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1 **Unraveling the genotype by environment interaction in a thermosensitive**  
2 **fish with a polygenic sex determination system**

3  
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19  
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22 M.P., N.S-B, and F.A. analyzed data; and B.G. wrote the paper.

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29 Main Text

30 Figures 1 to 6

31

32 **Abstract**

33 In most animals sex determination occurs at conception, when sex chromosomes are  
34 segregated following Mendelian laws. However, in multiple reptiles and fishes, this genetic  
35 sex can be overridden by external factors after fertilization or birth. In some species, the  
36 genetic sex may also be governed by multiple genes, further limiting our understanding of sex  
37 determination in such species. We used the European sea bass (*Dicentrarchus labrax*) as a  
38 model and combined genomic (using a SNPs chip) and transcriptomic (RNA-Sequencing)  
39 approaches to thoroughly depict this polygenic sex determination system and its interaction  
40 with temperature. We estimated genetic sex tendency (eGST), defined as the estimated  
41 genetic liability to become a given sex under a liability threshold model for sex determination,  
42 which accurately predicts the future phenotypic sex. We found evidence that energetic  
43 pathways, concerning the regulation of lipids and glucose, are involved in sex determination  
44 and could explain why females tend to exhibit higher energy levels and improved growth  
45 compared to males. Besides, early exposure to high temperature upregulated *sox3*, followed  
46 by *sox9a* in individuals with intermediate eGST, but not in individuals showing highly  
47 female-biased eGST, providing the most parsimonious explanation for temperature-induced  
48 masculinization. This gonadal state was maintained likely by DNA methylation and the  
49 upregulation of several genes involved in histone modifications, including *jmjd1c*. Overall,  
50 we describe for the first time a sex determination system resulting from continuous genetic  
51 and environmental influences in an animal. Our results provide significant progress in our  
52 understanding of the mechanisms underlying temperature-induced masculinization in fish.

53 **Significance Statement**

54

55 Traditionally, fish sex determination was considered to be governed by genetic or  
56 environmental factors. However, many teleost species defy this dichotomy. We combined  
57 genomic and transcriptomic approaches to characterize the temperature-dependent polygenic  
58 sex determination of European sea bass. We observed that the estimated genetic sex tendency  
59 (eGST) provides an accurate estimation of the phenotypic sex. Our data support the

60 hypothesis that sexually dimorphic growth is the consequence rather than the cause of sex  
61 determination. We also showed that temperature-induced masculinization involves the  
62 upregulation of *sox3* and *sox9a* for individuals in the middle of the eGST distribution. We  
63 unprecedentedly show that sex determination system is influenced by continuous genetic and  
64 environmental variation that results in variable proportions of males and females.

65

## 66 **Introduction**

67

68 Sex determination is a central biological process with consequences relevant for natural  
69 population dynamics and livestock production. A plethora of systems, from purely genetic sex  
70 determination (GSD) to environmental sex determination (ESD), have been described in the  
71 animal kingdom (1). Sex determination involves the interaction of pro-male and pro-female  
72 genetic pathways in birds and mammals (1), but interestingly, those pathways are often  
73 impacted by various environmental factors in reptiles and fish (2, 3). Undoubtedly, fishes  
74 represent the taxon exhibiting the widest diversity of sex determination systems (4), in which  
75 biotic (e.g. density) (5) or abiotic factors (e.g. pH and temperature) (6, 7) can interact with, or  
76 even override, the genetic background of sex.

77 These external factors affecting the sex of individuals are then transduced at the physiological  
78 level in different manners, depending on the species. Yet, two main routes have been  
79 identified in fishes, one involving the stress-axis pathway (8) and another one involving  
80 epigenetic mechanisms (differential methylation or histone modification) (9). Interestingly,  
81 the latter seems more conserved in reptiles (10, 11) when compared to the former (12).  
82 Temperature, the most studied environmental factor affecting fish sex, has been shown to  
83 either increase cortisol production (the main stress hormone) with cascading effects on sex  
84 (13) or to change methylation profiles in the promoters of key genes mostly involved in sex  
85 differentiation (9).

86 Temperature-dependent sex determination (TSD) has been detected in various fish species  
87 including the Atlantic silverside (*Menidia menidia*) (3), the Nile tilapia (*Oreochromis*  
88 *niloticus*) (14), the olive flounder (*Paralichthys olivaceus*) (15), the African spiny catfish  
89 (*Clarias gariepinus*) (16), the pejerrey (*Odontesthes hatcheri*) or the cobaltcap silverside  
90 (*Hypoatherina tsurugaen*) (17). In these species, natural temperatures within the thermal  
91 range of what fish usually encounter in the wild can impact sexual fate. Moreover, even in  
92 species with a supposedly strong GSD, extreme water temperatures outside the natural  
93 thermal range can sometimes override their sex determination pathway (18–20). In most of

94 the above-mentioned species, sex reversal (usually from female to male) induced by  
95 temperature fluctuations is relatively easy to detect since the genetic sex can be identified  
96 either at the gene (21–25) or at the chromosome level (26, 27), which enables the  
97 investigation of the underlying physiological mechanisms of sex reversal on an individual  
98 basis. Identifying cases of sex reversal becomes much more complicated when species exhibit  
99 a polygenic sex determination system (28). In such instances, each individual presents a  
100 specific combination of pro-male and pro-female genes involved in sex determination  
101 resulting in a genetic sex tendency (GST), defined as the genetic liability to become a male or  
102 a female under a liability threshold model (29) for sex determination. This GST is by  
103 definition continuous, as opposed to the dichotomic pattern found in species with a master  
104 sex-determining gene, in which the presence or the absence of such gene governs sex at  
105 conception.

106 The European sea bass (*Dicentrarchus labrax*) is a gonochoristic species that possesses a  
107 GST whereby the genetic architecture (likely involving many genes) interacts with  
108 temperature during a labile period where sex can be altered before the sexual fate of the gonad  
109 is definitively fixed (30–32). The labile period encompasses the larval and the juvenile stages  
110 (Fig. S1). Thus, as it occurs in many other fish species, exposure to relatively high  
111 temperature (  $> 17^{\circ}\text{C}$ ) during the larval stage promotes male differentiation (30–32).  
112 However, long-term exposure to relatively low temperature (  $< 17^{\circ}\text{C}$ ) before gonadal sex  
113 differentiation is complete (*i.e.*, during the juvenile stage) can also trigger masculinization  
114 (31, 33). In the European sea bass, future females are already bigger when compared to future  
115 males (34), and it has been shown that sex-related differences in growth are established well  
116 before the appearance of the first currently known molecular markers of sex (34). However,  
117 whether being female induces enhanced growth rate or, conversely, if high early growth rate  
118 promotes feminization need to be further studied.

119 The fact that this species possesses a polygenic sex determination system, where temperature  
120 also influences sex determination, has complicated the studies aiming at deciphering the  
121 underpinning mechanisms. Indeed, environmental effects have commonly been detected at the  
122 group level (35–37) or after the labile period (36), and genetic effects are deduced from the  
123 propensity of specific parents to produce a biased sex ratio (32, 38). While these earlier  
124 studies have improved our knowledge of the potential mechanisms involved, they did not  
125 allow the identification of the earliest molecular signs of environmental effects, when the  
126 gonad is not yet differentiated, even at the molecular level.

127 Here, we took advantage of the recently developed 57K SNP chip in the European sea bass  
128 (39) to determine the estimated genetic sex tendency (eGST) of individuals, exposed to either  
129 high or low temperatures. A prediction equation for eGST can be obtained by combining  
130 multilocus SNP genotypes and sex phenotypes in a training population, and then this equation  
131 can be used to estimate the eGST of fish for which only the SNP genotype is available, in a  
132 genomic evaluation framework (40). We predicted that the sex of individuals at both extremes  
133 of the eGST distributions would not be impacted by temperature, while those exhibiting  
134 intermediate values would be sensitive to temperature. To test this hypothesis, individually-  
135 genotyped fish were sampled at four key time points, during the labile period (*SI Appendix*,  
136 Fig. S1). We then used RNA-Sequencing (RNA-Seq) approaches, both at the whole-body and  
137 at the gonadal level, during gonadal sex differentiation. Based on the transcriptomic analysis  
138 of individually genotyped fish, we combined gene expression data with sex prediction  
139 through eGST data, enabling the investigation of the pathways involved in sex determination  
140 of the European sea bass at an early stage. Furthermore, our experimental design allowed us  
141 to test the overlooked hypothesis that masculinization by elevated temperature may result  
142 from sex-specific mortality of females rather than from induced female-to-male sex reversal,  
143 which could not be tested previously, as mortality in larval temperature treatments occurs  
144 before any phenotypic or molecular difference between sexes is visible.

145

## 146 **Results**

### 147 **Sex ratio analysis of control and high-temperature-exposed 1-year-old fish validates the** 148 **eGST model**

149 In individuals reared at 21 °C (high temperature, HT, n=493) from 8 to 390 days post hatching  
150 (dph), the sex ratio was highly biased towards males ( $75 \pm 8.5\%$  males). On the contrary,  
151 those fish that were kept at 16 °C (low temperature, LT, n = 537) from 2 to 59 dph before  
152 being switched to 21 °C had a more balanced sex ratio ( $46.5 \pm 1.4\%$  males), confirming a  
153 strong effect of early exposure to high temperature on the sex of European sea bass (z-value =  
154 -6.3, p-value < 0.001). When combining genomic relationships with the phenotypic sex in a  
155 single trait threshold model, where low and high temperatures were considered a fixed effect,  
156 sex was found to be highly heritable, with a heritability estimate of  $h^2 = 0.56 \pm 0.06$ . In the  
157 multi-trait analysis, where sex in each temperature treatment was considered a specific trait,  
158 both sex at LT (sex\_LT) and at HT (sex\_HT) were also found highly heritable:  $h^2_{\text{sex\_LT}} = 0.65$   
159  $\pm 0.06$  and  $h^2_{\text{sex\_HT}} = 0.51 \pm 0.08$ , with a strong genetic correlation between both temperature-

160 specific sex traits,  $r_G = 0.91 \pm 0.09$ . From a leave-one-out cross-validation approach, where  
161 we predicted the sex of an individual based on a genomic prediction equation established  
162 without providing information on its phenotype, the genomic prediction successfully  
163 classified animals in 74.5% of the cases for the fish reared at LT, and 72.5% for the fish at  
164 HT, based on the area under the curve values of the receiver operator characteristic (ROC)  
165 curves (*SI Appendix*, Fig. S2). Importantly, we did not detect any skew in the distribution of  
166 eGST over time (randomly sampled at the four time points, *SI Appendix*, Fig. S1) when  
167 comparing one temperature to the other (*SI Appendix*, Fig. S3), emphasizing that the skew in  
168 sex ratio observed at high temperatures is due to sex reversal, rather than to genotype-specific  
169 mortality. Based on the observed sex ratio of 1-year-old fish, we predicted that about 25% of  
170 the whole population had sex reversed at HT, and that this would likely concern those  
171 individuals with an eGST lower than 0.5 (i.e. “weak” genetic females exhibiting male sex  
172 differentiation at high temperature see *SI Appendix*, Fig. S10).

173 A genome wide association study (GWAS) identified five genomic regions explaining more  
174 than 2% of the total genetic variance of the GST (*SI Appendix*, Fig. S4), which we considered  
175 as putative quantitative trait loci (QTL). The region with the highest association with sex was  
176 in LG7, between positions 6.3 and 6.8Mb (*QTL\_LG7*) with a 8% variance explained. Two  
177 other regions in LG19 in the region 6.6-7.3Mb (*QTL\_LG19a*) and in the region 17.0-17.9Mb  
178 (*QTL\_LG19b*) explained 3.2% and 2.7% of the genetic variance, respectively. In LG13, the  
179 region 24.0-24.6Mb (*QTL\_LG13*) explained 2.3% of the genetic variance. A last region in  
180 LG1A (25.7-26.3Mb – *QTL\_LG1A*) explained 2.1% of the genetic variance for GST.

181

### 182 **Sox genes and genes linked to histone modification processes are affected by high** 183 **temperature in fish with intermediate eGST values at the “flexion” stage**

184 Whole-body RNA-Seq was performed at the “flexion” stage (25-40 dph), coinciding with the  
185 early stages of the labile period for sex determination (*SI Appendix*, Fig. S1). At this stage,  
186 only one germ cell per future gonad is observable in transversal fish sections (41). Ten  
187 individuals (five per treatment) were selected for their intermediate, though positive eGST.  
188 The DESeq2 analysis highlighted 341 differentially expressed genes ( $p$ -value < 0.05) between  
189 HT and LT individuals. The GOs response to steroid hormone and cellular response to steroid  
190 hormone stimulus, were among the biological processes upregulated at HT (*SI Appendix*, Fig.  
191 S5). Specifically, we found that two sox (Sry-related HMG box) genes classically involved  
192 in sex determination and differentiation, *sox3* and *sox9b*, were upregulated at HT (Fig. 1A,

193 B). Seven genes were linked to histone modification processes, two of them upregulated at  
194 HT (*ncoa6* and *lpin1*), and five upregulated at LT: *ube2a*, *zbtb7b*, *setd5*, *suz12*, and *auts2*.

195 Fig. 1. Both A) *sox3* and B) *sox9b* were differentially expressed following the DeSeq2  
196 analysis between neutral individuals ( $0 < eGST < 0.5$ ) at the “flexion” stage kept at high  
197 temperature (in red) and low temperature (in blue). The number of RNA-Seq transcripts of C)  
198 *sox3* and D) *sox9a* differed according to the temperature between groups of  $eGST < -0.5$ ,  $-0.5$   
199  $< eGST < 0$ ,  $0 < eGST < 0.5$  and  $eGST > 0.5$  in fish sampled at the “all fins” stage following  
200 the DeSeq2 analysis. The RNA-Seq analysis at the all fins stage revealed an overall negative  
201 and significant correlation between E) *sox3* and eGST and F) *sox9a* and eGST, as well as a  
202 temperature effect. Abbreviations: \*\*\*= p-value < 0.001; \*\* = p-value < 0.01, \* = p-value <  
203 0.05 ; ns= not significant.

204

### 205 **Sox genes and genes linked to energy regulation correlate with eGST at the “all fins”** 206 **stage**

207 Whole-body RNA-Seq was performed at the “all fins” stage (53-78 dph period) on 68  
208 individuals (29 LT and 39 HT). This period coincides with rapid primordial germ cell  
209 proliferation (41). Among the 17303 genes that respected our inclusion criteria (> 30 reads per  
210 gene), we detected 584 genes for which expression was correlated with the eGST (eGST p-  
211 value < 0.05;  $eGST \times T (^{\circ}C) > 0.5$ ). Twelve and two genes, respectively (*SI Appendix*, Table  
212 S1), were part of the GOs sex differentiation and sex determination, among which *sox9a* and  
213 *sox3* (p-value = 0.052) (Fig. 1E, F). For both these genes, we also detected a strong  
214 temperature effect on transcript number (p < 0.001), with a higher number of transcripts at HT  
215 compared to LT (Fig. 1C, D).

216 Sixteen genes were involved in the GO lipid biosynthetic process and 19 in the GO regulation  
217 of growth (*SI Appendix*, Table S1). The gene encoding the growth hormone (*gh*) was one of  
218 these genes, and was positively and significantly correlated with the eGST. Three other genes  
219 (*prkca*, *gf1b* and *eya2*) that are in close vicinity of the three previously detected QTL  
220 (*QTL\_LG7*, *QTL\_LG19b* and *QTL\_LG1A*) exhibited a significant correlation with the eGST,  
221 though their expression was independent of their SNP genotype of the QTLs (AA, AB or BB).  
222 The “response to glucose” was among the biological processes presenting a positive  
223 correlation with the eGST, and thus more expressed in females (*SI Appendix*, Fig. S6). Eleven  
224 genes involved in the GO histone modification were also significantly correlated to the eGST  
225 (*SI Appendix*, Table S1). Interestingly, the genes from the GO “histone H3-K27 methylation”  
226 and “histone H3-K4 methylation” were negatively correlated with the eGST (thus more  
227 expressed in males; *SI Appendix*, Fig. S6).



228 Using a more stringent significance threshold (p-value < 0.001), four genes (*spry1*, *egfr*, *dpp4*,  
229 and *dzip1*) were correlated to the eGST. Only the gene encoding the Daz interacting protein 1  
230 (*dzip1*), involved in spermatogenesis, showed a clear dimorphic expression higher for  
231 individuals with negative eGST. The three other genes have a role in growth rate (Epidermal  
232 growth factor receptor, *Egfr*; Sprouty rtk signaling antagonist 1, *Spry1*) and glucose (The  
233 dipeptidyl-peptidase IV, *Dpp4*) regulation (based on their gene ontology). The first axis of a  
234 PCA, representing these four genes (Fig. 2A), was highly correlated to the eGST (Fig. 2B).  
235 With a quadratic model, only seven genes (*SI Appendix*, Table S1) showed both an overall  
236 linear relationship with eGST (p < 0.01) and significant interaction with temperature, revealed  
237 by a temperature-specific quadratic component (p-value < 0.05). Three of these genes were  
238 involved in epigenetic processes: *sgsm2*, *entpd2*, and *map3k3*.

239

240 Fig. 2. A) Principal Component Analysis (PCA) of four genes (*dzip1*, *dpp4*, *egfr* and *spry1*)  
241 having a highly significant (p-value < 0.001) and linear correlation with the sex tendency  
242 (eGST) and detected from the RNA-Seq analysis of whole individuals at the “all fins” stage.  
243 B) The first component axis strongly correlated to the eGST. C) The energy content  
244 (joules.mg<sup>-1</sup> of tissue) of fish sampled at the “all fins” stage correlated positively with eGST,  
245 so that genetic females displayed slightly higher energy content than males. Fish kept at low  
246 temperatures also displayed higher energy content than those kept at high temperature.  
247 Individuals are represented with a color gradient, from maroon to yellow, representing their  
248 eGST. Circles represent fish kept at high temperature (HT = 21 °C; n = 39); and triangles  
249 those kept at low temperature (LT = 16 °C; n = 29). Abbreviations: ns, not significant.

250

## 251 **The juvenile gonadal transcriptome faithfully reflects the underlying eGST** 252 **independently of temperature influences**

253 RNA-Seq was performed on total RNA extracted from the gonads of 42 individuals (21 HT  
254 and 21 LT) sampled at the juvenile stage (117-124 dph), before the first signs of  
255 morphological sex differentiation (*SI Appendix*, Fig. S1). Among the 15724 genes that  
256 respected our inclusion criteria, 1297 showed a significant (p-value < 0.01) linear correlation,  
257 either positive or negative, between their expression level and the eGST, independently of the  
258 initial temperature treatment (HT vs LT). Among those genes, nineteen and six genes (*SI*  
259 *Appendix*, Table S3) were within the gene ontologies (GOs) of sex differentiation and sex  
260 determination, respectively, including *cyp19a1a* (gonadal aromatase), *foxl2* (forkhead box l2),  
261 *dmrt1* (doublesex and mab-3 related transcription factor 1), *gsdf* (gonadal soma derived  
262 factor), *amh* (anti-Müllerian hormone), *sox9a* (sry-related HMG box 9a), and *insr* (insulin  
263 receptor). Those genes, well described to be involved in sexual development, allowed to

264 distinguish two groups on the first axis of the Principal Component Analysis (PCA): the  
265 differentiating males as opposed to the differentiating females (Fig. 3A). As expected, the  
266 correlation was positive for genes involved in ovarian development and negative for those  
267 involved in testis development (*SI Appendix*, Fig. 3B). This was confirmed by genes involved  
268 in the GO steroids metabolic process, namely *hsd17b1*, *cyp26a*, and *3 $\beta$ -hsd* (*SI Appendix*, Fig.  
269 S7).

270 Fig. 3. A) Principal component analysis (PCA) of 7 genes involved in sex determination and  
271 differentiation. Data are from the RNA-Seq analysis of the gonads of fish at the juvenile stage  
272 ( $n = 42$ ). The PC1 separated the sex horizontally and explained 88.4% of the variance. The  
273 PC2 separated the variables vertically and explained 4.1% of the variance. The contribution of  
274 the variables (genes) are represented by the arrows. B) Significant ( $p < 0.01$ ) linear correlation  
275 between the estimated genetic sex tendency (eGST) and both *insr* and *sox9a* (relative number  
276 of transcripts on the y axis). For five genes, *cyp19a1a*, *foxl2*, *dmrt1*, *amh*, *gsdf*, and the PC1  
277 axis, a dichotomic distribution was observed and modelled with a “quasibinomial” function.  
278 Circles represent fish kept at high temperature (HT = 21 °C;  $n = 21$ ); and triangles those kept  
279 at low temperature (LT = 16 °C;  $n = 21$ ). Individuals are represented with a color gradient,  
280 from maroon to yellow, representing their lower or higher eGST. Abbreviations: \*\*\*= p-  
281 value  $< 0.001$ ; \*\* = p-value  $< 0.01$ .

282

283 Overall, this allowed ascertaining the high relevance of the GST estimated with the Gibbs  
284 model (eGST), especially for individuals at both extremes of the distribution, independently  
285 of the temperature. Our results were further validated at the group level (eGST  $> 0$  = genetic  
286 females vs eGST  $< 0$  = genetic males) with DESeq2 on the GO of sex determination (Fig.  
287 S8). Fifty-two genes involved in the GO histone modification were also significantly  
288 correlated to the eGST (*SI Appendix*, Table S2), which was confirmed with the “without *a*  
289 *priori* approach” showing that genes involved in histone methylation and acetylation were  
290 also up- or downregulated in differentiating gonads (*SI Appendix*, Fig. S9). Interestingly,  
291 other epigenetic processes such as those involved in the miRNA production, were negatively  
292 correlated with the eGST, and thus positively with maleness (*SI Appendix*, Fig. S9). With the  
293 quadratic model used for detecting changes linked to the temperature in the middle of the  
294 eGST distribution, only seven genes (*thop1*, *paxip1*, *sik3*, *jmjd1c*, *bcor*, *wiz*, and *auts2*)  
295 showed both an overall linear correlation with eGST ( $p < 0.01$ ) and a significant interaction  
296 with temperature for the quadratic term (p-value  $< 0.05$ ). Four of these genes are involved in  
297 epigenetic processes: *jmjd1c*, *bcor*, *wiz*, and *auts2*. The expression of these four genes  
298 increased in individuals with an eGST in the middle of the distribution and that were reared at  
299 HT, which are the ones with a weak genetic sex determination that are expected to be more  
300 influenced by the environment (Fig. 4).

301

302 Fig. 4. Quadratic correlation between the estimated genetic sex tendency (eGST) and genes  
303 involved in histone modification, detected from the RNA-Seq analysis of the gonads of fish at  
304 the juvenile stage. The four genes exhibit a significant (\*\*= p-value < 0.01) linear correlation  
305 with the eGST, plus a significant (\*= p-value < 0.05) interaction with the temperature for the  
306 quadratic term ( $T^{\circ}C^2$ ). Red and blue points represent respectively fish kept at high  
307 temperature (HT = 21 °C; n = 21) or low temperature (LT = 16 °C; n = 21).

308

### 309 DNA methylation levels of 1-year-old fish gonads

310 Reduced Representation Bisulfite Sequencing (RRBS) was conducted at the 1-year-old fish  
311 stage using gonadal tissue from 65 males and 42 females. The statistical analysis of  
312 methylation data showed several differentially methylated cytosines (DMCs) between fish  
313 reared at LT and HT in *sox3* and *sox9a* genes (Fig. 5). For *sox3* there was a decrease of  
314 methylation levels at HT in both males ( $P = 0.01844$ ), and females ( $P = 0.0001636$ ). In males,  
315 this gene showed seven hypomethylated DMCs in the first exon, close (< 200 bp) from the  
316 transcription start site (TSS; Fig. 5A). In the females, the same positions were  
317 hypomethylated in the first exon, with a total of up to 15 DMCs detected, among which two  
318 of them, found around 600 bp from the TSS, were hypermethylated (Fig. 5B). The methylation  
319 levels of *sox9a* showed an increase at high temperature in males (p-value = 0.01069), but no  
320 significant difference in females (p-value = 0.1866) between LT and HT (Fig. 5 C-F). In  
321 males, there were three hypermethylated DMCs towards the end of the gene body (Fig. 5C).  
322 However, three out of the five DMCs identified in this region were hypomethylated in  
323 females at HT (Fig. 5D).

324

325 Fig. 5. Boxplots of DNA methylation levels of *sox3* (A, B) and *sox9a* (C, D) in of 1-year-old  
326 fish testes (maroon) and ovaries (yellow), respectively. Individual DMCs identified within the  
327 gene region (left side), and average methylation levels of the gene body  $\pm 2000$  bp (right  
328 side). The black line within the box indicates the median of the distribution, and the lower and  
329 upper hinges display the distribution of values between the first and third quartiles. The upper  
330 whisker extends to the maximum value ( $1.5 * \text{interquartile range (IQR)}$ ), and the lower  
331 whisker extends to the minimum value ( $1.5 * \text{IQR}$ ). Individual DMCs are defined as CpGs  
332 with methylation differences > 15% and  $q$ -value < 0.01, while significant differences between  
333 average data were assessed with the  $t$ -test. Abbreviations: \*\* = p-value < 0.01; ns, not  
334 significant. Circles represent fish kept at high temperature (HT = 21 °C; n = 65); and triangles  
335 those kept at low temperature (LT = 16 °C; n = 42).

336

### 337 **Gonadal histology**

338 The sampling at the juvenile stage (117-124 dph; n = 10 fish per temperature) confirmed that  
339 gonads were still not morphologically differentiated. Nevertheless, some oocytes were  
340 sparsely observable, but it was impossible to conclude with confidence on the actual  
341 phenotypic sex of individuals based on histological analyses alone. Furthermore, the number  
342 of oocytes was not correlated to the eGST.

### 343 **Relationship between energy content, body size and eGST**

344 The energy content (joules.mg<sup>-1</sup>) was not significantly correlated to the eGST at any stage,  
345 though it almost reached significance (p = 0.054) at the “all fins” stage, with genetic females  
346 tending to have higher values than males regardless of their size (Fig. 2C). Individuals from  
347 LT presented significantly (p-value < 0.001) higher energy content than those from HT at the  
348 “all fins” stages (Fig. 2C), likely because LT fish were older. The size of fish was not  
349 correlated to the eGST (length: t-value = 1.56, p-value = 0.13; wet weight: t-value = 1.3, p-  
350 value = 0.2), while there was an effect of temperature (length: t-value = 4.6, p-value < 0.001;  
351 wet weight: t-value = 4.75, p-value < 0.001).

### 352 **Discussion**

353 Our analysis combining genomic and transcriptomic data allowed to shed new light on the  
354 mechanisms involved in temperature-induced masculinization of the European sea bass. The  
355 results confirm the high heritability of the GST. Furthermore, the high genetic correlation  
356 between sex\_LT and sex\_HT suggested low genotype-by-temperature interaction. In other  
357 words, the ranking of animals based on their eGST remains very similar at least across the  
358 two tested thermal environments. Hence, the effect of larval rearing temperature on sex was  
359 mostly additive. This appears to contradict previous results where such interaction occurred  
360 (31, 38). However, these previous results were obtained using between-family variation, while  
361 we used a genomic relationship matrix for the present study, which is expected to accurately  
362 estimate the true genetic parameters (42). Note that the low genotype-by-environment  
363 interaction for GST between the two temperature treatments accounts for what happens  
364 globally. But it does not impede local GxE interaction occurring at the genes level or the  
365 existence of GxE interactions in other populations and/or under other environmental  
366 circumstances. The prediction equation for GST, which allowed us to estimate the eGST of  
367 genotyped individuals, was established on phenotypically sexed individuals at one year of  
368 age, and predicted their sex with a 72.5-74.5 % success. The relevance of eGST was further

369 confirmed by transcriptomic analysis of gonads at 117 and 124 dph, i.e. when the first signs of  
370 molecular differentiation can be identified (43). Indeed, we detected a strong correlation  
371 between key genes involved in sex differentiation in the gonad and the eGST at this juvenile  
372 stage. This good match between phenotypic and genetic sex allowed us to determine, for the  
373 first time, the eGST of one to three-month-old individuals, when the temperature is known to  
374 act on the sex of European sea bass (30). Five putative QTLs, explaining a low but significant  
375 part of the variance of GST, were identified in four different chromosomes, (LG7, LG19,  
376 LG13 and LG1). Faggion et al. (44) already identified *QTL\_LG7* in Northern Atlantic and  
377 Mediterranean populations of European sea bass and the two LG19 QTLs in Mediterranean  
378 populations only (origin of the present population). The minor QTLs found in LG13 and  
379 LG1A were, however, specific of this study. None of the QTLs previously found in LG6,  
380 LG11 and LG18-21 (45) were detected in the present study. Overall, our results are consistent  
381 with those of previous studies, pinpointing a GST strongly driven by polygenic variation (~  
382 90% of the variance) with a low contribution of minor QTLs (~10%).

383 These QTLs however participate to the accuracy of the model, which appeared to be strong  
384 (100%) for individuals at both extremes of the eGST distribution. However, some mismatches  
385 were detected in the middle of the distribution, with some individuals with low negative  
386 eGST value that likely were phenotypic females (15% at HT and 29% at LT) based on the  
387 dichotomic expression of *cyp19a1a*, *foxl2*, *dmrt1*, *gsdf* and *amh*; and some individuals with  
388 positive eGST values that were likely phenotypic males (25% at HT and 14% at LT). The  
389 proportion of fish that are supposedly genetic females, but that exhibit a male phenotype  
390 (25%), could well be explained by precocious and relatively long-term exposure to HT (31,  
391 46, 47). It also corresponds well to the supposed percentage of masculinized genetic females  
392 observed at the end of the experiment: 25%, a figure within the range of masculinization  
393 typically observed in European sea bass exposed to HT (48). The mismatch occurring at low  
394 temperature (14%) could also be due to the masculinization of fish kept too long at this  
395 temperature, since there is what could be regarded as a second period of sensitivity to adverse  
396 environmental conditions, including prolonged exposure to low temperature, in European sea  
397 bass (31, 37, 46). However, temperature itself might not explain the pattern detected for  
398 individuals that are supposedly genetic males. This could come from the fact that some errors  
399 occurred in the estimation of eGST (as detected in one-year-old fish, with the leave-one-out  
400 approach), which is expected with a polygenic trait that typically has incomplete penetrance,  
401 when heritability is lower than 1, which is the case here (49, 50). It could also be that  
402 phenological events linked to gonad development are involved. In the European sea bass, the

403 sex is considered “fixed” once animals reach a size of 8 to 10 cm according to some studies  
404 (41) but at a size of 4 to 6 cm according to others (34). It is thus possible that individuals in  
405 the middle of the distribution can still develop a phenotypic sex not predicted by their eGST,  
406 according to the polygenic nature of sex determination in this species, and that their observed  
407 transcript values of sex-related genes remain transitory at this stage/age (7.2 cm and 4.5 g). In  
408 this sense, the histological analysis did not permit to unambiguously identify males and  
409 females at the early juvenile stage, but some intra-testicular oocytes were detected in some  
410 individuals, showing that they probably had undergone sex reversal (51). In European eels  
411 (*Anguilla anguilla*), individuals presenting intra-testicular oocytes showed higher levels of  
412 *cyp19a1a* than males (52). It is thus possible that the presence of both tissues in the same  
413 gonad drives the mismatch observed between transcript values of sex-related genes and eGST  
414 for intermediate individuals. A last plausible explanation involves the sampling design at this  
415 stage for transcriptomic analysis. To ensure having sufficient tissue quantity (> 100 ng total  
416 RNA), we sampled the biggest fish. Since early growth rate is known to impact sex, it is  
417 possible that some of the genetic males (eGST < 0) developed as phenotypic females at this  
418 stage.

419 Interestingly, the goodness of the linear fit between genetic and phenotypic sex was  
420 confirmed by the gene expression of *sox9a* and *insr*, even when considering individuals in the  
421 middle of the eGST distribution and at both temperatures. These two genes are within the GO  
422 of sex determination and play a key role in male sex differentiation (1, 53–55). None of them  
423 were pinpointed as essential for sex differentiation in previous studies on European sea bass  
424 (35, 43, 56). However, both genes are known to be overexpressed at high temperatures in  
425 TSD reptile species during sex determination (57–59). The fact that both genes present a very  
426 linear correlation (as opposed to the dichotomic expression of *cyp19a1a*, *foxl2*, *dmrt1*, *gsdf*,  
427 and *amh*) with the eGST suggests that they are involved early in the process of sex  
428 determination, while the other sex-related genes just transduce the future state of the gonad  
429 (male or female). This was confirmed at the “all fins” stage with *sox9a* exhibiting a negative  
430 correlation with the eGST, with higher expression at high temperatures for neutral fish. This  
431 gene was also shown to play a key role in the ovary-to-testis transition in the zebrafish (60),  
432 where it was strongly expressed in pre-Sertoli cells prior to oocyte apoptosis and  
433 degeneration. The DNA methylation levels of *sox9a* increased with high temperatures in  
434 testes of 1-year-old fish. This suggests that HT could affect *sox9a* gene expression already at  
435 the “flexion” stage and maintain its state through to adulthood in males. However, the higher  
436 expression of this gene found at HT for neutral eGST fish and the hypermethylation detected

437 at HT in males would not match the standard association of hypermethylation with  
438 downregulation of gene repression (61). This could be explained by the fact that the DMCs  
439 were identified towards the end of the gene body, while it is the methylation level of the first  
440 intron, and to a lesser extent, the first exon, which was shown to play an important role and  
441 inverse association with gene expression regardless of tissue and species (62).

442 Another gene that was also reported as a marker of germ cells and supporting cells that  
443 preferentially develop into testes in zebrafish juvenile ovary-to-testis transformation (60) was  
444 *dzip1*, which is concordant with our results where *dzip1* shows a clear dimorphic expression  
445 at the “all fins” stage, predominantly at HT. Three other genes were strongly correlated with  
446 the eGST at this stage, namely *egfr*, *dpp4*, and *spry1*. The specific function of these genes has  
447 not been described in fish and much of our knowledge comes from murine models and  
448 humans. Sprouty1 (*Spry1* gene product) is involved in fat and bone development (63). *Spry1*  
449 gene knockout in mice adipocytes results in decreased bone mass and increased body fat (63),  
450 while adipose tissue-specific expression of the *Spry1* gene in mice protects against fat  
451 accumulation and bone loss (64). EGFR is involved in protein kinase activity and the  
452 inhibition of such protein was shown to improve glucose tolerance and favor insulin action  
453 (65). Interestingly, its expression was shown to be regulated by *spry1* (66). DPP4 is also  
454 involved in glucose homeostasis and targeted inactivation of this gene in mice yielded  
455 individuals with enhanced insulin secretion and improved glucose tolerance (67). All those  
456 studies advocate for genetic males having less capacity to produce fat and display appropriate  
457 glucose levels. Although this could be viewed as only a conjunction of facts, this hypothesis  
458 is enforced by the tendency of genetic females (individuals with high eGST values) to display  
459 higher energy content in their tissue (in joules/mg), when compared to males (individuals with  
460 low eGST values) at the “all fins” stage. At this stage (53-78 dph), no correlation was found  
461 between size and eGST, and the earliest sexual size dimorphism was found at 103 dph in  
462 another experiment (68). Overall, all these results support the hypothesis that early growth  
463 differences are the consequence rather than the cause of sex differentiation. In that scheme,  
464 the polygenic sex determination of the European sea bass provides information that is later  
465 transduced at the phenotypic level, as exemplified by the positive correlation between eGST  
466 and the *gh* gene at the “all fins” stage.

467 Regarding the role of temperature, at both the “flexion” and the “all fins” stages we detected  
468 enhanced expression of *sox3* at a high temperature compared to a low temperature, while this  
469 was not detected for genetic females (eGST > 0.5) at the “all fins” stage following  
470 transcriptomic analysis. Sox3 is the evolutionary precursor of Sry (sex determining region Y)

471 in mammals (69) and a major master-sex determination gene in three medaka fish species  
472 (70). Interestingly, ectopic expression of Sox3 in the developing XX gonads resulted in the  
473 complete sex reversal of XX females to males in mouse (*Mus musculus*) (71), and loss-of-  
474 function of *sox3* caused sex reversal of XY males in the Indian rice fish (*Oryzias dancena*)  
475 (72). The expression of *sox3* also gradually increased during the protogynous sex change  
476 (female-to-male) of the hermaphroditic fish (*Epinephelus coioides*) (73). This gene is  
477 essential in the upregulation of downstream key-related genes for testicular differentiation,  
478 *gsdf* in the Indian rice fish (72) and *sox9* in mouse (71). Here we also detected that  
479 upregulation of *sox3* appeared chronologically before the upregulation of *sox9a* suggesting  
480 similar mechanisms. *Sox3* was strongly hypomethylated at HT in both males and females.  
481 These methylation levels were very close (< 200 bp) from the transcription start site and  
482 within the first exon. The methylation and gene expression data together suggest that HT  
483 could affect *sox3* expression at the flexion stage through DNA hypomethylation-mediated,  
484 unlocking of gene expression and continuing this state to adults through mitosis. The  
485 hypomethylation levels found in this gene at 1-year-old gonads and the higher levels of  
486 expression of this gene at HT found at earlier stages match the standard negative correlation  
487 between DNA methylation and gene expression (61). It is highly difficult to assess whether  
488 the observed methylation changes are the cause or the consequence. However, DNA  
489 methylation is known to contribute to the acquisition and maintenance of cell identity, making  
490 it possible that changes in DNA methylation during sex differentiation contribute to stabilize  
491 the gene expression program of each sex. Regarding the exact function of *sox9a* and *sox3*,  
492 only a proper experiment involving the knockout of these genes would allow to fully  
493 understand their role in temperature-induced masculinization.

494 Regarding other epigenetic mechanisms, genes from the “histone modifications” GO term  
495 were always differentially expressed in all our analyses, confirming their implication in the  
496 sex-specific response to temperature (75). However, the specific upregulation of DNA  
497 methyltransferases (DNMTs), which are key in the regulation of DNA methylation, was never  
498 detected. This was unexpected owing to the previous demonstration of specific methylation of  
499 promoters of both *cyp19a1* and *dmrt1* in males and females of European sea bass,  
500 respectively (9, 36). Indeed, epigenetic reprogramming mediated by changes in sexually  
501 dimorphic DNA methylation has been suggested to be a key mechanism in the determination  
502 of sexual fate in sexually isogenic species (76, 77). Further, we found that key Jumonji family  
503 (Jmj) gene *jmjdlc*, involved in histone demethylation at the H3K9 site, was upregulated in  
504 male gonads compared to female gonads. Additionally, *jmjdlc* expression was highly



505 impacted by temperature in individuals in the middle of the eGST distribution, with  
506 upregulation in the gonads of HT-exposed individuals and downregulation in those exposed to  
507 LT. Interestingly, this signal was still detectable in gonads of LT fish even after the LT  
508 treatment had ended (e.g. fish from the LT treatment were at 21 °C from day 60). It has been  
509 hypothesized that temperature may reset epigenetic marks thus redirecting sexual fate (1),  
510 supporting our observed results. In certain reptilian species, some specific and related histone  
511 demethylases (JMJD3, JARID2, and KDM6B), were shown to be crucial in the shaping of the  
512 gonadal phenotype during temperature-induced sex-determination (10, 78). Furthermore,  
513 although the results here presented are not sufficient to infer the functionality of genes *bcor*,  
514 *wiz*, and *auts2*, to the best of our knowledge this is the first study to suggest that these genes  
515 could be involved in the transduction of temperature signals influencing sexual fate in  
516 teleosts, which warrants further examination.

517 To conclude, we found evidence that sex reversal rather than genotype-specific mortality was  
518 the cause of some mismatches between the sex predicted by the eGST and the actual  
519 phenotypic sex. We did not find any evidence that stress-axis activation was involved in  
520 masculinization. Rather, our data supports the involvement of conserved sex-related  
521 pathways, epigenetic and energetic processes as key to understand temperature-induced  
522 masculinization in the European sea bass. We propose a model where the GST of individuals  
523 drives the specific expression of genes involved in lipid and glucose metabolism,  
524 independently of temperature (Fig. 6). In that scheme, individuals presenting higher energy  
525 content (transduced by higher transcript levels of genes involved in lipids and glucose  
526 production) would become females and those with lower energy would become males (Fig.  
527 6). This may explain the early sexually dimorphic differences in growth usually observed in  
528 European sea bass, and why domestication (and selection for growth) leads to an increased  
529 proportion of females (79). Once the sex is fixed, a classical cascade of genes involved in sex  
530 differentiation is activated, starting by *sox9a*. However, if fish are exposed very early to high  
531 temperatures, this first triggers an upregulation of *sox3* that is then followed by an  
532 upregulation of *sox9a*, determining the sex of individuals in the middle of the eGST  
533 distribution (Fig. 6). This signal is then maintained, likely thanks to epigenetic processes  
534 (DNA methylation and histone modifications, e.g. through *jmjdlc*), which leads to individuals  
535 with intermediate eGST values developing as males.

536 From an adaptive and evolutionary point of view, the conditions favoring the emergence of  
537 ESD over GSD have been extensively discussed (28, 80). But species where both strategies  
538 coexist, with additive effects, provide new challenges for evolutionary scientists.

539 Understanding how external factors can override genetic information, affecting an essential  
540 trait such as phenotypic sex is indeed of major importance in a global warming context.  
541 Furthermore, the insights provided by this study on European sea bass can help to illuminate  
542 other systems where temperature, stress or other environmental cues can override a GSD  
543 system, as reported in vertebrates, from fish to mammals (81). The present study, therefore,  
544 helps pave the way for our understanding of such mechanisms: the genetic architecture likely  
545 provides information linked to the regulation of energy and growth, constituting a strong  
546 example in support of the hypothesis linking metabolism and sex determination (82). Any  
547 environmental cues that might affect this relationship (*e.g.* temperature influencing  
548 metabolism) will trigger a specific expression of a cascade of genes that will, in turn, affect  
549 the phenotypic sex of sensitive genotypes.

550

551 Fig. 6. Summary of the polygenic sex determination system of the European sea bass. In the  
552 top panel (temperature-independent sexual phenotype), all fish have their sexual genotype  
553 (estimated genetic sex tendency, eGST) within a Gaussian like distribution, with those that  
554 present a male tendency (maroon) in the left extreme and those that present a female tendency  
555 (yellow) in the right extreme. The genes involved in lipid and glucose (in dashed square)  
556 regulation are correlated to the eGST, which likely explain the difference in energy content  
557 between future males and future females at the “all fins” stage (around 60 dph). This  
558 “energetic” information likely drives sex determination, starting with the overexpression of  
559 *sox9a*. At 120 dph, the gonad is undergoing molecular sex differentiation involving classical  
560 sex pathways. Those in the middle of the distribution can still change sex. Now, if European  
561 sea bass are kept at high T (°C), these results in the overexpression of *sox3* and *sox9b* for  
562 individuals with a relatively “low” female eGST at 30 dph and that is conserved at 60 dph,  
563 followed by an increase in the expression of *sox9a*. This sexual phenotype is then likely  
564 maintained thanks to the overexpression of key genes involved in histone modification at  
565 high, but not low, temperatures for individuals in the middle of the eGST distribution.  
566 Twenty-five percent of the population is then likely masculinized at HT following these  
567 processes.

568

## 569 **Material and Methods**

570

571 SI Appendix provides a detailed description of the materials and methods used in this study.

572

## 573 **Fish production and rearing**

574

575 The fish population used was the result of a mating design including eight males and one  
576 female from a West Mediterranean Sea strain of European sea bass, performed by artificial  
577 fertilization (22/03/2017). Fertilized eggs were incubated at 14 °C until 48 hours post-

578 fertilization. Eggs were then evenly dispatched in six tanks of 500 L each, and the  
579 temperature was gradually increased from 14 °C to 16 °C within one day. Following hatching,  
580 fish density was of 50 larvae per liter. Larvae were then exposed to 21 °C (HT) in triplicates  
581 or kept at 16 °C (LT) in triplicates, as described in (83). HT treatment consisted in gradually  
582 increasing temperature to reach 21 °C, from 3 dph to 8 dph. From 10 dph onwards, fish were  
583 fed *Artemia* nauplii for 40 days, then weaned on a commercial sea bass diet (Pro Start and Pro  
584 Wean, BioMar, Nersac, France). For the LT treatment, the temperature was also increased  
585 from day 59 (1 °C/day) to day 64, to reach 21 °C. Fish were reared at the Ifremer Plateforme  
586 Expérimentale d'Aquaculture (Palavas-les-Flots, France), accredited to use and breed  
587 laboratory animals (n°C341926), and the project was approved by the Animal Care  
588 Committee # 36 COMETHEA under project authorization number APAFIS 19676.

589

### 590 **Sampling**

591

592 Fish were evenly sampled in the triplicates of each temperature treatment, at four different  
593 developmental stages. Since fish growth is favored at high temperature, the development of  
594 fish generally greatly differs between thermal treatments. Hence, to enable data comparison  
595 between individuals kept at different temperatures, the first three samplings were carried out  
596 at the same sum of degree-day (base 10 °C, DD<sub>10 °C</sub>), a procedure previously used to allow  
597 standardized measurement of growth in fishes (84). Thus, larvae were not collected at the  
598 same date, but at the same DD<sub>10 °C</sub>: 77 DD<sub>10 °C</sub>, 242 DD<sub>10 °C</sub>, and 550 DD<sub>10 °C</sub> (see Table S4  
599 for details regarding stage, age and size). The fourth and last sampling of juveniles, aiming at  
600 obtaining developing gonads, was, however, based on size rather than age, since growth  
601 difference between treatments declined with age (Table S4, Fig. S1). For this reason, gonads  
602 were sampled on juveniles at 117 dph (HT) or 124 dph (LT). At the end of the experiment,  
603 after one year (at 390 dph), all fish were euthanized using benzocaine (150 mg/L), measured,  
604 weighed, and sexed by *in situ* gonad examination. All samples were genotyped with an SNP  
605 chip (see below), but only subsamples of those performed at 242 DD<sub>10 °C</sub>, 550 DD<sub>10 °C</sub>, and  
606 117-124 dph were used for molecular analysis (*SI Appendix*, Table S4).

607

### 608 **Genotyping**

609

610 We genotyped three generations of European sea bass using the ThermoFisher DlabChip  
611 European sea bass array of 57k SNP markers (Griot et al. 2021). Generation 0 (G0) includes

612 the parents of the 8 sires. Generation 1 (G1) corresponds to the parents (the dam and the 8  
613 sires). Generation 2 includes the 2030 offspring, composed of the larvae sampled at  
614 77 DD<sub>10</sub>°C (n = 192), at 242 DD<sub>10</sub>°C (n = 300) and at 550 DD<sub>10</sub>°C (n = 280), the juveniles  
615 collected at 117 dph (HT, n = 70) and 124 dph (LT, n = 70), and fish sexed at 390 dph  
616 (n = 1118). Fish were sampled at random at each stage, and the number of fish per family was  
617 known a posteriori following genotyping and parentage assignment (*SI Appendix*, Table S4).  
618 Complete details of the genotyping analysis can be found in *SI Appendix*, Materials and  
619 Methods.

620

### 621 **Heritability, estimate genetic sex tendency prediction and genome wide association scan**

622

623 We predicted the sex using a liability threshold model, which has been used in a large variety  
624 of settings, often with diseases (29, 85), but also for sex determination (28, 32). With this  
625 model the binary phenotype is the realization of an underlying continuous phenotype, the  
626 liability trait, here called phenotypic sex tendency (PST). When PST exceeds a given  
627 threshold, animals differentiate as females, while they differentiate as males when PST  
628 remains below the threshold (*SI Appendix*, Fig. S10). The phenotypic sex tendency is itself  
629 the addition of a genetic and an environmental sex tendency. In a polygenic sex determination  
630 system, the PST is considered a quantitative trait, influenced by many genes with small  
631 effects plus environmental effects (28). The genetic sex tendency (GST), which cannot be  
632 measured directly, is the genetic part of this PST, positive for individuals more likely to  
633 develop as females in a neutral environment and negative for individuals more likely to  
634 develop as males (*SI Appendix*, Fig. S10). Variance components and heritability were  
635 estimated for phenotypic sex tendency (PST), the underlying liability of the binary sex, with a  
636 threshold model using THRGIBBS1F90 (86). Complete details for the establishment of  
637 eGST, QTL presence and heritability assessment can be found in *SI Appendix*, Materials and  
638 Methods.

639

### 640 **Transcriptomics**

641

642 The transcriptomic approach aimed to detect genes whose expression would be correlated to  
643 the eGST. Since we expected to have fewer females at HT vs LT, we collected more  
644 individuals in the HT than in the LT treatment to ensure having a sufficient number of  
645 females to analyze. Overall, 70 larvae, 40 from the HT group and 30 from the LT group, were

646 randomly collected at the “flexion” (242 DD<sub>10</sub> °C,) and the “all fins” stages (550 DD<sub>10</sub> °C),  
647 euthanized (benzocaine 150 mg/L) and snap-frozen in liquid nitrogen. At these stages, the  
648 RNA extraction was performed on entire individuals because of the impossibility to neatly  
649 dissect the gonads of such small individuals. This was justified from two points of view: i) it  
650 has been previously shown that the chance of underestimating gene expression of sex-related  
651 genes using body trunks was negligible in sea bass (87) and ii) we aimed to have a whole  
652 picture of the physiological processes associated with TSD. At the juvenile stage (117-124  
653 dpf), the gonads of 35 euthanized (benzocaine 150 mg/L) individuals from each temperature  
654 treatment were collected and snap-frozen in liquid nitrogen. At this stage, the biggest  
655 individuals were collected to ensure the sampling/collection of enough gonadal tissue for  
656 transcriptomic analysis. All samples were stored at -80°C until RNA extraction. Complete  
657 details for RNA extraction, RNA-seq and RNA-seq data analysis can be found in *SI*  
658 *Appendix*, Materials and Methods.

659

## 660 **Histology**

661

662 At the juvenile stage, 20 gonads (10HT and 10 LT) were fixed in Bouin’s fluid for 6–8 h,  
663 rinsed in clear water for 1 h and stocked in a 70% alcohol solution. Each gonad was stained  
664 with eosin and then placed in agarose (to improve detection) before being dehydrated, and  
665 embedded in paraffin. Sections of 5–6 mm thickness were stained with Trichrome de Masson,  
666 Haematoxylin Groat, Fuschine Ponceau and Aniline blue using an automated device.

667

## 668 **Elemental analysis**

669

670 Elemental analysis was performed to estimate the energy content of fish with a known eGe.  
671 Elemental composition was determined using a microVario Elemental analyzer (Elementar),  
672 allowing to obtain the percentage of carbon, hydrogen, nitrogen, sulphur and oxygen  
673 (CHNSO) per milligram of dry weight. Seventy sampled fish were weighted and measured  
674 before being euthanized (benzocaine 150 mg/L) and kept at -20°C. Then, fish at each stages  
675 (“flexion”, “all fins” and juveniles) were lyophilized simultaneously for 24 hours. Dried fish  
676 were then individually homogenized using ball mills. About 1 mg was used for CHNS  
677 analyses. Another 1 mg of the same samples were used for oxygen analysis. The analysis was  
678 performed by the laboratory of Physical measures (<https://lmp.edu.umontpellier.fr/elem>) of  
679 Montpellier (certified AFNOR, Iso 9001). Individual elemental composition was then

680 transformed to a relative energetic content (cal.mg<sup>-1</sup> dry weight) using the Given (1986)  
681 formula, as recommended by the analyser program:

682

683 
$$\text{Relative energetic content (cal.mg}^{-1}\text{)} = ((78.3C + 339.1H - 33O + 22S) + 152)/1000$$

684 Where C, H, O, and S refer to the percentages of carbon, hydrogen, oxygen, and sulphur in a  
685 sample. It was then divided by 4.184 to obtain the energetic content in joules.mg<sup>-1</sup>.

686

### 687 **RRBS library preparation and analysis**

688

689 Genomic DNA was isolated from ~25 mg of frozen gonadal tissue from the 1-year-old fish  
690 with the Qiagen Blood and Cell Culture kit (cat. no. 13323). Genome-wide profiling of DNA  
691 methylation levels was performed by RRBS using the Premium RRBS Kit (cat. no.  
692 C02030033; Diagenode) according to the manufacturer's instructions. Complete details for  
693 RRBS sequencing and preliminary analysis can be found in *SI Appendix*, Materials and  
694 Methods.

695

### 696 **Data analysis**

697

698 Final sex ratio was analysed using a binomial mixed model, with “tank” added as a random  
699 factor and “initial temperature treatment” as a fixed factor. For the three transcriptomes  
700 datasets of fish at the “flexion” stage, “all fins” stage, and juvenile stage, we adopted a  
701 traditional approach based on group comparison, where differentially expressed genes are  
702 detected using the Bioconductor (88) package DESeq2 v.1.18.1 (89) in R (90) and data  
703 normalized using the default method of DESeq2. Regarding the 10 transcriptomes at the  
704 “flexion” stage, group comparisons were performed between LT (n=5) and HT (n=5) fish  
705 with a positive, but not extreme eGST (i.e.  $0 < \text{eGST} < 0.5$ ). Regarding the 68 transcriptomes  
706 at the “all fins” stage, individuals were grouped based on their eGST, within each initial  
707 temperature treatment (note that they were collected at the same T (°C)). Those at each  
708 extreme of the eGST distribution were considered as extremes genetic males (eM,  $\text{eGST} < -$   
709  $0.5$ ) or extreme genetic females (eF,  $\text{eGST} > 0.5$ ), respectively, and those in the middle of the  
710 distribution were considered neutrals males (nM,  $-0.5 < \text{eGST} < 0$ ) or neutrals females (nF,  $0$   
711  $< \text{eGST} < 0.5$ ). Group comparisons between temperature treatments with DESeq2 were thus  
712 performed between eight groups, eM-HT (n=6), nM-HT (n=10), nF-HT (n=12), eF-HT  
713 (n=11), eM-LT (n=8), nM-LT (n=6), nF-LT (n=13), eF-LT (n=3). For juveniles' gonads, we

714 considered only two groups, those with an eGST > 0 (genetic females, n=26) and those with  
715 an eGST < 0 (genetic males, n=17) to detect differentially expressed genes according to the  
716 genetic sex. For all comparisons, genes with an adjusted p-value less than 5% (according to  
717 the FDR method from Benjamini-Hochberg) were declared differentially expressed.

718 For the two transcriptomic datasets with the highest number of samples (68 and 43), we also  
719 considered another approach using our linear predictor, the eGST, as a fixed value in linear  
720 models. Using normalized (DESeq2) and filtered data (keeping those with more than 30 reads  
721 in average), we ran a loop in R (R version 4.0.4) to detect all genes that are significantly  
722 correlated to the eGST in a linear and a quadratic model where the temperature treatment (HT  
723 or LT) is added as an interaction term with the eGST (T x eGST). For the linear model, we  
724 considered only the genes without significant interaction with temperature. Once detected, we  
725 also ran a linear model with a quasibinomial distribution (link= Logit) for those interesting  
726 genes displaying a dichotomic pattern of expression. For the quadratic model, we considered  
727 either the genes with a significant correlation with eGST plus a significant quadratic term for  
728 the interaction (I(eGST<sup>2</sup>) x T) or those where the quadratic term (I(eGST<sup>2</sup>)) plus the  
729 interaction between the temperature treatment and the quadratic term (I(eGST<sup>2</sup>) x T) are  
730 significant. One outlier (LT) was removed since it was at the extreme for eGST with unusual  
731 intermediate values for all sex-related genes, though keeping this outlier resulted in similar  
732 outcomes for those genes (*SI Appendix*, Fig. S11). Linear models were also used to assess the  
733 effect of eGST on energy content (joules.mg<sup>-1</sup>) and the size of the fishes.

734 For all transcriptomic analyses, the GO dataset of the mouse was used as it gives a more  
735 complete overview of the genes involved in each pathway. We adopted two strategies: 1) with  
736 *a priori*, where GOs were selected based on our hypotheses (*SI Appendix*, Materials and  
737 Methods) and 2) without *a priori*, where GOs were automatically detected from a functional  
738 enrichment analysis. For this second approach, we used a functional enrichment analysis. The  
739 clusterProfiler version 3.16.1 (91), an R package, was used to analyze function profiles of  
740 genes to identify major biological functions of genes. GO terms were predicted based on  
741 differentially expressed genes (from the group comparisons and linear models), including  
742 biological process and cellular component categories. Enrichment analysis was performed and  
743 a p-value < 0.05 was considered to indicate a statistically significant difference. PCA was  
744 performed using gene expression levels and illustrated using the ggplot2 package. The same  
745 package was used for representing raw values of genes for linear, quadratic regressions and  
746 selected genes from group comparisons. The “lme4” package was used for mixed models.  
747 Heatmaps of standardized expressions (mean subtraction followed standard deviation

748 division) were created using the pheatmap package. Methylkit and Genomic Ranges v1.44.0  
749 (92) packages were used to filter percent methylation data for the target regions: gene body  $\pm$   
750 2000 bp. DMCs were defined as cytosines with a methylation difference of  $\geq 15\%$  between  
751 temperature treatments and a significance threshold of  $q$ -value  $< 0.01$ .

752

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754

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762

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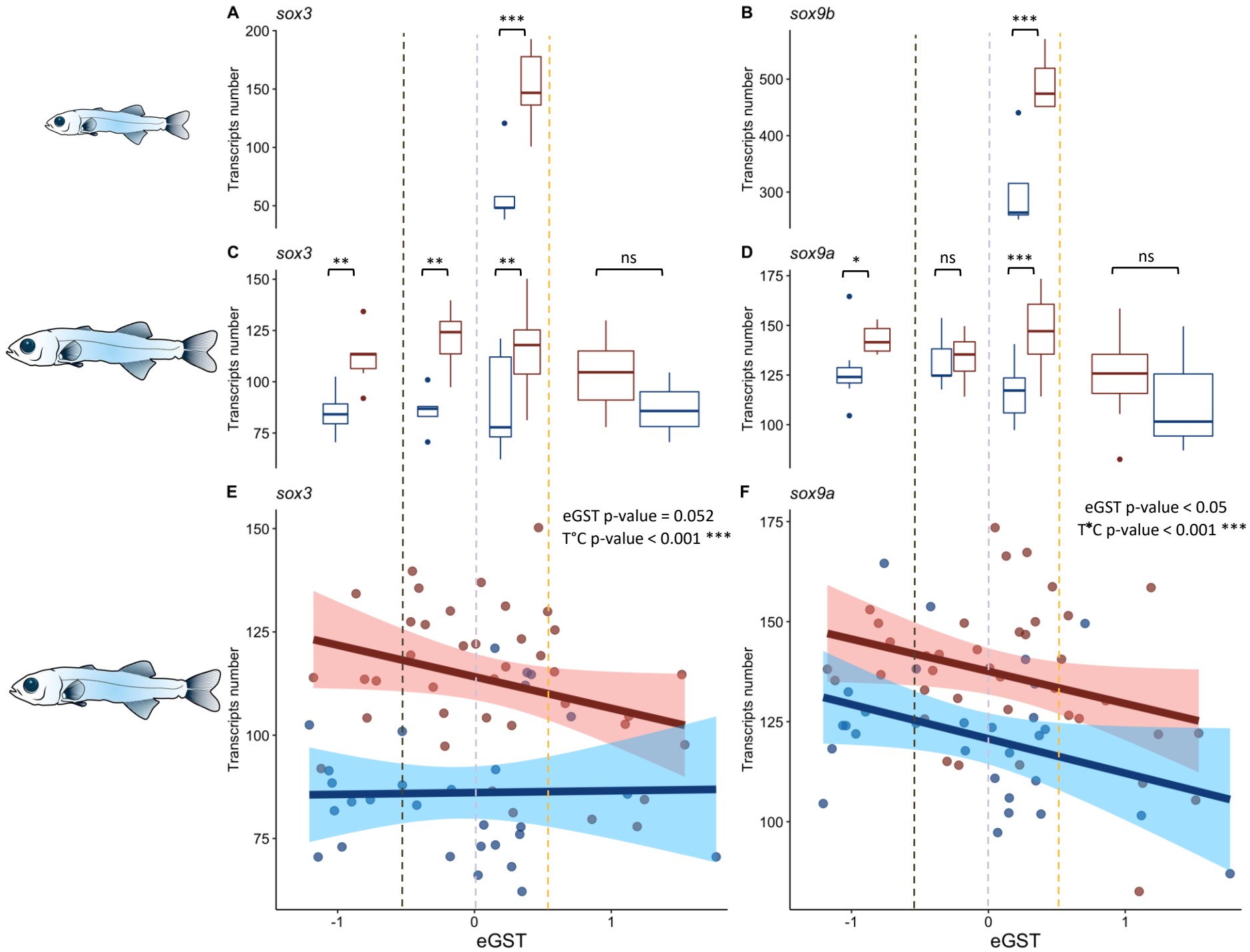
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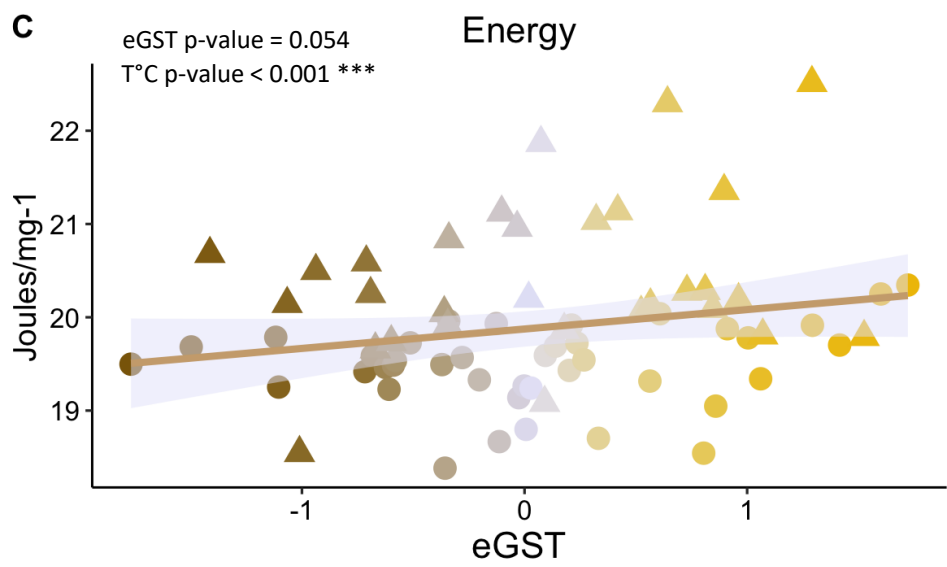
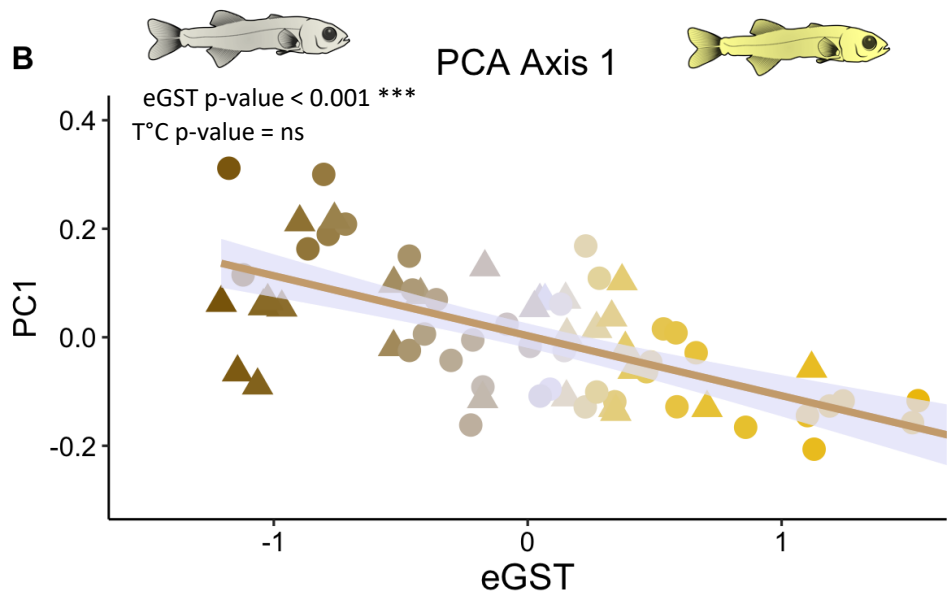
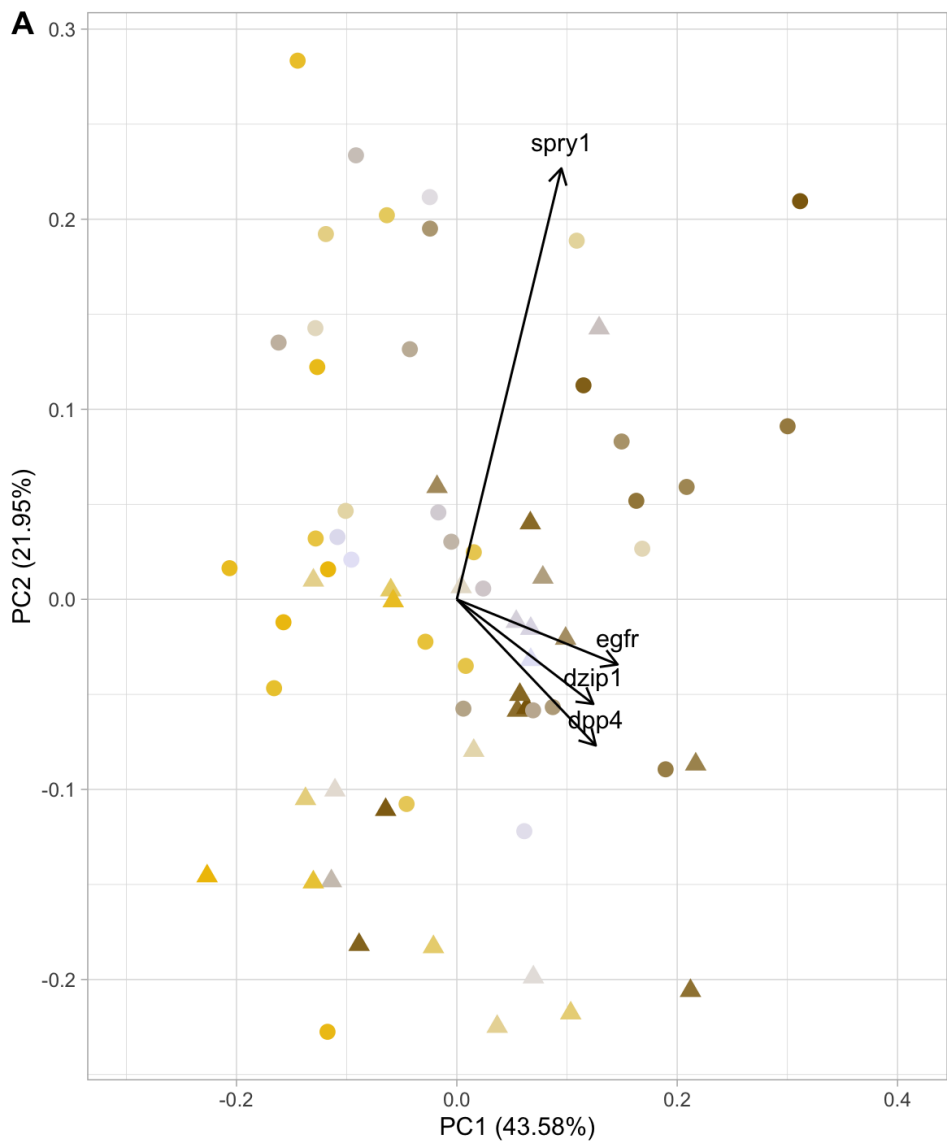
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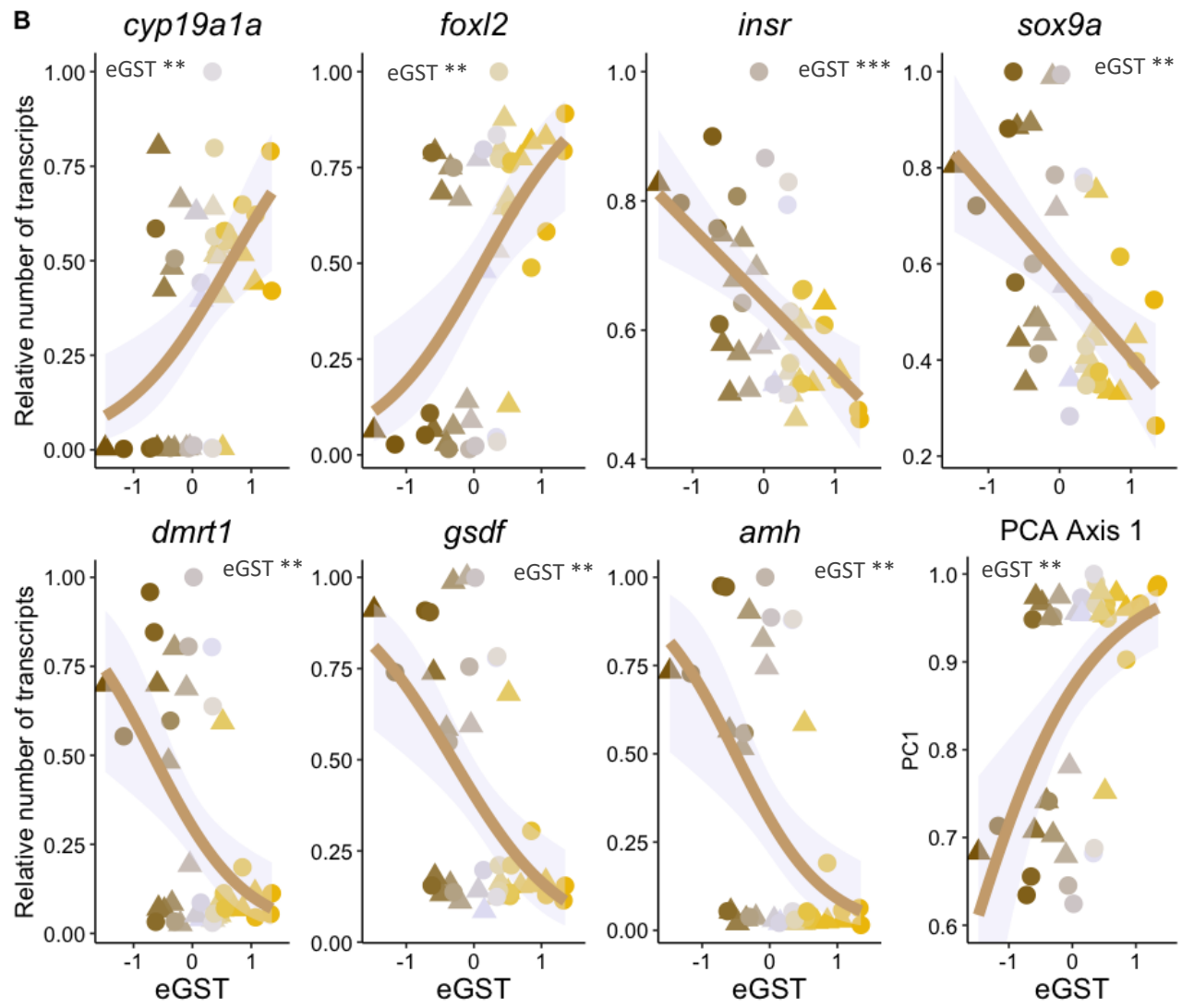
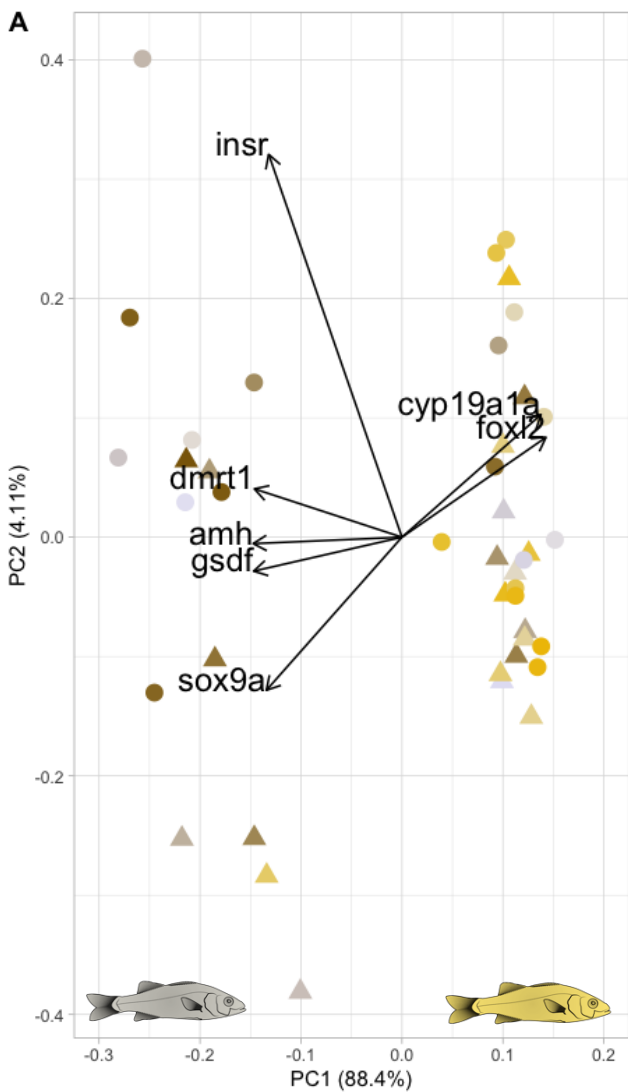
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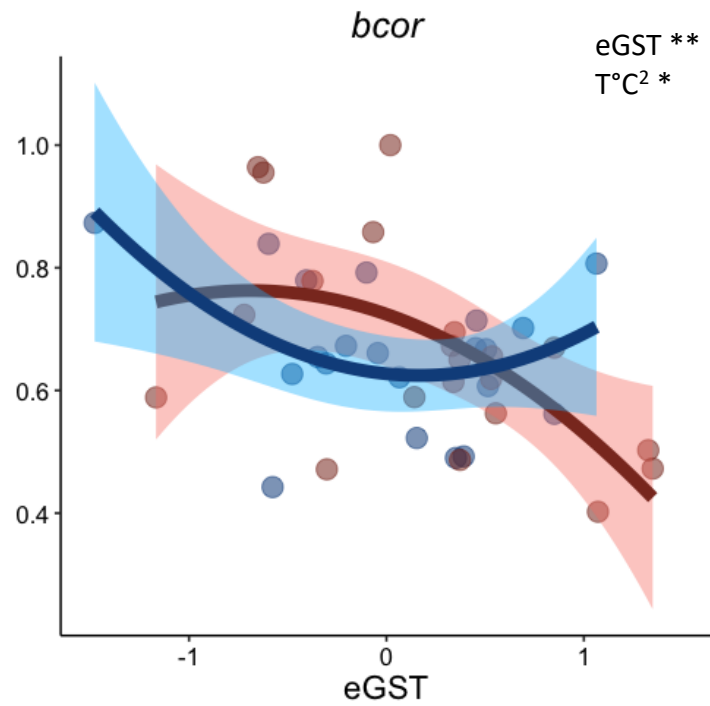
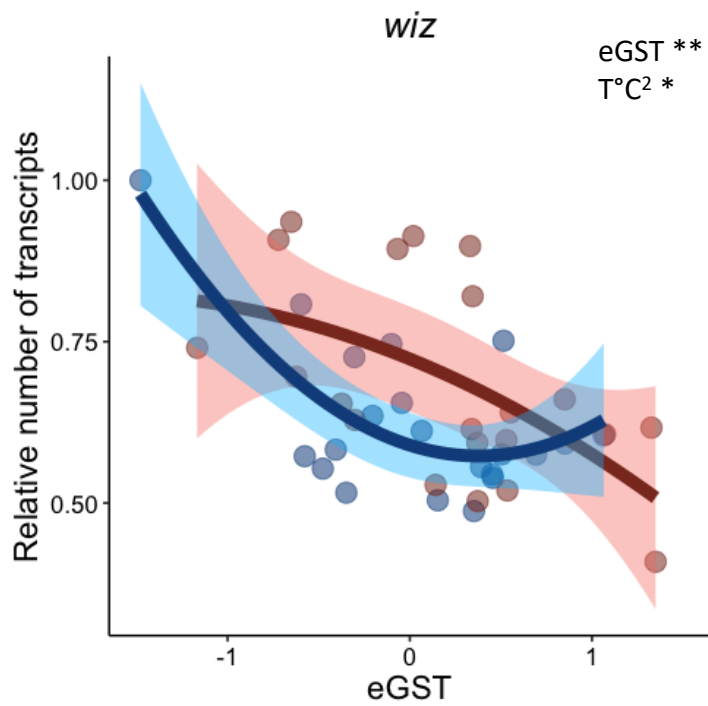
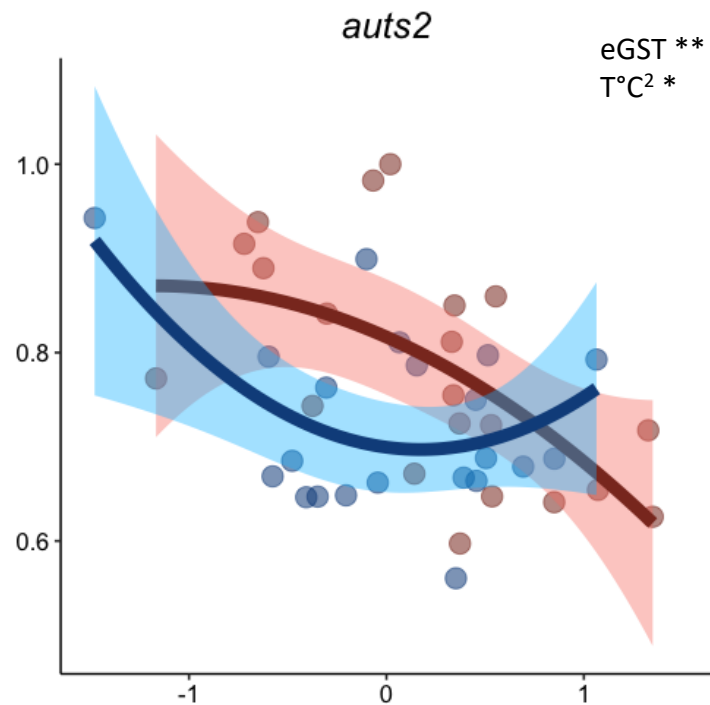
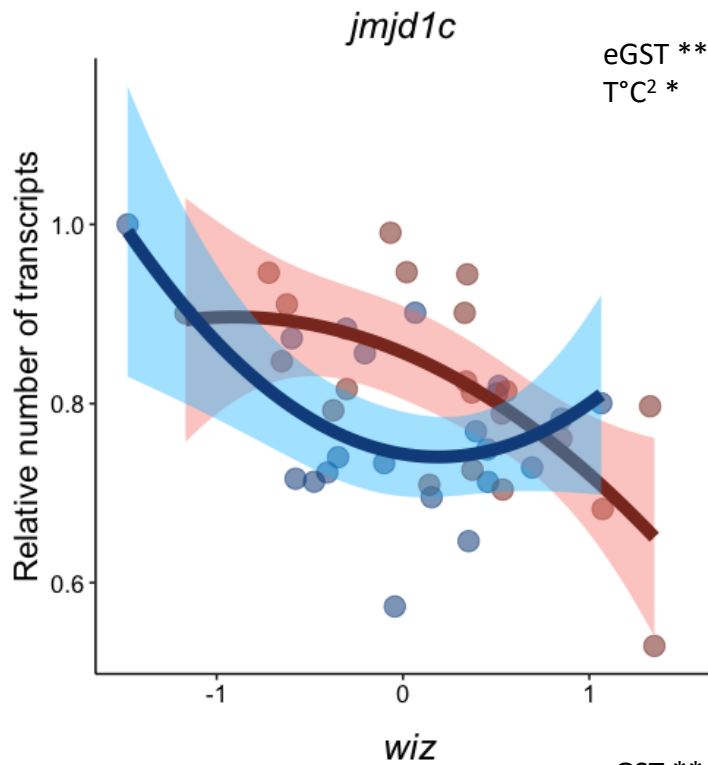


Fig. 5

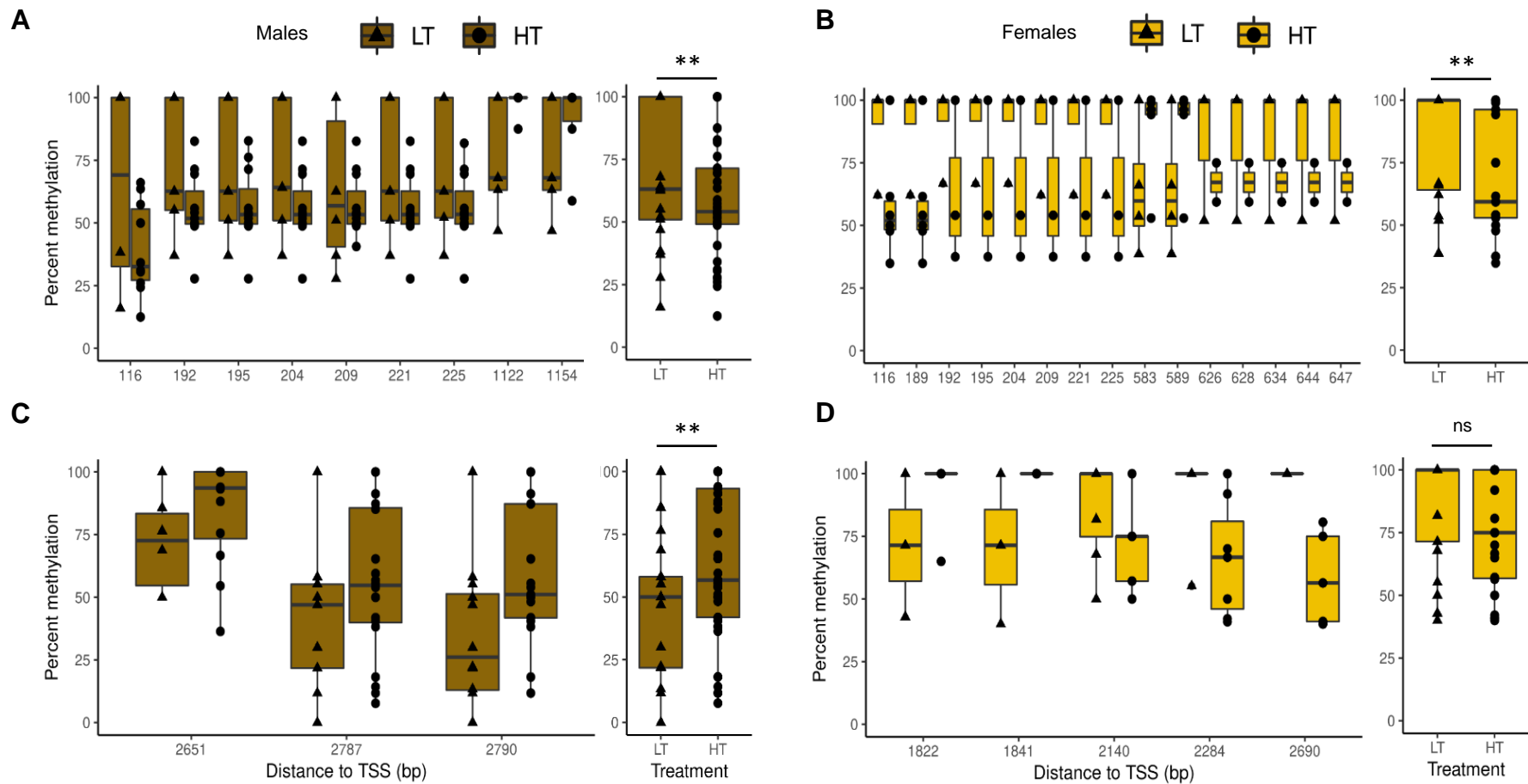


Fig. 6

Phenotype T°C

