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Development of epigenetic biomarkers with diagnostic and prognostic value to assess the lasting effects of early temperature changes in farmed fish

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ABSTRACT

Epigenetic changes such as DNA methylation modifications at specific loci in the genome in response to environmental cues can appear long before the appearance of transcriptomic or other phenotypic changes with potential consequences for performance. Thus, epigenetic DNA methylation-based biomarkers hold great promise in aquaculture and conservation biology because they can have prognostic value. However, to the best of our knowledge, a clear demonstration of such biomarkers has not been provided yet. Temperature during early stages of development is the main abiotic factor determining larval quality, with effects potentially persisting until adulthood and influencing performance. The objective of this study was to test whether epigenetic biomarkers could be developed as reliable indicators of the quality of the thermal environment during European sea bass (*Dicentrarchus labrax*) early development and their relation to long-lasting phenotypic consequences. We exposed European sea bass embryos and newly hatched larvae to different temperature regimes and sampled fish thereafter at three time points: at larval, juvenile and adult stages. Here, we provide an analytical strategy combining Reduced Representation Bisulfite sequencing (RRBS) and RNA-sequencing (RNA-seq) to develop epigenetic and transcriptomic biomarkers with both prognostic and diagnostic value. Specifically, we report a series of DNA-methylation biomarkers linked to short-term, mid-term, long-term, and very-long term effects of temperature in both somatic and reproductive tissues. Among the different biomarkers identified, one promising example is the methylation status of keratin-associated protein 10–4 (*krtap10–4*), which fulfills the criteria of an epigenetic biomarker with both prognostic and diagnostic value. This study constitutes a first step toward providing the European sea bass farming sector with a set of epigenetic biomarkers that can be used to screen batches of larvae to determine the quality of their thermal environment and to anticipate their performance when juveniles and adults. Our study provides the way for similar developments for other species, farmed or not.

1. Introduction

Larval rearing is one of the most crucial and delicate procedures during modern, intensive fish farming production and, thus, many factors, both biotic and abiotic, can influence its success (Conceicao and Tandler, 2018). Temperature affects many biological functions at different levels of organization (Schulte et al., 2011), being considered also the main abiotic factor determining fish larval quality and survival (Pepin, 1991). The early stages of development are particularly the most sensitive to temperature (Burggren and Mueller, 2015), while these effects can persist until adulthood influencing growth performance and product quality (Jonsson and Jonsson, 2014).

The European sea bass, *Dicentrachus labrax*, is, after the Atlantic salmon, *Salmo salar*, and rainbow trout, *Onchorhynchus mykiss*, the third most important species for finfish aquaculture in Europe in terms of volume, and the most important in the Mediterranean region, with over

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208,000 tons produced in 2019 (FEAP, 2020, https://feap.info/index.php/data/). The European sea bass is a eurythermal marine teleost (Pickett and Pawson, 1994). Its thermal tolerance in juveniles acclimated to 20 °C ranges from 4.7 °C to 34.8 °C (Dülger et al., 2012). In the Mediterranean sea, spawning typically takes place during winter. In hatcheries eggs are incubated and hatch at around 13–14 °C, and larval rearing takes place usually between 15 and 21 °C (Morretti, 1999). Temperature has a profound effect on the European sea bass ontogenetic plasticity (Koumoundouros et al., 2001). It is a gonochoristic species with sexual size dimorphism in favor of females and a polygenic system of sex determination (Vandeputte et al., 2007), where genetics and temperature contribute equally to sex ratios (Piferrer et al., 2005). Thus, proper temperature control during their embryonic and first larval stages of development is essential to minimize skeletal deformities (Georgakopoulou et al., 2007), prevent precocious male maturation (Felip et al., 2006), maximize growth, optimize sex ratios (Navarro-Martín et al., 2009), and to increase overall product quality (López-Albors et al., 2008).

Epigenetics concerns the study of phenomena and mechanisms that cause chromosome-bound, heritable changes to gene expression that are not dependent on changes to the DNA sequence (Deans and Maggert, 2015). Epigenetic mechanisms, namely, DNA methylation, histone modifications and non-coding RNAs, regulate gene expression by integrating genomic and environmental information, thus contributing to phenotypic plasticity (Duncan et al., 2014). Epigenetics holds great promise in aquaculture (Gavery and Roberts, 2017; Granada et al., 2018; Piferrer et al., 2015). Epigenetic alterations in response to environmental stimuli, for example, changes in DNA methylation levels in specific loci in the genome that correlate to a certain phenotype, can be used as epigenetic biomarkers. Of note, epigenetic biomarkers not only can have diagnostic but also prognostic value, since sometimes epigenetic changes appear long before gene expression or other phenotypic changes become apparent (Costa-Pinheiro et al., 2015). In the European sea bass, DNA methylation-based biomarkers have already been developed to predict sex (Anastasiadi et al., 2018), assess the early stages of domestication (Anastasiadi and Piferrer, 2019b), determine biological age (Anastasiadi and Piferrer, 2019a), assess the consequences of heat waves (Anastasiadi et al., 2021), and to evaluate the stress response associated with rearing density (Krick et al., 2021). However, and to the best of our knowledge, there are no epigenetic biomarkers in the European sea bass or, for that matter, in any other farmed fish species, that can be used to link the temperature regime during early stages of development with subsequent aquaculture performance. Developing epigenetic biomarkers that link adult phenotypic traits to early environmental conditions is a major goal of epigenetics in aquaculture as well as in conservation biology.

The objective of this study was to assess the effects of temperature changes during embryo and early larval development on the establishment of epigenetic and transcriptomic biomarkers that could correlate with phenotypic traits later in development in both somatic and reproductive tissues. Specifically, we tested whether these biomarkers could be developed as reliable indicators of the quality of the thermal environment during the most critical stages of development of fish, where temperature may have long-lasting phenotypic consequences. This constitutes a novel approach and a first step toward the ultimate goal of providing the aquaculture sector with a set of epigenetic biomarkers with diagnostic and prognostic value to improve management.

2. Materials and methods

2.1. Experimental facilities

Fish were reared at the Hellenic Center of Marine Research (HCMR) in Crete, Greece. The HCMR aquaculture facilities are certified from the national veterinary authority (code GR94FISH0001) and are licensed for operations of breeding and experimentation with fish issued by Region of Crete, General Directorate of Agricultural & Veterinary no. 3989/01.03.2017 (approval codes EL91-BIObr-03 and EL91-BIOexp-04). The experimental protocol was approved by the Veterinarian Authority of the Region of Crete with the 255,332/29-11-2017 document. Animal experiments were carried out in accordance with the EU Directive 2010/63/EU.

2.2. Egg incubation and temperature treatments

All eggs used in this study came from the same mass spawn of captive breeders. Approximately 240,000 eggs were distributed into six 500-l tanks at about 60,000 eggs per tank. Eggs were incubated at 14 °C. For the rearing recirculation systems, biological filters were used. Water temperature was adjusted using chillers and heaters, which were automatically controlled with electronic sensors. Hatching and larval rearing were performed by using the "pseudogreen water" methodology (Papandroulakis et al., 2001). With a hatching rate close to 95% the initial number of larvae was approximately 54,000 per tank.

Three groups of fish were subjected in duplicate to 14 °C, 17 °C, and 20 °C (two of the six tanks mentioned above randomly assigned to each group) from the stage of epiboly onset to the end of the yolk-sac larval stage and the beginning of exogenous feeding (Fig. S1A). Given that the protocols for European seabass larval rearing are applied with temperature around 17 °C, the choice of 3 °C difference for the upper and lower limit is considered sufficient for the purpose of the study and potentially applicable at commercial hatcheries.

In all cases, the experimental temperature was set 1 day post fertilization (dpf) and lasted until 8 dpf in the case of 17 °C and 20 °C, while for the 14 °C the duration was until 12 dpf (different durations compensated for the effect different temperatures on growth). In this study, therefore, and following the ARRIVE guidelines (Percie du Sert et al., 2020), there were three experimental groups each carried out in replicate (being the tanks the biological replicates), and the group exposed to 17 °C was the control group. Subsequently, all groups were kept under identical rearing conditions including temperature (17 °C) up to the end of the trial. Acclimation of the eggs from the spawning temperature (14 °C) to the test temperatures, and then to the common temperature for larval rearing (17 °C) was performed at the rate of 0.5 °C and 0.2 °C h^{-1}, respectively. Fish were weighed periodically at the times indicated below.

2.3. Larval and juvenile rearing

Larvae were successively fed on enriched (DHA Protein Selco, INVE) rotifers until they reached 5.5 mm total length (TL), enriched with rotifers and *Artemia* Instar 1 nauplii (AF, INVE) when they were between 6 and 7.5 mm TL, and enriched with *Artemia* nauplii (Easy DHA Selco, INVE) afterwards. Rotifer concentration in the tanks was adjusted three times daily to 6–8 individuals·ml^{-1}. The concentration of *A.* nauplii was adjusted three times daily to 0.5–2 individuals·ml^{-1}. During the rotifer-feeding period, live microalgae *Chlorella* sp. were added three times daily at a concentration of $3 \pm 1 \times 10^5$ cells ml^{-1}. Weaning to inert diets (O-range, INVE) started when fish were approximately 9.5 mm TL. In Table S1 the feeding regime per experimental condition is shown.

Air-blowing skimmers were installed since first feeding until 29 dpf to keep the water surface free from lipids and enhance swim bladder inflation. Water oxygen concentration was 7.68 ± 0.9, pH 8.37 ± 0.12, salinity 35–36‰ and photoperiod 12L:12D. After larval rearing (65 dpf) the survival was estimated and fish were transferred for pre-growing in 10 m^3 tanks, supplied with seawater from a deep well and operating in an open water system. The survival rate (mean ± SD) at the end of larval rearing was 19.6 ± 5.1% for the control group exposed to 17 °C (16.0% and 23.2% for the two replicates); 18.5 ± 2.4% for the group exposed to 14 °C (16.8% and 20.2% for the two replicates) and 17.2 ± 3.9% for the group exposed to 20 °C (14.5% and 20.0% for the two replicates). Water oxygen saturation was 7.0 ± 0.9, pH 7.6 ± 0.3, salinity 35–36‰, and

photoperiod natural. During pre-growing (65–130 dpf) the larvae were grouped per temperature treatment resulting in one group per experimental temperature. Following the standard husbandry procedures, the juveniles were sorted by size, removing the very small and the very large individuals in order to create a more homogenous size distribution among the three experimental groups and were also selected for swim bladder with a salinity test.

2.4. Coping style test

The test was performed following a standard procedure developed at Marine Biology, Biotechnology and Aquaculture (IMBBC) in the frame of the FP7 project "A new integrative framework for the study of fish welfare based on the concept of allostasis, appraisal and coping styles, COPEWELL, (Grant agreement ID: 265957)". From each temperature treatment, three subgroups were randomly created when fish had approximately 130 dpf and there was no size difference between the groups. We did not consider sex in this test because the earliest stage in which gonads can be sexed is around 150 dpf histologically (Papadaki et al., 2005). These groups were transferred to cages for on-growing after being subjected to a conditioned place preference test (CPP) to characterize fish coping style. Briefly, the methodology consisted in: (i) restraining the individuals in a 1 m^3 containment, placed within the holding tank, for 20 h, (ii) allowing the individuals to pass through three open holes (10 cm diameter), constructed close to the water surface while feed was distributed outside the containment by the use of an automatic feeder, and (iii) recording the number of individuals passed through the holes, during a time course of two hours. At the end of the test, six groups were made according to the temperature treatment and the coping style expressed, mentioned as "*Bold*" for the proactive individuals, i.e. the ones exiting the containment and "*Shy*" for the reactive individuals i.e. the ones remaining in the containment.

2.5. Rearing in sea water

The six original groups (from 2000 to 3000 individuals each) were then transported for further on-growing in net-open cages. At this time fish were approximately 140 dpf and had a mean weight of 1.72 ± 0.40 g. On-growing lasted for nine months and was performed in the pilot scale cage facility of the Institute of Aquaculture (HCMR) located at the North-West of Crete in Souda Bay (35°28′48.3"N 24°06′41.8″E). The mean water depth at the site is about 60 m (ranging from 30 to 90 m) with a continuous water circulation. Water temperature varied from 15 °C to 28 °C, and salinity was constant all year round at about 39 ‰. Water temperature and dissolved oxygen concentration were monitored daily using digital probes (HQ 40d; Hach SA, Loveland, CO, USA).

Rearing was performed in polyethylene cages (rectangular and/or circular) and standard rearing methodologies were applied (Pavlidis and Mylonas, 2011). The cage size at the beginning of the rearing was approximately 35 m^3 (3 m high x 3 m wide x 4 m depth) and increased to approximately 50 m^3 (3 × 3 × 6 m) when fish reached a mean weight of about 50 g. Rearing conditions were the standard followed for the European seabass with daily feeding using automatic feeders with standard commercial diets based on feeding tables available/adapted in the farm.

2.6. Samplings

The tissues selected for this study were sampled at three time points. After each sampling, tissues were kept in RNA-later solution (ThermoFisher Scientific) and stored at −80 °C until further analysis. Whole larvae at the first feeding stage (8 or 12 dpf depending on the temperature group) were sampled and four pools of ten larvae per temperature group were used for RNA-sequencing. Another four different pools of ten larvae each were made for DNA methylation analysis (see details below). At the past metamorphosis stage (90 dpf), the central part of the body trunk of juveniles, consisting mostly of muscle tissue was taken, and two different pools with four fish each were used for RNA-seq and DNA methylation analysis, respectively. Adult fish were sampled at 482 dpf, and testes of four individual fish per each temperature treatment were selected for RNA-seq and DNA methylation analysis. The testis was used for two reasons: first, because we had examined the very long-term effects of temperature on this organ in a previous study (Anastasiadi et al., 2021), and thus could compare results; and second because epigenetic information can be transmitted through the sires in the European sea bass (Anastasiadi et al., 2018). To avoid possible size-dependent differences that could confound the results, we selected larvae samples at the first feeding stage of the same size. Similarly, juvenile samples were weighted to ensure that all samples contributed equally to the pool. In addition, during the adult fish sampling at 482 dpf, the total weight was determined in 20 fish (ten males and ten females) randomly taken in each temperature treatment. Phenotypic sex was identified in the same fish by visual inspection, and gonads were weighted. Gonadosomatic index (GSI) was calculated as gonad weight (g) / total fish weight (g) × 100.

2.7. RNA and DNA extraction

Tissues for transcriptomic analysis were removed from the RNA-later® solution, samples were immersed into TRIzol® Reagent (ThermoFisher Scientific), and homogenized manually. RNA was extracted from larvae, juveniles, and adults according to the manufacturer's instructions. RNA quality was evaluated with Agilent RNA 6000 Nano Kit (Agilent), and samples with RNA Integrity Number (RIN) > 8 were used for RNA-seq library preparation. Genomic DNA was extracted using the Blood and Cell Culture DNA Mini kit (13,323, Qiagen) according to the manufacturer's instructions. The quality of the DNA was checked with NanoDrop ND-1000 (Thermo Fisher Scientific), and concentration was measured by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

2.8. Sea bass reference genome

The European sea bass reference genome (GCA_000689215.1) (Tine et al., 2014) was used for this study. For methylation analysis, the reference genome was bisulfite converted using Bismarck software (v. 0.22.3) applying the bismark_genome_preparation function (Krueger and Andrews, 2011). The start and end positions for each gene (gene body region) in the genome were taken from Biomart (Durinck et al., 2005, 2009).

2.9. RNA-sequencing (RNA-seq) and differential gene expression analysis

The RNA libraries of larvae, juveniles, and adults were prepared using the mRNA-Seq preparation kit (Illumina) according to the manufacturer's protocol at the Centre for Genomic Regulation (CRG) service within the Barcelona Biomedical Research Park (PRBB). Briefly, 0.5 μg of RNA were used for poly-A-based mRNA enrichment selection using oligo-dT magnetic beads followed by fragmentation of divalent captions at elevated temperature resulting into fragments of 80–250 base pairs (bp), with the major peak at 130 bp. First-strand cDNA synthesis by random hexamers and reverse transcriptase was followed by the second-strand cDNA synthesis. Double-stranded cDNA was end-repaired, 3′-adenylated, and the 3′-"T" nucleotide with the Illumina adapter was used to ligate the indexed adapters. The ligation product was amplified using 12 PCR cycles. Each library was sequenced using 50 bp paired-end mode on an Illumina HiSeq 2000 instrument following the manufacturer's protocol. The quality of raw reads was checked using FastQC software (v. 0.11.9) (Wingett and Andrews, 2018). Adapters and sequences with a Phred quality score lower than 30 were trimmed with the *Bbduk* software (v. 38.90) (Bushnell, 2014). The HISAT2 software (v. 2.1.0) (Kim et al., 2019) was used first to index the reference genome using hisat2-index build command and then to align the trimmed reads with default parameters. Finally, reads were counted with featureCounts

software (Liao et al., 2014) (v. 2.0.1) at the gene level. Detailed information of the samples for RNA-seq analysis in each tissue, treatment and control quality statistics are listed in Table S2.

Differential gene expression (DEGs) analysis was conducted using the *edgeR* package (v. 3.28.1) (McCarthy et al., 2012; Robinson et al., 2010). The lowly expressed genes were filtered using the "filterByExpr" function (Chen et al., 2016) among all samples. Retained counts were normalized using the upper-quartile "calcNormFactors" function (Anders and Huber, 2010). Estimates for negative binomial (NB) dispersion parameters were generated using "estimateDisp" function (Chen et al., 2014; Phipson et al., 2016). A quasi-likelihood binomial generalized log-linear model was fitted using "glmQLFit" (Lun and Smyth, 2016; Lund et al., 2012) and the "glmQFTest" function was used to compare the different contrasts within the matrix design (Lund et al., 2012; Chen et al., 2016; Lun and Smyth, 2016; Phipson et al., 2016; Lun and Smyth, 2017). Finally, a false discovery rate correction (FDR or Q-value, < 0.05) was applied in all contrasts (Benjamini and Hochberg, 1995).

2.10. Reduced representation bisulfite sequencing (RRBS)

The RRBS libraries of larvae, juveniles, and adults were prepared using the Premium RRBS kit (Diagenode, C02030032) according to the manufacturer's instructions. Qubit DNA High Sensitivity assays (ThermoFisher Scientific) were used to quantify the final concentrations of the libraries and their quality was checked using the DNA High Sensitivity Assay (Agilent). In total, 36 libraries (i.e., four biological replicates x three stages x three temperatures) were grouped in four pools of nine samples. The four pools of libraries were sequenced on an Illumina HiSeq 2500 instrument using single-end mode and a read length of 50 bp.

Quality control of reads were performed using the FastQC software (v. 0.11.9) (Wingett and Andrews, 2018). Trimmomatic software (v.0.36) (Bolger et al., 2014) was used for trimming the adaptors with parameters: sliding window 4, Phred score > 20, and adaptive quality trimming with largest length of 20 bp. Trimmed reads were aligned to the reference genome using the BSMAP software (v. 2.90) (Xi and Li, 2009) with RRBS mode and filtering for at least 10× coverage. In order to extract methylation data only from CG context (CpG) we used the methratio.py function from BSMAP. The bisulfite conversion efficiency of the libraries was checked following the instructions from the Premium RRBS kit.

The *methylKit* package (v. 1.14.2) (Akalin et al., 2012) was used to calculate differentially methylated cytosines (DMCs). To do so, coverage was normalized across samples for each tissue and only CpGs covered in all samples from the same tissue were maintained for further analysis. To detect DMCs a logistic regression model was applied with the F-test, which identifies methylation differences with the following chosen criteria: methylation differences larger than 15% and Q-value <0.01 adjusted from the SLIM method (Wang et al., 2011). A detailed information of the samples for the methylation analysis in each tissue and treatment and control quality statistics are listed in Table S3. Finally, the *GenomicRanges* package (v. 1.38) (Lawrence et al., 2013) was used to identify those genes that contained at least one DMC within their gene body region ±1000 bp.

2.11. Gene ontology terms and Kyoto encyclopedia of genes and genomes pathways analysis

The Gene Ontology terms (GO-terms) and the Kyoto Encyclopedia of Genes and Genomes pathways (KEEGs) analyses were carried using the *TopGO* package (v. 2.46.0) (Alexa and Rahnenfuhrer, 2010) adapted for the European sea bass GO-terms database. Only the Biological Process (BP) category was considered relevant for this study. The several GO-terms carried out were simplified using the R code script from Revigo software (Supek et al., 2011) with settings: medium (0.7) and SimRel semantic similarity method (Schlicker et al., 2006).

2.12. Data analysis

Sex ratio analysis and behavior tests were analyzed within each temperature treatment using Chi-squared test. A two-way ANOVA with Tukey post hoc test was applied to weight and GSI data. For methylomics and transcriptomics, the middle temperature, 17 °C, was considered the reference or control temperature. Therefore, two different comparisons at each one of the three sampling points were carried along in this study: the effects of cold temperature (i.e., 14 °C vs. 17 °C) and the effects of elevated temperature (i.e., 20 °C vs. 17 °C). In all analysis significant differences were considered when $P < 0.05$. Statistical analysis were performed with R software (v. 4.1.2) (R Core Team, 2020) using in Rstudio (v. 2021.09.0) (RStudio Team, 2020). Graphs were created using the *ggplot2* package (v. 3.3.5) (Wickham, 2011).

Samples for transcriptomic and DNA methylation analysis were selected based on the integrity of RNA (> 8 RIN level), the bisulfite conversion efficiency ($> 99.0\%$), and the aggrupation of the samples in a Principal Component Analyses (PCA) to detect the outliers for the same group. Those samples which did not meet these criteria were discarded. In total, 9 and 6 out of a total of 36 and 36 samples were discarded for RRBS and RNA-seq analysis, respectively. The number of samples used for each comparison by each tissue is presented in Table S4.

3. Results

3.1. Growth, gonadal development, sex ratio, and coping style

At 95 dpf, the weight (mean ± SEM) was 1.08 ± 0.26 g for the 17 °C-reared fish; 1.07 ± 0.28 g for the 14 °C-reared fish; and 1.07 ± 0.26 g for the 20 °C-rered fish, without statistically significant differences ($P > 0.05$). At 482 dpf, females tended to have a higher mean weight than males, but no significant sexual dimorphism was observed in any of the three temperatures tested. Although fish exposed to 14 °C were bigger than those exposed at 20 °C, (Two-way ANOVA: $F = 4.37$, $P = 0.017$) there were no statistical differences between fish exposed at 14 °C vs. those exposed at 17 °C and the same applied between fish exposed to 20 °C vs. those exposed to 17 °C (Fig. S1B). Analysis of the GSI showed that females exposed to 20 °C had significantly higher mean values ($P < 0.05$) than females exposed at 14 °C, but there were no differences in the GSI of males (Fig. S1C). Sex ratios progressively increased in favor of males with temperature, without significant differences (Fig. S1D). Regarding copying style, the number of bold fish increased in the group exposed at 20 °C (Fig. S1E).

3.2. Overview of DNA methylation and gene expression changes as a function of temperature and tissue

We carried out transcriptomic and DNA methylation analyses to identify DEGs and DMCs in larvae, juveniles and adults in two different comparisons: 14 °C vs. 17 °C, and 20 °C vs. 17 °C temperatures.

Regarding transcriptomic results for larvae, juveniles, and adults, we obtained 8490, 58 and 28 DEGs between 14 °C vs. 17 °C (Tables S5, S6 and S7, respectively), and 8128, 42 and 53 DEGs between 20 °C vs. 17 °C (Tables S8, S9, and S10, respectively). On the other hand, regarding DNA methylation, the number of DMCs were 27,286 and 22,166 in larvae (Tables S11 and S12), 9884 and 15,886 in juveniles (Tables S13 and S14) and 7254 and 49,610 in adults (Tables S15 and S16) from the 14 °C vs. 17 °C and 20 °C vs. 17 °C comparisons, respectively. Finally, for each tissue and contrast, we identified 7231 genes which had at least one DMC (Table S17).

Next, taking all gene lists together we identified 163 genes in the 14 °C vs. 17 °C comparison and 264 genes in the 20 °C vs. 17 °C comparison with DMCs that were common to larvae, juveniles, and adults (Fig. S2A and B). Furthermore, by comparing these 2 lists we identified

70 genes in common regardless of the direction of temperature change (Table S18). These are then genes, e.g., amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (*agl*), the methylation of which is affected in larvae and maintained in both juveniles and adults and represent indicators of long-term epigenetic (but not transcriptomic) effects of temperature on sea bass tissues. This list also includes jumonji domain containing 7 (*jmjd7*) and potassium voltage-gated channel subfamily H member 5 (*kcnh5b*), two genes that are related to environmental signal transduction, and zinc finger FYVE-type containing 9 (*zfyve9a*), which participates in the TGF-ß signaling pathway.

Finally, we were interested to know if there were genes that were considered both DEG and also contained at least one DMC in their body region. Results showed that 1350 (Table S19) and 723 (Table S20) genes in larvae, 6 (Table S21) and 7 genes (Table S22) in juveniles, and the gene potassium voltage-gated channel subfamily H member 3 (*kcnh3*), and two genes (ENSDLAG00005004413 and ENSDLAG00005021715) in adults were shared in the 14 °C vs. 17 °C and 20 °C vs. 17 °C comparisons, respectively (Fig. S2A and B). These are then genes, e.g., potassium voltage-gated channel subfamily H member 2 and 3 (*kcnh2a* and *kcnh3*), proprotein convertase subtilisin/kexin type 7 (*pcsk7*), protein kinase D4 (*prkd4*), in which temperature affected both their DNA methylation and expression and can be considered biomarkers of the persistent epigenetic and transcriptomic effects of temperature with potential functional consequences.

3.3. Genes whose expression is consistently up- or downregulated by temperature

In larvae, we wanted to know how many genes were consistently upregulated or downregulated by the temperature effects. There were 4839 DEGs that were common in the list of the 8490 DEGs between 14 °C vs. 17 °C and in the list of 8128 DEGs between 17 °C vs. 20 °C comparisons. From these 4839 genes, we filtered those that their transcripts levels were consistently upregulated (i.e.,153 genes) and downregulated (i.e., 58 genes) with the effects of temperature (Table S23). The raw transcript values from these 153 and 58 genes were normalized and plotted (Fig. 1A and B). These 211 genes (Table S23) included signal transducing adaptor family member 2 (*stap2b*), mannose receptor C-type 1 (*mrc1a*), and DAB adaptor protein 2 (*dab2*). Then, we searched for the biological role of these 153 and 58 list of genes separately and results showed 44 and 33 associated GO-terms, respectively (Fig. 1C and D). The most relevant GO-terms related with genes upregulated by

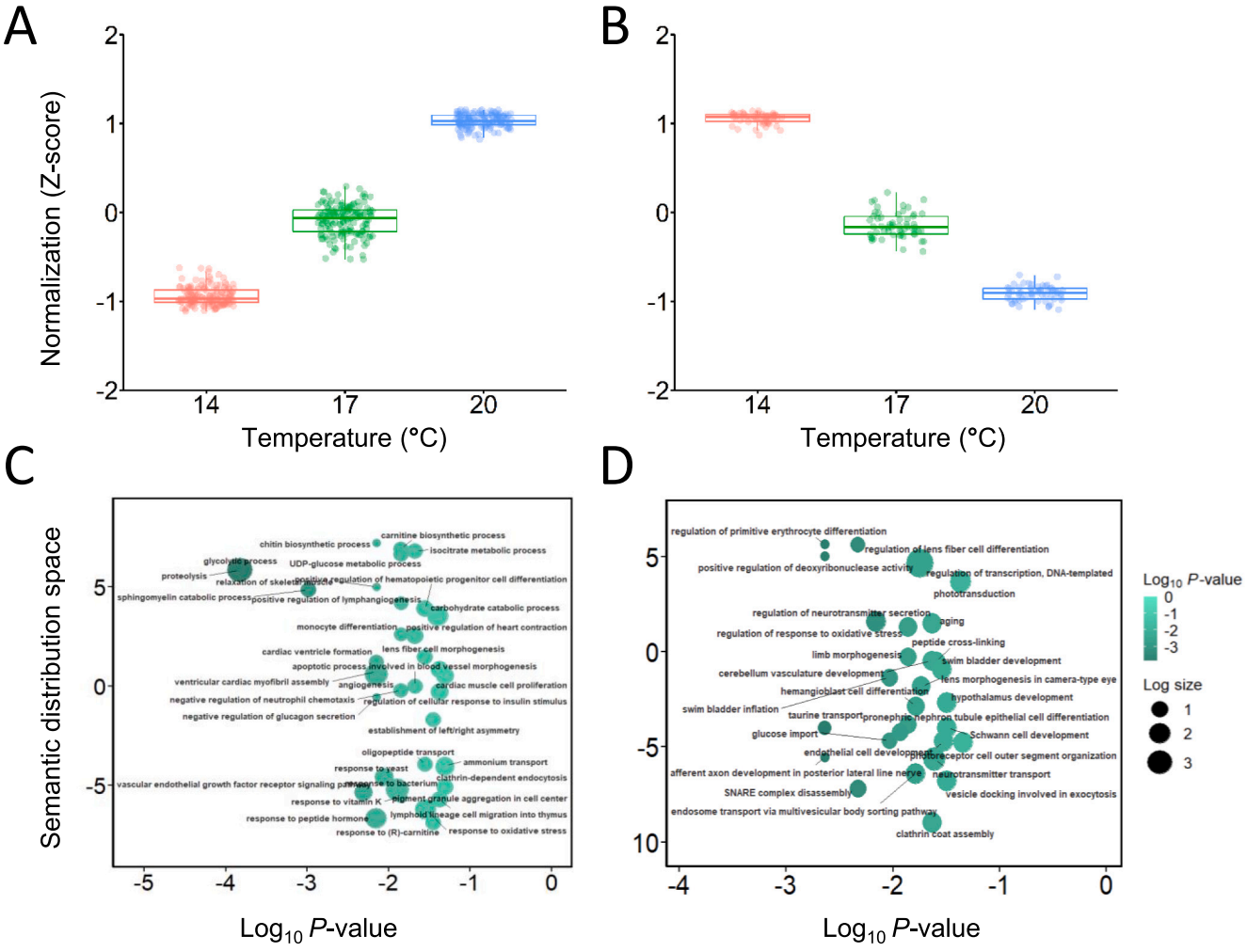

Fig. 1. (A-B) Box plots of the normalized (*Z*-score) gene expression of common differentially expressed genes (DEGs) between 14 °C vs. 17 °C ($n = 153$) and 20 °C vs. 17 °C ($n = 58$) that were consistently (A) upregulated (Kruskal-Wallis: chi-squared = 407.11; *P*-value <0.01) or (B) downregulated (Kruskal-Wallis: chi-squared = 153.78; *P*-value <0.01) along with the effects of temperature. (C-D) Semantic similarity scatter plots summarizing the enriched Gene Ontology (GO) terms of the Biological Process category (BP) from the (C) 153 DEGs into 44 GO-terms and from the (D) 58 common DEGs into 33 GOs for the genes that consistently increase or decrease expression with temperature, respectively. The scatterplots show GO-terms as circles. Significance was shown with the green gradient color palette (log10 *P*-value), and the sizes of the plotted circles were scaled by the frequency of GO-terms they represent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

temperature were: response to oxidative stress (GO:0006979), negative regulation of glucagon secretion (GO:0070093) and response to peptide hormone (GO:0043434). On the other hand, the most relevant GO-terms related with genes downregulated by temperature were: glucose import (GO:0046323), aging (GO:0007568) and photo-transduction (GO:0007602).

3.4. DEGs in larvae that contain at least one DMC regardless of temperature change

Next, we searched in larvae those genes that in addition of being considered DEGs also contained at least one DMC and appeared in both temperature comparisons (i.e., common genes between the 1350 genes at 14 °C vs. 17 °C and the 723 genes at 20 °C vs. 17 °C). We found 210 genes fulfilling this criterion (Table S24). To understand their relationship, these genes were filtered and their raw transcript counts data were normalized per individual sample and represented in a heatmap (Fig. 2A). The heatmap showed that samples clearly grouped according to temperature treatment with a close relationship between the 14 °C and 17 °C samples compared to samples from 20 °C treatment. Thus, of the 210 genes identified, 95 were clearly upregulated at 20 °C while they were mostly downregulated at 14 °C and 17 °C treatments. In contrast, 115 genes had just the opposite pattern, i.e., downregulated at 20 °C and upregulated at 14 °C and 17 °C (Fig. 2A). These 95 and 115 genes include: major intrinsic protein of lens fiber (*mipb*), ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1 (*atp2a1*), glutathione S-transferase omega 2 (*gsto2*), and calcium binding protein 2 (*cabp2a*).

We also searched for the biological role of these 210 genes to better understand the effects of temperature. Results showed that these genes

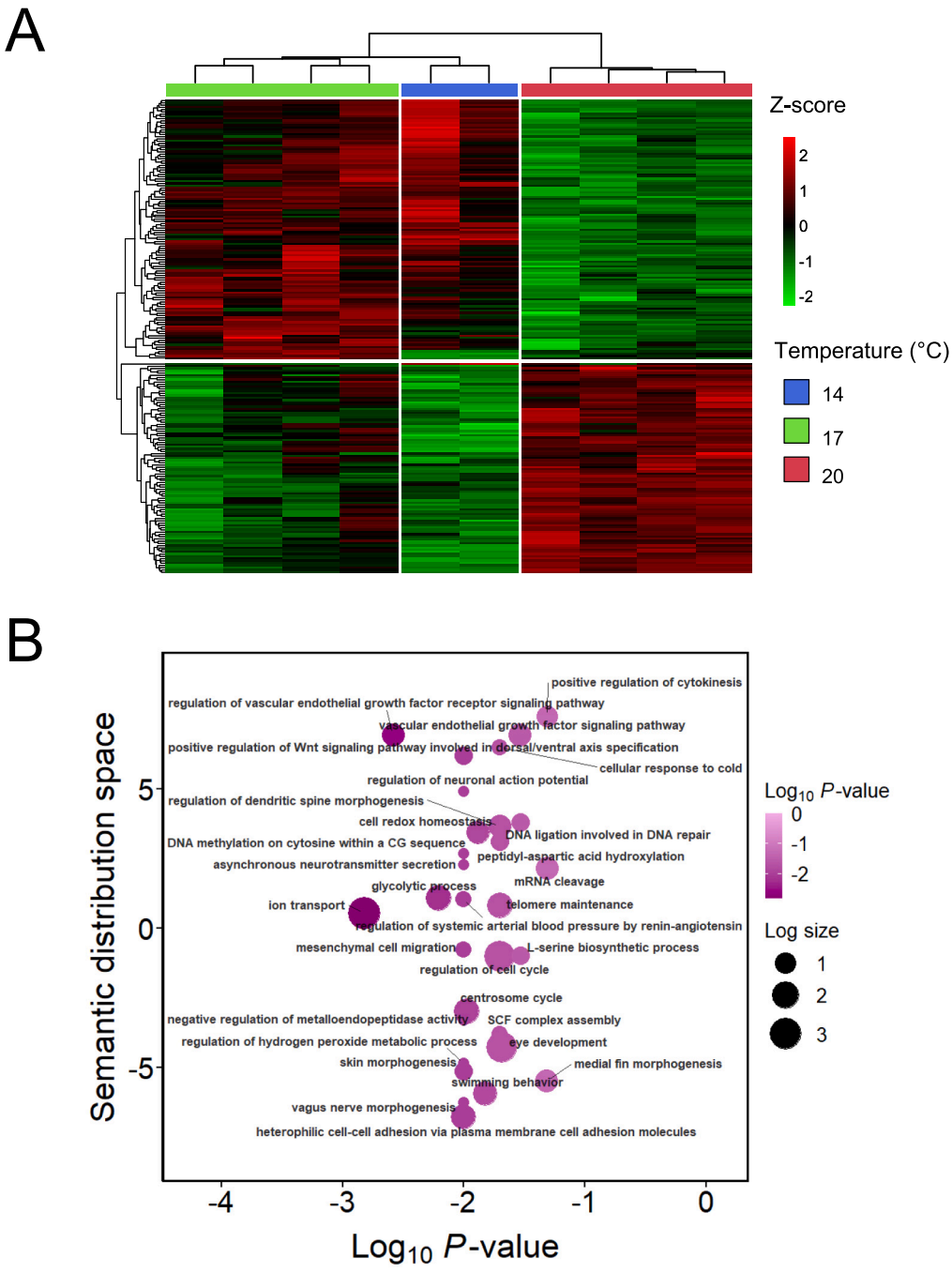

Fig. 2. (A) Heatmap of expression levels in larvae tissue from 210 common differentially expressed genes (DEGs) which at least contain one differentially methylated cytosine (DMC) between 14 °C vs. 17 °C ($n = 1350$) and 20 °C vs. 17 °C ($n = 723$). The color gradient represents the level of expression scaled by gene (red: high expression and green: low expression). The dendrograms indicate the similarity between genes and between samples. The bands on the top indicate the temperature treatment of each sample (blue: 14 °C, green: 17 °C, and red: 20 °C). (B) Semantic similarity scatter plot summarized the enriched GO-terms of the BP category from the 210 common DEGs in both temperature treatments with at least one DMC. The scatterplot showed GO-terms as circles and the significance was shown with the purple gradient color palette (log10 *P*-value). The sizes of the plotted circles were scaled by the frequency of GO-terms they represent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were participating in 30 GO-terms, of which the most relevant were: DNA methylation on cytosine within a CG sequence (GO:0010424), positive regulation of Wnt signaling pathway involved in dorsal/ventral axis specification (GO:2000055) and cellular response to cold (GO:0070417) (Fig. 2B).

3.5. Relationship between DNA methylation-gene expression in larvae

The 95 and 115 genes identified above in larvae are genes that not only their expression is affected by temperature but also their methylation levels are affected. However, whether those two changes are linked is not known. Thus, next we were interested to find out those genes whose expression was affected by the temperature treatment as well as their DNA methylation levels. To do so, we started with the list of 1350 and 723 genes that were DEGs and contained at least one DMC in the 14 °C vs. 17 °C and the 20 °C vs. 17 °C comparisons, respectively (Fig. S2A and B). For each selected gene, we calculated the average number of transcripts with logarithm transformation and also their mean methylation value by averaging the methylation values of all their DMCs among the samples belonging to the same temperature treatment. Next, for each temperature comparison we identified those genes whose expression was either significantly positively or negatively correlated with DNA methylation levels. Results showed that there were 577 (Table S25) and 344 (Table S26) genes with negative and positive correlation, respectively, in the 14 °C vs. 17 °C comparison (Fig. 3A and C), while there were 102 (Table S27) and 250 genes (Table S28) with negative and positive correlation in the 20 °C vs. 17 °C comparison (Fig. 3B and D). There were no common genes between the 577 and 102 gene lists, whereas only ENSDLAG00005009958 was common in the 344 and 250 gene list that were more methylated and upregulated by temperature.

3.6. Persistent temperature effects on the transcriptome of larvae, juveniles, and adults

Once epigenomic and transcriptomic changes resulting from

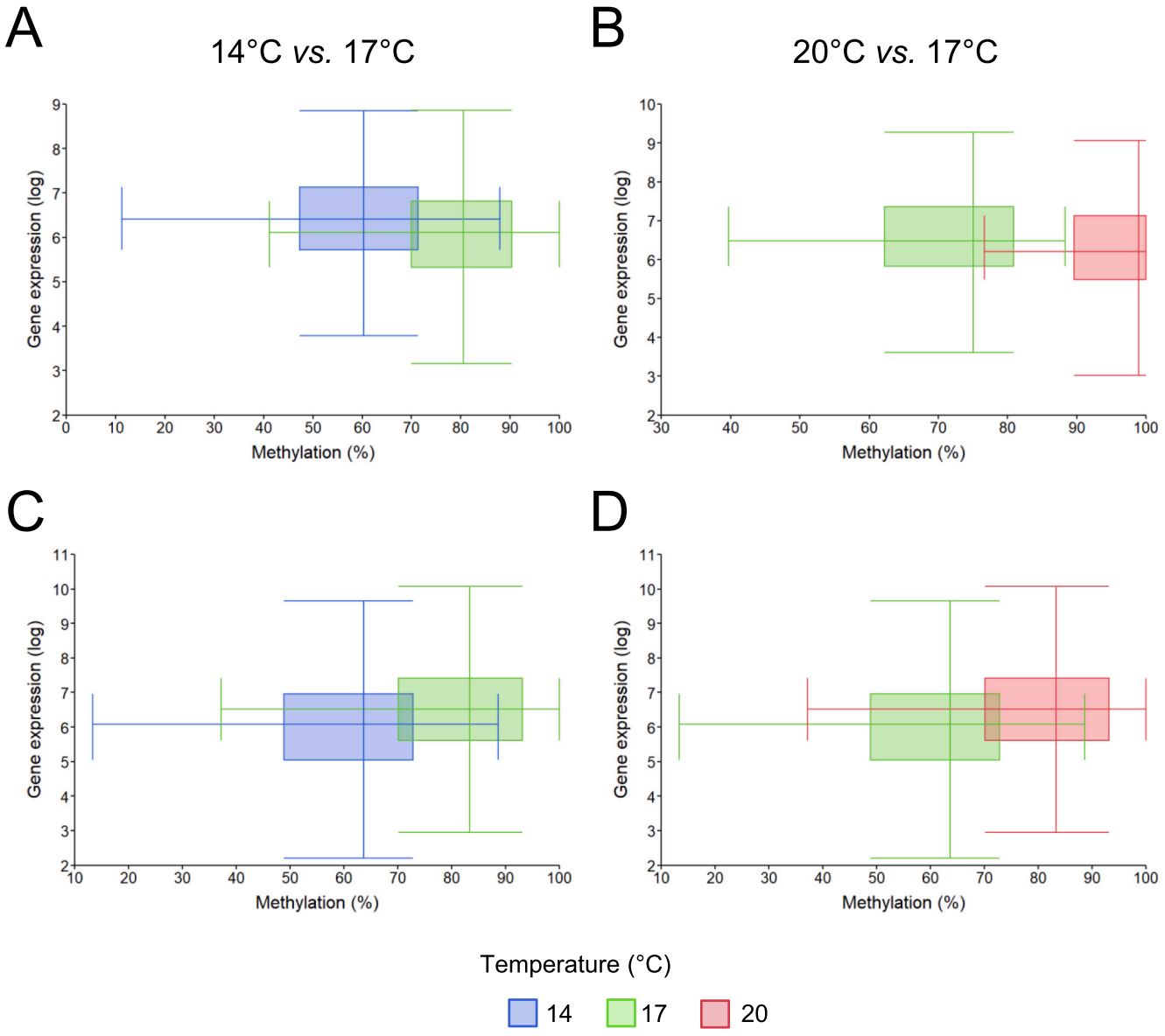

Fig. 3. Boxplots of the common differentially expressed genes (DEGs) between 14 °C vs. 17 °C and 20 °C vs. 17 °C which at least have one differentially methylated cytosine (DMC) in the gene body region (gene ±1000 base pairs, bp), and that on top showed DNA methylation negatively correlated with gene expression in (A) 14 °C vs. 17 °C, and in the (B) 20 °C vs. 17 °C comparison. The genes that met the same criteria with a positive correlation between DNA methylation and gene expression were plotted in (C) for the 14 °C vs. 17 °C comparison, and in (D) for the genes from the 20 °C vs. 17 °C comparison.

temperature effects had been well characterized in larvae, we were interested to know how persistent those changes were in other tissues. Specifically, we wanted to know which of the DEGs found in larvae were still affected in juveniles and in reproductive (testis) tissues long after the temperature treatments had ceased during larvae development. For this, we compared the DEGs lists from larvae, juveniles, and adults for each temperature comparison. Results showed that not a single gene was shared among three tissues in both comparisons. However, in the 14 °C vs. 17 °C comparison, we identified 29 and six genes between larvae-juveniles and larvae-adults, respectively (Fig. S2A and B). From these 29 DEGs that were common in larvae and juveniles, 16 genes, including chromobox 4 (*cbx4*), showed the same direction of change, either consistently upregulated or consistently downregulated (Fig. S3A). The same applied to three out of the six DEGs found in common between larvae and adults (Fig. S3B). In the 20 °C vs. 17 °C comparison, 21 DEGs in larvae-juveniles and 14 DEGs in larvae-adults were also identified. From these two gene lists, only five and six genes showed the same gene expression tendency, respectively (Fig. S3C and D).

We searched the GO-terms to better understand their biological function due to low temperature (Fig. 4). From the 29 and six common DEGs genes in larvae-juveniles and larvae-adults from 14 °C vs. 17 °C comparison, we found ten and three GO-terms, respectively (Fig. 4A and B). The most relevant GO-terms from larvae-juveniles were: response to hypoxia (GO:0001666), cell redox homeostasis (GO:0045454) and positive regulation of CREB transcription factor activity (GO:0032793), while from larvae-adults were: integrin-mediated signaling pathway (GO:0007229), cell-matrix adhesion (GO:0007160) and small GTPase mediated signal transduction (GO:0007264). From common genes in larvae-juveniles and larvae-adults in the 20 °C vs. 17 °C comparison, we identified 21 and 14 genes, respectively (Fig. S2C and D). We also searched the GO-terms to better understand their biological function due to the effects of elevated temperature (Fig. 4). We found twelve and three GO-terms from larvae-juveniles and larvae-adults, respectively (Fig. 4C and D). The most relevant GO-terms from larvae-juveniles were: cellular response to oxidative stress (GO:0034599), muscle cell cellular homeostasis (GO:0046716), and cardiac muscle tissue morphogenesis (GO:0055008), while from larvae-adults was: oocyte maturation (GO:0001556).

Additionally, we investigated in which pathways these set of GO-terms were participating. For juveniles-larvae we found metabolic and MAPK signaling pathways in 14 °C vs. 17 °C and 20 °C vs. 17 °C, respectively (Fig. 4E). In larvae-adults, for both comparisons we found 6 KEGG pathways of which the most relevant were: focal adhesion in 14 °C vs. 17 °C, and oocyte meiosis, P53 signaling and progesterone-mediated oocyte maturation pathways in 20 °C vs. 17 °C. Together, these results suggested that the biological functions and pathways affected were temperature-dependent and tissue specific.

3.7. Persistent temperature effects on the methylome in larvae, juveniles, and adults

We wanted to know the biological role of the genes found in larvae, juveniles, and adults that had DMCs within their body region due to temperature. For this analysis we selected the 163 and 264 list of genes found previously (Fig. S2A and B).

From the 163 genes in the 14 °C vs. 17 °C comparison, 25 GO-terms were identified (Fig. 5A) and the most relevant were: regulation of histone deacetylation (GO:0031063), cell adhesion (GO:0007155), smooth muscle tissue development (GO:0048745), and negative regulation of Wnt signaling pathway involved in dorsal/ventral axis specification (GO:2000054). On the other hand, from the 264 genes found in 20 °C vs. 17 °C, a total of 45 GO-terms were identified (Fig. 5B) and the most relevant were: neural crest cell migration (GO:0001755), negative regulation of transforming growth factor beta receptor signaling pathway (GO:0030512), and histone H3-K9 methylation (GO:0051567). Six and one KEGG pathways were present in 14 °C vs. 17 °C and 20 °C vs. 17 °C, respectively, for instance included TGF-beta signaling pathway and ECM-receptor interaction pathways (Fig. 5C). These results pointed at relevant pathways irreversibly affected by temperature changes and, in particular, to the contribution of histone modifications in maintaining certain environmentally-induced epigenetic changes.

3.8. Persistent temperature effects on transcriptome and methylome in larvae and juveniles

Given that we did analyses of the persistent DEGs between tissues and the persistent genes with affected DMCs between tissues, we were interested in those GO-terms and KEGG pathways. We only selected the 1350 and 723 genes from larvae and the six and seven genes from juveniles and we discarded the adults results due to very low number of genes found either in 14 °C vs. 17 °C or 20 °C vs. 17 °C comparisons (Fig. S2A and B).

In the 14 °C vs. 17 °C comparison, and from larvae and juveniles, a total of 36 and three GO-terms were identified from the 1350 and six genes, respectively (Fig. 6A and B). The most relevant GO-terms for larvae were: developmental growth (GO:0048589), somatic muscle development (GO:0007525), calcium ion transmembrane transport (GO:0070588), and regulation of multicellular organism growth (GO:004001), and for juveniles were: digestion (GO:0007586), sarcomere organization (GO:0045214) and embryonic hemopoiesis (GO:0035162). In the 20 °C vs. 17 °C comparison, and from larvae and juveniles, a total of 52 and three GO-terms were identified from the 723 and seven genes, respectively (Fig. 6C and D). The GO-terms for larvae included positive regulation of canonical Wnt signaling pathway (GO:0090263), methylation on cytosine within a CG sequence (GO:0010424), histone H3-K9 demethylation (GO:0033169) and neural crest cell differentiation (GO:0014033), and for juveniles tissue were: hemoglobin biosynthetic process (GO:0042541), iron ion transport (GO:0006826) and response to bacterium (GO:0009617).

We only found KEGG pathways in larvae but did not in juveniles due to the low number of genes represented in the two comparisons. Thus, in larvae, nine and eleven KEGGs pathways were identified in the 14 °C vs. 17 °C and 20 °C vs. 17 °C comparisons, respectively (Fig. 6E). KEGG pathways included Wnt signaling and MAPK signaling pathways in the 14 °C vs. 17 °C comparison and intestinal immune network for IgA production and biosynthesis of amino acids in the 20 °C vs. 17 °C comparison. Thus, in particular, Wnt signaling and histone H3K9 demethylation seemed to emerge as key targets of temperature effects.

3.9. Relationship with metastable epialleles and very-long term effects

Next, we wanted to know what of the genes identified in the present study coincide with the genes identified by Anastasiadi et al. (2021) that were differentially methylated still three years after exposing the European sea bass (adulthood) to a simulated heat wave. We did this to further determine the usefulness of the genes identified in the present study as putative biomarkers of the effects of temperature during larvae development that can have very long-term effects and thus potential lifelong consequences, i.e., well beyond the duration of this study.

First, we started with a quite stringent criteria and thus looked which ones of the genes we identified being DEGs and containing at least one DMC coincided with the nine genes containing metastable epialleles, i.e., loci the methylation of which changes in response to temperature and regardless of tissue being considered, in that case brain, liver, muscle and testis (Anastasiadi et al., 2021), of which the latter two coincide with the tissues investigated in the present study. We found two of these genes, *spata5l1* and *agl*, among the list of the 1350 DEGs that also had at least one DMC in larvae but not in juveniles or adults in the 14 °C vs. 17 °C comparison. None of the 9 metastable epialleles found by Anastasiadi et al. (2021) were found in larvae, juveniles or adults of the 17 °C vs. 20 °C comparison.

Given the low number of genes identified with the above stringent

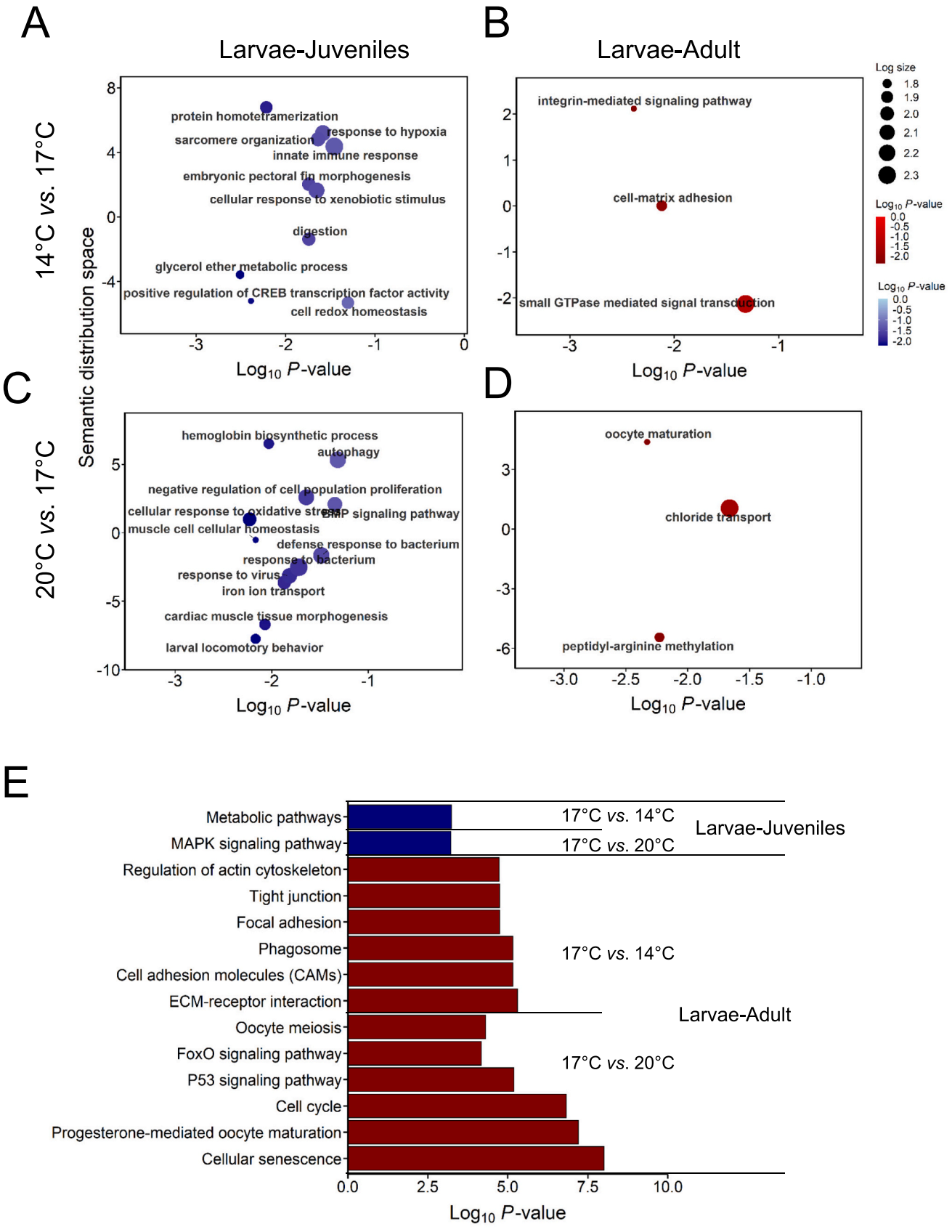

Fig. 4. Scatterplots of the enriched Gene Ontology (GO-terms) of the Biological Process (BP) category from the (A) Common differentially expressed genes (DEGs) in larvae and juveniles and (B) Common DEGs in larvae and gonad between 14 °C vs. 17 °C, (C) common DEGs in larvae and juveniles between 14 °C vs. 17 °C, and (D) Common DEGs in larvae and gonads between 20 °C vs. 17 °C. (E) The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of the DEGs persistently affected by temperature in larvae and juveniles or in larvae and gonads in each temperature comparison.

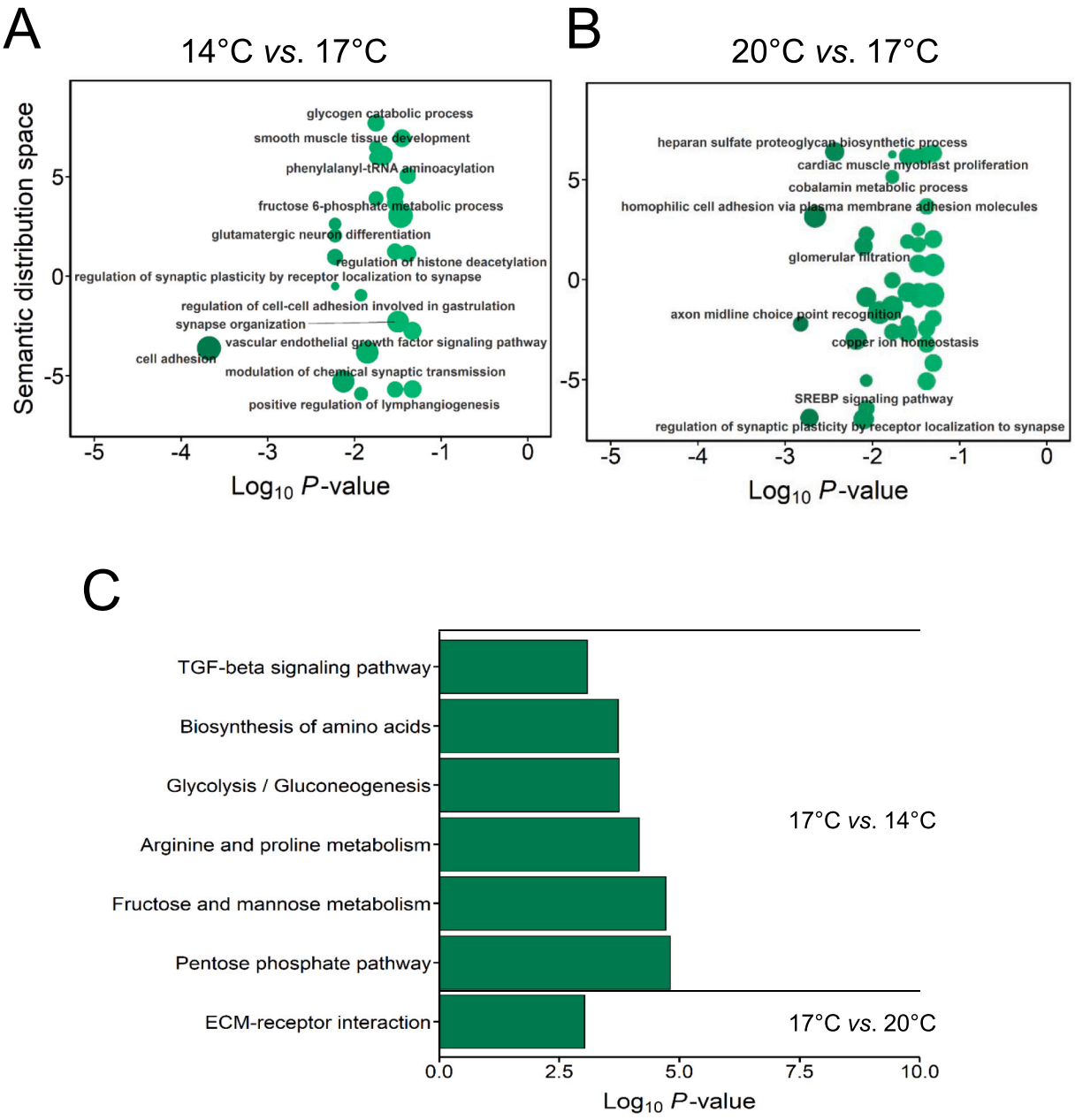

Fig. 5. Scatterplots of the enriched Gene Ontology (GO-terms) of the Biological Process (BP) category from the common genes which at least have one differentially methylated cytosine (DMC) in the gene body (gene ±1000 base pairs, bp) in larvae, gonads, and juveniles in (A) 20 °C vs. 17 °C comparison and in (B) 14 °C vs. 17 °C comparison. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the genes which at least have one DMC in the gene body (gene ±1000 bp) in larvae, juveniles and gonads in each temperature comparison.

strategy, we relaxed the criteria and selected the 527 genes that showed DMR in muscle from (Anastasiadi et al., 2021) and we compared to our 2199 (14 °C vs. 17 °C; Fig. S2A) and 2990 (20 °C vs. 17 °C; Fig. S2B) list of genes with at least one DMC in juveniles. We identified 64 genes in the 14 °C vs. 17 °C (Table S29) and 95 genes in the 20 °C vs. 17 °C (Table S30). Next, we wanted to know the numbers of genes present in juveniles regardless of temperature change comparing these 64 and 95 lists of genes. Results showed a total of 43 genes in common between these two lists (Table 1).

As we did in the previous section, we did the same analysis but in this case comparing with the list of genes with DMR in the list of 825 (14 °C vs. 17 °C) and 921 (20 °C vs. 17 °C) genes with at least one DMC in testis (Anastasiadi et al., 2021). We identified 16 (Table S31) and 20 genes (Table S32) in 14 °C vs. 17 °C and 20 °C vs. 17 °C lists, respectively. Next, we wanted to know the numbers of genes present in adults regardless of temperature change comparing these 16 and 20 lists of genes. We found seven genes in common between these two lists. These genes were: *agl*, heat shock protein family B (small) member 11 (*hspb11*), neuroligin 1 (*nlgn1*), serine/threonine kinase receptor associated protein (*strap*), ADP ribosylation factor guanine nucleotide exchange factor 2 (*arfgef2*), and regulatory factor X5 (*rfx5*).

3.10. Epigenetic biomarkers with both diagnostic and prognostic value

Finally, one of the interesting characteristics of epigenetic biomarkers is that they can have both diagnostic and prognostic value, i.e., epigenetic changes can anticipate transcriptomic changes. In order to explore whether we could find genes fulfilling these characteristics, we

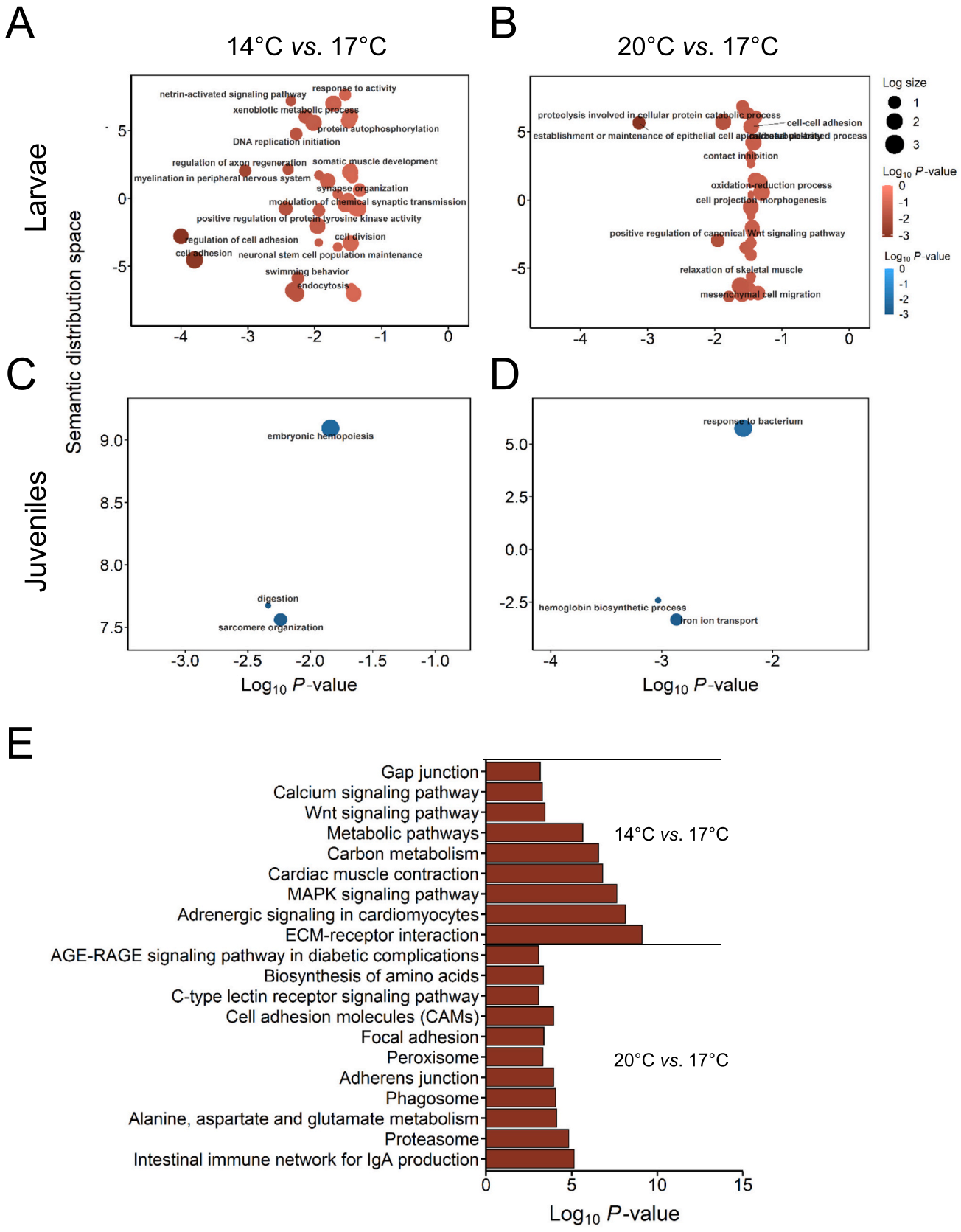

Fig. 6. Scatterplots of the enriched GO-terms of the BP category from the DEGs between (A) 14 °C vs. 17 °C comparison or (B) 20 °C vs. 17 °C comparison, which at least have one differentially methylated cytosine (DMC) in the gene body region (gene ±1000 base pairs, bp) in larvae tissue. GO-terms scatterplots of the DEGs between (C) 14 °C vs. 17 °C or (D) 20 °C vs. 17 °C comparison, which at least have one DMC in the gene body (gene ±1000 bp) in juveniles. (E) KEGG enrichment analysis of the DEGs between the two temperature comparisons and which at least contain one DMC in the larvae tissue.

Table 1
List of the 43 genes common between the 64 and 95 genes with at least one DMC in 14 °C vs. 17 °C and in 20 °C vs. 17 °C comparisons, respectively, that also contained differentially methylated regions (DMR) in juveniles tissue in the European sea bass after exposure to elevated temperature (Anastasiadi et al., 2021).

Gene ID	Gene name	Gene description
ENSDLAG00005009668	*abcc9*	ATP-binding cassette, sub-family C (CFTR/MRP), member 9
ENSDLAG00005019673	*agl*	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase
ENSDLAG00005002947	*astn1*	astrotactin 1
ENSDLAG00005011333	*atf7ip*	activating transcription factor 7 interacting protein
ENSDLAG00005007690	*bcas3*	BCAS3 microtubule associated cell migration factor
ENSDLAG00005017692	*cacna2d4*	calcium voltage-gated channel auxiliary subunit alpha2delta 4
ENSDLAG00005007417	*cadm1a*	cell adhesion molecule 1a
ENSDLAG00005015894	*csmd2*	CUB and Sushi multiple domains 2
ENSDLAG00005008332	*cxxc4*	CXXC finger 4
ENSDLAG00005016884	*dhrs13a.1*	dehydrogenase/reductase (SDR family) member 13a, tandem duplicate 1
ENSDLAG00005019105	*dusp16*	dual specificity phosphatase 16
ENSDLAG00005023582	*fgfr3*	fibroblast growth factor receptor 3
ENSDLAG00005013628	*fstl3*	follistatin-like 3 (secreted glycoprotein)
ENSDLAG00005021658	*gpc3*	glypican 3
ENSDLAG00005002969	*hivep1*	HIVEP zinc finger 1
ENSDLAG00005002265	*hoxc4a*	homeobox C4a
ENSDLAG00005004570	*hoxd4a*	homeobox D4a
ENSDLAG00005017996	*inhbaa*	inhibin subunit beta Aa
ENSDLAG00005009376	*kcnip2*	potassium voltage-gated channel interacting protein 2
ENSDLAG00005019431	*ksr2*	kinase suppressor of ras 2
ENSDLAG00005025443	*mcm5*	minichromosome maintenance complex component 5
ENSDLAG00005003802	*mios*	missing oocyte, meiosis regulator, homolog (Drosophila)
ENSDLAG00005011341	*mtss1*	MTSS I-BAR domain containing 1
ENSDLAG00005001244	*nckap5l*	NCK-associated protein 5-like
ENSDLAG00005014329	*ndufs4*	NADH:ubiquinone oxidoreductase subunit S4
ENSDLAG00005006976	*nlgn1*	neuroligin 1
ENSDLAG00005005359	*nup88*	nucleoporin 88
ENSDLAG00005023392	*ptk7*	protein tyrosine kinase 7 (inactive)
ENSDLAG00005003997	*rad54l2*	RAD54 like 2
ENSDLAG00005010100	*raly*	RALY heterogeneous nuclear ribonucleoprotein
ENSDLAG00005009049	*rbfox1*	RNA binding fox-1 homolog 1
ENSDLAG00005022557	*rnf25*	ring finger protein 25
ENSDLAG00005000657	*runx3*	RUNX family transcription factor 3
ENSDLAG00005018542	*samd4a*	sterile alpha motif domain containing 4A
ENSDLAG00005000254	*scap*	SREBF chaperone
ENSDLAG00005010637	*shf*	Src homology 2 domain containing F
ENSDLAG00005010165	*spata5*	spermatogenesis associated 5
ENSDLAG00005018273	*strn*	striatin, calmodulin binding protein
ENSDLAG00005019987	*syde2*	synapse defective Rho GTPase homolog 2
ENSDLAG00005009862	*tmcc1*	transmembrane and coiled-coil domain family 1
ENSDLAG00005009409	*ttc7a*	tetratricopeptide repeat domain 7A
ENSDLAG00005026035	*wnt9a*	wingless-type MMTV integration site family, member 9A
ENSDLAG00005013567	*xrn2*	5′-3′ exoribonuclease 2

first searched genes with at least one DMC in larvae (sampled at 5–8 dpf) and juveniles (sampled at 90 dpf) and differentially expressed in juveniles but not in larvae. In the 14 °C vs. 17 °C comparison we found one such gene, *prkd4*, while in the 20 °C vs. 17 °C we found two genes, PDZ Domain Containing 7 (*pdzd7*) and an unannotated gene, ENSDLAG00005003985. As for the adults, we found one gene, *kcnh3* in the 14 °C vs. 17 °C comparison and two unannotated genes, ENSDLAG00005021715 and ENSDLAG00005004413 in the 20 °C vs. 17 °C comparison. However, when we looked at the data at the single nucleotide level we found that we had data for the same exact DMC for only one unannotated gene, ENSDLAG00005004413. Regarding the DMC within this gene, the same CpG locus that was hypomethylated in larvae reared at 20 °C was also hypomethylated in the adults of fish reared at 20 °C at 482 dpf. Therefore, this gene can be considered an epigenetic marker with both prognostic and diagnostic value (Fig. 7). To investigate further the functionality of the biomarker, we searched for similar protein sequences across all genomes available in NCBI database (Database Resources of the National Center for Biotechnology Information, 2016) using blastp (Altschul, 1997) with the protein sequence of ENSDLAG00005004413. As a result, the keratin associated protein 10–4 (*krtap10–4*) from the brown trout, *Salmo trutta*, gave the highest sequence similarity (Percentage identity = 43%,) with high statistical significance ($P = 3E^{-25}$). This gene is associated with pathways of keratinization and developmental biology. Although the percentage of identity was low, the alignment was significant, allowing us to suggest the hypothesis that the identified biomarker is likely a coding gene that could be related to keratin production.

4. Discussion

In this study we provide a set of epigenetic and transcriptomic biomarkers associated with the quality of the thermal regime experienced by the European sea bass during early development, when temperature changes can have long-lasting phenotypic consequences for the performance of this species in aquaculture settings. Although different temperature regimes have been tested for longer periods (Bláquez et al., 1998; Person-Le Ruyet et al., 2004), to our knowledge, this study is one of the few that concentrates on the effects of temperature during the early development and, in particular, until exogenous feeding of larvae is initiated. We are aware that methylome and transcriptome is tissue-specific and that differences are not only stage-dependent but also, and importantly, tissue-dependent. Taking into account this, we wanted to know what changes persist rather where they persist because the goal of this study was to search for biomarkers. If the same change induced by temperature observed in whole larvae is also present in the muscle of treated juveniles (somatic tissue) and/or in testis of treated adults (germline tissue) but not in the muscle or testis of the control fish this means that the effect is due to temperature and not stage and therefore has informative value. Thus, as far as this lasting effect is robust it does not matter the contribution of tissue.

Before discussing the results proper, however, three considerations on the reliability of the data obtained seem appropriate. First, the temperature treatment was carried out during the thermosensitive period (Piferrer et al., 2005; Vandeputte and Piferrer, 2019) but, as regards to effects on sex ratio, there were no differences among the three tested temperatures, although higher temperature resulted in a slightly higher number of males. The lack of significant differences are likely due to the short duration of the temperature treatment, less than ten days, when treatments of thirty days or longer are necessary to elicit permanent sex ratio responses (Navarro-Martín et al., 2009), and are regarded as positive in the context of the present study because we wanted to assess the effects of temperature during the most critical phase of development, and not to elicit significant sex ratio changes. Hence, the deliberate short temperature treatment period used. Second, at 482 dpf, fish reared at 14 °C until 12 dpf were bigger than those reared at 20 °C until 8 dpf, a phenomenon that probably indicates better performance of the cold-exposed fish, a result that should be further studied without excluding the possibility of compensatory growth, as reported previously in the European sea bass (Díaz and Piferrer, 2015). However, the lack of differences in growth between fish reared at 14 °C, 17 °C and 20 °C at 90 dpf, nor between adult fish reared at 14 °C vs. 17 °C or between 20 °C vs. 17 °C (i.e., the two comparisons made in this study), coupled with the lack of differences in GSI values among the males of the different temperature regimes, provide confidence that the observed differences in DNA methylation or in gene expression were due only to temperature and not to differences in somatic or gonadal growth. Third,

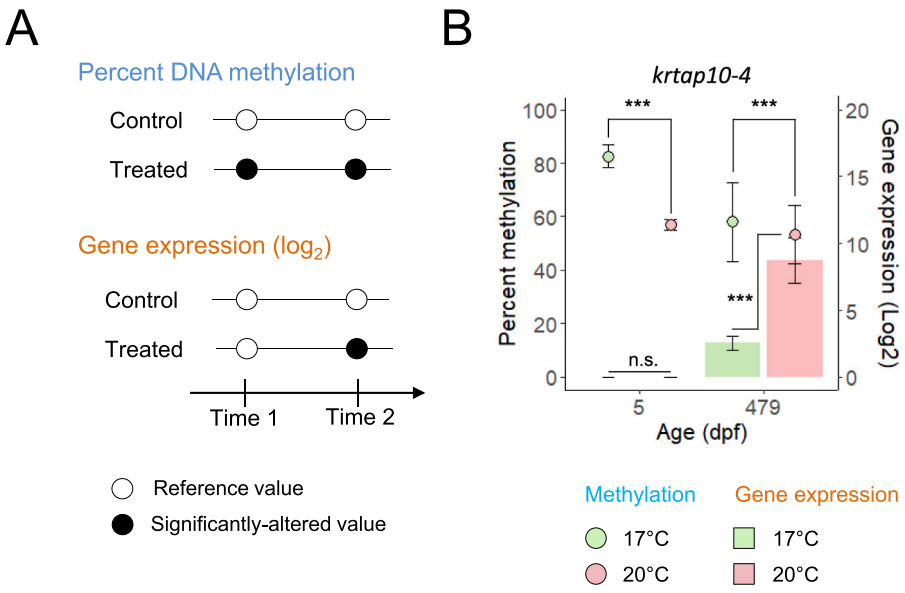

Fig. 7. Epigenetic marker with both prognostic and diagnostic value. (A) Conceptual diagram of an epigenetic marker. The integration of gene expression data and DNA methylation data in two time points under two conditions can reveal genes, regions or loci with prognostic and diagnostic value. (B) The dot plot with the primary y-axis (left) shows the DNA methylation data of the same DMC within ENSDLAG00005004413 gene body region ±1000 base pairs, bp in larvae at 5 and adults at 482 days post fertilization (dpf). The bar plot with the secondary y-axis (right) shows the gene expression data (log_2) for this gene in larvae at 5 and adults at 482 dpf. The color code indicates the temperature treatment: 17 °C in green, and 20 °C in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and regarding the analyses performed, transcriptomic data was of good quality since only samples with RIN > 8, average alignment rate > 88% and > 30 million assigned features were used. The same applies to the epigenomic data, based on an average > 70% alignment rate and > 99% bisulfite conversion quality indicators. Taken together, this indicates that epigenetic and transcriptomic results do indeed reflect temperature effects. It should also be noted that in this study we concentrated on DNA methylation-based biomarkers for their simplicity of measurement (Levenson, 2010) and because, once known the nature of the genes involved, no longer a whole-genome or genome-wide determination method is required. Instead, the measure of their DNA methylation can be achieved by a simple PCR-based method (Hernández et al., 2013), as done, for example, in the development of some epigenetic clocks, another type of DNA methylation-based biomarker (Aliferi et al., 2018; Naue et al., 2017). Other type of epigenetic markers do exist, like those based on histone modifications such as H3K4me3, H3K9me3, H3K79me, and H3K79me3, which have been used, for example, to assess proper oocyte development in cyprinid fishes (Zhou et al., 2019); and those based on the quantification of miRNAs, e.g., the differentially expressed miR-122, novel-miR6, miR-193a-3p, and miR-27a-5p, in the head kidney, the expression levels of which were linked to mechanisms of inflammation and injury during cadmium exposure in the common carp, *Cyprinus carpio* (Q. Liu et al., 2020). Concentrating in these other type of biomarkers was beyond the scope of study but efforts should be aimed at their identification in future studies. Finally, it is worth mentioning that we not only used epigenetic changes but also investigated gene expression changes, meaning that we reported epigenetic changes that also resulted in phenotypic (transcriptomic) changes, thus reinforcing the usefulness of the epigenetic biomarkers presented below. However, we are aware that beyond the proof-of-concept that this study represents, additional phenotypic endpoints would be desirable.

The relationship between markers and their usefulness to behavior is still poorly understood not only in the European sea bass but in fish in general. Regarding the coping style from our results, we found that in the group of fish treated at 20 °C there were more fish that were *Bold* than *Shy*. Therefore, the development of biomarkers that allow selecting fish reared at 14 °C and 17 °C (both groups fish performed similar results in the CPP test), are at the same time increasing the probability of selecting for *Shy* behavior, a trait preferred in an easily stressed species like the European sea bass. The study of the methylome and the transcriptome of the two different behavior types could hold interesting results for aquaculture purposes. These could be further explored in future studies since recent evidence showed differences in transcriptomic analyses between *Bold* and *Shy* copying styles for this species (Sadoul et al., 2022). However, a behavior assays study using the olive flounder (*Paralichthys olivaceus*) only found 144 DEGs out of the entire transcriptome when comparing *Bold* vs. *Shy* fish (Zou et al., 2021). For these reasons, we prioritized the development of biomarkers for thermal stress and not for the copying style.

Among all the genes identified that could potentially be used as biomarkers of the thermal quality of the early development, there are some that repeatedly showed up (Table 2) and thus we will focus on these genes. We classified the effects as short-term if they were present only in larvae, right at the end of the temperature treatment, mid-term effects if they were present both in larvae and juveniles at 90 dpf, long-term if they were present both in larvae and adults at ~500 dpf, and very-long term if they were affected in larvae, 90 or ~ 500 dpf. In addition, we studies whether the affected genes coincide with the genes the methylation of which was found to be affected in adults three years after the end of the exposure to elevated temperature as is shown in a previous study with the European sea bass (Anastasiadi et al., 2021).

Among the 70 genes that were differentially methylated regardless of the direction of the temperature change and that were affected in larvae, juveniles and adults, it is worth mentioning *jmjd7*, *kcnh5b,* and *zfyve9a*. The former encodes a highly conserved enzyme with a JmjC domain that recognizes and cleaves monomethylated and dimethylated arginine residues of histones H2, H3 and H4. Other members of this family include: (i) *jmjd3* (also known as lysine demethylase 6B, *kdm6b*), which has been implicated in environmental signal transduction in connection to temperature-dependent sex determination in reptiles, including the Australian bearded dragon,*Pogona vitticeps*, (Deveson et al., 2017), the red-eared slider turtle, *Trachemys scripta* (Ge et al., 2018), and involved in sex-related processes in the orange-spotted grouper fish, *Epinephelus coioides* (Zhang et al., 2021); and (ii) jumonji and AT-rich interaction domain containing 2, *jarid2a*, upregulated in response to elevated temperature in the European sea bass (Díaz and Piferrer, 2015) but downregulated when fish were also exposed to estrogen (Díaz and Piferrer, 2017). On the other hand, *kcnh5b* encodes a member of voltage-gated potassium channel, also involved in environmental signal transduction in different situations (Yang and Zheng, 2014). However, whether these genes are involved in capturing temperature effects on sex ratios is still not known. Lastly, *zfyve9a* encodes a double zinc finger motif-containing protein that interacts directly with SMAD2, and recruits SMAD2 to the TGFB receptor (Tsukazaki et al., 1998). The *zfyve9a* gene regulates the

Table 2
Summary of the biomarkers identified in this study related to the effects of early temperature according to their informative properties. Abbreviations: T = transcriptomic; E = epigenetic; B = both; L = larvae; J = juvenile; A = adults.

Properties	Type	Tissue	Lower temperature	Elevated temperature
Short-term effects (L)	E	L	*agl, kcnh2a, depdc5, prkd2, gemin7;* (Table S17)[a]	
	T	L	*cabp2a, eloal, serpina7,mrc1a, gpx1b, sncga;* (Table S23)[a]	
	B	L	*hivep1, kcnq5a, pcdh1b, hmgcra, dusp16;* (Table S24)[a]	
Mid-term effects (L & J)	E	L & J	*agl, dlgap1a, kcnh2a, scap, rsu1;* (Table S17)[a]	
	T	L & J	*ENSDLAG00005005752, ENSDLAG00005023917* (Tables S5, S6 and S8, S9)[a]	
	B	L & J	*capn9, ENSDLAG00005003493, ENSDLAG00005005752,*(Tables S19, S21)	*tfa* (Tables S20, S22)
Long-term effects (L & A or L & J & A)	E	L & A	*agl, pvalb7, brinp2, astn1, nup98;* (Table S17)[a]	
	E	L & J & A	*agl, pvalb7, brinp2, astn1, xdh;* (Table S17)[a]	
	T	L & A	*ENSDLAG00005000425* (Tables S5, S7 and S8, S10)[a]	
Very long-term effects (L or J or A & Anastasiadi et al., 2021)	E	J	*agl, spata5, fgfr3, cacna2d4;* (see more examples in Table 1)[a]	
	E	A	*agl, hspb11, nlgn1, strap, arfgef2, rfx5*[a] (Tables S31, S32)[a]	
	B	J	*spata5l1, agl,*	N/A
Putative prognostic and diagnostic	B	L & J	*prkd4*	*pdzd7, ENSDLAG00005003985*
	B	L & A	*kcnh3*	*ENSDLAG00005021715, krtap10–4*
Strong prognostic and diagnostic	B	L & A	N/A	*krtap10–4*

[a] Genes affected by both lower (14 °C) and higher (20 °C) temperatures with respect to the control temperature (17 °C).

proliferation of hepatic cells during zebrafish embryogenesis (Liu et al., 2013).

The 211 genes identified in which their expression either consistently increased or decreased from 14 °C to 17 °C and also from 17 °C to 21 °C included *stap2b*, *mrc1a* and *dab2*. These genes constitute, therefore, robust short-term only transcriptomic biomarkers of the effects of temperature during European sea bass early development. Their expression levels could be used to assess the on-the-spot quality of the egg incubation thermal regime and larvae until first feeding. Among the most relevant GO-terms related with the above mentioned genes upregulated by elevated temperature was the response to oxidative stress. It is well established that elevated temperature accelerates developmental rate as does mitochondrial respiration, leading to reactive oxygen species (ROS) accumulation and oxidative stress (Sun et al., 2015). Changes in ROS status and oxidative stress have been implicated as key elements in environmental signal transduction in poikilothermic vertebrates (Castelli et al., 2020). Identifying the mechanisms that regulate these molecular oxidative stress responses can help to develop new approaches to monitor the appearance of undesirable conditions, especially in an aquaculture context (Lazado et al., 2020).

There were 210 genes that in addition of being DEGs in larvae they also contained DMCs. Of these, 95 genes were upregulated at 20 °C and downregulated at 14 °C and 17 °C treatments, while the remaining 115 genes had the opposite pattern. Two considerations are worth being made here. First, the grouping in the heatmap of the 14 °C and 17 °C treatments, on one hand vs. the 20 °C treatment, on the other, suggests that in terms of DNA methylation and gene expression the temperature of 20 °C is perceived as altered when compared to the "natural" temperature range of 14–17 °C, appropriate for larval development in the European sea bass (Vazquez and Munoz-Cueto, 2014). In fact, this observation matches and supports the observations that temperatures > 17 °C alters sex ratios in this species while temperatures < 17 °C do not (Navarro-Martín et al., 2009). Second, these 210 genes, among which we find several genes related to calcium signaling such as *atp2a1*, *gsto2* and *cabp2a*, involved also in environmental signal transduction (Castelli et al., 2020), constituted robust short-term both epigenomic and transcriptomic biomarkers of the effects of temperature during the European sea bass early development. As above, they can be used to assess the quality of the egg incubation thermal regime and larvae until first feeding. Furthermore, the most relevant GO-terms linked to these 210 genes were DNA methylation, regulation of Wnt signaling pathway and cellular response to cold. All of these GO-terms results are consistent with the fact that DNA methylation, Wnt signaling and cellular response to cold has been previously implicated in other studies examining the effects of temperature on animals (Al-Fageeh and Smales, 2006; Risha et al., 2021; Yao et al., 2022).

Regarding the 577 and 344 genes with negative and positive correlation, respectively, in the 14 °C vs. 17 °C comparison and the 102 and 250 genes with negative and positive correlation, respectively, in the 20 °C vs. 17 °C comparison it is interesting to note that gene expression changed on average <10-fold (less than one log value). In contrast, DNA methylation changes were around 20–25%. These differences are of sufficient magnitude suitable to use them as biomarkers of effects of temperature during early development. There were no common genes between the 577 and 102 gene lists, meaning there were no genes the expression of which is epigenetically and consistently downregulated in response to temperature. As for the 344 and 250 genes more methylated and upregulated by temperature, only one gene, ENSDLAG00005009958, was common, implying that its expression is epigenetically regulated in a consistent manner in response to temperature changes and thus constitute a powerful biomarkers of the short-term effects of early temperature changes in larvae.

We only found two genes, *spata5l1* and *agl*, that coincided with the metastable epialleles identified previously in the European sea bass (Anastasiadi et al., 2021). This low number is not surprising given the stringent criteria we used and the requirements needed to be considered a metastable epiallele. Using instead genes with DMCs in juveniles and/or adults that coincided with genes differentially methylated in the same tissues in the above mentioned study returned other gene lists (43 shown in Table 1) that constituted robust epigenetic biomarkers for the very long-term effects of temperature alterations during early development on European sea bass juveniles and adults. Some of them are independent of the direction of temperature change and hence can be used as a reliable method to determine whether animals had the optimal temperature during larval rearing. *Agl* and *nlgn1* genes are present in both lists. *Agl* encodes the glycogen debrancher, an enzyme involved in glycogen degradation and *agl* mutations are linked with glycogen storage disease (Sentner et al., 2012) found differentially expressed in fast- vs. slow-growing rainbow trout, *Onchorynchus mykiss* (Danzmann et al., 2016) but no effects due to temperature have been reported.

One of the most important insight of this study is the report, to our knowledge for the first time, of epigenetic biomarkers with prognostic and diagnostic value. Of the five genes that in principle met the criteria for having these properties the affected DMCs were not consistently on the same CpGs position and thus can be considered as potential epigenetic markers with both prognostic and diagnostic value. The gene

ENSDLAG00005004413, for which we had gene expression data and DNA methylation data on exactly the same CpG position in two time points in larvae and adults, was not differentially expressed either at 14 °C nor at 20 °C when compared to 17 °C at 5 dpf but became it was upregulated in the adults in fish reared at 20 °C at 482 dpf. Regarding the DMC within this gene, the same CpG that was hypomethylated in larvae reared at 20 °C was also hypomethylated in the adults of fish reared at 20 °C at 482 dpf. Thus, hypomethylation of this gene in larvae exposed to elevated temperature precedes higher expression in adults ~500 dpf. Therefore, this gene can be considered an epigenetic biomarker with both prognostic and diagnostic value and hence their full identification is warranted. However, further analysis on the same gene and all the other biomarkers listed in the present study should be performed in a larger number of individuals from different families and from an independent experiments to confirm their usefulness as biomarkers. Given the protein sequence alignment of ENSDLAG00005004413 with *krpa10–4* gene in the brown trout, we suggest that the identified biomarker could be related to keratin production. This could be of relevance for the fitness of the sea bass since they produce keratin in the skin mucus (Cordero et al., 2015). The skin mucosa is a crucial first barrier of protection from both biotic and abiotic factors because it protects the skin epidermis, where fish in general have few or no keratinized cells (Brinchmann, 2016). Thus, since methylation is maintained, measuring the methylation of this gene in fish samples from a given batch at any time would indicate whether that the fish of that batch had been exposed to an abnormally high temperature during the critical stages of early development.

In this study, we focused on the most studied environmental factor, i.e., temperature, which has a huge impact on stress in many vertebrate and invertebrate marine farmed species. However another environmental factor, with detrimental effects on European sea bass development is rearing density (Sammouth et al., 2009), albeit without effects on sex ratio (Saillant et al., 2003) as temperature does. In an experiment with 6 month-old European sea bass exposed to short intervals of acute density stress, 57 DMCs in 51 distinct genes measured in blood samples were identified (Krick et al., 2021). Interestingly, among these 51 genes, two genes were also present in our list of genes with DMCs in three tissues: roundabout homolog 2-like (*robo3*) in 20 °C vs. 17 °C and zinc finger matrin-type 4b (*smat4*) in 14 °C vs. 17 °C. These two genes seem to be involved in behavior linked with stress conditions (Rey et al., 2013).

In conclusion, we report, to be best of our knowledge, the first identification of epigenetic biomarkers with both diagnostic and prognostic value to anticipate the long-lasting effects of thermal alterations during early development in fish. One can envisage the determination of DNA methylation levels (e.g., by a PCR-based approach) of CpGs within some of the genes identified in this study as a rapid and economic means to monitor the quality of the early environment, in our case the thermal environment, to screen samples of the European sea bass larvae as a diagnostic and prognostic tool for present and future performance. Our study shows the way for similar developments for other farmed species as well as for applications to conservation biology.

Data availability

BS-Seq and RNA-Seq reads are available at the NCBI-SRA under the BioProject accession number: PRJNA888946.

CRediT authorship contribution statement

Alejandro Valdivieso: Methodology, Investigation, Formal analysis, Writing – original draft. **Núria Sánchez-Baizán:** Methodology, Investigation, Formal analysis, Writing – original draft. **Nikos Mitrizakis:** Methodology, Investigation, Formal analysis. **Nikos Papandroulakis:** Conceptualization, Resources, Funding acquisition, Supervision, Methodology, Investigation, Formal analysis, Writing – original draft. **Francesc Piferrer:** Conceptualization, Resources, Funding acquisition, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare they have no competing interests.

Data availability

Data enclosed in the Supplementary Information

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738918.

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