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RESEARCH ARTICLE

Epigenomic patterns in fish with divergent cortisol reactivity

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Abstract

Glucocorticoids (GCs), such as cortisol, bind to receptors in most tissues, triggering a stress response that enables individuals to adapt to stressors physiologically and behaviorally. GC signaling has been associated with plastic, stress-induced epigenetic modifications. However, the epigenetic profiles of individuals with divergent cortisol reactivity before and after a common challenge test have rarely been considered. Cortisol levels were measured in the blood plasma of 400 European seabass (*Dicentrarchus labrax*) to select 80 individuals exhibiting low- and high-responsiveness phenotypes. Weight, lysozyme activity, and plasma glucose and lactate levels were also measured. The methylome of blood samples from these individuals was investigated at 41,132 CpG positions before and after a 3-mo stress challenge test. We identified 273 differentially methylated cytosines, which mapped to 159 annotated differentially methylated genes (DMGs). We categorized differential methylation as "constitutive" or "induced," distinguishing between the two phenotypes in pre- and post-stress situations, respectively. Methylation observed at DMGs may also remain hypo- or hypermethylated in both contexts; however, seven of these showed a rewiring from hypo- to hypermethylation. Many DMGs are relevant to GC signaling, connecting to the non-genomic and genomic actions of cortisol. They can be considered as stress biomarkers monitored in the blood of this highly-prized species. Although our current study has limitations, we demonstrate the potential of a genome-wide epigenetic approach to studying endocrine divergence between stress-responsive fish phenotypes.

NEW & NOTEWORTHY The association of DNA methylation with glucocorticoid signaling has attracted considerable interest. However, studies investigating genome-wide methylation differences in individuals with different cortisol responses before and after exposure to stress have been overlooked. Here, we demonstrate that the blood methylomes of fish with distinct cortisol responsiveness exhibit distinct pre-and post-stress methylation profiles, indicative of the cellular and organismal actions of cortisol possibly triggering the adaptive stress response of each phenotype in the face of stress.

blood methylome; epigenetic rewiring; epigenotyping-by-sequencing; European seabass; glucocorticoids

INTRODUCTION

Cortisol is a glucocorticoid (GC) hormone and the end-effector of the hypothalamic-pituitary-interrenal (HPI) axis in fish, amphibians, and reptiles. HPI is responsible for the primary neuroendocrine response to stressors, operating similarly to that observed in birds and mammals, in which the adrenal gland replaces the interrenal tissue (HPA axis). Cortisol regulates cellular homeostasis through membrane-initiated and cytosolic (non-genomic), as well as nuclear (genomic) actions (1–3). The effect of cortisol is primarily mediated by the activation of the glucocorticoid receptor (GR), a ligand-bound transcription factor distributed in most tissues. By cooperating with a set of transcription factors, the genomic actions of GR control the transcriptional activity of hundreds of genes operating in most tissues and exerting pleiotropic effects that activate or repress downstream regulatory networks in development, metabolism, cognition, and inflammation (2, 3). This triggers a general stress response in most body systems, enabling individuals to adapt physiologically and behaviorally to stressors.

Endocrine cascades have a heritable basis in vertebrates (4). Nevertheless, the heritability of GCs is poorly understood in humans (for review, see Ref. 5) and is often estimated to be low to moderately high (h^2 ~0.02–0.50) in other vertebrates [fish (6); birds (7); mammals (8–10)]. Consequently, the stress response is a highly plastic trait whose regulation is influenced by epigenetic factors, enabling it to resist allostatic load and trait-environment mismatch. It has been especially demonstrated that these factors add regulatory

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layers to GC signaling by regulating chromatin accessibility and gene transcription (11–13). Given a key role in gene transcription, the association of DNA methylation (i.e., the addition of a methyl group to cytosines without altering the DNA sequence) with the stress response, GR, and GCs has attracted considerable interest (for reviews, see Refs. 14–16). Initially, methylation studies focused on a few specific genes responsible for, or associated with, GC signaling. These include *NR3C1*, which encodes GR; *FKBP5*, which limits the translocation of the GR complex to the nucleus; and some significant genes associated with the neuroendocrine stress axis (e.g., corticotropin-releasing hormone *CRH*, CRH-binding protein *CRHBP*, proopiomelanocortin *POMC*), as well as a few others that interact indirectly with GCs (e.g., brain-derived neurotrophic factor *BDNF*; oxytocin receptor *OXTR*; serotonin transporter *SLC6A4*) (reviewed in Refs. 14, 17–19). Thanks to advances in molecular techniques, the scope of GC signaling epigenetic studies has expanded beyond genes related to the primary stress axis to include the genome level (20–24). Nevertheless, studies focusing on candidate genes or genome-wide epigenetic patterns have primarily *1*) investigated methylation differences between stressed and non-stressed individuals, and *2*) been interested in the epigenetic programming of the HPA axis during early development when organisms are susceptible to stress. Early-life stressors can favor increased epigenetic plasticity (i.e., the gain or loss of methylation marks through a methylation-demethylation process) and influence somatic and behavioral phenotypes later in life (25–28). However, if early-life adversity affects long-term phenotypic outcomes by shaping epigenomic profiles, additional epigenetic plasticity may also develop during the lifespan through episodes of stress exposure (29, 30).

Few studies have compared genome-wide methylation patterns in individuals with distinct constitutive cortisol reactivity before and after exposure to a common stressor when raised in a similar environment. Both Del Corvo et al. (31) and Corbett et al. (32) studied differences in methylation between individuals with low versus high cortisol reactivity in cows and piglets, respectively. However, Del Corvo et al. (31) did not subject cows to a stressor, meaning there was no post-stress comparison. In contrast, Corbett et al. (32) only compared the methylation profiles of low- and high-responder piglets after weaning—an event that combines dietary and social stress—and did not perform any pre-stress comparison. Miller et al. (24) compared the salivary methylomes of humans before and after exposure to acute stress. Although the authors assessed individual cortisol reactivity, they did not contrast extreme cases, and the study cohort exhibited moderate differences in salivary cortisol reactivity. Nevertheless, Miller et al. (24) reported plasticity of individual epigenetic profiles, either as a gain and loss of methylation marks or as consistent shifts in DNA methylation. These shifts were more precisely referred to as stress-induced "epigenetic rewiring," i.e., when methylated cytosines moved from hypo- to hypermethylated states and vice versa. This rewiring was only observed in a limited number of CpG sites (C = cytosine, G = guanine, and p = phosphate bond), and only a single site was reported to be associated with cortisol signaling (24). Although care was taken to select individuals with specific characteristics, none of these studies—whether involving humans, cows, or piglets—controlled the rearing and/or experimental environment. Therefore, our knowledge is limited regarding whether individuals with divergent cortisol reactivity, when raised in controlled conditions, exhibit constitutive methylation differences and how these differences indicate significantly enriched, tissue-specific functional pathways associated with GC signaling in a pre-stress condition. Similarly, our understanding of whether stressors induce post-stress epigenetic rewiring at differentially methylated genes and of changes in pathway distribution between phenotypes compared with the pre-stress condition remains limited. Finally, it is unknown whether epigenetic differences that potentially develop during a stress challenge could result in differences in other traits that reflect the actions of GC signaling.

In fish, differences in cortisol stress reactivity have allowed the identification of consistent low- and high-responder individuals (LR and HR, hereafter) who exhibit repeatable, low and high post-stress plasma cortisol over time when submitted to stressors [rainbow trout, *Oncorhynchus mykiss* (33); cod, *Gadus morhua* (34); European seabass, *Dicentrarchus labrax* (35)]. These LR and HR profiles are often associated with proactive and reactive behavioral phenotypes, respectively, and are included in standard definitions of coping styles (36, 37). Nevertheless, the LR and HR stress-responsive phenotypes have been overlooked in epigenetic studies of fish. Aravena-Canales et al. (38) manipulated plasma cortisol levels by injecting exogenous GCs to investigate differential methylation of skeletal muscle in rainbow trout that had or had not received the injection. However, they did not compare individuals with divergent cortisol reactivity. Krick et al. (39) investigated the pre- and post-stress blood methylomes of European seabass, but also failed to exploit the LR and HR phenotypes that have been repeatedly described in this species (35, 40, 41). This species is a key marine fish model that offers an opportunity to study any differences in methylation that may exist between LR and HR fish before and after they are subjected to a stressor.

Using the epigenotyping-by-sequencing (epiGBS) protocol developed by Krick et al. (39), this study uses pre- and post-stress blood samples from European seabass to investigate: *1*) how European seabass with LR or HR cortisol reactivity may differ in their genome-wide blood epigenetic profiles; *2*) which biological pathways are involved, and whether these differ before and after a common stress challenge test; and *3*) whether genes associated with these pathways have been identified as being associated in the fish HPI axis and, more generally, GC signaling.

MATERIALS AND METHODS

Experimental Mating and Conditions

All procedures involving fish in this study were approved by the Departmental Animal Care Committee in accordance with the Three Rs principle and Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals. All sample procedures were performed by researchers certified by the Federation of European

Laboratory Animal Science Associations (FELASA; http://www.felasa.eu/).

Fish were produced in the hatchery facility of AVRAMAR Aquaculture S.A. at Chalkida (Evvoia Island, Greece) from breeders maintained in this company for scientific purposes. They resulted from a single crossing experiment (12 dams, 20 sires) that took place in late January 2018. Egg and sperm collections were standardized with equal quantities of 20 g eggs and 1 g sperm to produce twenty different families (labeled alphabetically from A to T), each with a different father. Each of the 12 dams mated at least once, but eight families shared the same mother. The families were raised individually in open circulation tanks (cylindrical, conical shape; volume: 150 L; diameter: 70 cm), which were housed in the aquaculture installation. At 60 days post-hatch (dph), fish of each family were netted and checked for a fully inflated swim bladder. The sample size of each family was then adjusted to include these fish ($n = 1{,}000$ per tank). Fish with swim bladder deficiency and other remaining fish were discarded. The experimental phase involving the stress protocol began at ~180 dph and lasted for approximately three months (see, *Stress Protocol and Blood Sampling*).

Soon after mouth opening (2–3 days after hatching), fish transitioned to exogenous feeding and were first fed with Artemia and rotifers until 50 dph. During the remainder of the initial rearing prior to the experimental period, fish were hand fed between 3 and 18 times a day depending on the fish size (i.e., bigger fish fewer feeding times), seven days a week. During the experimental period, fish were fed twice a day, six days per week, using a commercially available diet (Blue Line 45:20 3.5 mm, Feedus S.A., Greece). During the experiment, all husbandry activities, including feeding, were carefully planned to minimize stressors (e.g., no additional noise, slow movements). All along the experiment, the photoperiod was set at 12L:12D, and the water temperature and salinity were held constant (18.1 ± 0.2°C and 28 ppt, respectively). We did not control for mortality before the implementation of the stress protocol. Dead fish were removed from tanks when observed.

Stress Protocol and Blood Sampling

At ~180 dph, 20 individuals were randomly selected from each fish family. They were weighed and individually tagged using a passive integrated transponder (PIT) in the left anterior part of their bodies, then distributed among 20 tanks of the same size and volume as those used previously. To prevent environmental effects during this experimental phase, one fish from each family was randomly assigned to a tank, resulting in 20 fish per tank. After PIT-tagging and two weeks before implementing the stress protocol, blood samples were collected from the caudal vein of all fish that had not previously been exposed to acute stress (hereafter referred to as "pre-stress samples"). During the stress challenge, the fish were exposed to high-density stress by lowering the water level in the tank to one-third of the original volume, followed by chasing the fish with a net for 5 min. This acute stress protocol was applied to all fish and repeated three times at 3-wk intervals. Plasma cortisol levels were found to return to the basal state in approximately one day in seabass after enduring a similar stress protocol (42). Between each stress challenge, the fish were returned to their host tank to recover. Post-stress blood samples were collected 30 min after each stress exposure. However, only blood samples from selected fish, collected after the final stress exposure, were included in the epigenetic analysis (hereafter referred to as "post-stress samples"). PIT tagging and all blood sampling were performed on anesthetized fish (2-phenoxyethanol, 300 ppm; Merck). Sterile syringes were used for blood sampling throughout the experiment. Four investigators wearing gloves to prevent contamination by skin-derived cortisol worked in parallel to reduce the time delay between netting and blood sampling (less than 2 min). Recovery was performed in a separate tank with an air provision before the fish were returned to their holding tank. Only three fish (0.075%) died during the stress challenge.

Plasma and blood cells from the pre- and post-stress samples were separated by centrifugation (2,000 *g* for 10 min) and carefully fractionated. Plasma cortisol levels were measured in all samples at each time point using a commercial enzyme immunoassay kit (DRG Cortisol ELISA, DRG International Inc., Germany), the performance of which has been previously evaluated in European seabass plasma samples (40, 43). Fish were ranked based on standardized (*z*-score) cortisol levels (43), and 40 individuals were retained at each extreme of the cortisol reactivity distribution (low vs. high cortisol reactivity). Eight fish were also retained randomly from the middle of the distribution as an internal control (Co) for cortisol data. This resulted in a sample size of 88 individuals per stress period, meaning that twice as many blood samples (176) were included in the genome-wide analysis of 5-methylcytosine (5mC) DNA methylation. DNA was extracted from blood cells of these individuals using the Macherey Nagel NucleoSpin Tissue DNA Kit, and its quantity was determined using a Qubit fluorimeter (Qubit dsDNA BR Assay Kit, Invitrogen).

In addition, blood glucose and lactate were measured using validated commercially available enzyme colorimetric assays (glucose: Biosis, 001733, Greece; lactate: Spinreact, 1001330, Spain). Plasma lysozyme activity was quantified using the turbidimetric assay with *Micrococcus lysodeictikus* (M3770, Sigma-Aldrich, St. Louis, MO) as substrate (44). Lysozyme activity was expressed as $kU \cdot L^{-1}$ compared with a lysozyme (L6876, Sigma-Aldrich, St. Louis, MO) standard curve. The weights of individual fish were also monitored during the stress challenge.

Library Preparation and Sequencing

Two genomic libraries were prepared using samples from fish that were either pre- or post-stressed or low- or high-responsive, as well as internal controls (see *Bioinformatics and Methylation Analysis*). Individuals were distributed across the libraries to minimize any sequencing bias. Library preparation was performed according to the epiGBS protocol developed by Krick et al. (39), a two-enzyme implementation of the protocol proposed by van Gurp et al. (45) for detecting differences among individuals. The quality of each library was verified on an Agilent 5200 Fragment Analyzer (Santa Clara, CA) using the following criteria: fragment size distribution, absence of adapters or primers remaining, and a fragment size range of 300–800 base pairs (bp). The libraries were sequenced (paired end, 150 bp) across four lanes of an

SP flow cell using an Illumina NovaSeq 6000 at the MGX sequencing facility in Montpellier, France.

Bioinformatics and Methylation Analysis

Methylation analysis was restricted to 5mCpG methylation, as methylation in the CHG and CHH contexts (H being any nucleotide but a cytosine) is negligible in the European seabass (39). The raw sequencing data were trimmed of low-quality reads and adapter residues using Trim Galore! (v.0.6.5; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The trimmed reads were then demultiplexed using the "process_radtags" command in STACKS (46) (RRID:SCR_003184). The disable_rad_check option was applied to prevent reads containing bisulfite-modified restriction sites from being discarded. The demultiplexed reads were trimmed a second time at the 5′ and 3′ ends using the -clip_R1, -clip_R2, -three_prime_clip_R1, and -three_prime_clip_R2 options to remove introduced methylated cytosines that may have resulted from adapter ligation. The bismark_genome_preparation function of the Bismark program (47) was used to prepare a bisulfite-converted version of the European seabass genome (48), against which the demultiplexed and trimmed reads were mapped.

The R package MethylKit (49) (RRID:SCR_005177) was used to determine the differential 5mC methylation of individual LR, HR, and Co fish in pre- and post-stress conditions. To account for PCR bias, the 5mCpG dinucleotides with a read depth of less than 30 and a frequency greater than 99.5% of the read count distribution were filtered out. Coverage was normalized across samples, and only 5mCpGs present in at least 60% of samples were retained for further analysis. Differentially methylated cytosines (DMCs) at 5mCpGs were determined using logistic regression [calculateDiffMeth function implemented in Methylkit (49)]. In each of our two comparisons, differential methylation between LR and HR fish was identified in both pre- and post-stress situations using the corresponding methylation levels observed in Co fish in the pre- and post-stress conditions, respectively. Cytosines were considered to be differentially methylated if there was at least a 15% difference in methylation between LR (or HR) fish and the Co fish. A nominal q value (false discovery rate; FDR) of <0.001 was considered.

Reads presenting DMCs were extracted using Geneious (v.11.0; RRID:SCR_010519) and mapped onto the linkage groups (LGs) of the European seabass genome (48). Gene annotation was based on the European seabass genome annotation available on ENSEMBL (RRID:SCR_002344), as described by Tine et al. (48). Gene names and models were verified, including a TBLASTN search in cases of discrepancy. A DMC was considered to be associated with a gene—referred to hereafter as a differentially methylated gene (DMG)—if it mapped within that gene, including the 5′ and 3′ untranslated regions (UTRs). As several DMCs can map to a single gene, we only considered the gene once. The gene symbols used in this study are from GeneCards (RRID:SCR_002773).

Enrichment Analyses

To determine which pathways were enriched in our blood-derived datasets, we performed three distinct enrichment analyses on sets of identified differentially methylated genes (DMGs) using Metascape v.3.5 (50) (RRID:SCR_016620). Our goal was to determine whether a systemic, organism-wide stress response could be detected based on the enriched pathways statistically supported by Metascape. First, we conducted two enrichment analyses between LR and HR fish independently for the pre- and post-stress periods, including sites of differential methylation common across stress periods. We also independently analyzed only these common DMGs whose methylation was not or poorly affected by the stress challenge test. Ontologies were performed using the human genome as a reference. For each list, cumulative hypergeometric P values and enrichment factors were calculated and used for filtering by Metascape. The remaining significant terms were then hierarchically clustered based on kappa (κ) statistical similarities (51) between their gene memberships. Clusters are made up of members that are queried by Metascape in standard databases, including Gene Ontology (GO; https://www.geneontology.org/), reactome (R-HSA; https://reactome.org), Kyoto Encyclopedia of Genes and Genomes (KEGG) (52) (RRID:SCR_012773), WikiPathways (WP) (53), and the Pathway Interaction Database (PID) (54). A standard score of $\kappa = 0.3$ was used as the threshold to identify clusters. Within each cluster, the enriched member with the highest significance [i.e., highest $-\log_{10}(P)$] was given its generic name by Metascape v.3.5 to describe that cluster and the DMGs it contained. Therefore, other members within each cluster are considered non-generic enriched clusters. The results were also screened for further appreciation.

RESULTS

DNA extraction or bisulfite conversion of some samples failed to pass quality filters during library preparation, and only samples for which quality filters met quality criteria in both pre- and post-stress conditions were retained for further analysis (i.e., if a pre- or post-stress sample from an individual failed, the individual was discarded from further analyses regardless of the quality of the second sample it was associated with). Nine individuals (18 samples) were discarded based on the aforementioned criterion, and a total of 79 individuals (158 samples) were considered for both cortisol and methylation data used in this study (Supplemental Table S1; see, https://doi.org/10.6084/m9.figshare.32586156).

LR and HR Fish

The LR and HR fish were distributed in 19 of the 20 European seabass families considered in this study (Fig. 1*A*; Supplemental Table S1). Fish characterized as LR covered 11 families, with only two families (G, R) representing 44.44% of the individuals (16 of 36; 4 LR fish discarded according to the aforementioned criteria). Representatives of 13 families were found in HR fish, with two families (E, O) representing 30.55% of the individuals (11 of 36; also 4 HR fish discarded). Five families have representatives in both the HR and LR groups of European seabass, indicating that most families reflect a tendency to characterize either LR or HR fish.

Cortisol Reactivity and Association with Other Variables

Mean cortisol reactivity for LR and HR fish in both the pre- and post-stress samples are given in Table 1, together

Table 1. *Phenotypic profiles of LR and HR fish in the pre- and post-stress contexts of the three-month challenge test*

Phenotype	Stress Period	Cortisol, ng·mL^{-1}	Glucose, mmol·L^{-1}	Lactate, mmol·L^{-1}	Lysozyme Activity, kU·L^{-1}	Weight, g
LR	Pre	78.1 ± 66.0	4.2 ± 0.93	1.9 ± 0.73	387.0 ± 147.1	46.93 ± 12.60
	Post	230.4 ± 41.7	6.5 ± 2.48	4.7 ± 2.08	583.8 ± 225.9	86.15 ± 25.14
HR	Pre	177.3 ± 125.7	4.6 ± 1.36	2.0 ± 0.85	386.6 ± 211.7	48.76 ± 11.72
	Post	454.7 ± 45.46	6.6 ± 1.48	5.1 ± 1.82	546.3 ± 245.2	86.17 ± 22.11

Values are means ± SD values; n = 35–36. The details of the levels of significance associated with each variable in the two-way repeated-measures ANOVA are provided in Supplemental Table S2. Graphical representations can be found in Supplemental Fig. S1, with the exception of cortisol (Fig. 2). HR, high-responsive; LR, low-responsive.

with means ± SD values for other variables considered in this study (for individual data, see Supplemental Table S1).

Blood plasma from fish sampled at each extreme of the distribution showed significant variation (two-way repeated measures ANOVA) in circulating cortisol in both pre- and post-stress situations [phenotype: $F(1,70) = 164.5$; $P < 0.0001$; stress: $F(1,70) = 265.6$; $P < 0.0001$; Supplemental Table S2]. The lower and upper limits of this distribution were characterized as LR and HR European seabass, respectively (Fig. 2). The blood plasma cortisol concentrations of the control (Co) fish included in this study showed variable but intermediate values compared with the LR and HR extremes in both pre- and post-stress assessments (Fig. 2). Despite an observed increase in each variable in both LR and HR fish between the pre- and post-stress contexts, this study found no effect of the phenotype on blood glucose and lactate levels, lysozyme activity, or weight (Table 1; Supplemental Fig. S1 and Table S2). Plasma cortisol was not found to significantly correlate with other variables in either the pre- or post-samples for both LR and HR fish (range of r^2 values [0.002–0.158]; all P values >0.05; NS).

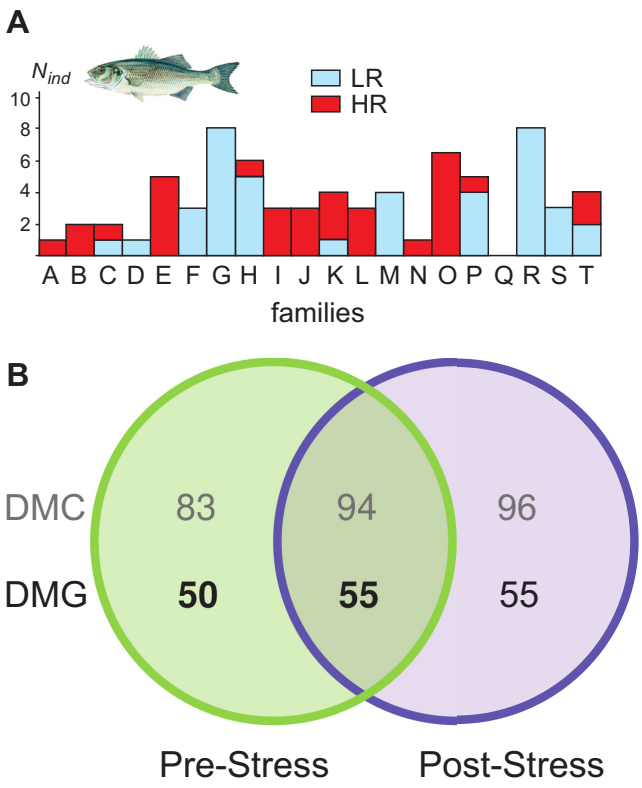

Figure 1. *A*: distribution of low-responsive (LR) and high-responsive (HR) fish in the 20 families (A to T) considered in this study. *B*: Venn diagram summarizing the numbered of differentially methylated cytosines (DMCs) and differentially methylated genes (DMGs) specific to the pre- and post-stress contexts, and those that were shared across stress periods (referred as "common" in the main text). The full list of DMCs and DMGs is given in Supplemental Table S3).

Library Sequencing

We obtained 504,271,331 [NCBI's Gene Expression Omnibus (GEO) Datasets accession: GSE153838] and 510,660,255 (NCBI's GEO Datasets accession: GSE156637) total sequencing reads, of which 99.4% (501,108,033) and 99.1% (505,553,652) were obtained after trimming the two libraries, respectively. After demultiplexing, the number of reads per sample ranged from 530,335 to 16,319,237 reads, with a mean of 4,405,922 reads per pre-stress sample, and from 503,579 to 11,039,473 reads, with a mean of 4,539,487 reads per post-stress sample (Supplemental Table S1). Demultiplexed samples were mapped against the *D. labrax* reference genome with a mean mapping efficiency of 74.2 ± 0.5% (72.7 ± 0.4% for unique best hits). The mapping efficiency was then similar to that reported by Krick et al. (39) and Blondeau-Bidet et al. (55), also on *D. labrax*. Sequencing reads were mapped across all 24 LGs of the European seabass genome. The average read depth per base pair was 230×.

Methylation Analysis

Of the 10,368,945 CG dinucleotides present in the EpiGBS reduced-representation of the *D. labrax* genome obtained, 41,132 5mCpG coordinates were extracted with a minimum of 30× read depth and presence in at least 20 individuals for both the pre- and pre-stress blood samples. They were filtered out using a 15% methylation difference threshold for at least one of the phenotypes relative to Co fish, and a FDR-corrected cut-off value of $q < 0.001$ for the comparisons analyzed in this study. Only 273 distinct DMCs positions over 41,132 (0.66%) in our data were detected as differentially methylated between LR and HR fish in the pre- and/or post-stress conditions. Positions of these DMCs along European seabass LGs and other information (sense/antisense strand, location in intergenic regions, UTR or gene body) are given in Supplemental Table S3. However, as 58.24% of all DMCs were found to be genic or located in UTRs ($n = 4$), and mapped to 160 distinct protein-coding genes, we did not expand further on unannotated DMCs in this study. A total of 159 DMGs were annotated and distributed across all LGs (Fig. 1*B*; Supplemental Tables S3, S4, S5, and S6). The exception was an unannotated protein-coding gene, *dkey-288a3.2*

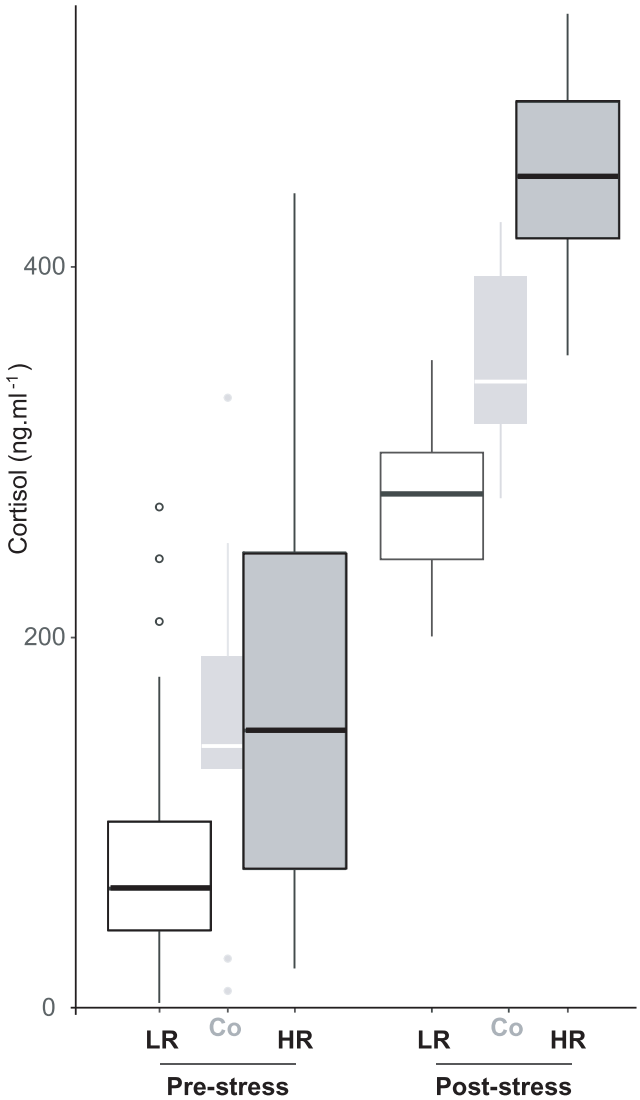

Figure 2. Box-plots of blood plasma cortisol concentration (ng·mL^{-1}) of the low-responsive (LR) and high-responsive (HR) European seabass in pre- and post-stress conditions (n = 36 for each phenotype and stress period). Light gray boxes represent distributions of blood plasma concentrations for internal controls (Co) fish.

(Supplemental Table S3). Twelve DMGs were identified by more than one single DMC (n = 2 or 3). Only the Ankyrin Repeat Domain 9 (*ANKRD9*) DMG was found to contain >10 DMCs within 300 bp along the "seabass unknown" (SB-UN) LG in the study by Tine et al. (48) (red in Supplemental Table S3). A suite of DMCs on LG15 was also found to cluster on 200 bp (red in Supplemental Table S3). Similar numbers of DMGs have been detected in the pre- and post-stress comparisons (n = 105 and n = 109, respectively) (Fig. 1*B*; Supplemental Tables S4 and S5, respectively). Only 55 DMGs (34.37%; 94 DMCs; Supplemental Tables S3 and S6) were common to the LR-HR comparison in both the pre-and post-stress period (Fig. 1*B*). These DMGs are referred to as "common" hereafter. Fifty-four common DMGs were annotated, and included three of the four DMGs located in UTRs (Supplemental Table S3). Twenty-three of the 159 annotated DMGs (14.46%) were identified as being involved in the transcription process as transcription factors or as regulators of transcription (bold in Supplemental Table S3).

The majority of the 94 common DMCs and 55 DMGs were found to be hypomethylated, with no change in methylation status observed between the pre- and post-stress conditions. Indeed, 66 DMCs were associated with the 55 DMGs; 46 of these were hypomethylated in the pre-stress condition versus 39 in the post-stress condition (Fig. 3; Supplemental Table S6). It means that only seven DMGs reported cytosine that became hypermethylated in the post-stress condition, when hypomethylated relative to the Control fish in the pre-stress condition (Fig. 3; bold in Supplemental Table S6). This includes cytosine located in the Vav Guanine Nucleotide Exchange Factor 2 (*VAV2*), Rho GTPase Activating Protein 32 (*ARGHAP32*), Insulin Like Growth Factor Binding Protein 5 (*IGFBP5*), CDC42 Small Effector 1 (*CDC42SE1*), ATP Binding Cassette Subfamily A Member 1 (*ABCA1*), Meningioma-Expressed Antigen-5 (*MGEA5*; *aka* OGA: O-GlcNAcase), and Zinc Finger Protein 107 (*ZNF107*) genes (Fig. 3). One unannotated DMC on LG2 was also found to shift from a hypo- to a hypermethylated state (green in Supplemental Table S6).

Functional Enrichment

To illustrate a potential organism-wide stress response through the analysis of methylation patterns, we analyzed three distinct sets of differentially methylated genes (DMGs) relative to the pre- and post-stress periods, as well as DMGs common to both periods, using Metascape.

During the pre-stress period, the top 20 significantly enriched generic terms and pathways detected with the set of 105 DMGs (50 specific to pre-stress; Fig. 1*B*) were related to different processes relevant to, e.g., the nervous system (synapse assembly, cell morphogenesis, neuron fate commitment), the regulation of fat and glucose (maturity-onset diabetes of the young, NR1H2- and NR1H3-mediated signaling, adipogenesis) (Table 2). Numerous enriched terms appeared significantly associated with terms related to intracellular processes and trafficking (regulation of small GTPase-mediated signal transduction, protein homo-oligomerization, Golgi to membrane transport, posttranslational modification: synthesis of glycosylphosphatidylinositol (GPI)-anchored proteins), or cellular and tissue integrity (response to wounding, negative regulation of wound healing) (Table 2; Supplemental Table S7).

In the post-stress data set (109 DMGs; 55 specific to the post-stress condition; Fig. 1*B*), the top 20 enriched generic terms were found to be associated with molecular and cellular processes, including phosphorylation, ubiquitination, and AMPK signaling, or the cellular response to oxygen levels (Table 3; Supplemental Table S4). Nevertheless, analysis of the pre- and post-stress DMGs revealed significantly different numbers of enriched terms for a similar number of DMGs. Indeed, Metascape reports the term with the highest significance and uses only its generic name to describe the cluster. The pre- and post-stress DMGs represent 93 and 276 DMG clusters, respectively (Supplemental Tables S4 and S5, respectively). This suggests that, in the post-stress case, the generic names of the top 20 clusters could not fully represent the full set of non-generic DMG clusters, as they do in

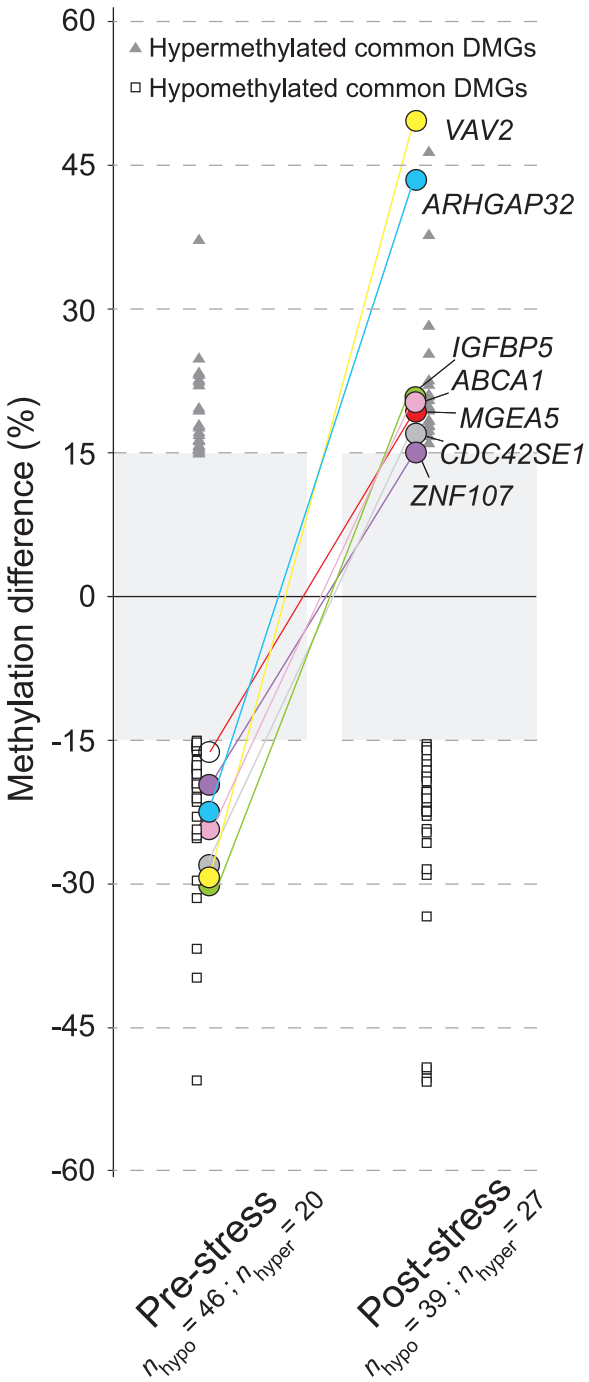

Figure 3. Methylation differences for the common differentially methylated genes (DMGs) found in this study. Hypo- and hypermethylated DMGs (15% threshold) in both the pre- and post-stress contexts are represented by gray stars and white squares, respectively. The numbers (n_{hypo}, n_{hyper}) of individual differentially methylated cytosines (DMCs) contained in common DMGs are given at the bottom of the figure. Rewired DMGs are labeled and represented by colored circles. See Supplemental Table S6 for details.

the pre-stress case. Non-generic enriched terms therefore had to be considered in greater detail. For instance, the platelet-derived growth factor (PDGF) pathway was identified as the most significantly enriched post-stress cluster in Metascape (Table 3), encompassing 83 distinct enriched clusters with $\log_{10}(P)$ values ranging from −6.50 to −2.05. These 83 enriched terms are associated with numerous immune-related pathways (B cell receptor signaling, macrophage-stimulating protein signaling, interleukin-1 and interleukin-17 signaling, Toll-like receptors, and infection-related pathways; Supplemental Table S8). Two immunity- and inflammation-related generic enriched terms were also found: immune response-regulating signaling pathway and protein kinase RNA-activated (PKR)-mediated signaling. Another generic, post-stress enriched term that could suggest involvement in immune functions is the occurrence of the complex prolactin signaling pathway (Table 3). Indeed, some of the non-generic terms associated with the prolactin signaling pathway ($n = 52$) were also indicative of a possible relationship with immune functions, such as, e.g., the cellular response to cytokine stimulus, oncostatin M signaling (i.e., affiliated to the interleukin 6 family of cytokines), or the FGF (fibroblast growth factor) pathway (Supplemental Table S5).

Finally, of the 54 annotated common DMGs between the pre- and post-stress conditions, 10 generic terms were significantly enriched across 57 clusters (Supplemental Tables S9 and S10, respectively). The generic and non-generic enriched terms reflect some detected in pre- or post-stress analyses (Supplemental Table S10).

DISCUSSION

The blood methylome of fish with different levels of cortisol reactivity has not yet been investigated. This study investigated the relationship between 5mC epigenetic variation and cortisol blood plasma levels in low- and high-responsive European seabass (*D. labrax*), as characterized in previous studies (35, 40). We demonstrated that LR and HR fish exhibit distinct genome-wide methylation profiles in both pre- and post-stress conditions. DMCs are associated with DMGs that are relevant to signaling pathways representative of cortisol actions at the cellular and organismal levels.

Constitutive and Stress-Induced DMGs

This study identified several dozen DMCs pointing to 159 annotated DMGs, which are distributed across all European seabass LGs. These DMGs differentiate between LR and HR fish in both pre- and post-stress conditions. Around 58% of the DMCs are genic, consistent with values reported in previous studies. For instance, Moghadam et al. (56) found 51% genic DMGs in Atlantic salmon (*Salmo salar*) following repeated acute stress events, whereas Zuloaga et al. (13) found around 65%–70% in rainbow trout after manipulating cortisol levels. In genome-wide methylation studies of *D. labrax* in response to osmoregulatory stress, Blondeau-Bidet et al. (55) reported genic methylation levels of between 51% and 53%. As it has previously been demonstrated in fish in different experimental contexts (13, 57–61), the results revealed that the epigenetic profiles are dynamic and likely facilitated alternative regulatory pathways depending on the individual's experience of the stressor. However, previous studies of fish that reported dynamic methylation mark profiles among individuals considered different physical environments characterized by varying degrees and types of stressors (e.g., stress vs. non-stress situations) and not a similar stressor experienced by different individual phenotypes.

Table 2. *Top 20 significantly enriched generic terms detected with Metascape for pre-stress DMGs detected between LR and HR European seabass submitted to a three- month challenge test*

Generic Term	Term Description	Log *P*	Metascape Associated DMGs
GO:0051056	Regulation of small GTPase mediated signal transduction	−5.0195	*ABCA1, VAV2, KALRN, ARHGAP32, ARFGEF2, AUTS2, CDC42SE1, ARHGAP12, ITGA4, EHD4, SLC39A6*
hsa4950	Maturity onset diabetes of the young	−4.0276	*HNF4A, NKX6-1, HNF1A, IGFBP5, IRS1, PCK1, CDH2, MAP2K4*
GO:0007416	Synapse assembly	−4.0271	*CDH2, PLXNA1, SHANK2, PCDHB2, NTNG2, NFIA, CNTN4, SETD5, ARHGAP12*
GO:0051146	Striated muscle cell differentiation	−3.9190	*CDH2, IGFBP5, LMNA, MYOD1, MAP2K4, MYO18B, SCN5A*
WP2118	Arrhythmogenic right ventricular cardiomyopathy	−3.8948	*CDH2, ITGA4, ITGB5, LMNA, CLASP1, EXOC6, VAV2, TRIM63*
GO:0009611	Response to wounding	−3.8230	*HNF4A, ITGB5, NFIA, PLG, MAP2K4, VAV2, SULF2, DGKH, MYOD1*
GO:0006516	Glycoprotein catabolic process	−3.7535	*OGA, EDEM2, NCCRP1, CUL9, CLN6, RNF123, ZNRF3, ANKRD9*
WP3850	Factors and pathways affecting insulin like growth factor IGF1 Akt signaling	−3.5996	*IGFBP5, IRS1, TRIM63, LMNA, SCN5A*
R-HSA-9024446	NR1H2 and NR1H3-mediated signaling	−3.5293	*ABCA1, PCK1, EEPD1, GNB1, IGFBP5, MYOD1, RYR3*
GO:0003012	Muscle system process	−3.2632	*MYOD1, RYR3, SCN5A, MAP2K4, SULF2, TRIM63*
hsa04814	Motor proteins	−3.2376	*DNAH5, KIF11, MYO10, MYO18B, MYO3B*
GO:0000902	Cell morphogenesis	−3.1602	*CDH2, ITGA4, NFIA, PLXNA1, KALRN, AUTS2, DPYSL5, NTNG2, CNTN4, MAP2K4, MYO10, SCN5A, CLASP1, SULF2*
GO:00051260	Protein homooligomerization	−3.1406	*RYR3, NLRP1, EHD4, KCTD9, KCNG4*
GO:0006893	Golgi to plasma membrane transport	−3.0319	*ARFGEF2, CCDC93, EXOC6, EHD4, ABCA1, CCKBR, HNF1A, MICAL3, SYT9, CDH2, ITGA4, LMNA*
WP236	Adipogenesis	−2.9739	*IRS1, LMNA, PCK1, HNF1A, GNB1, IGFBP5, ITGA4, NKX6-1, RYR3, ITGB5, OGA, HNF4A*
GO:0046683	Response to organophosphorus	−2.9255	*DNTT, IGFBP5, RYR3, TRIM16*
GO:0048663	Neuron fate commitment	−2.7337	*JAG2, NFIA, MYT1L*
GO:0061045	Negative regulation of wound healing	−2.6822	*PLG, CLASP1, CD109, SULF2, ADAMTS17*
WP4239	Epithelial to mesenchymal transition in colorectal cancer	−2.6358	*CDH2, JAG2, MAP2K4, CDKL2*
R-HSA-163125	Post-translational modification: synthesis of GPI-anchored proteins	−2.4966	*NTNG2, CD109, CNTN4*

Database labels for generic terms: GO, gene ontology; R-HSA, reactome; WP, wikipathways; hsa, Kyoto encyclopedia of genes and genomes (KEGG) pathways. DMG, differentially methylated gene; GPI, glycosylphosphatidylinositol; HR, high-responsive; LR, low-responsive.

The latter was the main advantage in the present study, as well as the LR and HR fish used came from different families and, as in the study by Krick et al. (39) in another experimental context, the results also supported a family-based component in *D. labrax* methylation profiles. A transcriptomic study by Samaras et al. (40) also supported cortisol reactivity depending on families in European seabass. Although the rearing environment was distinct among families before 180 dph, we hypothesized that fish differing in cortisol reactivity have distinct basal, constitutive epigenetic profiles that may respond to the three-month challenge test. Given the moderate heritability of cortisol reactivity reported in European seabass [h^2 ~0.35 (5, 62)], it can be hypothesized that differences in DNA methylation between LR and HR fish could contribute to trait heritability (63), as has been demonstrated in three-spine stickleback (*Gasterosteus aculeatus*) (64). Although the concept of heritability may not be entirely applicable to epigenetic studies, a significant impact of genotype on DNA methylation patterns has been demonstrated in species such as rainbow trout (65). Further investigation into European seabass is required, as it is a valuable aquaculture species. The process of its domestication, which currently relies on breeding programs, has already been shown to depend on genome-wide methylation alterations in this species (66, 67).

The observed differences in the methylation profiles of LR and HR fish certainly depend on the nature, context, strength, and duration of the challenge test (68, 69), as well as on thresholds used to identify DMCs. In this study, around a third of the DMGs that characterized LR and HR individuals were specific to either the pre- or post-stress condition. The remaining one-third of DMGs were common across

Table 3. *Top 20 significantly enriched generic terms detected with Metascape for post-stress DMGs detected between LR and HR European seabass submitted to a three-month challenge test*

Generic Term	Description	Log *P*	Metascape Associated DMGs
WP2526	PDGF pathway	−6.5044	*JUN, NFKB1, MAP2K4, SRC, VAV2, ITGA4, ABCA1, CAMK2A, MYL11, FGF18, ANGPT4, SETD5, IGFBP5, RYR3, ZBTB16, TRIM63, LMNA, PPP2CB, PPP2R5E, SMAD9, IRS1, RPL13A, FGD5, PHB2, NCOA7, TUBB6, HMGCR, IFT80, MIGA1, CPS1, EHD4, JAG2, OGA*
WP2037	Prolactin signaling	−5.1574	*IRS1, JUN, NFKB1, SRC, VAV2, CAMK2A, IGFBP5, PHB2, FGF18, CCKBR, MYO1B, MYO10, TUBB6, CPS1, MYOD1, NKX6-1, BCAS3, ABCA1, RYR3, PLG, ZBTB16, ITGA4, TRIM25, RPL13A, TRIM63, NCOA7, SULF2, JAG2, ANGPT4, MAP2K4, PPP2CB, HNF4A, ARHGAP32*
WP3850	Factors and pathways affecting insulin like growth factor IGF1 Akt signaling	−5.0835	*IGFBP5, IRS1, NFKB1, TRIM63, LMNA, MYOD1, MAP2K4, ITGA4, MYL11*
GO:0002764	Immune response-regulating signaling pathway	−4.9024	*BTN1A1, NFKB1, MAP2K4, SRC, VAV2, TRIM25, PHB2, NLRP1, SLC39A6, HNF4A, CAMK2A, NKX6-1, PPP2CB*
GO:0051056	Regulation of small GTPase mediated signal transduction	−4.8678	*ABCA1, CHN1, PPP2CB, SRC, VAV2, ARHGAP32, CDC42SE1, FGD5, HNF4A, PLG, ANGPT4, DGKH, MAP2K4, SULF2, ITGA4, MYO1B, NECAP1, EHD4, JUN, MYL11, SLC39A6, FGF18, NME2, CPS1, NKX6-1*
hsa04814	Motor proteins	−4.1109	*DNAH5, KIF11, MYO1B, MYO10, MYL11, TUBB6*
hsa04152	AMPK signaling pathway	−4.1068	*HMGCR, HNF4A, IRS1, PPP2CB, PPP2R5E, JUN, NFKB1, VAV2, ITGA4, KIF11, PLG, ANGPT4, TUBB6, CD109, DGKH, CAMK2A, FGF18, JAG2, BTN1A1, ZBTB16, TRIM63, LMNA, CHN1, ARHGAP32, SHKBP1, FGD5*
GO:0007167	Enzyme-linked receptor protein signaling pathway	−4.0612	*CHN1, IGFBP5, IRS1, JUN, SMAD9, SRC, VAV2, FGF18, SULF2, IFT80, BBS1, ZBTB16, JAG2*
GO:0003012	Muscle system process	−4.0116	*MYOD1, RYR3, MAP2K4, SLMAP, MYL11, SULF2, TRIM63*
GO:0005160	Protein homooligomerization	−3.9927	*RYR3, NLRP1, EHD4, KCTD9, SHKBP1, KCNG4*
GO:0071453	Cellular response to oxygen levels	−3.4943	*LMNA, MYOD1, SRC, PHB2, ANGPT4, NUP98, ZBTB16, DCLK1, BBS1, IFT80, IMMP2L, C1QL2, EHD4*
GO:0050673	Epithelial cell proliferation	−3.3547	*IGFBP5, NKX6-1, PHB2, IFT80, CD109, BBS1, SRC, ZBTB16, FGF18, HNF4A, LMNA, MYOD1, PLG, ITGA4, NME2, SULF2*
M92	Angiopoietin Receptor Pathway	−3.1420	*NFKB1, PLG, ANGPT4, ABCA1, HMGCR, MAP2K4, NUP98, TRIM25, CAMK2A, EEPD1*
WP4787	Osteoblast differentiation and related diseases	−3.0523	*JAG2, SMAD9, MYOD1, FGF18*
GO:0051668	Localization within membrane	−3.0367	*BBS1, ITGA4, LMNA, SLMAP, EHD4, EXOC6, IFT80, C1QL2, DNAH5, VAV2, FGD5, CFAP184, CAMK2A, NFKB1, TUBB6, MYO1B*
GO:0016310	Phosphorylation	−3.0269	*CAMK2A, MYOD1, SRC, DCLK1, ERN2, DGKH, PIM3, BBS1*

Continued

Table 3.— *Continued*

Generic Term	Description	Log *P*	Metascape Associated DMGs
R-HSA-9833482	PKR-mediated signaling	−2.9500	*PPP2CB, TRIM25, TUBB6, BBS1, JUN, ERN2, CAMK2A, IRS1, MAP2K4*
GO:0051223	Regulation of protein transport	−2.9360	*HMGCR, HNF4A, IRS1, NKX6-1, SRC, SH3TC2, PIM3, IGFBP5, DCLK1*
GO:0016567	Protein ubiquitination	−2.9019	*ZBTB16, TRIM25, DCAF4, KCTD9, IFT80, TRIM63, ANKRD9, RNF180*
GO:0010595	Positive regulation of endothelial cell migration	−2.8733	*PLG, FGF18, ANGPT4, BCAS3, IGFBP5, ITGA4, SRC, ELP3*

Database labels for generic terms: GO, gene ontology; R-HSA, reactome; WP, wikipathways; hsa, Kyoto encyclopedia of genes and genomes (KEGG) pathways; M, pathway interaction database (PID). DMG, differentially methylated gene; HR, high-responsive; LR, low-responsive.

periods, with the methylation of cytosines in these genes being independent of the stress period considered. This enables methylation changes to be categorized broadly, each category reflecting a different level of plasticity. The first category comprises those that exceed the 15% threshold only once during the challenge test and includes either pre- or post-stress-specific DMGs. These are temporally plastic over the duration of the challenge test. The second category comprises common DMGs that were found to be hypo- or hypermethylated relative to the 15% threshold in both pre- and post-stress conditions. This category exhibits more limited plasticity and maintenance of their methylation state. These could be explored further as potential candidate biomarkers for distinguishing between low- and high-risk European seabass when exposed to various stressors, if they are shown to be independent of the nature, context, strength, and duration of the challenge test. Methylation-based biomarkers have already been proposed for this species (57, 70). The third category comprises common stress-induced DMGs that transitioned from a hyper- to a hypomethylated state in both pre- and post-stress conditions (Fig. 3). These DMGs also demonstrate temporal plasticity throughout the challenge test. As in the study by Miller et al. (24), we consider these DMGs illustrate "epigenetic rewiring," this issue being discussed in *Epigenetic Rewiring*.

GC Signaling

Collectively, although blood serves as the template, results highlighted DMGs that have been recognized as involved in GC signaling for each category mentioned earlier and characterized by plastic, common, or rewired methylation at a 15% threshold. Results also showed that some of these DMCs gathered in enriched terms that are relevant to the stress response. Notably, this includes terms associated with the nervous system or the regulation of fat and glucose in the pre-stress condition, whereas immune-related pathways characterized were more prone to characterize differential methylation between LR and HR seabass in the post-stress situation. These observations have to be critically considered. Indeed, Klughammer et al. (71) have demonstrated higher inter-tissue variation in methylation patterns in teleosts compared with other vertebrates. At the cost of sacrificing individuals, future studies are undoubtedly needed for a better understanding of how fish blood can reflect and/or aggregate tissue-specific patterns into a whole body epigenetic status. Relevant tissues such as, e.g., brain, liver, muscle, as well as immune cells may yield more direct insights into GC reactivity of LR and HR seabass. Nevertheless, although this is a current limitation to interpretations of results presented in this study, several other observations suggest that a peripheral tissue such as blood provides insights on the ability to develop methylation-based differences associated with stress. First, there has generally been rather consistent agreement between epigenetic patterns of blood and, at least, brain tissues in humans, rodents, and birds (21, 72–75; but see Ref. 76). Second, other studies using peripheral tissues and interested in the association between circulating GCs and genome-wide methylation were also found to be enriched for brain- and immune-related functions (31, 32). Third, studying LR and HR seabass differing in cortisol reactivity, Samaras et al. (40) showed transcriptional differences in liver for functions associated with the immune system (e.g., PDGF pathway), fat and glucose metabolism, together with, e.g., signal transduction, phosphorylation, ubiquitination, and response to oxygen levels that were also found in this study. Thus, if blood methylation in LR and HR seabass does not accurately reflect the processes driven by methylation levels in various stress-responsive tissues affected by cortisol and GC signaling, we propose that these patterns, obtained via a noninvasive approach in a tissue also used for GC titration and the assessment of stress-related parameters such as glucose, lysozyme, and lactate, may provide valuable, complementary insight into the molecular basis of stress in both seabass phenotypes.

Numerous DMGs reported in this study have been shown to engage in GC signaling and be associated with various disorders resulting from a stress-induced imbalance. We discuss below only a few of them, whether they are either constitutive or specific to one of the two stress conditions. Among pre-stress DMGs, this includes, e.g., *NFIA* encoding nuclear factor 1A that cooperates with GCs after they bind receptors (77, 78). Constitutive, pre-stress differential methylation of genes expressed in the brain and involved in neurodevelopmental, cognitive, or psychotic disorders has also been found in this study. The molecular drivers of cognitive and neurodevelopmental disorders have attracted interest in European seabass (79, 80) and in the general literature over the past decades (16, 68, 81). These include DMCs within, e.g., the *AUTS2, DPYSL5, KLRNA,* and *SHANK2* genes, and,

to our knowledge, only *DPYSL5* has not been found to be associated with GC levels or exposure. *AUTS2* (activator of transcription and developmental regulator AUTS2) acts via chromatin remodeling and modification of histones, and its expression was shown to depend on GC levels in zebrafish (82). *AUTS2* is a key gene involved in the regulation of synapse organization and differentiation, translating into various neurological disorders and behavioral deficits. For example, *AUTS2* was shown to be associated with anxiety-like behavior and environment recognition capabilities in zebrafish (83). Dihydropyrimidinase like 5 (*DPYSL5*) also contributes to neurodevelopmental or mental disorders (72, 84). In fish, *AUTS2* and *DPYSL5* genes were shown to be associated with locomotor fear-related responses in offspring of the rainbow trout whose mothers were exposed to stress (85). The methylation of *SHANK2* (SH3 and multiple ankyrin repeat domains 2), a gene affecting *N*-methyl-D-aspartate (NMDA) postsynaptic signaling and associated with autism syndromes, hyperactivity, and anxiety, is known to respond to cortisol reactivity (86). A correlation between blood and brain methylation levels has been reported for *SHANK2* (87). KALRN (kalirin RhoGEF kinase) is a central regulator of synaptic function, interacting with BDNF, which is the most extensively studied GC-dependent molecule in the brain and central to the stress response (88, 89). KALRN was shown to be responsible for anxiety-like behaviors and fear learning by interacting with the HPI-associated gene, *POMC* (90). Other examples include transcription factors such as HNF1 homeobox A (*HNF1A*) and microtubule-associated monooxygenase, calponin and LIM domain-containing 3 (*MICAL3*) that are also related to GC-responsive genes (91, 92). *MICAL3* is considered as an epigenetic marker of stress exposure (93). The expression of sodium voltage-gated channel alpha subunit 5 (*SCN5A*) is controlled by GCs. It plays a role in sodium homeostasis and the regulation of stress-induced cardiac fibrillation, which can lead to arrhythmic cardiomyopathies (94, 95). Stress-induced heart arrhythmia and cardiomyopathies are interesting topics for study in fish (96–98), with cortisol involved in the regulation of cardiac hypertrophy (96, 97). As a last example, we also found pre-stress differential methylation for the phosphoenolpyruvate carboxykinase 1 (*PCK1*) gene that has been shown to participate with *FKBP5* to systemic GC deficiency in zebrafish (99).

Numerous common DMGs are also related to GC signaling. For example, the insulin receptor substrate 1 (*IRS1*) is involved in glucose homeostasis and was shown to respond to GCs by competing with GR and involving signal transduction via the PI3K/Akt pathway. Other candidate common DMGs—all of them encoding for transcription factors—deserving attention include, e.g., hepatocyte nuclear factor 4 alpha (*HNF4A*), myelin transcription factor 1 like (*MYT1L*), NK6 homeobox 1 (*NKX6-1*), tripartite motif containing 63 (*TRIM63*), and zinc finger and BTB domain containing 16 (*ZBTB16*). These DMGs were all shown be associated with GC signaling (100–104). GC-induced differential methylation is especially well supported for *ZBTB16* (104–107). *ZBTB16* was shown to limit GR-mediated activating effects and enhancing repressive effects in zebrafish (108). Ryanodine receptor 3 (*RYR3*), that encodes for a protein involved in calcium homeostasis, is modified by levels of GC (109, 110). A change in the level of intracellular calcium is considered an initial non-genomic action of cortisol (2). Furthermore, *RYR3* has been shown to be associated with the release of adrenocorticotropic hormone along the HPA axis (111). *RYR3* is also one of the numerous genes associated with stress resilience in rodents subjected to stress (112–114). Another interesting common DMG is SET domain containing 5 (*SETD5*), which encodes an epigenetic enzyme that has been shown to regulate chromatin methylation and preserve transcription fidelity. This was shown to result in differences in socio-behavioral deficits in mice (115). In a high-throughput RNA sequencing study, it was found that *SETD5* and the aforementioned pre-stress DMG *AUTS2* were among the most influential expressed genes in the polygenic pathway leading to the masculinization of European seabass (116). This warrants further epigenetic examination, as the HPI and the hypothalamo-pituitary-gonadal axis are strongly associated, and stress is effectively being a driver of sex determination in seabass (117).

In the post-stress condition, differential methylation observed at *JUN* [Jun proto-oncogene, Activator Protein-1 (AP-1) transcription factor subunit, also known as *c-Jun*] and nuclear factor kappa B subunit 1 (*NFκB1*) is noteworthy. Indeed, GCs are the most potent anti-inflammatory agents, acting via interactions between the ligand-activated GR and the proinflammatory transcription factors NF-κB and AP-1, of which *NFκB1* and *JUN* are elements of their respective protein complexes. These genomic actions lead to transrepression that promotes the anti-inflammatory effects of GCs by altering their transcriptional activity (1, 2). Differences in cortisol reactivity have been shown to influence the expression of the NF-κB1 gene (118), and both *NFκB1* and *JUN* have been shown to be differentially expressed in response to stressors in fish (119, 120). Therefore, stress-induced differences in methylation at post-stress DMGs reinforce our hypothesis of the induction and regulation of immune pathways in LR and HR seabass. Although NF-κB/AP-1 is a hub in the regulation of inflammation with complementary roles in—relevant to this study—the formation and consolidation of fear memory for NF-κB (121, 122), other post-stress DMGs have been found to be associated with GCs. Another example is the SRC proto-oncogene (*SRC*, also known as *c-Src*), which encodes a tyrosine kinase that plays a fundamental role in translating signals from growth factor receptors on the cell membrane to activate growth pathways, including the IGF1 (123) and the PDGF pathways (124, 125) mentioned in this study. *SRC* is also known to interact with the mineralocorticoid receptor (126) and with PI3K in several signaling cascades [e.g., "clock genes" involved in the rhythmicity of GC production and the regulation of GR activity (127)]. Epigenetics of circadian rhythmicity has recently attracted attention in seabass (128). Nevertheless, *SRC* primarily contributes to the assembly and conformation of the multi-protein complex, which also includes heat shock proteins (HSP70, HSP90), immunophilins (FKBP5), and various cofactor molecules (e.g., histone acetyltransferases, histone deacetylases) that bind to or participate in the GR complex to maintain its high affinity to GCs (2, 129). Anchoring of this complex to GR is a fundamental step in the non-genomic actions of GCs. Otherwise, tripartite motif-containing 25 (*TRIM25*) encodes a GC-regulated ubiquitin ligase that acts jointly with *NFκB1* to promote cell survival (130, 131). Specific

post-stress immune-related DMGs such as Tubulin beta 6 class V (*TUBB6*) and Sortilin-related VPS10 domain-containing receptor 3 (*SORCS3*) have also been shown to be associated with anxiety- or depression-like behaviors depending on GC exposure (132, 133). The methylation state of *SORCS3* was shown to respond to GC in zebrafish (82).

It results that although there is a need for a better understanding of how fish blood can reflect and/or aggregate tissue-specific patterns into a whole body epigenetic status in LR and HR seabass, the observed differential methylation at common, pre- and post-stress DMGs found in this study suggests an organism-wide response to the stress challenge test. As access to blood is minimally invasive to respect the Three Rs principle, it should be considered as a source of molecular biomarkers of interest in the study of the stress response.

Epigenetic Rewiring

As in the study by Miller et al. (24), which investigated individuals presenting different levels of saliva cortisol reactivity in humans, we also found a low number of DMGs undergoing epigenetic rewiring. Indeed, of the 54 common DMGs, only seven showed the methylation state of their respective cytosines to switch from hypomethylated in the pre-stress context to hypermethylated in the post-stress condition (*VAV2, ARHGAP32, IGFBP5, CDC42SE1, ABCA1, MGEA5/OGA*, and *ZNF107*) (Fig. 3). Most of these seven genes have been shown to respond to GC. The exceptions are *ZNF107* and *VAV2*. Nevertheless, zinc finger proteins have been shown to be associated with GC and the stress response (22), and *VAV2* regulates the small GTPase-mediated signal transduction pathway, which is a pre-stress-enriched term in this study (Table 2) and has pronounced effects on GR regulation (134, 135). The effector gene *CDC42SE1*, on the other hand, has been shown to inhibit GTPase activity. It is involved in, e.g., cell proliferation (136) and cardiovascular traits (137). *ARHGAP32* and *ABCA1* have been shown to respond to changes in GC levels (138, 139), as are the actions of IGFBP proteins represented by the *IGFBP5* gene in this study (140). Basal levels of *IGFBP5* in the blood have been shown to be strongly influenced by stressors (141). Rewired *ABCA1, IGFBP5*, and *VAV2* have also been shown to be differentially methylated in the blood of LR- and HR-like piglets (32) and are not restricted to seabass. Their role as epigenetic biomarkers of stress has to be investigated further.

The O-GlcNAcylation process, which involves the rewired *MGEA5* DMG that encodes for the OGA enzyme, is especially interesting. Basically, O-linked β-*N*-acetylglucosaminylation (O-GlcNAcylation) is a key process of systemic glucose homeostasis by interacting with GCs (142, 143). At the cellular level, O-GlcNAcylation also favors the import of cytosolic molecules to the nucleus by interacting with the nuclear pore complex. To do so, *MGEA5/OGA* acts with *NUP98* (nucleoporin 98 and 96 precursor), a DMG referred as "common" in this study (Supplemental Table S6). *NUP98* is essential to the nuclear pore complex assembly and nuclear import/export. These DMGs directly interact in the functioning of the "sieve"-like structure of the nuclear pore to large molecules (142). This includes the import of cytosolic GR to the nucleus (144, 145), with consequences on cognitive disorders, including in zebrafish (146, 147). Therefore, rewired differential methylation at *MGEA5* may potentially regulate some of the genomic and downstream physiological actions of GR by regulating its nucleocytoplasmic dynamics. Furthermore, *MGEA5* and *NUP98* have also been shown to alter chromatin structure to promote transcriptional memory, and to work in association with epigenetic "writers" and "erasers" of the histone code to provide a link between cellular metabolic status and the epigenetic machinery (148–150). *MGEA5,* or more generally O-GlcNAcylation seem promising candidate for improving our understanding of stressors on epigenetic rewiring in European seabass.

As other aforementioned DMGs, the actions of rewired *ARHGAP32*, *ABCA1, MGEA5*, *IGFBP5*, and *VAV2* are also associated with the IGF1-PI3K-Akt pathway (151–155), or the IGF1-PI3K-CDC42 pathway for *CDC42SE1* (156, 157). Other DMGs briefly illustrated in this discussion also depend on PI3K-Akt (e.g., *IRS1*) (158). Signal transduction by PI3K/Akt signaling mediates some of the non-genomic actions of GCs, including protein synthesis and cell proliferation that promote the maintenance of homeostasis during stress (2, 159, 160). PI3K/Akt signaling is active to regulate organism-wide GC-related processes such as, e.g., neurogenesis (161), muscle atrophy (99), cardiac hypertrophy (162), glycemia (163), inflammation (164), or bone regeneration (165).

Overall, it results that analyses of individual pre-stress, post-stress, common, and rewired DMGs found in this study may contribute to differentially regulate the non-genomic or genomic actions of cortisol in LR and HR fish. Indeed, our findings report an activation of membrane-bound receptors by GCs (e.g., IGF1 pathway), completed by cytoplasmic signaling, import of GR to the nucleus, then genomic signaling through differentially methylated transcription factors. We hypothesize that these initiate an adaptive response (allostasis) to provide a specific "energetic drive" to each phenotype in response to stress.

Limitations

Although our study examined genome-wide methylation associated with stress response and circulating GCs, and was found to cover the main cellular mechanisms of GC/GR actions, it remains a descriptive study with limitations. The first one has already been addressed earlier: the methylation and gene expression patterns resulting from differences in methylation distribution between phenotypes are tissue-dependent. More specific epigenetic and expression studies in which individuals will be sacrificed are essential to better understand the stress response of each phenotype. A second limitation is methodological. EpiGBS is similar to double-digest restriction-site associated DNA sequencing in genomics (39). It covers only a small fraction of the genome and is based on short reads between restriction sites. Therefore, it poorly detects differentially methylated regions (DMRs) that are standard and considered more robust (87), whereas functional consequences of single CpG methylation remain ambiguous (166). However, GC-related epigenetic studies have already prioritized genome-wide distributed DMCs and reported association with the stress response (22, 167). Whole genome bisulfite sequencing, previously employed in *D. labrax* (55), should be implemented to identify more DMRs, alongside an investigation into other

relevant patterns of the epigenetic control of its stress response (e.g., acetylation of histones, 5-methylhydroxycytosine profiles, noncoding RNAs).

Conclusions and Future Directions

In this study, we demonstrated that HR and LR European seabass with divergent cortisol reactivity exhibit genome-wide differences in blood methylation before and after exposure to a stress challenge test. These differences were most likely either constitutive or induced by the challenge test, possibly contributing to the organismal stress response of each phenotype when facing a common adverse experience and helping them to adjust to stressors. Although further investigations are required, it is certainly of prime interest to integrate epigenetic studies with behavioral or gene expression data that have already been jointly investigated in *D. labrax* (168). As heritable circulating cortisol levels have been demonstrated in European seabass, approaches such as epigenome-wide association studies that necessarily integrate aspects of transgenerational inheritance must be considered for this aquaculture species (169). Finally, *D. labrax* exhibits a more pronounced stress response and is less resilient to stress than other species (42, 170) and comparing interspecific methylation profiles of blood across fish species with different physiological stress sensitivity may shed light on what might shape the stress response in this highly diverse clade of vertebrates.

DATA AVAILABILITY

Source data for the blood methylome study are openly available as NCBI's Gene Expression Omnibus [GEO] Datasets with accessions GSE153838 and GSE156637. Cortisol, glucose, lactate, lysozyme activity data as wells as pre- and post-stress body weights of individual fish are available on FigShare (https://doi.org/10.6084/m9.figshare.32586156).

SUPPLEMENTAL MATERIAL

Supplemental Tables S1–S10 and Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.32586156.

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DISCLAIMERS

The authors are solely responsible for the content, which does not necessarily represent the official views of the funders, AVRAMAR Aquaculture S.A., which hosted the experiments, or the institutions to which the authors are affiliated.

DISCLOSURES

No other conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.S.T. and B.G. conceived and designed research; A.S., M.V.K., A.D., E.D., and L.P. performed experiments; S.B.C., A.S., M.V.K., A.D., E.D., M.P., and B.G. analyzed data; S.B.C., A.S., M.V.K., A.D., E.D., M.P., and B.G. interpreted results of experiments; S.B.C., A.S., and B.G. prepared figures; S.B.C., A.S., and B.G. drafted manuscript; S.B.C., A.S., C.S.T., and B.G. edited and revised manuscript; S.B.C., A.S., M.V.K., A.D., E.D., L.P., M.P., C.S.T., and B.G. approved final version of manuscript.

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