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RESEARCH

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Multigenerational temperature effects on paternal epigenetic inheritance in European sea bass

Núria Sánchez-Baizán^{1,2}, François Allal², Marc Vandeputte² and Francesc Piferrer^{1*}

Abstract

Environmental changes can induce epigenetic modifications, influencing gene expression, phenotype, and species adaptation. This study investigates how temperature affects genome-wide DNA methylation patterns, particularly in genes crucial for sex development and whether these modifications can be transmitted across generations. Using the European sea bass—a fish model with both genetic and environmental sex determination—we analyzed DNA methylation at single nucleotide resolution using reduced representation bisulfite sequencing in 64 individuals from five families across two generations (F0 and F1). Parental fish (F0) were exposed to either control (16 °C, C) or elevated (21 °C, T) temperatures from 12 to 60 days post-fertilization. Their offspring (F1) were then subjected to four thermal regimes: control (CC), ancestral exposure via sires (TC), developmental exposure in offspring (CT), and dual exposure (TT). We determined the length of differentially methylated regions (DMRs) using a conservative, reproducible, and species-specific method adapted from plant epigenetics. To disentangle ancestral and developmental temperature effects, DMRs were classified according to their association with F0, F1, or F0 x F1 interaction effects. This allowed us to quantify the relative contribution of each treatment, separately for testes and ovaries in the F1 generation. While the proportion of additive DMRs showing cumulative temperature effects (e.g., 2.1% in testes, 1.4% in ovaries) was relatively rare, a substantial proportion of DMRs (37% in testes, 31.1% in ovaries), exhibited opposing methylation changes with F0 and F1 treatments, indicative of compensatory epigenetic interactions. These interactions were also reflected at the phenotypic level: TT individuals showed body weights comparable to CC, and the sex ratio in TT approached statistical significance when compared to CC ($P=0.051$), suggesting a link between epigenetic regulation and phenotypic plasticity under elevated temperatures. Finally, we also investigated the inheritance of epimarks from sires to offspring. While most epimarks remained stable across generations, ~5% of all DMRs were both temperature-induced and inherited, offering direct evidence for environmentally responsive multigenerational epigenetic inheritance. This study demonstrates the role of temperature in shaping the epigenome and highlights the potential of epigenetic plasticity and inheritance in species adaptation and conservation amid global warming.

Keywords DNA methylation, Sex ratio, Sex reversal, Polygenic sex determination, Reduced representation bisulfite sequencing, Epigenetic buffering and traps

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Introduction

Epigenetics is the study of reversible changes in gene expression that are mitotically and/or meiotically transmitted to subsequent generations and that occur without alterations to the DNA sequence [24, 73], [93]. Epigenetic variability arises from genetic factors, environmental influences, gene-environment interactions (G x E), and stochastic processes [4, 50, 69], each shaping the epigenome [4]. The proper characterization of the sources of variability in epigenetic patterns is crucial for understanding the diverse phenotypic outcomes and their response to a changing environment. However, the proportion and mechanisms by which epimarks, particularly those environmentally-induced, are inherited remain largely unknown.

Many studies on epigenetic inheritance (see Anastasiadi et al. [3] for review) involve model organisms, short life cycle species, or simulated data. Studies with bacteria [31], protists [32], fungi [49], plants [35, 53], or invertebrates [23] offer practical advantages such as lower maintenance costs and less technical challenges and a short time period to generate large sample sizes and multiple generations [38].

Some studies on epigenetic inheritance have also involved vertebrates, including mammals [30, 45, 98] and fishes [2, 12, 34, 37]. Zheng et al. [98], for instance, quantified epigenetic alterations in mouse sperm due to paternal psychological stress, showing intergenerational inheritance: 11.4% from F0 to F1 and 0.48% of the initially inherited from the exposed F0 generation were conserved in the F2 generation. In fishes, Heckwolf et al. [34] and Hu et al. [37] studied the threespine stickleback (*Gasterosteus aculeatus*), separating stable from random epimarks, advancing our understanding of epigenetic inheritance dynamics. In this species, genetic variation explained an average of 24–35% of the methylation variance [37]. Moreover, they considered the underlying genetic architecture of the two ecotypes by identifying methylation quantitative trait loci —genomic loci where genetic variation influences DNA methylation levels. This analysis revealed two key CpG sites that were differentially methylated between the marine and freshwater ecotypes, suggesting an important role of DNA methylation in the adaptation to different environments [37].

The extent to which epimarks can be transmitted across generations may be related, in part, to epigenetic reprogramming [59]. In mammals, this process is well characterized by two waves of DNA demethylation: one during the early stages of embryo development, and the other during germ cell specification [51, 79]. In the mouse model, recent evidence confirmed the inheritance of DNA methylation at CpG islands in the promoters using epigenetic editing [85]. In fishes, however, this phenomenon appears to differ significantly from mammals,

at least in the species studied to date. In zebrafish (*Danio rerio*), there is a gradual transformation of the maternal methylome to resemble that of sperm, facilitating zygotic genome activation, rendering demethylation waves unnecessary [39], [63]. Similarly, in medaka (*Oryzias latipes*), recent studies have described a lack of global DNA methylation erasure [71]. The mangrove killifish (*Kryptolebias marmoratus*), undergoes a more extensive epigenetic reprogramming compared to other vertebrates [26]. Overall, these findings suggest that epigenetic reprogramming in fishes could exhibit a stronger propensity for inheritance through DNA methylation, with potential ecological implications [71]. This raises important questions regarding the mechanisms of epigenetic inheritance and the extent to which epimarks are transmitted across generations.

Regarding specific mechanisms in epigenetic inheritance, DNA methylation has understandably received most attention. However, other epigenetic factors such as small non-coding RNAs (ncRNAs) and chromatin modifications also contribute to transgenerational epigenetic changes [9, 10, 46, 82]. In chickens (*Gallus gallus domesticus*), maternal inheritance of ncRNA expression has been reported [46]. In mammals, epigenetic transgenerational inheritance through sperm is mediated by the integration and interplay of the three mechanisms: DNA methylation, non-coding RNA, and histone modifications [9, 10, 82].

This study focused on DNA methylation to investigate its role in shaping inheritance patterns, particularly in response to temperature. We used the European sea bass (*Dicentrarchus labrax*), a species with polygenic sex determination [88] and temperature influences on sex ratios, i.e., a model of a mixed genetic and environmental sex determination [57]. Elevated temperatures during early development are known to skew sex ratios by inducing sex reversal, transforming genetic females into phenotypic males [13, 15]. This makes this species an ideal model to study how environmental cues, through epigenetic mechanisms, influence gene expression and phenotype, and how these changes might be transmitted to subsequent generations.

While transcriptomic studies have identified genes involved in sexual phenotype acquisition and maintenance [13, 14], [21, 22, 29, 67, 76], methylation changes have only been shown for cytochrome P450 family 19 subfamily a member 1 (*cyp19a1a*) [57] and doublesex and mab-3 related transcription factor 1 (*dmrt1*) [2]. The relationship between genome-wide methylation changes, sex, and temperature remains unexplored. However, Shao et al., [81] showed that gonadal DNA methylation levels of sex determination genes were the main targets of substantial methylation modification during sexual reversal in the half-smooth tongue sole (*Cynoglossus semilaevis*),

a species with chromosomal sex determination (ZW/ZZ).

In this study, we aimed to identify sex-related genes and genomic regions, other than *cyp19a1a* and *dmrt1*, affected by temperature in the European sea bass, a long-lived species which typically takes two to three years to reach sexual maturity [95]. This provides a unique opportunity to investigate not only immediate but also multi-generational epigenetic inheritance in a vertebrate. Thus, a single female was crossed with five males (F0) of the same genetic background, categorized into two types: (1) two males reared at control temperature (C) during the thermosensitive period, and (2) three males exposed to high temperature (T) during the same window. The offspring (F1), were also exposed to either C or T during their own thermosensitive period, resulting in four groups, identified by two letters, the first indicating the temperature regime of F0 and the second of the F1: CC, CT, TC and TT.

By crossing all males with the same female, we minimized maternal effects, which can significantly influence offspring development and fitness [78, 80, 91]. We focused on paternal contributions for three reasons: (1) paternal epigenetic effects are less explored [3, 80], (2)

to maintain a feasible sample size, and (3) sperm can be cryopreserved, offering practical applications for aquaculture and conservation [18, 91].

We investigated temperature-induced epigenetic changes by comparing genome-wide methylation profiles of fish exposed to control temperature both in the F0 and F1 (CC), ancestral exposure in the F0 and control temperature in the F1 (TC), developmental exposure in offspring (CT), and dual exposure (TT) to elevated temperature. Specifically, we quantified the number of temperature-induced and inherited DNA methylation marks acquired in the sperm of F0 males and evaluated their potential transmission to the gonads (testes and ovaries) of F1 offspring not directly exposed to temperature. Thus, there were a total of eight groups of F1 gonads for analysis: 4 temperature regimes \times 2 sexes (Fig. 1A).

F1 fish were sampled at one year of age, when the gonads are fully differentiated [67], allowing unambiguous recording of their phenotypic sex. Based on genetic sex tendency and phenotypic data, we selected individuals that were representative of each group (sex and temperature treatment), avoiding those with extreme values. In total, we produced 64 RRBS libraries: parents' gametes (F0, $n=6$; gametes of the five males, 2 C, 3 T, plus one

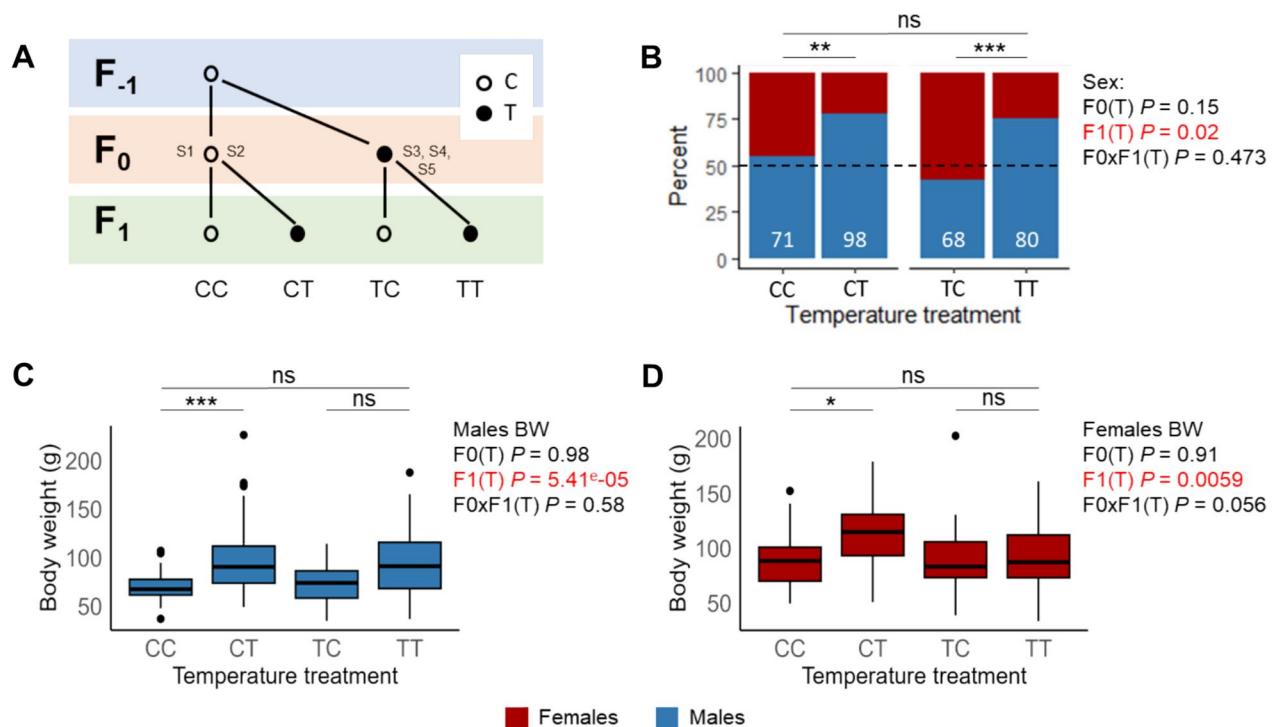


Fig. 1 Experimental design and effects of temperature on sex ratio and body weight. **A** Diagram of the experimental design including three generations: F₋₁, F₀, and F₁. The F₀ males (S1–S5) were exposed to either control (C, open circle) or elevated temperature (T, solid circle), producing five families (C: families 1–2; T: families 3–5). The F₁ offspring were also reared at C or T, resulting in four treatment groups based on F₀–F₁ combinations: CC, CT, TC, and TT. **B** Stacked bar plot showing the percentage of males (blue) and females (red) in each F₁ treatment group. Statistical differences were tested using a generalized linear model (GLM); ns = not significant, ** $P < 0.01$, *** $P < 0.001$. **C, D** Body weight of F₁ males (**C**) and females (**D**) by treatment group. Box-plots display the median (line), interquartile range (box), and $1.5 \times$ IQR range (whiskers); outliers are shown as individual points. Significance of GLM results is indicated in black (ns) or red (significant)

dam), and offspring juvenile gonads (F1, $n=58$, Supplementary Table 1). The overall objective was to elucidate the multigenerational temperature dependent sex-specific effects and their inheritance in the European sea bass. This work contributes to the understanding of how epigenetic mechanisms mediate environmental influences and how such modifications might support adaptive responses the current scenario of global warming.

Results

Phenotypic traits

All F1 fish were unambiguously assigned to their respective sires through genotyping. Sex-reversed individuals (genetic sex tendency ≥ 0) were excluded from this study. The generalized linear model (GLM) analysis of phenotypic sex, using treatments for the F1 and F0 generations, showed that the main effect of the F1 treatment was significant ($P=0.02$), while the main effect of the F0 treatment ($P=0.15$) and the interaction between F1 and F0 treatments ($P=0.47$) were not statistically significant. Pairwise comparisons of the estimated marginal means revealed a significant increase in males reared at T compared to the control in both F0 and F1 (CC = 54.9%, CT = 77.6%; $P=0.012$, and TC = 42.6%, TT = 75.0%; $P=0.0005$; Fig. 1B). Although the contrast between CC and TT treatment groups was near significance, it did not reach the conventional threshold ($P=0.051$).

Regarding body weight, F1 individuals reared at T exhibited significantly higher weight (99.0 ± 2.26 g) compared to their siblings reared at C (82.13 ± 1.68 g; $P=0.04$) regardless of the family, temperature treatment and sex. F1 males exposed to high temperature during their own development (CT) were significantly heavier than CC males ($P=0.0005$; Fig. 1C). On the other hand, F1 CT females showed higher weight (114.37 ± 0.67 g; $P=0.04$) when compared to CC females (88.84 ± 0.78 g; Fig. 1D). Additionally, in both sexes, there was no significant difference between the body weight of CC and TT (males: $P=0.54$; females, $P=0.99$). A similar pattern was observed for body length, with the smaller fish being CC males (176.17 ± 0.14 g; Supplementary Fig. 1).

Global methylation patterns

As expected, global mean methylation levels were tissue-specific and similar to previously reported values for the sea bass. Global mean methylation was 93.8% and 86.1% in the F0 sperm and unfertilized eggs, respectively. In the F1 generation, it was 92.2% in the testes and 84.4% in the ovaries. Comparisons of the four different temperature treatments in F1 (CC, CT, TC and TT) revealed no significant differences in global methylation between treatments, neither in testes (Supplementary Fig. 2A), nor in ovaries (Supplementary Fig. 2B).

Effect of sex and temperature in F0 and F1

To determine the effect of temperature on the methylome, we first accurately defined the size of regions to be studied according to the nature of our data. We employed an autocorrelation algorithm previously developed for studying epimutation rates in *Arabidopsis thaliana* [20]. This approach allowed us to balance resolution and statistical power while considering region sizes specific to sea bass methylation patterns. We identified non-overlapping 200 bp regions containing at least 4 CpGs, with a median of 8 CpGs per region. This window size was selected as it maintained correlation coefficients above 0.75 for most samples, with correlation dropping significantly below this threshold at larger sizes (Supplementary Fig. 3). The methylation level for each region was calculated as the mean of all reads within the window, enhancing the robustness of our analyses. Using 200 bp windows, we performed the following three type of analyses: *Type I*) applied GLMs separately for each sex to evaluate the effects of developmental and ancestral temperature exposure, as well as their interaction, on DNA methylation at each region; *Type II*) identified sex-specific DMRs by comparing F1 ovaries and testes; and *Type III*) detected temperature-associated DMRs across the different F1 treatment groups.

Regarding the Type I analysis, and to assess methylation responses to thermal history, we modeled methylation counts using GLMs with the formula: methylation counts \sim F0 treatment * F1treatment, analyzing each region and sex separately. The strong treatment-tissue interaction ($P < 2e-16$) justified the need of separate models for each sex. Genomic regions were classified into seven categories: no effect, F0 effect only, F1 effect only, additive (F0 + F1), opposing F0 and F1 effects, and regions with significant interactions (quantitative or qualitative interactions), as shown in Fig. 2 (see also Supplementary table S2). By quantitative interaction (also known as ordinal or scaling), we referred to the regions for which the magnitude of the effect of one factor depends on another, but the direction remains the same across groups (e.g., treatment changes always in the same direction, but more strongly under one factor) [25]. In qualitative interactions (also known disordinal), the direction of change in one factor depends on the interacting factor (e.g., treatment increases in one group but decreases in another) [17, 25]. The distribution of categories differed significantly between sexes ($P < 0.001$ Chi-square test). In testes, the most represented category was qualitative interaction (37.0%, cyan), followed by no effect (26.4%, grey), F0 only effect (11.2%, orange), quantitative interaction (9.5%, fuchsia), F1-only effect (8.8%, green), additive effect (5.1%, yellow), and finally opposite-direction effects (2.1%, beige). In ovaries, the most abundant category was no effect (36.2%, gray), followed by qualitative interaction

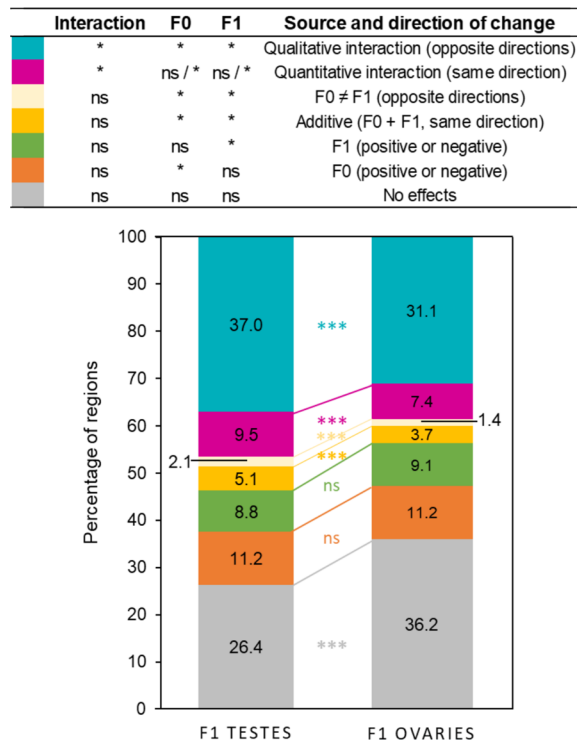


Fig. 2 Percentage of regions classified into different categories of temperature effect from F0, F1 and the interaction, in testes and ovaries. Bars represent the percentage of regions out of the total number of studied regions for each sex. Colors indicate different effect categories. Asterisks between the bars denote statistically significant differences between sexes for each category, *** = $P < 0.001$, Chi-squared test. In the legend, asterisks indicate significant model terms ($P < 0.05$); ns = non-significant

(31.1%, cyan), F0-only effect (11.2%, orange), F1-only effect (9.1%, green), quantitative interaction (7.4%, fuchsia), additive effect (3.7%, yellow), and opposite-direction effects (1.4%, beige).

These results highlight sex-specific patterns in methylation responses to ancestral (F0) and developmental (F1) temperature exposures. The testes exhibited a higher proportion of regions with complex interactions, particularly qualitative and quantitative effects, while ovaries had a greater proportion of regions unaffected by thermal history, alongside strong qualitative effects as well.

To further illustrate the different methylation region categories identified by the GLMs, we plotted representative regions for three of the categories that took our attention because of the overall methylation change when comparing between CC and TT groups for each sex (Fig. 3). Regions classified as additive and quantitative generally exhibited cumulative effects, with methylation levels in the TT group being higher or lower than those in the CC group. In contrast, regions showing qualitative interaction displayed a compensatory effect, as methylation levels in the TT group returned and closely resembled the levels observed in the CC group.

To visualize the genome-wide distribution of temperature-associated epigenetic effects, we generated Manhattan plots for each term of the generalized linear model—parent (F0), offspring treatment (F1), and their interaction—in F1 testes and ovaries (Fig. 4). Numerous significant loci were dispersed throughout the genome, and in certain linkage groups (LG4, LG6, LG10, LG13, LG18-21, LG19 and LG20) notable clusters of highly significant regions (the top 20 most significant regions are shown in grey) influenced by temperature treatments were observed in both sexes. Additional highly significant regions were identified in LG7 and LGX, but these were exclusive to ovaries. Most of these regions were classified as showing qualitative interaction in both sexes. Altogether, these results reveal in an unprecedented and detailed manner the complexity of temperature-induced epigenetic modulation across generations and highlighted genomic regions potentially involved in compensatory or adaptive mechanisms.

Regarding Type II analysis to identify DMRs, when examining the gonads of the F1 fish, the largest differences in DNA methylation levels were found, as expected, when comparing ovaries with testes, regardless of the temperature treatment. Thus, we found 5,619 DMRs between ovaries vs. testes at C, of which 94% were hypomethylated in ovaries (Supplementary Fig. 4A) and most of them (71.3%) mapped to intergenic regions (Supplementary Fig. 5A). In contrast, the hypermethylated DMRs in ovaries were mostly found at coding gene regions: 15.5% at the promoter, 7.3% at the first exon, 29.9% in the rest of exons, 10.1% in the first intron, and 36.6% in the rest of introns and mapped to either the gonadotropin-releasing hormone (GnRH) receptor, Wnt, heterotrimeric G-protein, integrin, and fibroblast growth factor (FGF) signaling pathways. Among the hypomethylated genes, we identified the same pathways and additional ones such as the chemokine and cytokine signaling pathway, the CCKR PDGF cadherin pathway, and the TGF-beta and EGF receptor signaling pathways (Supplementary Fig. 6A). Furthermore, when comparing ovaries vs. testes at different temperature treatments (CC, CT, TC and TT), the effect of temperature exposure led to a gradual increase in the number of DMRs (5,619, 7,049, 7,630 and 8,432, respectively, Supplementary Fig. 4A; Supplementary Table 3).

The analysis of DMRs between groups exposed to different temperature treatments within the same sex, revealed a sex-specific effect. CT vs CC testes (from unexposed sires—see Fig. 1 A for terminology) had 1,730 DMRs, of which 61% were hypermethylated (Supplementary Fig. 4B; Supplementary Table 4). Approximately half of these DMRs (both hyper- and hypomethylated) were found within gene regions: ~10% in the promoters, 4.5% in the first exon, ~17% in the rest of exons, 12% in the

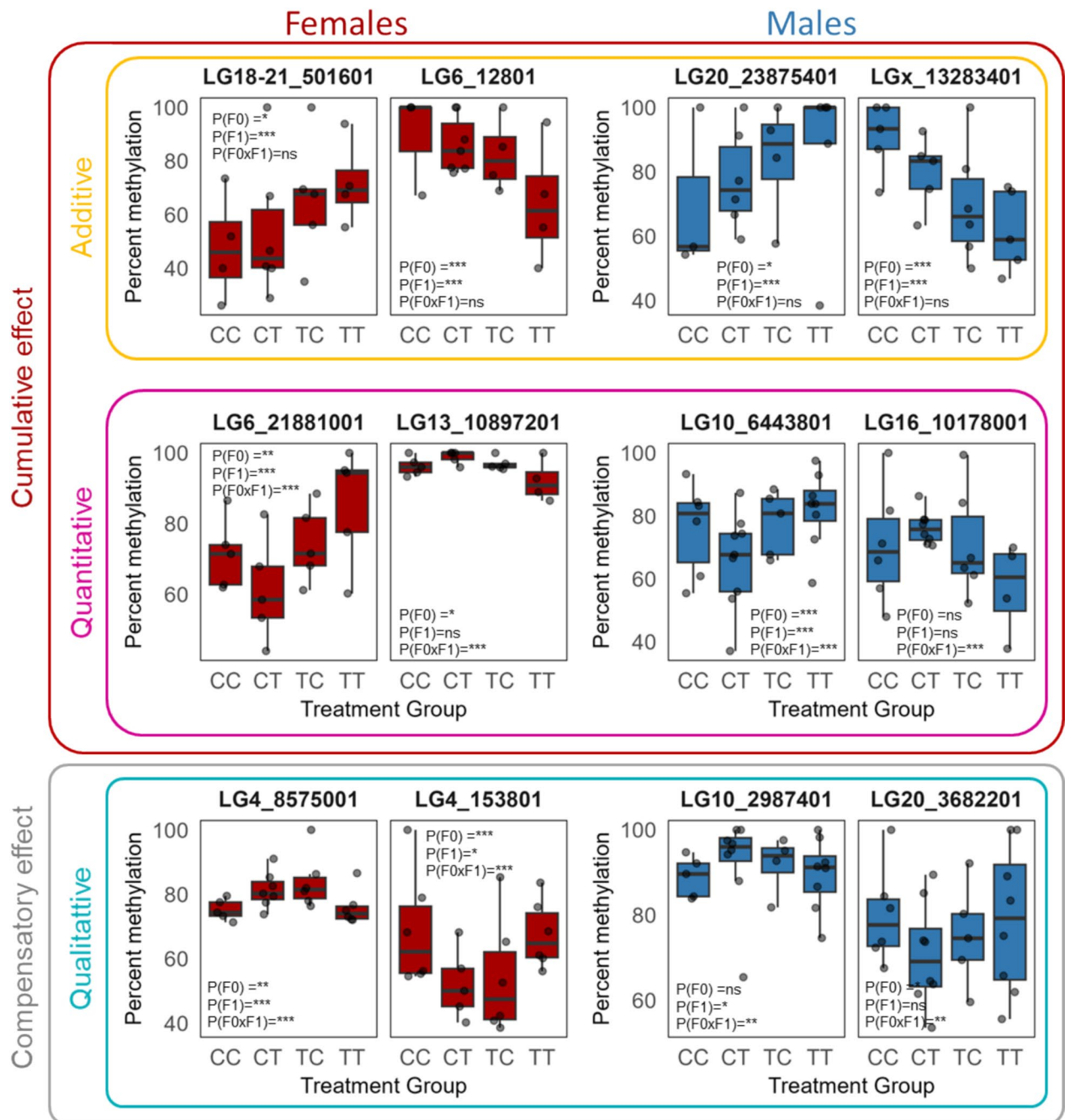


Fig. 3 Examples of classified methylation regions by effect category. Representative boxplots illustrating percent methylation at selected genomic regions, classified by the type of temperature-associated effect, in F1 ovaries (red) and testes (blue). Each panel displays methylation levels across four treatment groups (CC, CT, TC, TT). Regions are grouped by effect category: additive (yellow border) and quantitative interaction (magenta border) were grouped into a larger category named cumulative effect (red border); Qualitative interaction (cyan border), where the direction the F1 effect was opposite from the F0 treatment resulting in similar methylation levels between TT and CC (compensatory effect). For each region, the boxplots show the distribution of percent methylation for each treatment group. Statistical results from the generalized linear model are indicated: P(F0) for ancestral effect, P(F1) for developmental effect, and P(F0x F1) for interaction. Asterisks denote significance levels (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$), and "ns" indicates non-significance

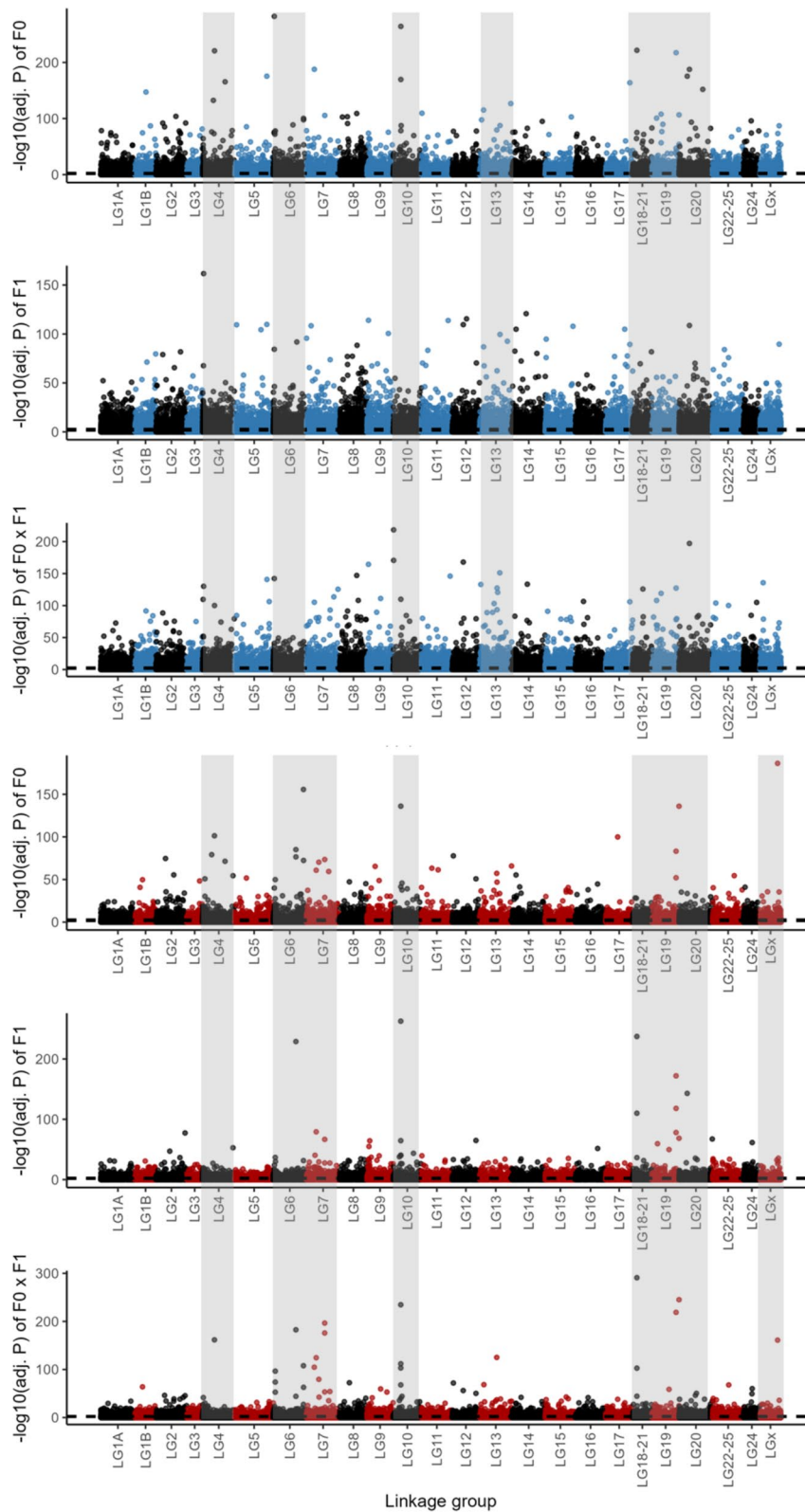


Fig. 4 Manhattan plots showing the genome-wide distribution of temperature-associated methylation effects in F1 testes (upper panels in blue) and ovaries (lower panels in red). Each group of panels represents the results for a different term of the generalized linear model: top, parental (F0, indirect) treatment; middle, offspring (F1, direct exposure during development) treatment; and bottom, the interaction between F0 and F1 treatments. The y-axis displays the negative log10 of the adjusted *P*-value for each region, while the x-axis indicates the genomic position, grouped by linkage group (LG). Alternating colors distinguish adjacent linkage groups, and chromosomes shaded in grey highlight those containing the top 20 most significant regions

first intron and 30% in the rest of introns (Supplementary Fig. 5B). Enrichment analysis of differentially methylated genes between CT and CC testes revealed their involvement in GnRH receptor pathway, Integrin, and chemokine and cytokine signaling, Wnt signaling, angiogenesis and cholecystokinin receptor (CCKR) signaling pathways (Supplementary Fig. 6B). In CT vs CC ovaries, 909 DMRs were identified, of which 51.2% were hypermethylated (Supplementary Fig. 4C).

Comparing TT vs CC testes revealed 2,709 DMRs, mostly hypomethylated (73.8%), whereas ovaries had a higher number of DMRs (5,877), predominantly hypermethylated (83.3%). This pattern was consistent in the TT vs TC and in the TC vs CC comparisons (Supplementary Fig. 4B and C). The offspring from exposed sires exhibited the highest number of DMRs when comparing TT vs TC, with 7,002 DMRs in testes (65.7% hypermethylated) and 7,093 DMRs in ovaries, of which 96.6% were hypermethylated (Supplementary Table 5).

The effect of temperature was not just evident in the higher number of DMRs, but also on the magnitude of the percentage methylation differences. For instance, in the CT vs CC testes comparison, the methylation difference of the DMRs ranged from over 10% to 50% in absolute value (Supplementary Fig. 4D). However, in the TT vs CC testes comparison this range increased up to 65%. This trend was more noticeable in the ovaries. Methylation differences ranged similarly across all comparisons (CT vs. CC, TC vs. CC, TC vs. CT, TT vs. CT, and TT vs. TC), falling between 10 and 65%. However, most differentially methylated regions (DMRs) showed larger differences, with an average change of around 45% (Supplementary Fig. 4E).

Methylation levels of key sex-related genes

Among 42 genes previously known to be involved with the gonadal development of the sea bass [67, 68], we found the methylation levels captured on our experiment for six sex-related genes: follicle stimulating hormone receptor (*fshr*), SRY-box transcription factor 9a (*sox9a*), Wnt family member 4 (*wnt4*), hydroxysteroid 17-beta dehydrogenase 10 (*hsd17β10*), estrogen receptor alpha (*era*), and prostaglandin-endoperoxide synthase 2 b (*ptgs2b*). However, there were significant differences in methylation across groups in four of them: *fshr*, *sox9a*, *wnt4*, and *hsd17β10* (Fig. 5).

These genes illustrate some of the categories of temperature effects. Methylation levels in *fshr* were lower in ovaries than in testes, except in the CT group. At TC and TT, the methylation levels of *fshr* were similar to CC group. Thus, this gene was classified as ‘No effect’ category in the ovaries and as qualitative interaction in the testes. For *sox9a* TC-testes were hypermethylated when compared to TC-ovaries and CC-testes. In the TT group,

this gene was hypermethylated in ovaries and hypomethylated in testes. This gene contained regions of qualitative interaction class in both F1 testes and ovaries. For the *wnt4* gene, methylation levels were lower in ovaries with respect to testes in the CC and CT groups, with the TC group showing slightly higher levels, although not significant. In TT ovaries it was hypermethylated with respect to the other temperature groups of ovaries. The GLM of the overlapping regions for this gene were not able to capture significant effect and it was classed as “No effect” in both sexes. Regarding the regions overlapping *hsd17β10* gene, lower levels of methylation were found in the ovaries when compared to testes. Across ovaries treatment groups, the CT group showed a strong hypomethylation when compared to CC, and the TC and TT groups showed intermediate levels, lower than in CC but higher than CT. The *hsd17β10* gene overlapped with a total of 6 regions: two classified as “no effects”, one showing an F1 treatment effect, one with a scaling interaction where the F1 effect was stronger than F0 effect, and two regions with qualitative interaction (compensatory effect).

Temperature-induced and inherited DNA epimarks

In the previous sections (Types I and II analysis), we used 200 bp genomic regions to characterize the epigenetic landscape and assess genome-wide temperature effects. For the Type III analysis, focused on inheritance, we also employed single-nucleotide resolution but this time without considering regions, as this fine-scale approach is essential to uncover the yet unknown mechanisms of epigenetic inheritance and, to date, there is no evidence that DNA methylation marks are inherited collectively as groups or regions. Thus, we assessed the transmission of temperature-induced epimarks to the next generation by determining the number of: (1) stable CpGs between F0 and F1 to identify loci where the methylation levels are constitutive or unchanged across generations; (2) environmentally-induced epimarks in F0 sperm transmitted to F1 gonads using standard differentially methylated cytosines (DMCs) analysis (1 permutation); and (3) environmentally-induced epimarks verified using a permutation test. Stable CpGs were defined as those that were not DMCs between F0 and F1, i.e., with a methylation difference < 15% and *q*-value > 0.01. We found that a large proportion of CpGs maintained stable methylation levels between F0 gametes and F1 offspring gonads in both comparisons: 88% from the C sires to the CC offspring and 80% from the T sires to the TC offspring, with similar values for both F1 testes and ovaries.

To determine the number of environmentally-induced epimarks, we determined the number of temperature-induced DMCs between C and T F0 sire sperm samples, which amounted to 10,042 DMCs (Fig. 6A;

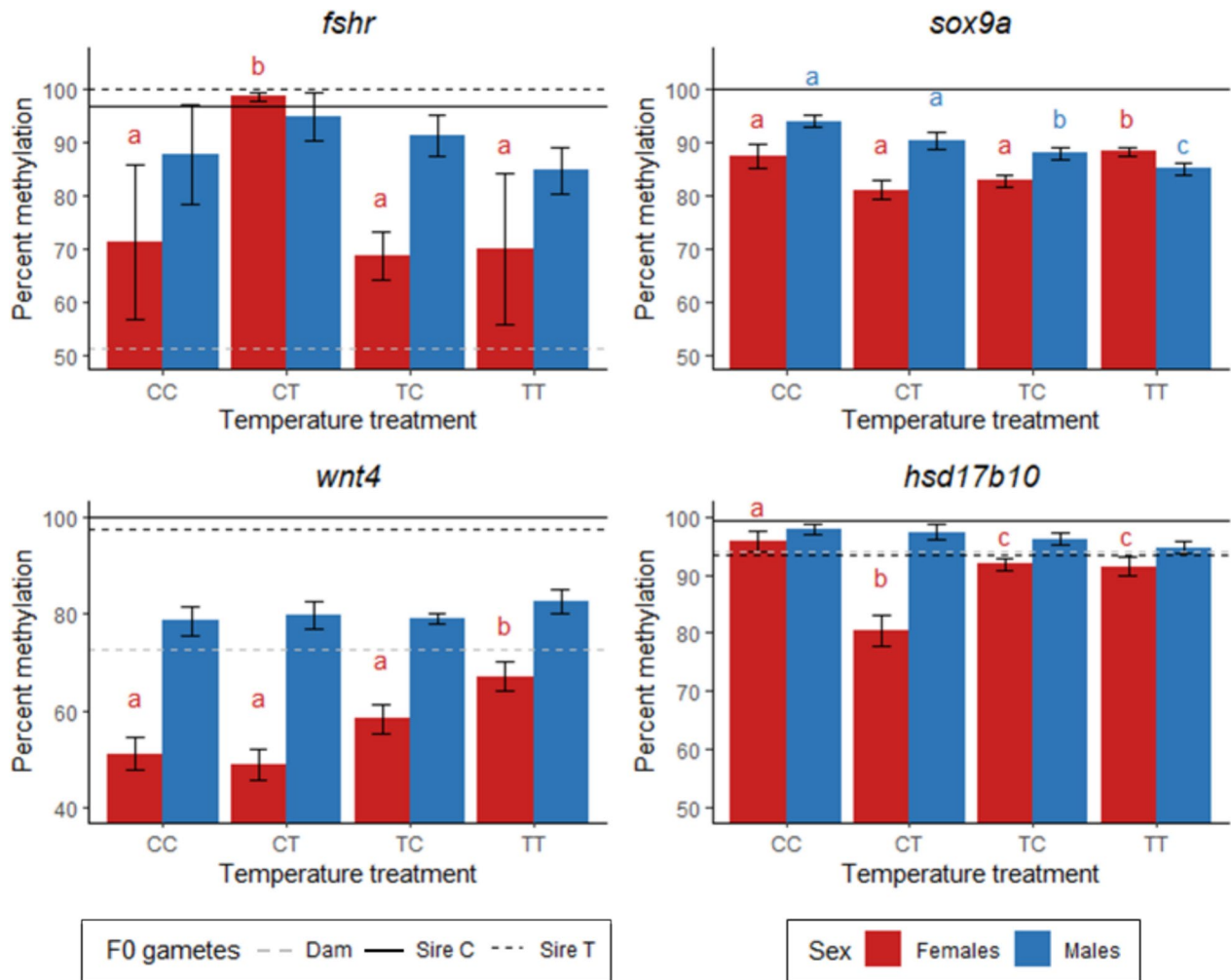


Fig. 5 DNA methylation levels of key sex-related genes in F1 ovaries (red) and testes (blue) according to temperature treatment: CC (control), CT (developmental), TC (ancestral) and TT (developmental and ancestral). Data as mean \pm SEM. For reference, levels in the F0 gametes are also indicated by horizontal lines (solid lines, F0 unfertilized eggs; dashed and dotted lines, F0 sperm). In *sox9a* gene, the three horizontal lines overlapped. Different letters denote significant methylation differences between groups of the same sex (logistic regression, $q < 0.05$). Absence of letters indicate no significant differences

Supplementary Table 6), representing 1.94% of the 516,651 CpGs captured by RRBS in F0 samples. These DMCs were significantly affected by the temperature treatment, applied only during early development, and remained in their gametes when the fish were two years old. These DMCs were annotated against the reference genome, and 13% were found in promoters, 5.2% in the first exon and 18.4% in the rest of exons, 8.9% in the first intron and 26.5% in the rest of introns, while the rest were mapped in intergenic regions (Supplementary Fig. 5C).

Then, we compared F1 TC with CC gonads to identify DMCs induced by the temperature experienced by F0 sires. We found 39,390 DMCs in F1 testes (69.4% hypomethylated) and 26,032 DMCs in F1 ovaries (73.8% hypermethylated). A total of 647 DMCs were found in common between the F0 T vs C sires' sperm and F1 TC vs CC testes (Fig. 6A, B, Supplementary Fig. 5D, Supplementary Table 7). When using these 647 DMCs

for hierarchical clustering analysis, it was revealed that clustering of the samples was based on temperature treatment and then by generation, further highlighting the previously described genotype-by-environment interaction in relation to sex determination in sea bass [29]. Those DMCs, overlapped with 422 genes (Supplementary Table 8 enriched in biological processes such as DNA integration (GO:0015074), nucleobase-containing compound metabolic process (GO:0006139) and cellular nitrogen compound metabolic process (GO:0034641; Supplementary Table 9 and 10). In the F1 ovaries, 870 DMCs were identified in common with the F0 T vs C sires' sperm overlapping with 210 genes which were enriched for DNA integration (GO:0015074, similar as in the testes), metabolic and primary process (GO:0008152, GO:0044238). Thus, 6.4% and 4.5% of the temperature-induced epimarks in the F0 sperm of the sires reared at T were transmitted to F1 testes and ovaries, respectively

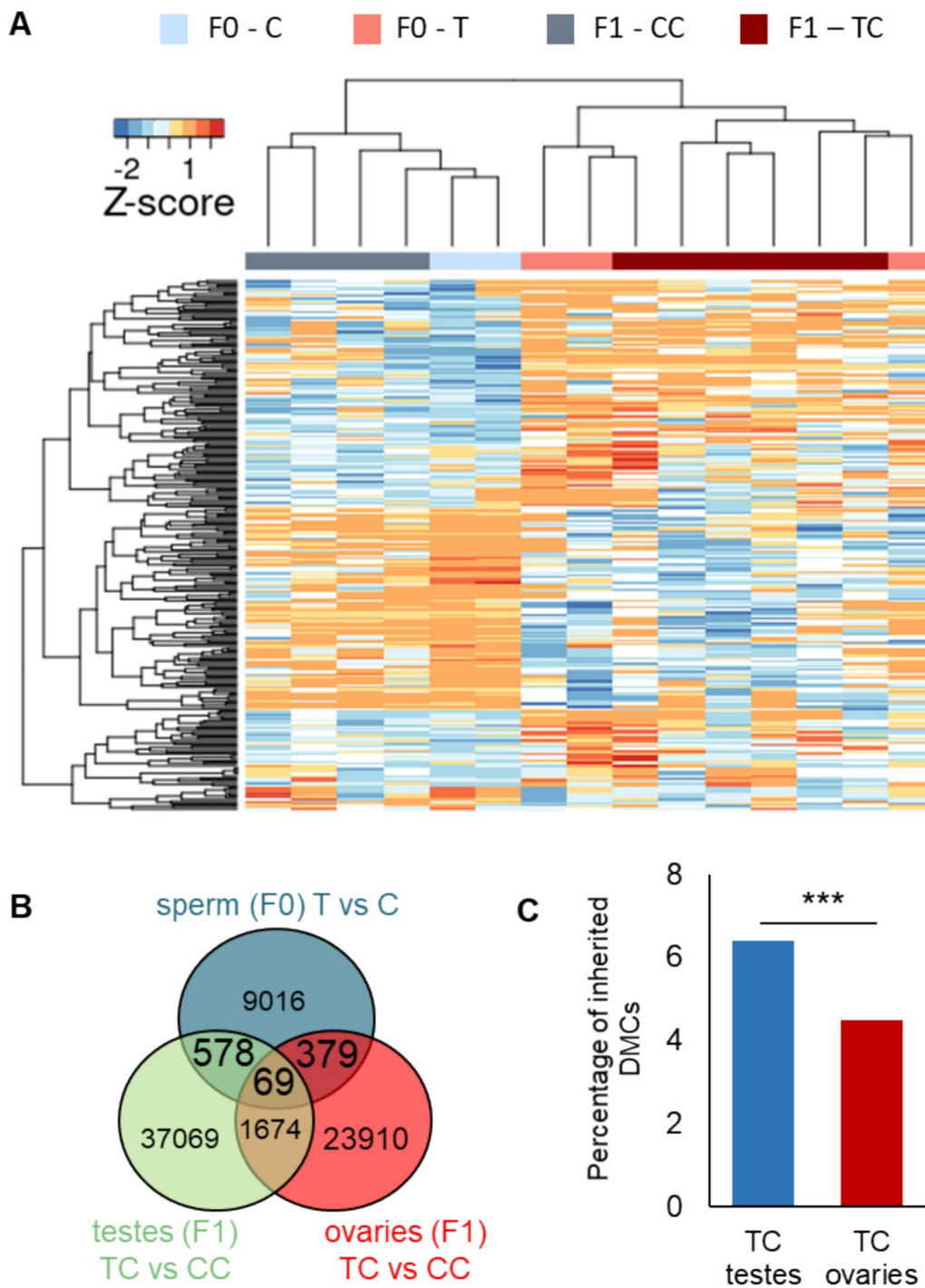


Fig. 6 **A** Heatmap of methylation levels of the 647 differentially methylated cytosines (DMCs) inherited from the sires reared at high temperature (T, not neomales) vs. sires reared at control temperature (C) and transmitted to the TC F1 testes, which were never directly exposed to temperature. The color of the top x-axis indicates the temperature treatment and the generation: F0 at C (light blue) F0 at T (light red), F1 testes at CC from unexposed sires (dark blue) and F1 TC testes (dark red). Clustering of the samples was based on temperature treatment and then by generation. **B** Number of DMCs between T and C sperm of the F0 that overlapped with DMCs in the F1 gonads between TC and CC (ancestral and control). **C** Percentage of temperature-induced and inherited epimarks transmitted from F0 sperm to F1 gonads. Statistical comparison between proportions was performed using a two-proportion z-test; $P = 6.22 \times 10^{-10}$

(Fig. 6C). The percentage of temperature-induced marks transmitted from sires to offspring was sex-specific (two-proportion z-test; $P = 6.22 \times 10^{-10}$).

Finally, from the identified temperature-induced DNA methylation marks inherited from the F0 sperm, we filtered out any potential marks acquired by chance using a permutation test using the *MethylInheritance* package. We ran 4,000 permutations with the threshold of 0.05, using the testes and ovaries of the F1 generation separately against the F0 sperm samples. For each permutation, DMCs were identified for each generation separately using the same parameters as previously described. In F1 testes, 85 hypermethylated and 53 hypomethylated DMCs were identified as inherited from the F0 sperm (Supplementary Fig. 6A and B). In the F1 ovaries, there were 166 hypermethylated DMCs and 7 hypomethylated DMCs identified as temperature-induced and inherited from the F0 sperm (Supplementary Fig. 6C and D). However, the level of significance was higher than 0.05 in both F1 testes and ovaries (Supplementary Fig. 6E and F).

Discussion

In this study, we used the European sea bass to compare the epigenetic profiles of fish never exposed to high temperature (control, CC) against those exposed at different life stages: once, either through their parents (ancestral exposure, TC) or during their early development (developmental exposure, CT), or twice (ancestral and developmental exposure, TT). Further, we discriminated among temperature effects by classifying them into two major types: without or with interaction between the effects of the two generations. Those without interaction included effects in just one of the two generations (F0 or F1) or in both generations, which, in turn, could be either additive or with opposite effects. On the other hand, interaction effects could be either quantitative or qualitative. This approach—to the best of our knowledge, used for the first time in this type of studies—revealed that multigenerational exposure to high temperature predominantly induced qualitative, compensatory interaction effects (~35%) on the gonadal methylome, and exhibiting sex-specific patterns. While cumulative effects were limited (additive ~4%, quantitative interaction ~8%), they were also detected. Moreover, some of these temperature effects were observed in genes involved in gonadal sex development, and the percentage of temperature-induced and inherited DNA methylation levels were determined in the testes and ovaries of one-year-old European sea bass.

The effect of sex and temperature on the sea bass methylome

Our results revealed that qualitative interactions—in which ancestral (F0) and developmental (F1) exposures

to high temperature induced opposing effects on the gonadal methylome of the F1 generation—were the predominant response across thousands of genomic regions, in a sex-specific manner. This compensatory epigenetic pattern may reflect a buffering mechanism that helps organisms adjust to temperature changes across generations, potentially contributing to short-term adaptation. Although such responses are infrequently documented in vertebrates, similar patterns have been proposed in other fish species. For example, DMRs between sulfidic and non-sulfidic populations of *Poecilia mexicana*, a fish species inhabiting hydrogen sulfide-rich springs, were largely retained across two laboratory generations, even in the absence of the original environmental stressor. This transgenerational transmission of methylation was suggested as a mechanism that may contribute to adaptation in this species [40].

Venney et al. [92], investigated how parental and offspring thermal environments influence DNA methylation in brook charr (*Salvelinus fontinalis*), a thermally sensitive salmonid of both ecological and economic importance. They found that parental temperature during sexual maturation induced significant and persistent changes in offspring DNA methylation, whereas the offspring's own rearing temperature had minimal effect. Although the study did not explicitly test for interactions between parental and offspring temperature, it strikingly showed that the parental environment was the main driver of offspring's epigenetic profile, at least in brook charr. This suggests the presence of a multigenerational priming mechanism in this species. However, the extent to which organisms can modify or reset inherited methylation marks remains a key factor in determining the adaptive value of epigenetic inheritance under environmental change. For instance, in the self-fertilizing mangrove rivulus (*Kryptolebias marmoratus*), experimental manipulation of both parental and offspring environmental enrichment revealed that most DNA methylation changes induced by the parental environment were not maintained when offspring experienced contrasting conditions [11].

DNA methylation is often studied in relation to short-term acclimation to environmental change, with growing evidence that it can modulate gene expression [90]. While the relationship between methylation and transcription is complex, numerous studies in fish and invertebrates demonstrate that methylation can respond within hours or days to environmental stressors, and often reverts once the stress is removed [5, 36, 37]. This plastic and reversible nature of methylation has been interpreted as a potential buffering mechanism, allowing organisms to maintain regulatory stability during acute stress without requiring permanent genetic alterations [84, 90].

To the best of our knowledge, our results provide the first evidence suggesting that such a potential buffering effect may operate under changing environmental conditions across generations. However, caution is warranted in interpreting these findings, since our dataset includes five families derived from a single female. Future work should incorporate a larger number of genetically diverse families, and even considering individuals from distinct populations, to improve the robustness and generalizability of these findings.

While the temperature regimes used in our study may not reflect current environmental conditions, the use of treatments that are commonly used in the aquaculture of this species, allowed us to investigate the underlying mechanisms of the response to high temperature. Moreover, these temperatures are not entirely unrealistic: by the year 2100, sea surface temperatures in the eastern Mediterranean, a region within the natural range of the European sea bass, are projected to reach up to 26 °C [75], lending ecological relevance to our findings.

A limited but important proportion of genomic regions showed cumulative effect, classified as either additive or with quantitative interaction. Previous research had shown additive effects of prolonged exposure to high temperatures on reproductive outcomes in both female and male fish, potentially impacting population sustainability [83]. An environmentally-induced additive effect on DNA methylation, albeit in a different context, has been reported in mice exposed to dietary zearalenone, an estrogenic mycotoxin, for multiple generations. This resulted in a cumulative impairment of fertility and delayed puberty in females [97]. In another study, Ryu et al., [74] evaluated if changes in DNA methylation were associated with within-generational thermal plasticity in a coral reef fish (*Acanthochromis polyacanthus*), and compared these methylation profiles with those of a population exhibiting transgenerational thermal plasticity. In both cases elevated temperature induced DMRs in genes related to metabolism, development, cardiovascular and heat-response functions. However, the minimal overlap between the two populations suggested that different epigenetic toolkits were used to cope with temperature and, notably, no cumulative effects were reported in the population with transgenerational thermal plasticity. Thus, to the best of our knowledge, our findings represent the first documented additive effect of temperature on a limited portion of the sea bass methylome. This observation has broader implications for fields such as animal production and conservation biology, and offer insights on how species may adapt to changing environmental conditions.

Temperature effects on sex ratio and growth

The observed increases in body weight and length in temperature-exposed fish suggest that high temperature

positively influenced growth traits, especially in males and CT females. In European sea bass, sex and growth are strongly linked complex traits, both influenced by a combination of genetic and environmental factors, including epigenetic regulation [21], [29, 57]). Although these results indicate an association rather than direct causation, epigenetic mechanisms are known to play a role in sex reversal in European sea bass [2, 57], [60, 89], as has also been described in other species such as the half-smooth tongue sole [81] and the Nile tilapia (*Oreochromis niloticus*, [94]). The significant increase in the proportion of males in the CT group, together with the enrichment analysis of differentially methylated genes between CT and CC samples in key pathways related to gonadal development—such as GnRH receptor, integrin, chemokine and cytokine signaling, Wnt signaling, angiogenesis, and cholecystikinin receptor (CCKR) signaling— supports the hypothesis that DNA methylation is involved in sex reversal in this species, consistent with the model of conserved epigenetic regulation of sex (CERS, [62]).

In line with this, certain genomic regions showed DNA methylation patterns in the TT group similar to those of the CC group, but not to the CT group. This may be explained by the fact that, although the TT group had a higher proportion of males, the difference in sex ratio compared to the CC group was not statistically significant ($P=0.051$). This convergence toward the control phenotype further supports the hypothesis of a compensatory effect resulting from multigenerational exposure to high temperature. Similarly, for body weight and length, temperature exposure triggered sex-specific responses. In males and females, both traits increased in CT when compared to the controls, while repeated exposures (TT) showed no significant difference from controls. Although the causal link between methylation and phenotype remains unclear, the observation of the majority of regions following a compensatory pattern is noteworthy. This pattern, also reflected in the phenotype, is suggestive of short-term adaptation or plasticity, as it indicates a phenotypic convergence toward control levels despite environmental change. Future research will be required to further elucidate the relationship between the observed methylation patterns and the phenotypic growth related traits.

At the level of global methylation profiles, the largest differences were found between ovaries and testes, regardless of the temperature treatment. This is in line with the known role of DNA methylation in the acquisition and maintenance of tissue identity [66, 96] and, in particular, in establishing the sexual phenotype [62]. A predominantly hypermethylated profile was found in ovaries, while both hypo- and hypermethylation were found in testes. These findings support the complex

interplay between sex, environmental history and DNA methylation landscape in this species.

Epigenetic trap versus epigenetic buffering in non-exposed offspring

A major difference was found in the comparison the TT and TC groups, especially in F1 testes. This finding underscores two opposing concepts: epigenetic buffering and the epigenetic trap hypothesis. Epigenetic buffering refers to the capacity of epigenetic modifications to enhance population persistence under environmental stress, either by stabilizing phenotypes or by generating heritable variation that can facilitate adaptation. In this framework, epigenetic modifications may contribute to evolutionary rescue (i.e., the avoidance of extinction through rapid adaptive responses), by enabling the inheritance of environmentally induced phenotypes. Such mechanisms can act as a form of heritable bet-hedging, maintaining or enhancing a population's evolutionary potential [58]. In contrast, the epigenetic trap hypothesis posits that inherited epigenetic modifications can become maladaptive when they are no longer aligned with the offspring's environment. This mismatch can hinder adaptive responses and potentially reduce fitness [58].

Epigenetic traps have been proposed as possible mechanisms behind environmentally-driven changes in population sex ratio, potentially posing risks amid a global warming scenario [19]. In species with Environmental Sex Determination (ESD) such epigenetic mechanisms can allow rapid adaptation to fast environmental changes. A similar process may be at play in the sea bass, a species in which sex is determined by both polygenic and environmental (particularly thermal) influences. In this study, the F1 testes methylome from TC group (ancestral exposure only) exhibited the most significant divergence, suggesting a mismatch with the environment in which the offspring developed. This mismatch may represent an instance of an epigenetic trap, in which inherited DNA methylation changes no longer confer a fitness advantage under new environmental conditions.

While both epigenetic buffering or trap could occur in relation to fitness-related traits such as growth or body weight, determining their relative impact would require further analysis at the population level—beyond the scope of this study. Nonetheless, our findings may represent the first evidence supporting the epigenetic trap hypothesis in a species with polygenic sex determination, where inherited DNA methylation changes could lead to maladaptive alterations in subsequent generations [61].

DNA methylation in sex-related genes

We characterized methylation levels in key genes involved in early gonadal development in sea bass and

other vertebrates [54, 67, 76]. Specifically, we focused on *hsd17β10* [67] and *sox9a* [77], recognized biomarkers of early ovarian and testes differentiation, respectively. Although the interplay between DNA methylation and gene expression remains complex, inverse correlations between epigenetic silencing and gene expression were noted. This underlies the CERS model proposed by Piferer et al. [62] in which pro-male genes show high expression combined with low DNA methylation in males, while pro-female genes exhibit high expression and hypomethylation in females.

In our study, *sox9a* was differentially methylated in both the TC and TT groups. In the TT group, it was hypomethylated in the testes and hypermethylated in the ovaries of temperature-resistant females (i.e., fish that still develop as phenotypic females despite the T treatment). Given that higher expression in testes than ovaries under control conditions was previously reported [67], our results are consistent with the CERS model [62]. This suggests that the reduced methylation in testes as a consequence of T could lead to higher expression of *sox9a* in genetic males, while hypermethylation in resistant females could be associated with lower expression, preventing them from undergoing sex reversal. As a recognized biomarker of early testes differentiation and a hub gene in the regulatory network [77], *sox9a* appears to play a key role in the environmentally-induced modulation of sexual phenotype (including sex reversal) via methylation changes.

The *hsd17β10* gene is a biomarker of ovarian differentiation and thus a pro-female gene [67]. In the F1, females under control conditions (CC) exhibited hypomethylation relative to males, again aligning with the CERS model. However, as the number of temperature exposures increased, DNA methylation in females converged toward those observed in males. Of note, in the TT group, females exhibited intermediate methylation: hypomethylated when compared to males but more methylated than control females. These findings suggest that *hsd17β10*, like *sox9a*, may play an important role in temperature-induced sex reversal of the sea bass, and that its methylation status is sensitive to both developmental and ancestral thermal environments.

Epigenetic inheritance of temperature-induced epimarks

Epigenetic inheritance is a burgeoning field, but there is still much work to be done in establishing mathematical models and criteria to accurately identify and classify instances for when it occurs, particularly in long-lived animals where large sample sizes are difficult to obtain. Currently, many studies base their claims solely on the detection of DMCs between treatments and generations, without fully considering underlying genetic factors, genetic-environment interactions, or randomly-acquired

changes. For instance, a study on the inland silverside (*Menidia beryllina*), reported that early-life exposure to endocrine disrupting chemicals (EDCs) resulted in multigenerational and transgenerational epigenetic changes [48]. However, this conclusion was drawn from identifying DMCs common across generations, potentially overlooking the complex interplay between genetic, environmental factors and random factors. Additionally, the concept of epigenetic inheritance is often conflated with stable epimarks, loci that do not exhibit differential methylation.

In our study, we first quantified the proportion of such stable CpGs, then determined which environmentally-induced marks in the parental generation (F0) were maintained in the filial (F1) generation. We found that 88% and 80% of CpG sites were stably methylated across generations in C and T groups, respectively. This was in accordance with findings in the threespine stickleback, where 96% of CpGs remained stable using a similar analytical approach and identical statistical thresholds [37]. Importantly, both studies used a threshold of 15% and a q -value < 0.01 , allowing meaningful cross-study comparisons. However, while they compared the fin tissue in both generations, we compared the F0 sperm with the F1 testes, which could explain our lower number of shared CpGs.

Our study found that approximately 5% of temperature-induced DNA methylation marks in F0 sperm were also present in the gonads of the F1 generation. Specifically, 6.4% of temperature-induced marks in F0 sperm were inherited by F1 testes, and 4.5% in ovaries. While this aligns with previous studies, it is important to note that direct comparisons are challenging due to differences in tissues, species and techniques. For example, [98] reported 11.4% of stress-induced DMRs in mice sperm were transmitted to F1. Similarly, Rodriguez Barreto et al. [70] found that 13.9% of DMRs between wild and hatchery Atlantic salmon (*Salmo salar*) were inherited. Although these studies used different methylation profiling techniques (WGBS, in the mouse and a targeted approach focusing on regions with high levels of methylation, in the salmon), they are among the few to identify environmentally-induced and inherited epimarks, consistently suggesting that only a small proportion are transmitted across generations. Despite this, even a small proportion of environmentally-induced marks could contribute to rapid adaptation to environmental change [6, 34].

Importantly, genetic background can influence methylation responses to environmental stimuli [16, 42]. In light of this, the design of our study provides a unique perspective by minimizing this confounding factor since all F1 individuals were half-siblings (same dam) and derived from full-sibling males. This design significantly

reduced genetic variation and allowed for a more precise examination of temperature-induced epigenetic changes with minimal, yet still present, genotype by environment interactions (as observed from the sample clustering of the heatmap in Fig. 6A). Besides, only five DMRs overlapped with single nucleotide polymorphisms, emphasizing the potential importance of stochastic variation.

We could not quantify the proportion of marks from stochastic source, as significance was not achieved with the recommended number of 4000 permutations (Belleau et al., 2018). This limitation may stem from the fact that the *methylInheritance* package was originally designed for three-generation studies using the same tissue type. Our two-generation study using different tissues may have introduced analytical bias. This underscores the necessity for standardized methodologies to study inherited epimarks across generations, enabling better comparison in future studies. Nonetheless, it is worth to note that the study by Belleau et al., identified only three inherited sites, reinforcing the idea that the number of true environmentally-inherited marks is likely very small.

Limitations of the study, future perspectives and conclusions

The analysis of 64 methylomes yielded valuable insights on how sex and temperature shape the epigenome of European sea bass. However, there are areas for further improvement and investigation. Notably, integrating additional regulatory layers such as transcriptomic data and other epigenetic mechanisms, could further enhance our understanding. For instance, Feng et al., [27], demonstrated role of long non-coding RNA doublesex and mab-3 related transcription factor 2 (*dmrt2*) as a transcriptional regulator of *dmrt2* in sex differentiation in the half smooth tongue sole. Incorporating such analyses would broaden our understanding of the regulatory interactions relevant to environmental sex determination.

To further clarify the extent of transgenerational epigenetic inheritance and its influence on phenotype, it will be crucial to examine the methylome and transcriptome of the F2 generation. Given that this study has revealed the effects of sex and temperature on the epigenome at a genome-wide level, along with initial evidence of their inheritance, future research should focus on minimizing potential confounding factors. Specifically, analyzing the same tissue across generations is crucial to avoid tissue-specific variability that could obscure inheritance patterns. Another priority is to better understand the dynamics of DNA methylation reprogramming of the sea bass. This would provide insights on how epimarks are transmitted from gametes to developing embryos and how reprogramming may modulate this transmission. To date, it is clear that both parental methylation patterns contribute to offspring epigenetic landscapes [34, 78, 80,

86, 91], but their roles and relative influence can vary. Unlike mammals, zebrafish and medaka largely preserve paternal methylation during early development [39], [63, 71]. Ross *et al.* suggested that embryonic DNA methylation reprogramming is conserved across distantly related teleost species. Thus, the persistence of paternal methylation in teleosts, combined with our findings of temperature-induced and sperm-inherited epimarks, underscores the need for further research to elucidate the relative roles of maternal and paternal methylation inheritance. Understanding these mechanisms is essential for predicting how fish populations may respond to rapid environmental changes such as global warming.

Conclusions

This study investigated how temperature-induced epigenetic changes, specifically DNA methylation, are influenced by multigenerational exposure and whether these changes can be inherited in the European sea bass. We analyzed methylation patterns both at the genome-wide level and in key genes crucial for sex development. Methodologically, we optimized sea bass methylome analysis by iteratively adjusting region lengths and correlating methylation levels, enhancing both resolution and biological relevance. Notably, we found that multigenerational exposure to high temperature had a predominantly qualitative, compensatory effect (~35%) on the gonadal methylome, with clear sex-specific patterns and evidence of intergenerational inheritance. In contrast, cumulative effects, both additive (~4%) and quantitative interactions (~8%) were limited, but detectable. Moreover, regarding the inheritance of those marks, we found that despite a high stability of constitutive methylation levels (80–88%) between F0 and F1 generations, the proportion of temperature-induced methylation marks transmitted from gametes to the gonads of the next generation was relatively low (~5%). All in all, this study provides valuable insights into the role of epigenetics in short-term adaptation, which may constitute early stages of evolutionary change and have important implications for species conservation under global warming.

Materials and methods

Fish and rearing conditions

The European sea bass used in this study were from the West Mediterranean Sea strain. We used samples from two generations: the parental generation (F0, the first generation exposed to temperature), and the filial generation (F1). The F0 hatched larvae (March 2014) were split into two groups at 13 days-post-fertilization (dpf): one group was reared at 16.5 °C (C), the control group, and the other at 21 °C (T, Fig. 1A). Temperature treatments were applied between 13 and 65 dpf, a thermal regime known to cause environmentally induced sex

reversal in previous studies [2], [15, 55, 56]). Each treatment was replicated in two separate tanks. Afterwards, temperature was gradually raised to 21 °C and from day 135 onwards, it was maintained at 20–22 °C (more details on the rearing conditions of F0 can be found in Anastasiadi *et al.*, [2]).

Once the F0 individuals reached three years of age (March 2017), sperm was collected from five males (all derived from "male d" in the F-1, a male known for producing balanced sex ratios when reared at C; Anastasiadi *et al.*, 2018): two F0 males reared at C and three F0 males reared at T. One aliquot of sperm was flash frozen and stored at –80 °C for further analysis of the methylome, and another aliquot was used for artificial fertilization and creation of the F1 generation. The five F0 sires were crossed with the same female (not the same female used in F-1) and the fertilized eggs (F1 generation) were incubated at 14 °C until 2 dpf before being evenly distributed into six tanks of 500 L each. The temperature was gradually increased from 14 °C to 16 °C within one day and larvae were exposed to the same temperature treatment as the parental generation: T and C groups, each in triplicate. These treatments were applied from 8 to 62 dpf (Fig. 1A).

Fish were reared at Ifremer Plateforme Expérimentale d'Aquaculture (Palavas-les-Flots, France), accredited to use and breed laboratory animals (no. C341926), and the project was approved by the Animal Care Committee no. 36 under project authorization no. APAFIS 19676. After one year, 1,256 F1 fish were euthanized using benzocaine (150 mg/L) and randomly sampled. From 476 fish, we measured standard length, weight, and sexed them by *in situ* gonad examination. Gonads of the F1 generation were dissected, snap frozen and stored at –80 °C until genomic DNA extraction. A total of 1,118 fish were genotyped, including the parents and the 476 offspring for which the gonad tissue was kept. Genotyping was performed using the Thermo Fisher DlabChip European sea bass array, with 57,000 single nucleotide polymorphisms [29, 33].

To assess the effect of parental (F0) and offspring (F1) temperature treatments on sex ratio, we used a GLM. We initially assessed the influence of tank and family (each sire crossed with the same dam) as random effects, but they were found to be negligible and therefore excluded from the final model. The model included fixed effects for F0 treatment, F1 treatment, and their interaction. For body weight and length, we used linear mixed-effects models using the *lmerTest* package in R [41]. Body weight and length data were log-transformed prior to analysis to meet the assumptions of normality and homoscedasticity. We also assessed tank as a random effect, but it was negligible and we excluded it from the final model. A likelihood ratio test (ANOVA) was performed to

compare both models for each trait separately. The final model for body weight and for body length included fixed effects for F0 treatment, F1 treatment, and their interaction, and a random intercept for family to account for non-independence within family groups. Significance of fixed effects were assessed using Satterthwaite's method for approximating degrees of freedom, as implemented in *lmerTest* [41]. Post hoc pairwise comparisons between F0 and F1 treatment combinations were conducted using estimated marginal means. All analyses were conducted in R 4.3.1 using *lme4* (v.1.1.37, [7]), *emmeans* (v.1.8.8, [44]) and *stats* packages in R software [64].

Sample selection for methylome analysis

To ensure that genome-wide methylome analysis included samples representative of each group (two sexes, two temperatures in F1 and two temperatures in F0), we selected three individuals from each group based on multiple phenotypic traits: body weight, body length, and genetic sex tendency. First, we selected individuals with body weight and standard body length around the group mean. For the purpose of this study, we used males and females resistant to sex reversal. Neomales, i.e., genotypic females turned into phenotypic males by temperature [55, 56], were excluded based on their genetic sex tendency, i.e., the probability of developing as male or female under the threshold model for sex determination [29]. To identify them we selected fish representative of the distribution of the GST values of the group. The genetic sex tendency, estimated based on the 57,000 single nucleotide polymorphisms data for the same batch of fish in a previous study [29], can take a range of values around zero, usually from -1.5 to $+1.5$, with negative values associated with genetic males and positive values with genetic females or sex reversed males (Supplementary Fig. 8A). To select samples representative of each group, we calculated the mean and standard deviation of GST from each group. Then, we selected three individuals per group: 1) the sample closest to the mean, 2) the sample closest to the mean minus one standard deviation, and 3) the sample closest to the mean plus one standard deviation (Supplementary Fig. 8B). By selecting these samples, we reduced the selection of outliers and increased the likelihood that our findings accurately reflected the characteristics of each experimental group. In this study, we focused on the effects of sex and temperature in the gonads of the offspring (F1), in males and females that were resistant to sex reversal. The sex-reversed individuals, i.e., neomales, will be reported elsewhere (Sánchez-Baizán et al., in prep.).

In F1, the chosen tissue for analysis was the gonads. While directly comparing gametes across generations would have been valuable, several technical challenges made this unfeasible. First, most males were not yet

mature, making it difficult to obtain sufficient sperm from all individuals. Second, the primary aim of the study was to investigate the effect of temperature on the methylome of each sex separately. Unfertilized eggs pose several technical challenges, as they contain a high proportion of 5S RNA and an elevated mitochondrial-to-nuclear DNA ratio, making it difficult to meet the stringent DNA quality and integrity requirements for generating RRBS libraries. Therefore, to include both sexes in F1, we opted to sample gonadal tissue instead of gametes.

RRBS library preparation

Genomic DNA was isolated using the Qiagen Blood and Cell Culture DNA Mini kit (catalog no. 13323) according to the manufacturer's instructions. The quality of DNA was checked with NanoDrop ND-1000 (Thermo Fisher Scientific), and the concentration was measured by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Reduced Representation Bisulfite Sequencing (RRBS) libraries were prepared using the Premium RRBS kit v.1 (Diagenode, C02030032) according to the manufacturer's instructions. The final concentration of the libraries was measured and their quality checked using the DNA High Sensitivity Assay (Agilent). A total of 64 RRBS libraries were produced, including five samples of sperm from sires of the F0, the unfertilized eggs of the F0 female and 58 F1 gonad samples (three replicates x two sexes x two temperatures x five sires), which resulted in a total of 60 samples, except two females reared at T that were not available. The neomales (males with genetic sex tendency ≥ 0), were removed for future further analysis. Sequencing of the libraries was performed using Illumina HiSeq 2500 instrument at single-end and read length of 50 bp.

Bioinformatic analysis

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 the following 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 $10\times$ coverage. In order to extract methylation data only from CG context (CpG), other types being negligible, we used the *meth-ratio.py* function from BSMAP. Bisulfite conversion efficiency was calculated following the instructions from the RRBS kit. Stringent criteria were implemented to ensure high-quality data by enforcing a minimum alignment percentage ($> 85\%$) and a robust bisulfite conversion rate ($> 98\%$). Any samples failing to meet these criteria were excluded from further analysis. Detailed information on

the reads, sequencing and quality control statistics for all samples are listed in Supplementary Table 11. Subsequent statistical analysis described in the following sections were performed using R software (v. 4.2.2 [65]) and Rstudio (v. 2021.9.0.351, [72]).

Effect of temperature on the genome-wide level

To assess the effect of temperature treatments on DNA methylation, we applied generalized linear models using methylation counts within 200 bp windows as the response variable and tissue and temperature treatments in F0 and F1 as fixed effects, for each region separately. For this analysis, we used the same matrix employed for DMR identification, retaining only regions with data available in at least three samples per group (CC, CT, TC, TT). As tissue-fixed factors showed significant interactions with treatments ($P < 2e-16$), separate analyses were performed for ovaries and testes samples. Thus, a generalized linear model was used to analyze methylation counts for each region, in each sex separately, with the following formula: $\text{glm}(\text{cbind}(\text{numCs}, \text{numTs}) \sim \text{F1 treatment} * \text{F0 treatment}, \text{family} = \text{binomial}, \text{data} = \text{df_pos})$, where numCs and numTs represent the counts of cytosines and total counts, respectively. False discovery rate was controlled for multiple testing using Benjamini-Hochberg (BH) adjustment ($P < 0.05$). In the F1 ovaries ($n = 28$), 47,975 regions (1,343,300 observations) were tested, while in the F1 testes ($n = 30$), 80,679 regions (2,420,370 observations) were analyzed. In regions where the interaction was not significant, regions were classified based on the effect of the sire and offspring treatment: 1) no effect, 2) F0 effect (positive/negative), 3) F1 effect (positive/negative), and 4) additive, both F0 and F1 effects (positive/negative). When the interaction was significant, *emmeans* and *contrast* functions from the *emmeans* package (v.1.8.8, [43]) were used for group means and pairwise comparisons. Significant contrasts between CC vs. CT and TC vs. TT were used to interpret the directionality of sire and offspring treatment effects.

To understand the effect of temperature on sex-related genes, we checked how many of 42 key genes previously known to be involved with sex development and/or maintenance in sea bass [67, 68], were captured in the RRBS libraries. We also checked whether these genes overlapped with DMCs in the CC F1 ovaries vs. testes, at the gene body ± 5000 bp of the transcription starting site (TSS).

Differentially methylated cytosines

The *methylKit* package (v. 1.14.2, Akalin et al. 2012) was used to identify DMCs. CpGs with coverage $> 10\times$ or less than 99.9% of the distribution of read counts were kept for further analysis. Then, coverage was normalized across all samples using *normalizeCoverage* function

with median method. DMCs were detected using a logistic regression model with the family information as covariate and the Chi-squared test with the following criteria: methylation differences larger than 15% and q -value < 0.01 adjusted from the SLIM method, a non-parametric method to adjust p-values and estimate q-values in high-throughput data analyses (Wang et al. 2011). The *genomation* package (v.1.30.0, [1]) was used to annotate the DMCs to the reference genome annotation file.

Differentially methylated regions (DMRs)

DMRs capture the methylation changes in genomic regions, providing more biologically relevant information than isolated changes at individual cytosine sites [28]. The process of identifying DMRs is not fully standardized, as it depends on various factors such as the reference genome and the type of libraries used (whole genome vs RRBS). We chose the MethylKit tools because, as shown in a study by Liu et al. [47], which compared seven bioinformatics tools for DMR detection in RRBS data, MethylKit ranked among the top choices. This was partly due to its ability to avoid over-merging regions or reporting larger DMRs than necessary, as it uses fixed windows and is better suited to the sparse coverage typical of RRBS data.

Additionally, we implemented a custom improvement to this method based on the work of [20] in *Arabidopsis thaliana*. Instead of selecting a window size based on trial-and-error or a standard size (e.g., 100 or 1000 bp), we chose the window size based on additional information from the nature of the samples being studied. The window size was determined by analyzing the Pearson correlation between each CpG site and its neighboring sites iteratively within a 1000 bp window, across all samples. We observed that, across all samples, the correlation generally decreased after 200 bp, leading us to select this as the optimal window size for DMR calculation using MethylKit for our dataset (Supplementary Fig. 3). Such strategy is more precautionary than considering larger regions as DMRs because the 200 bp regions can still be merged in downstream analyses if justified. Besides, this strategy allowed to strike a balance between overly fine-scale analyses at the single nucleotide level and avoiding excessively large DMRs that might obscure meaningful treatment-induced changes. Thus, we selected non-overlapping 200 bp regions with at least 4 CpGs. The median number of CpGs per region was eight. At a window size of 200 bp most of the samples showed correlation above 0.75 dropping significantly with larger window sizes. The methylation level obtained was the mean of all reads included in the window. With the established window size, we performed two sets of comparisons, using regions with data available in at least three samples per group (CC, CT, TC, TT). The first set included four

comparisons of the methylome of ovaries vs. testes of F1 individuals: Control (CC), Developmental (CT), ancestral (TC), and developmental and ancestral (TT). The second set included six comparisons between the different temperature groups of the F1, for each sex separately: CT vs CC, TC vs CC, TC vs CT, TT vs CC, TT vs TC and TT vs TC. We used logistic regression model using family information (sire) as covariate. DMRs were defined with methylation differences higher than 10% and a q -value < 0.05 .

Environmentally induced and inherited epimarks

To study methylation levels across generations, DMCs were used as the unit of analysis, because the mechanisms of inheritance remain unknown and therefore the maximum resolution used to study this is still required. We defined two levels to be considered: *i*) CpGs with stable DNA methylation levels across generations. We classified CpGs using similar criteria than in previous studies [37], considering as *i*) stable CpGs when not differentially methylated (i.e., $< 15\%$ methylation difference, q -value < 0.01); *ii*) CpGs environmentally-induced and inherited when they met the following criteria: the CpGs were DMCs between sires (F0) at C and T and also DMCs between F1 gonads of comparison TC vs CC, analyzed for each sex separately; *iii*) environmentally-induced, inherited and not randomly acquired CpGs. We used the *MethylInheritance* package (v. 1.22.0) [8] to evaluate the number of conserved DMCs between T and C in the parental generation (F0) with those of the filial generation (F1) at C that are due to an inherited environmental effect rather than random chance. This analysis utilized a Monte Carlo algorithm to determine whether DMCs identified in the F0 sperm between C and T groups were transmitted to F1 CC testes and ovaries separately. To assess the statistical significance of the observed number of conserved DMCs, we performed 4,000 permutations for each sex to generate a null distribution by randomly shuffling the sample labels. Statistics were calculated at every 500 permutations during the permutation analysis. By comparing the observed number of conserved DMCs to this null distribution, we aimed to reject the null hypothesis ($P < 0.05$), that the number of conserved DMCs could have been obtained by chance.

Annotation and enrichment analysis of DMCs and DMRs

For the annotation of DMCs and DMRs we used the COMBINED ANNOTATION track of the European sea bass genome (dicLab v1.0c, July 2012). For enrichment analysis we retained only the gene bodies plus regions 5,000 bp upstream of the transcription start site and 5,000 bp downstream of the 3' UTR containing at least one DMC or DMR. To determine over-represented Gene Ontology (GO) categories in the biological processes we

used the PANTHER tool [52] using Fisher's test, which evaluates the null hypothesis that the proportion of genes annotated to a given GO term is the same in the test set as in the background set. For this analysis, the background set included only feasible genes, i.e., those that were covered by RRBS in each comparison, as recommended by Timmons [87]. The human gene database was used as the reference, based on orthology mapping. To account for multiple hypothesis testing, a false discovery rate correction was applied using the Benjamini–Hochberg procedure, and terms with adjusted p -values (q -values) below a significance threshold (e.g., $q < 0.05$) were considered enriched. Additionally, the PANTHER tool was used to map genes to biologically relevant pathways, offering functional insights beyond GO annotation.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13072-025-00630-5>.

Additional file 1.

Additional file 2.

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Author contributions

Núria Sánchez Baizán: Conceptualization, Methodology, Investigation, Formal analysis, Writing—original draft. François Allal and Marc Vandeputte: Provided the genotyping data, Conceptualization, Resources, Funding acquisition. Francesc Piferrer: Conceptualization, Methodology, Resources, Funding acquisition, Supervision, Investigation, Writing—original draft.

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Data availability

The data associated with this study are available on the NCBI platform in the Sequence Read Archive (BioProject PRJNA1060909).

Declarations

Competing interests

The authors declare no competing interests.

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