Genome-wide epigenomic stress response in the European sea bass (*Dicentrarchus labrax*, L.)

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Abstract

Background: While the stress response inspired genome-wide epigenetic studies in vertebrate models, it remains mostly ignored in fish. We modified the epiGBS (epiGenotyping By sequencing) technique to explore changes in genome-wide cytosine methylation to a repeated acute stress challenge in the nucleated red blood cells (RBCs) of the European sea bass (*Dicentrarchus labrax*). This species is widely studied in both the natural and farmed environments, including issues regarding health and welfare.

Results: We retrieved 501,108,033 sequencing reads after trimming, with a mean mapping efficiency of 73.0% (unique best hits). Fifty-seven differentially methylated cytosines (DMCs) close to 51 distinct stress-related genes distributed on 17 of 24 linkage groups (LGs) were detected between RBCs of pre- and post-stress individuals. Literature surveys indicated that thirty-eight of these genes were previously reported as differentially expressed in the brain of zebrafish, most of them involved in stress coping differences. DMC-related genes associated to the Brain Derived Neurotrophic Factor, a protein that favors stress adaptation and fear memory, are especially relevant.

Conclusion: We provide an improved epiGBS protocol with increased multiplexing and sequencing capacities that offer new opportunities to improve data acquisition and to investigate important biological processes at a genome-wide level, such as the stress response. Minimally invasive RBCs deserve more attention to investigate the epigenetic response to stress without sacrificing fish.

Background

The study of stress is of large interest in biological sciences. Stress affects cells as well as ecosystems and influences processes that span from seconds to evolutionary time scales [1,2]. Reaching a common definition is quite complex [2-5], but stress often refers to the non-specific, total response of one organism to answer to internal and/or external stimuli acting as stressors. Accordingly, the stress response represents the repertoire of physiological, metabolic and behavioural changes developed during and observed after a threat to counteract the effect of stressors and recover or approach the homeostatic equilibrium. In teleost fish – the largest group of vertebrates - an integrative understanding of the stress response is still expected [1,2]. This concerns natural populations that may develop plastic and adaptive response to stressors [1,6], but also cultured species in order to increase fish health and welfare in stressful farming environments [7-9].

Classically, stress studies have to integrate physiological, immunological, neurobiological and behavioural components that altogether shape the stress response in fish [8, 10-12]. The stress response implies - among others - a primary neuroendocrine and a secondary immune response, with interacting roles of neuropeptides and cytokines in each respective system [13-15]. The Hypothalamus-Pituitary-Interrenal (HPI) axis plays a crucial role in the manifestation and regulation of the stress response through the release of neuropeptides, catecholamines and glucocorticoids. In teleosts, cortisol is the primary circulating glucocorticoid and species-specific differences in the timing, magnitude and peak duration of post-stress plasma cortisol concentrations have been reported [16]. Glucose - another classical biomarker of stress - is also under control of hypothalamus, and its blood level depends on glycogen metabolism as well as

68 gluconeogenesis, themselves triggered by interrenal glands and catecholamines [17, 18].
69 Beyond the classical HPI axis, several mediators of allostasis, organs and systems (e.g. serotonin
70 and the serotonergic system; osteocalcin and the parasympathetic nervous system) are involved
71 in stress and anxiety [19-21]. A proper regulation of the stress response requires the
72 consideration of a complex regulatory network of non-linear actions to integrate the whole
73 organism response to stressors [22, 23].

74 In the past few years, the stress response largely inspired genome-wide epigenetic
75 studies in model organisms [24-28]. For animal models, it was established that stress can
76 especially reshape the stress axis/network through epigenetic modification of genes in the
77 hippocampus, hypothalamus and other stress-responsive brain regions [23,29,30]. Human stress
78 studies have shown that blood cells responded to DNA methylation in the brain [31-33, but see
79 34], and more generally that blood is useful whenever target tissues cannot be easily sampled
80 or biopsied [35]. Because blood cells are mobilized as part of the stress response in fish [36, 37],
81 it is appealing to investigate if an epigenomic signature of stress is present in their nucleated
82 red blood cells (RBCs). The use of RBCs in fish transcriptomics or proteomics has received
83 attention over the last decade [38-40]. However, fish epigenetics has not embarked on this path
84 yet and genome-wide epigenetic studies using RBCs are quite rare in fish [41]. This contrasts
85 with the over-increasing interest to investigate epigenomic variation in teleosts [41–72]
86 Nevertheless, documenting the epigenomics of the stress axis/network also remains scarce [58],
87 while authors asked for greater attention on this topic [73,74].

88 After salmonids, the European sea bass (*Dicentrarchus labrax*) is certainly the most
89 investigated marine fish species in Europe using molecular tools. It has been extensively studied

90 over the last three decades, for both natural and farmed populations (reviewed in [75]). This
91 includes the sequencing of its genome [76] and an increasing number of epigenetic studies [77-
92 84]. The stress response of European sea bass remains evaluated using blood parameters that
93 have been shown to reflect multiple components of its stress response [16,85], despite authors
94 have proposed alternatives [86,87]. How the European sea bass methylome may capture such
95 components is actually missing.

96 We adapted the epiGenotyping By sequencing (epiGBS) protocol originally proposed by
97 Van Gurp et al. [88] to assess the genome-wide epigenomic variation in the RBCs of *D. labrax*
98 submitted to periods of acute stress. EpiGBS targets variation in cytosine methylation – the
99 covalent addition of a methyl group to cytosine nucleotides – that has long been accepted as an
100 important epigenetic modification in many organisms [89,90]. This modification integrates a
101 second restriction enzyme and further multiplexing of individuals, saving costs. Our aims were
102 to explore the epigenomic landscape of the stress response in the European sea bass using this
103 protocol, and to investigate if nucleated RBCs from minimally invasive blood samples could
104 provide relevant information on this issue in an economically important fish like sea bass.

105

106

107 **Results**

108

109 Nineteen out of 20 sea bass families were represented by at least one individual among the 74
110 samples analyzed in this study. Fish number per family ranged from one (families A, D, N) to
111 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 thus analyzed for both pre- and post-challenge stress conditions. A total of 70 distinct fish has been analyzed in this study.

EpiGBS library construction and sequencing

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

---------- Table 1 here ----------

Methylation analysis

Out of the 10,368,945 CG dinucleotides (0.46% of the whole) present in the *MspI-SbfI* reduced-representation of *D. labrax* genome we obtained, 47,983 CpG coordinates were extracted with a minimum of 30X read depth and presence in at least 20 individuals. They were filtered out using a 15% methylation difference threshold and a nominal cut-off value of $q < 0.001$. With these parameters, only a total of 57 cytosines in CpG context were defined as DMCs between pre- and post-stress sea bass (Table 1). Methylation differences ranged up to 46.4% for hypermethylated

134 cytosines, and down to -27.5% for hypomethylated cytosines. Hyper-methylation was more
135 frequently detected than hypomethylation (11 [19.30%] hypo- *vs* 46 [80.70%] hypermethylated
136 DMCs) in post-stress sea bass. DMCs were distributed on 17 out of 24 LG groups and in or close
137 to 51 distinct genes. Further information is provided in Additional File 2 (e.g. gene annotations,
138 CpG context).

139 Most identified DMCs were found located within identified gene bodies (44 out of 57,
140 77.19%), one in the 3'UTR regions of the Solute Carrier family 22 Member 2 (*SLC22A2*) gene on
141 LG17, and one in a repeated region (a non LTR Retrotransposon Element on LG20). In the
142 remaining cases (*n* = 11), DMCs are intergenic and located in a window ranging from 0.9kb to
143 51kb to the closest gene (respectively: *SASH1A* on LG12 and *TRMT11* on LG16; Table 1). Two
144 pairs of overlapping, but inversely oriented genes share on their sense vs. antisense strand an
145 identical DMC: *PLG* and *SLC22A2* on LG17, and *SART3* and *FICD* on LG20 (Table 1)

146 When located on the same LG, DMCs were usually distant by at least 30kb from each
147 other. In only four instances, DMCs occurred as differentially methylated regions (DMRs) that
148 grouped 2-3 DMCs separated by only few hundreds of base pairs (Table 1). This includes three
149 hypo-methylated cytosines grouped in a DMR located ~1500 bp downstream of the predicted
150 Kelch Repeat and BDB domain 13 (*KBTBD13*) gene with at most 88 bp between the cytosines.
151 Another DMR includes three hypomethylated cytosines (>20%) on LG1A in the second intron of
152 the forkhead box J3 (*FOXJ3*) gene. Two other groups of two cytosines were found either in the
153 same exon (distant by 3 bp) of the *BTR30* gene or in two different introns (distant by 3.8Kb) of a
154 predicted CREB regulated transcription coactivator 2 (*CRTC2* or *TORC2*: Transducer Of Regulated
155 cAMP Response Element-Binding Protein [CREB] 2) (Table 1). In these two latter cases, one

156 cytosine was found hypomethylated while the other is hypermethylated. A single DMC was
157 associated to a repeat region and two DMCs were found to refer to the same gene (homologous
158 to the *Gasterosteus aculeatus* paralogue of *COL4A5*, a collagen gene of type IV mostly
159 implicated in the protein network of the basement membrane) (Table 1). For this gene, one
160 DMC is located in the first intron while the second is 16.5kb upstream of the start codon. They
161 were not considered as a DMR.
162
163 **Clustering**
164 Hierarchical clustering showed a strong family effect in methylation patterns (i.e. individuals
165 within family clustered together; Fig. 3). The four individuals that were caught twice clustered
166 together by pairs in all four cases. These individuals have the lowest levels of dissimilarity in
167 hierarchical clustering, suggesting very few differences in their methylation profiles in pre- *vs*
168 post-stress condition. Despite this strong family effect and clues of low impact of the stress on
169 methylation, pre- (T0) and post-stress (T4) groups can be distinguished based on their DMC
170 profile in PCA. Mean loading scores of individuals were found significant among T0 and T4 for
171 PC1 that explained 7.2% of total variance (Student *t*-test; $p < 0.005$, Fig. 4). No significant
172 difference was found for loading scores along PC2 (3.0% of total variation; $P = 0.404$).
173
174 **Literature surveys**
175 Several literature surveys (see the Methods section for details) were performed to better
176 understand if DMC-related genes detected in sea bass RBCs could effectively *(i)* represent real

177 candidates to investigate the stress response if fish, and *(ii)* be indicative of processes occurring
178 in the brain.
179 *Thematic survey* - Our first literature survey showed that DMC-related genes detected in this
180 study were found involved in one of the eight themes we retained to describe the stress
181 response in fish (Fig. 2 and Additional File 3). Most of them were previously analysed or
182 detected in human or mice stress studies, in few cases in other model organisms (see Additional
183 File 3). Additionally, the *GLI2* gene encoding for a zinc-finger protein and the *BTR30*
184 (bloodthirsty-related gene family member 30, also known as E3 ubiquitin-protein ligase *TRIM39*-
185 like) gene (Table 1) were found differentially regulated in sea bass cultured-cells submitted to
186 viral infection and thus involved in the immune response [91].
187
188 *Transcriptomics survey in zebrafish* - In a second time, we searched for differential expression of
189 sea bass DMC-related genes in studies using high-throughput transcriptomics in fish blood. Only
190 four studies were retained [38-40,92], and none of our DMC-related genes was found to be
191 differentially expressed in these studies.
192 After curation of PubMed lists and addition of few undetected studies, we surveyed 22
193 zebrafish studies analyzing the transcriptomics of the stress response in six tissues: brain (n =
194 10), liver (n = 7), muscle (n = 2), gonads (n = 1), intestine (n = 1), skin (n = 1) (no high-throughput
195 transcriptomics study in gill, kidney, one in muscles of eye that was accounted for 'muscle';
196 Additional File 4). The main outcome of this survey was to document 38 out of 51 RBC's DMC-
197 related genes (74.51%) in stress-related studies. Strikingly, these 38 DMCs were reported mainly
198 from few neurotranscriptomics zebrafish studies that dealt either with reactive-proactive

199 behavioural response to stress [93, 94] or with changes in social regulation that may promote
200 stressful behaviour among congeners [95, 96] (Fig. 2 and Additional File 3). Some genes are
201 shared among these studies. A recent studies recorded five of them as also differentially
202 expressed in Siamese fighting fish (*Betta splendens*) brain during male contests [97] (see
203 Additional File 3), *FBOX33* was not formerly reported in zebrafish, suggesting that 39 DMCs
204 found in sea bass RBCs could be associated to stress.

205 Only four of these DMC-related genes were reported in the former RNA sequencing
206 study of Anastasiadi and Piferrer [83] in sea bass. This included *CILP1* and *PTGFRN* in muscle,
207 *PRKCQ* in testis, and *RRM2* in liver (blood and brain not investigated) (Fig.2). In comparison, the
208 twelve other zebrafish studies investigating other organs than brain retrieved only 15 of the sea
209 bass DMC-related genes detected in RBCs (see Additional File 4).

210

211 *Epigenomic survey* - We finally looked at former studies dealing with epigenomics in fish. The
212 studies retained (*n* = 31) are presented in Additional File 5. Twenty studies reported differential
213 methylation at genes also found differentially methylated in sea bass RBCs (see Additional File
214 5). Data sets were highly heterogeneous for different reasons (e.g. experimental set-ups, nature
215 of stressors if any, thresholds considered to define a DMC/DMR). Main observations might be
216 summarized as follows: all studies considering brain tissue for which gene annotations were
217 made available (*n* = 6) reported at least one match with DMC-related genes found in the present
218 study. More specifically, data of the single former sea bass epigenetic study performed at a
219 genome-wide level [83] reported five genes of the present study as differentially methylated in
220 the brain (*GLG1a, GFRA2, CELF2, PRKCQ, ROBO3*), three in muscle (*CILP1, FURIN, KBTBD13*), one

221 in testis (*GLG1a*), and any in liver (total: eight distinct genes). This indicated slightly more
222 matches with the brain than with other organs investigated so far. However, the single previous
223 study analyzing RBC methylome in fish so far [41] shared no DMC-related gene with the present
224 study. Finally, the study that reported the largest number of genes also found as DMC-related
225 genes in sea bass (*n* =15) was by Gavery et al. [45]; These authors were interested in the liver of
226 hatchery *vs* wild rainbow trout during a so-called 'temporal analysis' (see Additional File 5).
227 Another study on zebrafish reported 11 genes in common with the present study, but was not
228 organ specific (see Additional File 5) [53].
229
230 **Protein-protein interactions**
231 Further database mining screening for specific protein interactions on the STRING server revealed
232 few other possible pairs of associations (*n* = 8): *ROBO3-CHN1*, *ROBO3-PRKCQ*, *ROBO3-LRRC3*,
233 *DLG1-NCBP2*, *FURIN-PLG*, *PLG-MMRN2a*, *CELF-RRM2*, and *CRTC2-DENND4B* (see Additional File
234 6). The *FURIN-PLG* and *DLG1-NCBP2* associations were also detected in our literature survey.
235 The first one will be presented in the 'Discussion' (see Additional File 3 for the second
236 association). However, most of these associations retrieved only poor information relative to the
237 stress response. *ROBO3* and *CHN1* have been shown to interact in the brain with poorly-
238 understood implications of *CHN1* in neurological disorders [98] (*CHN1* may have other stress-
239 related functions, see Additional File 3). *CELF2* (CUGBP Elav-like family member 2) and *RRM2*
240 (Ribonucleotide reductase M2 polypeptide) are both known to participate to messenger RMA
241 (mRNA) metabolism [99]. *CELF2* acts to post-transcriptionally stabilize mRNAs by relocating
242 them to stress granules in the cytosol. *CELF2* interferes with *RRM2* that modulates its splicing

243 activity. As post-transcriptional activities are at the core of methylation studies, the detection of
244 this association is relevant to our study. To illustrate some limits of these associations: *CRTC2*
245 and *DENN4B* were detected apparently because they are located close to the sex determining
246 region of *Onchorhyncus mykiss* [100]. No relationship with stress is suspected. The relationship
247 between multimerin (*MMRN2*) and plasminogen (*PLG*) via its co-activator remains poorly
248 investigated and supported [101]. This automatic search using STRING did not really enrich our
249 findings.

250

251

252 Discussion

253

254 We showed that a modified epiGBS protocol originally proposed by Van Gurp et al. [88] was
255 applicable to further analyze patterns of cytosine methylation at a genome-wide scale in *D.*
256 *labrax*. This is the first use of epiGBS in fish and the second in an animal species (Canadian lynx
257 [102]). The addition of a second restriction enzyme illustrates the flexibility of the epiGBS and
258 more generally of reduced-representation bisulfite sequencing (RRBS) protocols to improve data
259 acquisition and impact [103]. This is however not the first RRBS protocol dedicated to the
260 addition of a second restriction enzyme [104], but this is hereby proposed in a context of
261 improved multiplexing of samples and increased sequencing capacities, thus offering more
262 opportunities to explore important biological processes, such as the stress response. Data
263 allowed us to explore how RBC's DNA methylation may respond to stressors in the European sea
264 bass and - albeit some limitations discussed below - was shown to complement both the

265 traditional evaluation of the stress response based on analyses of blood serum samples
266 [85,105], or on gene expression variation, often using invasive brain samples [93,94,106-111].

267 **Mining the epigenome…**

268 The information provided in this study is based on the analysis of 47,983 distinct methylated
269 sites distributed over all sea bass LGs. The mapping efficiency was high (74.5%) when compared
270 to early values retrieved in human (~65%) [112], or in some recent fish studies screening for
271 genome-wide methylation (e.g. 55-60% in [59]; 40% in [41]). Other studies reported similar
272 mapping efficiencies, but reported percentages of mapping for unique best hits that were
273 generally lower. For example, in *Kryptolebias marmoratus*, Berbel-Filho et al. [43] reported a
274 mean mapping efficiency of 74.2% but 61.1% unique best hits while, in this study, this latter
275 percentage reached 73.0%. This reflects a more robust mapping of the DMCs we detected and
276 significantly enlarge the breadth of the sites that can confidently exploit to retrieve functional
277 information. Taking advantage of the epiGBS protocol that allow to process more samples [88],
278 the number of individuals considered in this study is rather high (n = 70 distinct individuals),
279 when most epigenomic studies in fish dealt with less than 30 individuals (range: n = 3 in [60]; n
280 = 106 in [47] for a population study). In sea bass, Anastasiadi and Piferrer [83] previously
281 reported a study that used 27 samples and as many libraries to be sequenced while our data
282 were obtained from a unique library preparation. Our modified epiGBS protocol provides a
283 considerable amount of information, certainly at a reasonable cost, to decipher methylation
284 landscapes of sea bass or other species.

285 The operational and statistical thresholds used in the successive steps of this study are
286 large, resulting in the discovery of a rather low total number of methylated sites. For example a

287 threshold of 30X and nominal cut-off value of 0.001 are quite conservative, when some studies
288 might consider a threshold of 5X or 10X for a CpG to be analysed and associated cut-off values
289 of 0.05 or 0.01 (e.g. [46,59,83]). Relaxing thresholds would enable to retrieve more DMCs, but
290 elevated thresholds should normally ensure that access to relevant information is reached.
291 Thus, only 57 DMCs have been found in RBCs of pre- and post-stress European sea bass, and
292 only few were grouped as DMRs. Considering DMRs rather than DMCs is common in genome-
293 wide methylation studies, including sea bass [83]. Methylation changes grouped in clusters (i.e.
294 DMRs) are more likely to influence transcriptional activity at nearby loci than single cytosines
295 [113]. However, other experimental issues associated to the flexibility of RRBS protocols (e.g.
296 single- *vs* paired-end sequencing, likelihood of annotation, selection and size of fragments) can
297 made the advantages of considering DMRs over DMCs perhaps more disputable [103] and
298 DMRs are not always accessible [114]. In this study, DMCs were found mostly hypermethylated
299 in post-stress individuals compared to the pre-stress individuals, and mostly located in gene
300 bodies of fifty-one different genes (44 out of 57; 77.2%). It has been shown that such location of
301 differential methylation may regulate splicing and/or act as alternative promoters to reshape
302 gene expression [115-117]. Thus, despite a low number of DMRs in this study, a large fraction of
303 DMCs points to the transcriptionally active portion of the genome and to important stress-
304 related genes.

305 In addition to DMCs located in gene bodies, a dozen of DMCs were found in intergenic
306 regions (21.0%). Intergenic cytosine methylation has been frequently described, including in
307 stress studies [118], but its role remains poorly understood [119,120]. While numbers of genic
308 *vs* intergenic DMCs may greatly vary, a ratio of ~80% of DMCs located in gene bodies and ~20%

309 located in other genomic regions has been reported in other fish studies (e.g. [46]). Sometimes
310 proportions are approximately reversed (~80% intergenic *vs* ~20% in gene bodies; e.g. [50]).
311 While under-represented in our data, the role for differentially methylated transposable
312 elements is also poorly understood [121, 122], while crucial in the stress response [123].
313
314 **… but mining the unknown**
315 Studies looking at the epigenomic landscape of RBCs in fish are scarce. Gavery et al. [41]
316 reported 85 DMRs between hatchery and wild fish in *O. mykiss*. This study suggested that blood
317 DNA methylation patterns are indicative of the stressors experimented in the environment, but
318 we are still currently mining the unknown to gain supporting information.
319 We nevertheless demonstrate that fish RBCs are relevant candidates to investigate the
320 stress response at the epigenomic level in the European sea bass. Numerous DMC-related genes
321 reported here have been previously shown to respond to stress or implicated in variation of the
322 stress response in animal models. Overall, our comparative surveys showed that the DMC-
323 related genes we detected in sea bass using RBCs have more connections with results found in
324 the brain than in other tissues. This relationship is however weak at the epigenomic level and
325 about one third of our DMC-related genes have also been detected in liver of trout [45]. This
326 relationship is far more pronounced when genome-wide gene expression difference is
327 considered. Indeed, genome-wide neurotranscriptomics studies dealing with personality and
328 stress coping styles [93,94], or social regulation [95] collectively reported thirty-eight of our 51
329 DMC-related genes as expressed and potentially associated to the stress response in zebrafish
330 (one added when including a study on *B. splendens* [96]). As differential methylation is

331 classically interpreted as leading to differential gene expression (but see, e.g., [124, 125] for a
332 more nuanced consideration), this observation is one interesting signal indicating that RBCs
333 might monitor events occurring in the brain. However, it remains unclear why most of them are
334 related to coping and personality, and to studies where fish were under stress for few minutes
335 [93,94], hours or days [95]. How distinct stress contexts and challenges may apparently rely on
336 similar stress circuitry has to be investigated further.
337

338 **A brain-derived neutrophic factor (BDNF) network**

339 The roles of the DMC-related genes have to be exposed more extensively to illustrate their
340 possible involvement in the stress response of sea bass. This is not place to fully expand on all
341 DMC-related genes (see Additional File 3 for details), but one single selected example can be
342 developed. The best representative is a set of DMC-related genes from the BDNF network
343 whose role in the stress response is commonly reported. BDNF is a protein synthesized in the
344 brain that offers resistance to neurodegenerative diseases and favors stress adaptation and
345 resilience [126,127], but also energy homeostasis [128]. BDNF has emerged as one of the most
346 important molecule in memory [129, 130]. It consolidates both the within- and between-
347 generation fear memory owing to epigenetic regulation [131,132]. Its activity is strongly linked
348 to glucocorticoid stress to imprint neurogenesis [133] and it acts as both a regulator and a target
349 of stress hormone signaling [127]. In fish, cortisol binds the glucocorticoid receptor (*gr*) and
350 controls BDNF expression in the brain. With *gr* and relatively few other genes, BDNF has thus
351 emerged as one of the target genes of interest in stress studies, as shown for zebrafish [134],
352 sea bream [135] and sea bass [86,136-138]. BDNF is also involved in other aspects of the stress

353 response in fish (e.g. regulation of the microbiota-gut-brain axis [139]). Variation in BDNF
354 methylation status could not be investigated in this study as no *SbfI* restriction site is present
355 within or close to this gene. Nevertheless, a methylation signature of stress associated to BDNF
356 is likely present in our data and illustrates how blood methylation may provide a snapshot of
357 events studied in the brain.

358 Indeed, several DMC-related gees are involved in the maturation of proBDNF to mature
359 BDNF or the regulation of its activities (*ABLIM2, ADCY1b, CRTC2, FURIN, NPAS3, PLG,* and
360 possibly *SLC22A2*). Most of these DMC-related genes were already found differentially
361 expressed in the zebrafish brain [93-95], but an outline of their interaction was not presented.
362 The adenyl(ate) cyclase (AC, *ADCY1b* gene) is a brain-specific signaling enzyme that synthesizes
363 the cyclic AMP [140]. In the brain, AC is activated by PACAP (Protein Adenylate Cyclase
364 Activating Protein) and stimulates memory and long term potentiation [141]. This inducible
365 signaling pathway participates to the synthesis of the active form of BDNF (proBDNF to mature
366 BDNF) [142]. ProBDNF is processed by furin and the plasminogen system [128], including
367 processing steps that necessitate actions of actin-binding LIM kinases (ABLIM) [143]. Furin -
368 encoded by *FURIN* - is a subtilisin-like protein proconvertase ubiquitously expressed in fish
369 [144]. It presents an intracellular cleaving activity of numerous biologically relevant molecules in
370 the trans-Golgi network. This includes BDNF in the brain, notably in pre-synaptic axons. In mice,
371 furin has been shown to participate to dendrite morphogenesis and modulate learning abilities
372 and memory [145]. Stress imprinting at *FURIN* is likely and it has recently been shown that
373 transgenerational epigenetic effects of furin activity were active in brain of mice [146]. ProBDNF
374 cleavage by furin depends on brain AC and CREB (cAMP response element-binding protein)

375 signaling [147,148] and plasminogen (*PLG*) [149]. This activity is modulated by stress hormones
376 (corticosteroids) and is essential to brain hippocampal plasticity in mammals [22,150]. One
377 interesting supplementary observation is that CREB signaling necessary to furin is associated to
378 *CRTC2* - a CREB co-activator – found as one of the four DMRs reported in this study. In mice,
379 *CRTC2* is known to act as a switch for BDNF and glucocorticoids to direct the expression of
380 corticotropin-releasing hormone (CRH) in the hypothalamus [151].

381 Addiitonally, in the brain, plasminogen encoded by *PLG* is converted to plasmin that
382 cleaves BDNF in the extracellular synaptic domain [128,152]. *PLG* has also been shown to
383 regulate pro-opiomelanocortin (POMC) in the hypothalamic-pituitary axis, then the production
384 of peptides hormones such as the adrenocorticotropic hormone (ACTH) [153].

385 In relation to BDNF, two other DMC-related genes should be mentioned: *NPAS3*
386 (neuronal PAS domain containing protein 3) and *SLC22A2*. *SLC22A2* - also known as *OCT2*
387 (organic cation transporter 2) – associated to the unique 3'UTR DMC found in this study is
388 implied in numerous transmembrane transports [154], including at the blood-brain barrier
389 [155]. It was found involved in memory in mice [156,157] or *Drosophila* [158], and associated
390 itself to neurodegenerative diseases [159]. Substrates of *SLC22A2* include neurotransmitters
391 such as norepinephrine, but also dopamine, serotonin and a wide-variety of internal or
392 exogenous compounds [160,161]. Functional links of *SLC22A2* with BDNF remain however
393 poorly documented [162], while they have been found co-expressed and co-regulated in the
394 brain during drug administration experiments (e.g. methamphetamine, mimicking the action of
395 catecholamines) [163]. In this study, *PLG* and *SLC22A2* are associated to the same DMC; the
396 functional significance of this situation should be investigated further. Finally, *NPAS3* has a well-

397 established action in memory [164,165]. It participates to neurogenesis in the hippocampus
398 within a network that also includes BDNF [166]. *NPAS3* is also associated to the glial cell line-
399 derived neurotrophic factor (GDNF) receptor-alpha2 gene (*GFRA2*) detected in this study and
400 related to stress and anxiety [167]. The *DLG1* (Disk-large homolog 1) gene could be also
401 associated to BDNF, but this will be not develop further (see Additional File 3).

402 Overall, results suggest that even in absence of BDNF sequencing reads, some
403 DMCs/DMRs with a potential impact in fear memory and anxiety response as expected in fish
404 after an acute stress challenge certainly did not occur only by chance in this study. They are also
405 related to processes that regulate the HPI axis and hormones. The measurement of methylation
406 difference now used to monitor the BDNF activity and its relationship to stress [132,168] could
407 thus potentially depend on the methylation status observed at or close to *ADCY1b, ABLIM2,*
408 *FURIN, CRTC2*, *PLG* and other genes such as *SLC22A2* and *NPAS3*. This should be investigated
409 further to improve our understanding of changes in the directionality of multigenic epigenetic
410 modifications and inter-individual variation in stress coping. Candidate genes coming from other
411 fish or more generally vertebrate studies with role in memory consolidation could be added
412 (e.g. [112,169] for proposals).

413 As at least *ADCY1b, FURIN, CRTC2*, *PLG* and *SLC22A2* have other well-recognized
414 relationships with stress and the stress response (especially the glucose metabolism and the
415 immune function; see Additional File 3), this also illustrates the complex non-linear network
416 modulating the stress response in fish with a pivotal role in the brain associated to BDNF and
417 secondary additional outcomes in other tissues and organs.

418

419 **Family effect, development and few individuals**

420 While the PCA showed that pre- and post-stress individuals have in average distinct methylation
421 profiles that may represent stress imprinting during the challenge test, a strong family effect
422 was also detected. Family effects were already reported in fish epigenetic studies [51,54,63],
423 and sea bass families certainly do not have the same sensitivity to stress. This may suggest that
424 (*i*) their genomic backgrounds alter how family will develop an epigenetic stress response over
425 the period covered by the challenge test, (*ii*) that the developmental period during which
426 families were distributed over different tanks was a "critical time window" [170] sufficient to
427 imprint individuals and lead them to develop a familial response to stress during the challenge,
428 or that (*iii*) some degree of transgenerational inheritance in methylation profiles is present.
429 These proposals are not mutually exclusive. Transgenerational inheritance has gained interest
430 over the last years [171], notably in marine species [50,172,173], and including sea bass [83].
431 Parents and/or the germline were unfortunately not sampled in this study for further analysis.
432 As Gavery et al. [41] reported that *BTR30*, *DLG1*, *MMRN2*, and *NOL4B* - also found in this study -
433 were differentially methylated in the sperm of *O. mykiss*, these differentially methylated genes
434 could be interesting candidates for transgenerational inheritance study of the stress response.
435 Other candidates are possible. For example, Ghalambor et al. [108] reported rapid evolution in
436 brain gene expression of the Trinidadian guppy (*Poecilia reticulata*) for the *TNKS* and *NCBP2*
437 genes, found as DMC-related genes in this study. It would thus be interesting to investigate how
438 gene expression and cytosine methylation at these genes may co-vary within and between
439 species over generations.

440 Anastasiadi and Piferrer [83] showed that the domestication process in *D. labrax* resulted
441 notably in genome-wide methylation differences for genes involved in the nervous system and
442 neural crest cell differentiation. In our study, *GLI2* [174], *PBX1* [175], *DLG1* [176] and *FOXJ3* [177]
443 are DMC-related genes involved in neural crest cell proliferation and differentiation. *ROBO3* is
444 involved in the immune response, but also implied in early neurogenesis [178], as well as *NPAS3*
445 [179]. With *BMP3* [180], *FURIN* [181]; *NOL4B* [182], and *Myf5* [183,184], most of these genes
446 are engaged in the development of the anterior region and/or the craniofacial skeleton which is
447 modified during sea bass farming [83]. This suggests that methylation patterns observed in sea
448 bass could also potentially rely on the ontogenetic regulation of a particular phenotype
449 between pre- and post-stress fish, rather than being directly related to the stress challenge.
450 Variation in gene expression or methylation accompanying ontogeny are reported in fish [45,
451 185] and, as for other organisms, cytosine methylation is known to vary with age in sea bass
452 [84]. Collateral effects of stress might thus be present in our data as suggested by the fact that
453 fifteen genes we found differentially methylated in RBCs were reported as also differentially
454 methylated in the liver of the rainbow trout [45] (see Additional File 5).

455 On another hand, the epigenetic profiles of four individuals were analyzed in the pre-
456 and post-stress situations and clustered very closely from each other in each case. While based
457 on few observations, this suggests that neither the stress challenge itself, nor development have
458 a strong impact on the cytosine methylation landscape in sea bass. How a transgenerational and
459 within-generation stress-imprinting events influenced by ontogeny may interact to shape both
460 the plastic and the heritable component of the stress response in relation to environmental
461 stimuli require in depth evaluation [186,187]. Nature and strength of family-based epigenomic

462 variation are of considerable importance for selection breeding improvements in a cultured fish
463 like sea bass [188], including also issues about health and welfare [189].

464 **Blood and RBCs: extending the perspective**

465 As fish engaged in aquaculture programs as sea bass are costly and based on breeders that
466 cannot be easily sacrificed, epiGBS using blood samples may offer a genome-wide assessment of
467 stress-induced epigenetic marks over a significant number of individuals and at reasonable cost.
468 Blood is widely used in human epigenomics, but not in fish despite the presence of nucleated
469 cells and minimally invasive sampling that allows for multiple measurements. Using RNA-
470 sequencing, authors [38,39] already suggested that fish erythrocytes would be useful to provide
471 insights on the innate immunity or response to pathogens. Our study suggests that blood could
472 effectively offer an in depth insight for the evaluation of the stress response from a novel
473 perspective, accompanied by classical physiological measurements (e.g. cortisol, glucose,
474 lactate) and may provide a more complete picture of the stress response in fish than currently
475 performed.

476 The link between brain and blood epigenomics remains however to be explored more
477 deeply in fish and requires careful evaluation and validation to correct for tissue specificity, as
478 requested in human [190]. Our attempts to survey fish studies undoubtedly showed that
479 considerably more knowledge has to be accumulated before to reach this issue. In human, the
480 lack of access to brain tissues have been shown to impede some epigenomic studies and
481 naturally raised ethical issues certainly also valid for fish [191,192]. *In situ* hybridization
482 techniques in brain have been shown to be especially important to link neurobiological activities
483 and stress coping in fish [134,193]. Their use and thus sacrifice of individuals cannot be ruled

484 out. Actually, methylation differences among full brain or brain areas have been rarely studied in
485 fish, mostly in zebrafish [194], but recently in a tilapia [48], a goby [56], and a killifish [43].

486 Conclusion

487

488 The European sea bass has become one of the most studied species in fish epigenetics [77-84].
489 Because of the availability of its genome and the development of selection programs, efforts
490 should be dedicated to further explore its epigenomic landscape regarding the stress response,
491 especially in an aquaculture context. This should be done over a multigenerational scale to
492 identify pathways structuring the stress response at key life stages, on different tissues to better
493 delineate stress-responsive cellular networks, and investigating the effects of the nature, the
494 duration, and the number of stressors. Studies in epigenomics including phenotypic information
495 [195,196] could increase the panel of diagnostic tools for breeding selection in aquaculture if
496 blood could be – at least partly - substituted to brain or other tissues when studying the stress
497 response. Screening for genome-wide epigenomic variation in natural populations as already
498 done for genomic variation [197] is now also conceivable in this species. Our modified version of
499 the original epiGBS protocol seems to be a powerful and affordable method to screen a
500 significant number of individuals with sufficient depth and coverage to reach meaningful
501 conclusions. While it should be compared to others (e.g. [104,198,199]), our protocol is close to
502 the double digestion restriction site DNA sequencing protocol used in genomics. Their parallel
503 use certainly deserves attention to design more integrated epigenomic-genomic studies
504 [200,201], and, more generally, multi-omics investigations of stress, health and welfare
505 [202,203].

506

507

508 # Methods

509

510 **Rearing, stress challenge, and blood sampling**

511 Four hundred European sea bass were initially used in this study. Fish were produced in the
512 hatchery facility of Nireus S.A. from breeders maintained in this company for scientific purposes.
513 They resulted of a single crossing experiment (12 dams, 20 sires) that took place in January
514 2018. During the larval phase, 20 different families labelled from A to T were raised. Each family
515 was reared separately in open circulation tanks at Nireus S.A. research facilities (Greece). To
516 avoid environmental effects, fish were tagged at ~280 days post-hatch (July 10-13th 2018) and
517 distributed in 20 tanks; each tank receiving 1 fish from each family (i.e., 20 fish per tank). Fish
518 were fed twice a day, for 6 days a week, using a commercial diet (Blue Line 45:20 3.5 mm,
519 Feedus S.A., Greece). Throughout the experimental period, the photoperiod was set at 12L:12D,
520 the water temperature and the salinity held constant (18.1 ± 0.2°C and 28 ppt, respectively).
521 Fish weights (mean ± SD) were 48.1 ± 12.8g and 86.2 ± 24.1g in pre- and post-stress individuals,
522 respectively.

523 Fish were submitted to one acute challenge test per month for three consecutive
524 months, from July to October 2018. During this challenge, fish were exposed to high density
525 stress by lowering water levels in the tank to 1/3 of the original volume, followed by chasing of
526 the fish with a net for five minutes and a 30 min waiting period before sampling. These stressors

527 are classical in sea bass studies regarding response to acute stress [16]. This protocol took place
528 in each tank then for each fish individual entering the experiment. It was repeated for 3
529 consecutive times at 20-21 day intervals (period long enough for fish to recover).

530 An initial blood sampling occurred two weeks prior to the implementation of the stress
531 challenge (hereafter T0, July 19th, 2018, pre-stress/control group) then at the end of the
532 challenge test (hereafter T4, October 5th, 2018). Blood samplings were performed in
533 anesthetized fish. Specifically, fish were anesthetized in 2-phenoxyethanol (300 ppm; Merck;
534 807291; USA). Recovery was performed in a separate tank with provision of air before fish
535 returned to their holding tank. Blood from the caudal vessel was collected. Plasma and RBCs
536 were separated by centrifugation at 2,000g for 10 min. Careful separation of the plasma and
537 RBCs was performed using 200 µl pipettes. RBC extracts were heparinized (heparin sodium;
538 Sigma-Aldrich), transferred in microtubes and conserved at -20°C in 1 ml of RNA later. DNA was
539 extracted using the Macherey Nagel Nucleo Spin Tissue DNA kit and quantified using a Qubit
540 fluorometer (Qubit dsDNA BR Assay Kit, Q32853, Invitrogen). Thirty-seven blood samples at T0
541 (pre-stress) and thirty-seven additional samples at T4 (post-stress) have been randomly selected
542 for the downstream epigenomic analysis.

543

544 **Library preparation and sequencing**

545 We followed the epiGBS protocol published by van Gurp et al [88]. As the method was
546 developed for plants (with methylation occurring in CpG, CHH, and CHG context; H being any
547 nucleotide but a cytosine) and used a single digestion approach, the protocol was modified to
548 make it more suitable and straightforward for our vertebrate system. Particularly, a double

549 digest instead of the single digest approach was implemented. We chose the restriction enzyme
550 *MspI*, a standard choice in reduced representation bisulfite sequencing-like (RRBS) studies, as its
551 recognition site targets CpG rich regions [204]. A second enzyme (*SfbI*) with a recognition site
552 length of 8 bp was used to reduce fragment numbers, and thus to increase read depth per
553 fragment. The choice of this enzyme was guided by *in silico* digestion of the European sea bass
554 genome [76] using *simRAD* [205]. This genome is available at:
555 https://www.ensembl.org/Dicentrarchus_labrax/Info/Index.

556 One single library was prepared for a set of 74 samples. For this library, 200 ng of DNA of
557 each sample were digested in a 40 µl reaction, using 0.25 µl *MspI* (NEB, 20,000 U/ml R0106S),
558 0.25 µl S*bfI*-HiFi (NEB 20,000 U/ml R3642L) and 4 µl of 10X cutsmart buffer. The reaction was
559 run overnight at 37°C. Unique forward and reverse adapter combinations allow multiplexing
560 samples in the library. We added forward and reverse adapters in unique combinations (1 µl of
561 adapter, 2.5 µM), 0.5 µl T4 Ligase (NEB, 400,000 U/ml m0202L), 6 µl of 10X T4 ligase buffer and
562 11.5µl water were added directly to the digested DNA. Sequences of adapters are provided as
563 Additional File 1. Adapters were ligated for 3 h at 23°C followed by 10 min of enzyme
564 inactivation at 65°C. After ligation, all samples were pooled and one third of the total volume
565 was used in the following step. The mixture volume was reduced using a Qiaquick PCR
566 purification kit (28104 Qiagen). The resulting product was cleaned a second time to ensure the
567 removal of small fragments and adapter remnants using CleanPCR paramagnetic beads
568 (Proteigene, CPCR-0050) with a ratio of 0.8X [sample : beads].

569 Since oligonucleotides were not phosphorylated (see Additional File 7), a nick translation
570 was performed to repair the nick of the DNA at the restriction site and to fully methylate the

571 hemi-methylated adapters. To do so, 19.25 µl of the concentrated and purified ligation pool
572 were used in a 25 µl reaction, including 0.75 µl DNA Polymerase I (*E. coli*, 10,000 U/ml M0209S),
573 2.5 µl of 10 mM 5-Methylcytosine dNTP mix (Zymo Research, D1030), and incubated at 15 °C for
574 one hour. The library was then treated with sodium bisulfite using the EZ DNA Methylation Gold
575 Kit (D5005, Zymo Research) following manufacturer instructions to convert unmethylated
576 cytosines to uracil, paying attention to the optimal DNA amount per reaction. After bisulfite
577 conversion, 14 cycles of PCR (95°C for 1 min, 91°C for 10s, 65°C for 15s, 72°C for 10s and a final
578 elongation step at 72°C for 5 min) were performed, followed by a paramagnetic bead clean up
579 with a 0.8X ratio. The library quality (fragment size distribution, no adapters left, no primers
580 left; fragment size range from 300 to 800 bp; see Additional File 8) was verified on an Agilent
581 5300 Fragment Analyzer (Santa Clara, USA). It was sequenced (paired-end, 150 bp) on one lane
582 of a SP flow cell on an ILLUMINA™ NovaSeq 6000 at the MGX sequencing facility in Montpellier,
583 France.
584
585 **Bioinformatics analysis**
586 Raw sequencing data were trimmed of low quality reads and adapter residues using *Trim*
587 *Galore!* (v.0.6.4; available at
588 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The trimmed reads were
589 then demultiplexed using the `process_radtags` command of STACKS [206]. The option
590 `disable_rad_check` was applied to avoid reads with bisulfite-modified restriction sites to
591 be discarded. The demultiplexed reads were trimmed a second time on both 5' and 3' ends with
592 the options `--clip_R1` `--clip_R2` and `--three_prime_clip_R1` `--`

593 `three_prime_clip_R2` to remove introduced methylated cytosines during adapter ligation.
594 Using the `bismark_genome_preparation` function of the program *bismark* [112], a
595 bisulfite converted version of the European sea bass genome was prepared against which the
596 demultiplexed and trimmed reads were mapped.
597
598 **Methylation analysis**
599 The R package MethylKit [207] was used to determine differential methylation between pre-
600 and post-stress fish. CpGs with less than 30 read depth and with coverage >99.9% of the
601 distribution of read counts were filtered out to account for PCR bias. Coverage was normalized
602 across samples, and only CpGs present in at least 20 out of 37 samples per group (56%) were
603 kept for further analysis. Differentially methylated CpG sites were determined between pre- and
604 post-stress individuals using logistic regression (`calculateDiffMeth` function). Pre-stress
605 individuals were considered as the baseline. Cytosines were considered as differentially
606 methylated when presenting at least 15% methylation difference (as in [83] for epigenomic
607 variation in *D. labrax*), and a nominal *q*-value < 0.001 between pre- and post-stress fish. Reads
608 presenting differentially methylated cytosines (DMCs) were extracted using GENEIOUS (v.11.0;
609 available at https://www.geneious.com/) and mapped along LGs of the European sea bass
610 reference genome to produce a primary annotation of potential candidate genes. As the
611 annotation of the European sea bass and the sea bream (*Sparus aurata*) reference genomes
612 have been recently released on ENSEMBL (http://www.ensembl.org/index.html), gene names and
613 models have been controlled, eventually including a TBLASTN search in case of discrepancy.
614

615 **Literature surveys and protein-protein association**

616 A first literature survey was performed in order to decipher if genes found associated to
617 European sea bass DMCs were candidates related to stress in vertebrates. We searched PUBMED
618 (https://pubmed.ncbi.nlm.nih.gov/) for studies in which they could have been mentioned as
619 responding to stress. This approach was preferred to, e.g. gene ontology or enrichment analyses
620 that are useful summaries of functional knowledge, but less suitable when searching how one
621 or a specific set of genes might be involved more precisely in the stress response, if they are
622 known to be associated to each other and/or how they were found related to stress (e.g. in
623 which organism, context, study, organ or tissues). As relatively few DMCs were detected (see
624 Results), this detailed approach was amenable.

625 The data mining considered a DMC relevant to the stress response when associated to a
626 gene related to a stress phenotype (typically issued from genome-wide association studies,
627 knock-out experiments or empirical evidence of impairment and dysfunction when facing
628 stressors). Eight stress response themes were identified and used to structure the mining:
629 '*blood*' (including issues on red blood cells, blood homeostasis, blood volume, hemato- and
630 erythropoiesis, platelets, hypo- and hypertension, angiogenesis), '*stress*' (including stress-
631 related genes, physiological disorders, HPI/HPA axis, adrenals, head kidney, stress
632 resistance/response), '*immunity*' (including issues on all types of leukocytes and immune
633 response, cytokines, cytotoxicity and response to pathogens), '*hormones*' (including issues
634 related to catecholamines, corticosteroids, glucocorticoids, neuro- and peptide transmitters,
635 other hormones related to the HPI axis in fish), '*brain*' (including issues on hypothalamus and
636 pituitary themselves, forebrain, limbic brain, neuronal development and plasticity, signaling

637 pathways in the brain, serotonergic/dopaminergic activities), '*glucose*' (including issues on
638 glucose metabolism [systemic and brain], lactate, glycolysis, gluconeogenesis, [pro]insulin,
639 diverse receptors, pancreatic β-cells), and '*behaviour*' as stress-induced behavioural changes
640 and not only physiological changes. Diverse health disorders related to stress often well-studied
641 in mammalian models were screened, as well as some genetic disorders that affect behaviour.
642 This may include changes in mood, emotional and affective states (e.g. fear, anxiety), cognitive
643 traits (e.g. memory, learning abilities), or psychiatric disorders and neurodegenerative diseases
644 (e.g. bipolar disorders, autism, schizophrenia). One additional category ('*others*') was included
645 to report significant items known to be important for, e.g., epigenetic regulation that could not
646 be directly addressed with previous themes. Cellular issues of stress such like, e.g., oxidative,
647 chromatin or endoplasmic reticulum stress where not covered.

648 A second literature survey focused on the literature dealing with stress, stress response
649 and high-throughput transcriptomics or epigenomics in fish. We searched PubMed for relevant
650 articles over the period 2010-2020 (June) and, when available, their associated supplementary
651 materials, and searching for difference in gene expression or methylation. Our goal was to
652 evaluate if DMC-related genes detected here in RBCs are relevant to brain and/or processes
653 specific to brain or if they are commonly detected whatever the target tissues and the nature of
654 stressors. For this survey, we first mined gene expression studies that used blood within fish
655 species in order to verify whether sea bass DMC-related genes were not commonly
656 overexpressed in this tissue. In a second time, we focused on zebrafish for which functional
657 annotation is well established. We searched for transcriptomics (RNA sequencing) studies
658 dealing with stress in various tissues (liver, kidney, gill, brain, gonad, muscle, eye and skin). The

659 following search string was used: '*zebrafish*' AND '*RNA seq**' AND '*stress*' AND ['*tissue name*'].
660 As mRNA studies often consider several tissues, results were manually curated. Studies whose
661 title referred to single gene families (e.g. heat shock proteins) or microRNAs were discarded.
662 Finally, fish epigenomic studies were also analyzed. About this issue, we first analyzed gene
663 transcripts and differentially methylated genes in the single genome-wide epigenomic study
664 performed so far in sea bass [83]. As genome-wide methylation studies remain relatively few in
665 fish, this was extended to other species in a second time. Transcriptomics and methylation data
666 sets that did not provide for gene names and/or gene symbols were discarded. Reported gene
667 names and symbols were considered to be exact.

668 Finally, in order to complete these manually curated surveys, we used STRING
669 (https://string-db.org/) to investigate if DMC-related genes encoded for proteins are known to
670 interact together. We used our gene list as input and the zebrafish genome as a reference for
671 annotation. This search was done without reference to the thematic stress categories
672 mentioned above, and when one interaction was provided we specifically explored the
673 literature for confirmation and relevant experimental evidence.

674

675 **Data analysis**

676 A principal component analysis (PCA) on the methylation profiles of individual samples at DMCs
677 was performed to analyse the potential grouping structures within our data set. Differences in
678 the distribution of mean individual loading scores for pre- and post-stress fish were tested along
679 each principal components axis using a Student *t*-test. Additionally, a hierarchical clustering was

680 performed using Ward's linkage method on Euclidean distances in order to explore whether sea
681 bass families structure the data set.
682

683 **Abbreviations**

684 ABLIM: actin-binding LIM kinase; AC: adenyl(-ate) cyclase; ACTH: adrenocorticotropic hormone;
685 BDNF: Brain Derived Neurotrophic Factor; cAMP: cyclic adenosine monophosphate; CpG:
686 cytosine-phosphate-guanine; CREB: cAMP response element-binding; CRH: corticotropin-
687 releasing hormone; DMC: differentially methylated cytosine; DMR: differentially methylated
688 region; epiGBS: epiGenotyping By sequencing, GDNF: glial cell line-derived neurotrophic factor;
689 HPA: Hypothalamus-Pituitary-Adrenal; HPI: Hypothalamus-Pituitary-Interrenal; LG: linkage
690 group; mRNA: messenger RNA; NCBI: National Center for Biotechnology Information; PACAP:
691 Protein Adenylate Cyclase Activating Protein; POMC: pro-opiomelanocortin; RBC: red blood cell;
692 RRBS: reduced representation bisulfite sequencing, UTR: untranslated region.

693

694

695

696 **DECLARATIONS**

697

698 **Ethics approvals and consent to participate**

699 All experiments were performed in accordance with relevant guidelines and regulations. Nireus
700 S.A. research facilities are certified and have obtained the codes for the rearing and use of fish
701 for scientific purposes (EL04-BIOexp-01). All procedures on fish used in this study were
702 approved by the Departmental Animal Care Committee following the Three Rs principle, in
703 accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use

704 of experimental animals. All sample procedures were performed by FELASA certified researchers
705 (http://www.felasa.eu/).
706

707 Consent for publication

708 Not applicable
709

710 Availability of data and materials

711 Individual Illumina raw reads (one round of trimming) and processed files have been deposited
712 in NCBI's Gene Expression Omnibus [208] and are accessible through GEO Series accession
713 number GSE153838 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153838).
714

715 Competing interests

716 None declared

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721 study and collection, analysis, and interpretation of data and in writing the manuscript.
722

723 Author contributions

MVK, ED, BG developed the protocol, MVK and ED conducted the lab work, EG ensured technical assistance and supervised sequencing. AS, AD, MP and CST established the study design used during the challenge test. Experiments with live fish were conducted by AD, AS, MP, with technical contribution of MVK. MVK performed data analyses under supervision of ED and BG. MVK, ED, BG drafted the manuscript; others authors reviewed drafts. CST and BG coordinated this study. All authors have read and approved the manuscript.

Author details

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Figure Legends

Figure 1: Coverage of the 24 linkage groups (LGs) of the sea bass genome by sequencing epiGBS reads. Ordinates represent the mean number of sequencing reads. LG numbers are those used in [76]. Complex IDs resulting from the merging of previously defined LGs during final assembly (e.g. LG22-25 resulted in the fusion of previous LG22 and LG25). SB-UN (sea bass – unknown; hereby SB) indicates a composite LG made from originally unassembled scaffolds [76].

Figure 2: Diagrammatic representation of the involvement of the 51 DMC-related genes found in this study and issues regarding the stress response. One additional issue was added for few genes that are involved in the regulation, the control and the dynamics of epigenetic process (*NCBP2, GTPF*), and acid-base homeostasis in kidney without formal proof of actual relation with a stress issue (*CLDN4*). DMCs located within gene bodies are indicated in black, in brown for intergenic regions including promoters. The hatched symbols refer to genes also reported in

1237 high-throughput transcriptomic studies in zebrafish [93-96] (main text and Additional File 3).
1238 This does not mean that our literature survey was supported only by these studies, but it
1239 illustrates their importance over results. Stars with full line indicate genes detected as
1240 differentially expressed (*BTR30, GLI2*) in one immunity-related transcriptomic study in sea bass
1241 [91], while stars with broken lines indicate genes with methylation differences (*PTPRB, SASH1A*)
1242 (stickleback: [54]; rainbow trout: [58]; respectively). Reports outside of the above-mentioned
1243 studies are indicated by filled symbols. On the right are indicated genes found differentially
1244 expressed (RNA) or differentially methylated (CpG) in the genome-wide study by Anastasiadi
1245 and Piferrer (*A&P 2019*) on European sea bass [83]. Tissues in which genes were found
1246 differentially expressed/methylated are indicated (brain not investigated in transcriptomics).
1247 Association with the *LRRC3* gene is reported in grey as leucine-rich repeat (LRR) gene family is
1248 classically involved in neuroimmunity. Variations in transcription or methylation have been
1249 shown to impact behaviours after stress events within this family (see e.g. *LRRTM4L2* in this
1250 study), but this relationship remains presumptive for *LRRC3*. Additional support including
1251 references for these DMC-related genes is reported in Additional File 3, as well as comparisons
1252 with other epigenomic studies.
1253
1254 **Figure 3:** Hierarchical clustering based on of the 57 differentially methylated cytosines (15%
1255 threshold) of the randomly caught 74 pre- and post-stress sea bass samples considered during
1256 this study. Nineteen out the twenty families (A to T) used in this study are present in this graph
1257 and each family is associated to one single colour. Capital letters refer to the family, while T0
1258 and T4 are for pre- and post-stress individuals, respectively.

1259 **Figure 4:** PCA based on the methylation profiles of the 57 differentially methylated cytosines
1260 (15% threshold) reported in this study (Table 1). Pre- and post-stress individual sea bass (in red
1261 and blue, respectively) differ significantly along PC1 ($p < 0.001$), but not PC2. The insert illustrate
1262 the distribution of individual scores along PC1. Ellipses represent the 95% confidence limits over
1263 PC1 and PC2.
1264

Table 1: Differentially methylated cytosines (DMCs) found in this study between pre- and post-stress sea bass

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	*GRPT1*	growth hormone regulated TBC protein 1	*gb*
LG6	HG916846.1	6,44E-245	20,44	DLAgn_00164450	*GLG1α*	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	*PBX1*	pre-B-cell leukemia homeobox 1	*gb*
LG10	HG916827.1	6,98E-125	21,55	DLAgn_00006210	*ADCY1*	adenylate cyclase 1 (brain)	*gb*
LG11	HG916828.1	0	27,70	DLAgn_00016970	*MMNR2*	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	*CSMD3α*	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	*ZMAT4*	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	*GLI2*	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*

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 in bold. 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 Additional File 2.

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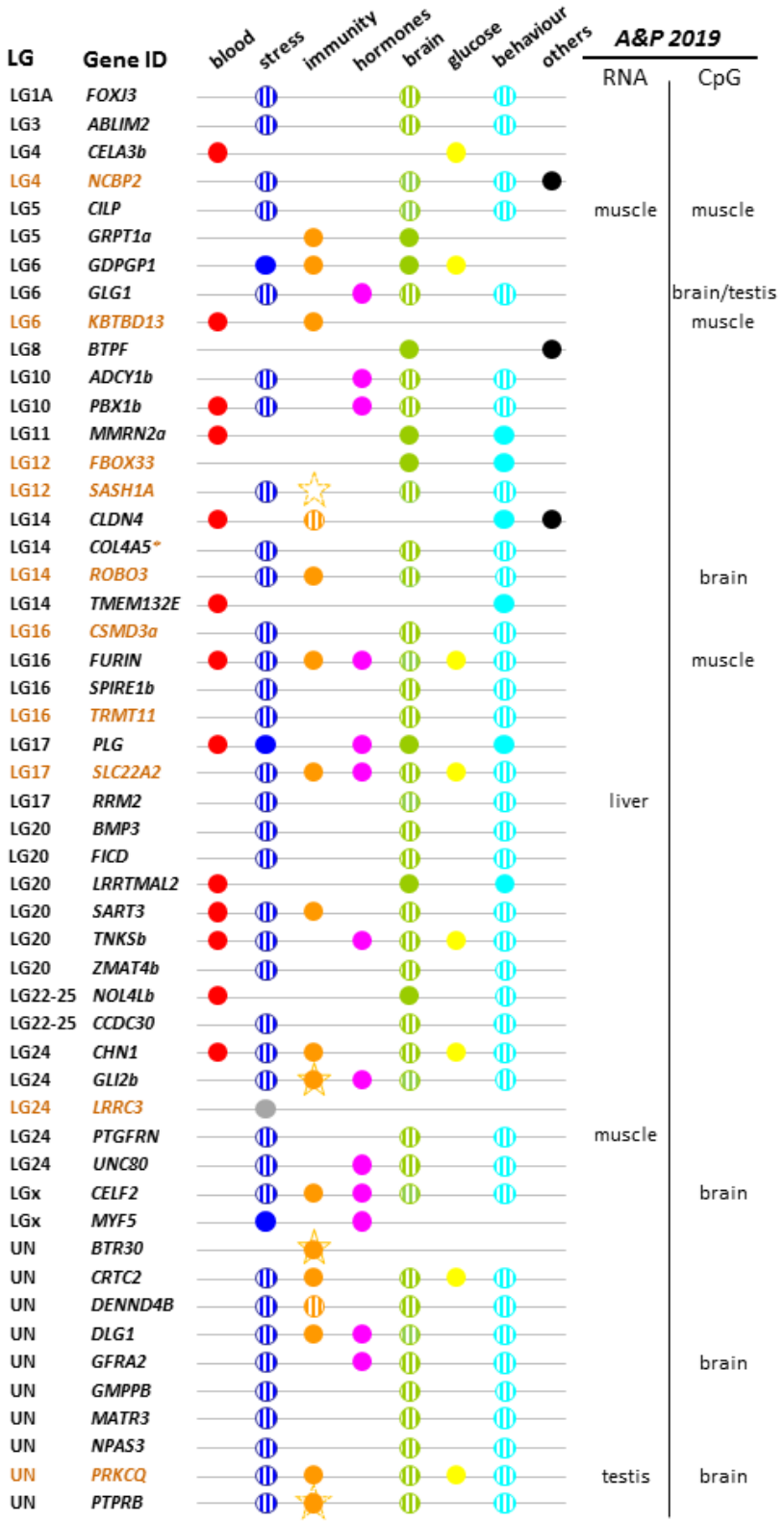
Figure 1:

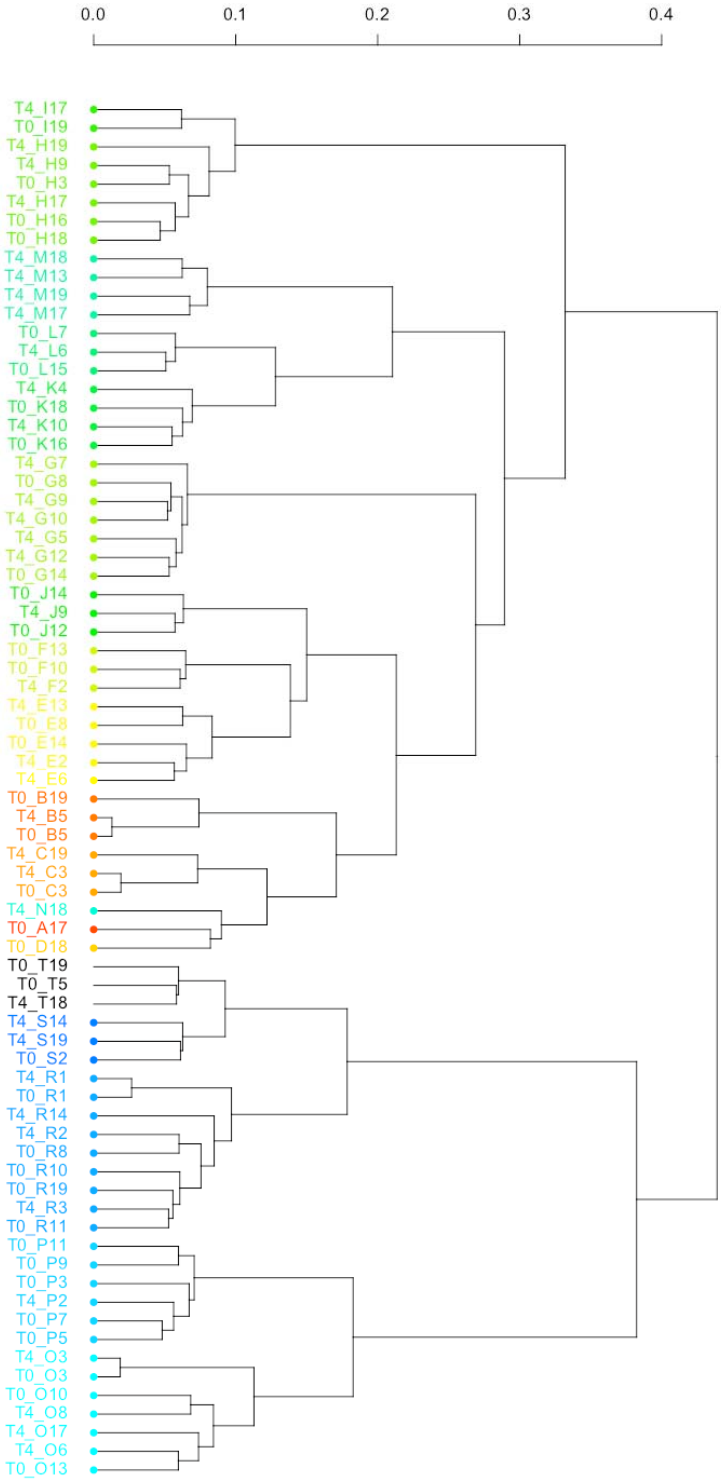
LG10
LG11
LG12
LG13
LG14
LG15
LG16
LG17
LG18-21
LG19
LG1A
LG1B
LG20
LG22-25
LG24
LG3
LG4
LG5
LG6
LG7
LG8
LG9
LGx
SB

1270 **Figure 2**



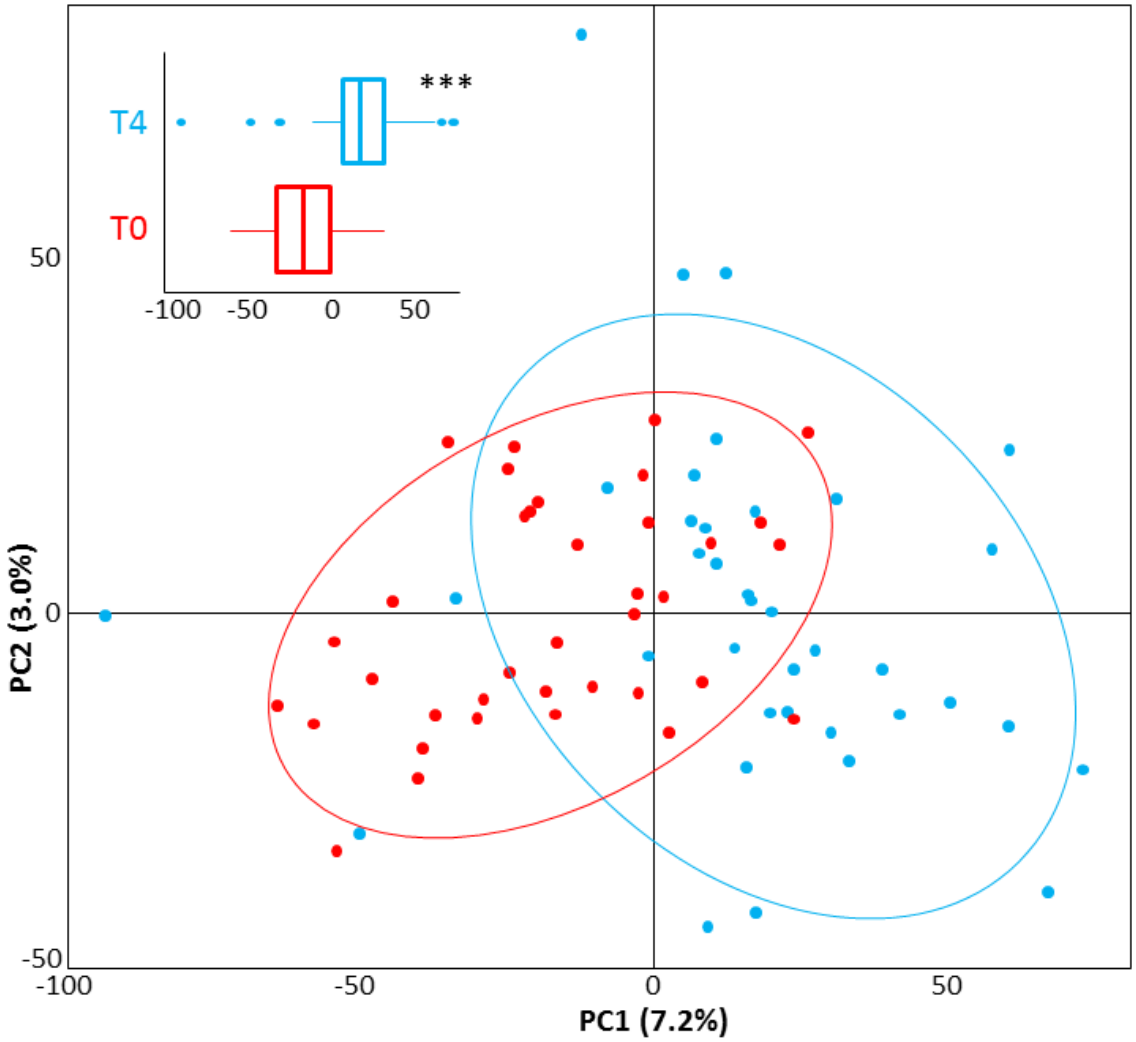
1271

Figure 3


0.0
0.1
0.2
0.3
0.4
T4_I17
T0_I19
T4_H19
T4_H9
T0_H3
T4_H17
T0_H16
T0_H18
T4_M18
T4_M13
T4_M19
T4_M17
T0_L7
T4_L6
T0_L15
T4_K4
T0_K18
T4_K10
T0_K16
T4_G7
T0_G8
T4_G9
T4_G10
T4_G5
T4_G12
T0_G14
T0_J14
T4_J9
T0_J12
T0_F13
T0_F10
T4_F2
T4_E13
T0_E8
T0_E14
T4_E2
T4_E6
T0_B19
T4_B5
T0_B5
T4_C19
T4_C3
T0_C3
T4_N18
T0_A17
T0_D18
T0_T19
T0_T5
T4_T18
T4_S14
T4_S19
T0_S2
T4_R1
T0_R1
T4_R14
T4_R2
T0_R8
T0_R10
T0_R19
T4_R3
T0_R11
T0_P11
T0_P9
T0_P3
T4_P2
T0_P7
T0_P5
T4_O3
T0_O3
T0_O10
T4_O8
T4_O17
T4_O6
T0_O13

1274 **Figure 4:**

1275



1276