (Chavez-Pozo et al. 2019), while stars with broken lines indicate genes with
1250 methylation differences (*PTPRB, SASH1A*) (stickleback: Sagonas *et al.* 2020; rainbow trout: Uren-
1251 Webster et al. 2018; respectively). Reports outside of the above-mentioned studies are
1252 indicated by filled symbols. On the right are indicated genes found differentially expressed (RNA)

1253 or differentially methylated (CpG) in the genome-wide study by Anastasiadi and Piferrer (2019)
1254 (*A&P 2019*) on European sea bass. Tissues in which genes were found differentially
1255 expressed/methylated are indicated (brain not investigated in transcriptomics). Association with
1256 the *LRRC3* gene is reported in grey as leucine-rich repeat (LRR) gene family is classically involved
1257 in neuroimmunity. Variations in transcription or methylation have been shown to impact
1258 behaviours after stress events within this family (see e.g. *LRRTM4L2* in this study). However,
1259 *LRRC3* is amongst the most poorly known gene within this family (Söllner and Wright 2009), and
1260 this remains presumptive. Additional support including references for these DMC-related genes
1261 is given in Supplementary Materials Table S4, as well as comparisons with other epigenomic
1262 studies.
1263
1264 **Figure 3:** Hierarchical clustering based on of the 57 differentially methylated cytosines (15%
1265 threshold) of the randomly caught 74 pre- and post stress sea bass samples considered during
1266 this study. Nineteen out the twenty families (A to T) used in this study are present in this graph
1267 and each family is associated to one single colour. Capital letters refer to the family, while T0
1268 and T4 are for pre- and post-stress individuals, respectively.
1269
1270 **Figure 4:** PCA based on the methylation profiles of the 57 differentially methylated cytosines
1271 (15% threshold) reported in this study (Table 1). Pre- and post stress individual sea bass (in red
1272 and blue, respectively) differ significantly along PC1 ($p < 0.001$), but not PC2. The insert illustrate
1273 the distribution of individual scores along PC1. Ellipses represent the 95% confidence limits over
1274 PC1 and PC2.
1275

1276 **Table 1**

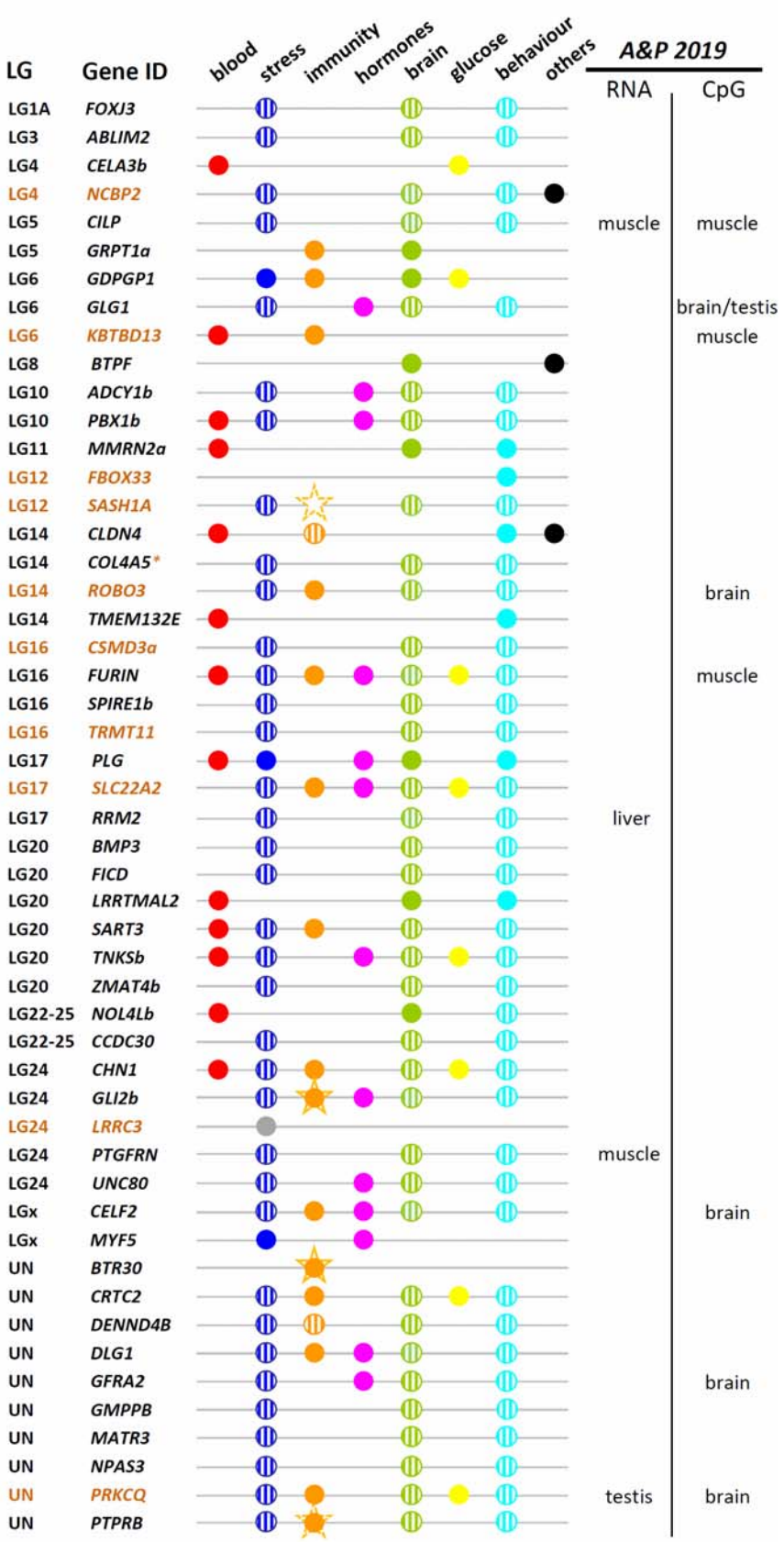
D. labrax LG	Ensembl LG	*q* -value	methylation difference (%)	*D. labrax* Name	Gene ID	Gene Name	Location
LG1A	HG916837.1	0	-21,40	DLAgn_00094580	*FOXJ3*	forkhead box protein J3	*gb*
LG1A	HG916837.1	0	-21,67				*gb*
LG1A	HG916837.1	0	-15,51				*gb*
LG3	HG916843.1	1,54E-95	18,14	DLAgn_00141440	*ABLIM2*	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*

1277

Figure 1

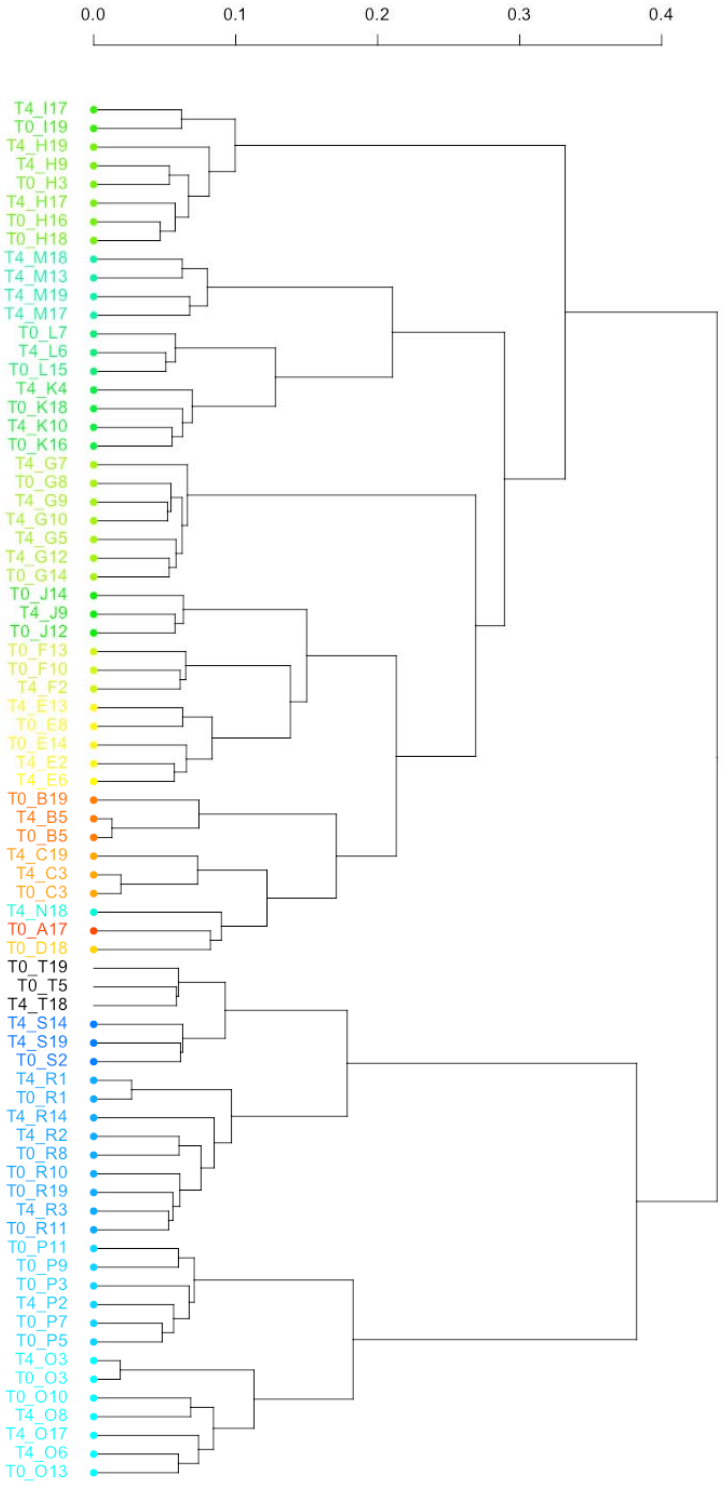
LG10
LG11
LG12
LG13
LG14
LG15
LG16
LG17
LG18-21
LG19
LG1A
LG1B
LG20
LG22-25
LG24
LG3
LG4
LG5
LG6
LG7
LG8
LG9
LGx
SB

1282 **Figure 2**



1283

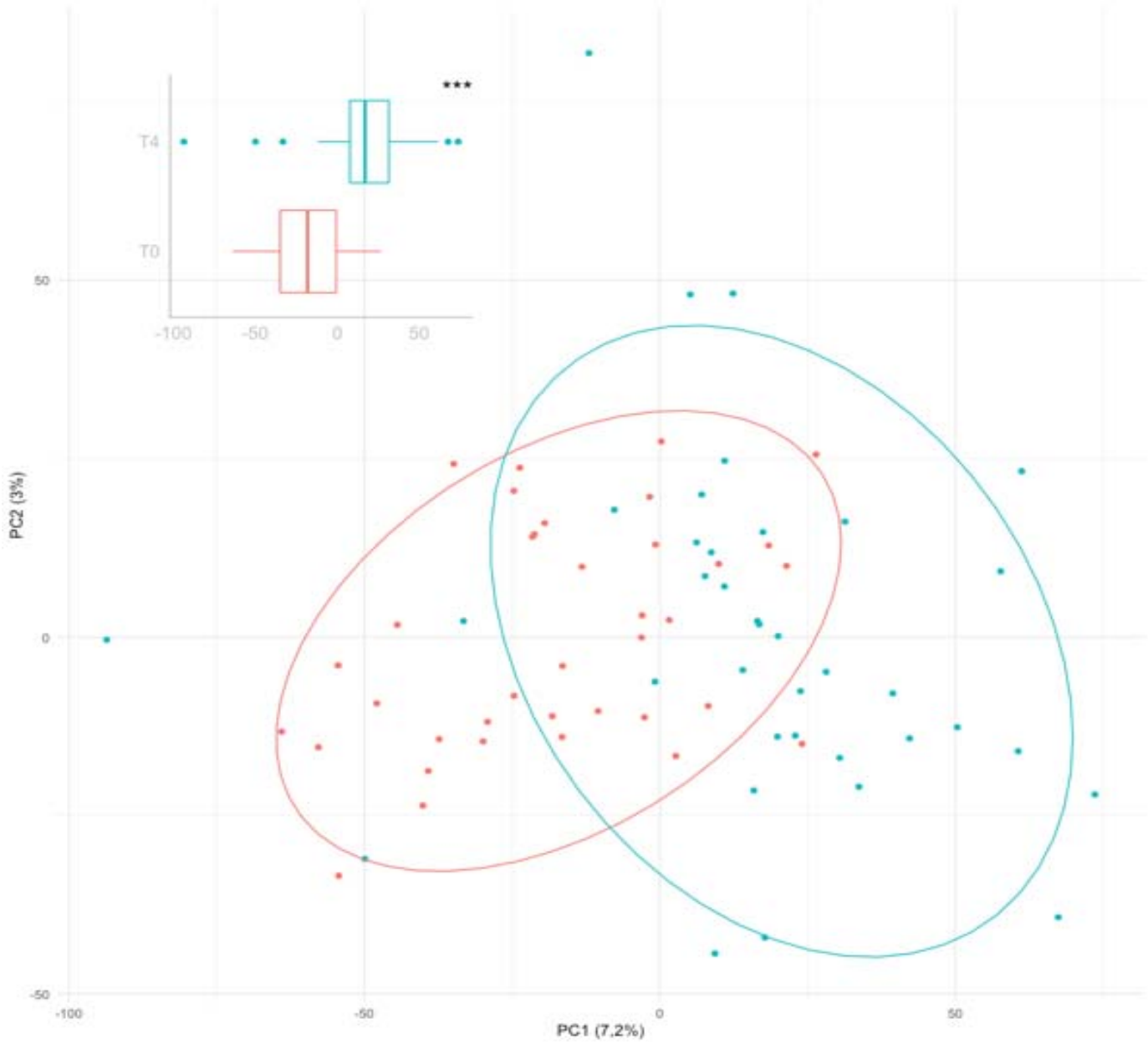
1284 **Figure 3**



1285

1286 **Figure 4**

1287



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