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

Benjamin Geffroy[a,1], Mathieu Besson[b,c], Núria Sánchez-Baizán[h], Frederic Clota[a,c], Alexander Goikoetxea[a], Bastien Sadoul[a,d], François Ruelle[e], Marie-Odile Blanc[e], Hugues Parrinello[f], Sophie Hermet[g], Eva Blondeau-Bidet[g], Marine Pratlong[f], Francesc Piferrer[h], Marc Vandeputte[a,c] & François Allal[a]

[a]MARBEC Univ Montpellier, CNRS, Ifremer, IRD, Palavas-Les-Flots, France

[b]SYSAAF, Station LPGP/INRAE, Campus de Beaulieu, 35042 Rennes, France

[c]Université Paris-Saclay, INRAE, AgroParisTech, GABI, 78350 Jouy-en-Josas, France

[d]ESE, Ecology and Ecosystem Health, Institut Agro, INRAE, Rennes, France,

[e]Laboratoire Service d'Expérimentations Aquacoles, Ifremer, Palavas Les Flots, France

[f]MGX, BCM, Univ Montpellier, CNRS, INSERM, Montpellier, France.

[g]MARBEC Univ Montpellier, CNRS, Ifremer, IRD, Montpellier, France

[h]Institut de Ciències del Mar, Spanish National Research Council, Barcelona, Spain

[1]To whom correspondence may be addressed. Email: bgeffroy@ifremer.fr

Author contributions: B.G., F.P., M.V. and F.A. designed research; B.G., M.B., F.C., A.G., B.S., F.R., M-O.B., H.P., S.H. and E.B-B. performed research; B.G., M.B., E.B-B., F.P., M.P., N.S-B, and F.A. analyzed data; and B.G. wrote the paper.

Competing Interest Statement: The authors declare no conflict of interest.

Classification: Biological Sciences, Developmental biology

27 **Keywords:** sex determination, genomics, temperature, fish, epigenetic

28 **This PDF file includes:**

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 definetively 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°C) during the larval stage promotes male differentiation (30–32).
112 However, long-term exposure to relatively low temperature (< 17°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 ± 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 ± 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: $h^2_{sex_LT} = 0.65$
159 ± 0.06 and $h^2_{sex_HT} = 0.51 \pm 0.08$, 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,

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

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

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

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

Sixteen genes were involved in the GO lipid biosynthetic process and 19 in the GO regulation of growth (*SI Appendix,* Table S1). The gene encoding the growth hormone (*gh*) was one of these genes, and was positively and significantly correlated with the eGST. Three other genes (*prkca, gfi1b* and *eya2*) that are in close vicinity of the three previously detected QTL (*QTL_LG7, QTL_LG19b* and *QTL_LG1A*) exhibited a significant correlation with the eGST, though their expression was independent of their SNP genotype of the QTLs (AA, AB or BB). The “response to glucose” was among the biological processes presenting a positive correlation with the eGST, and thus more expressed in females (*SI Appendix,* Fig. S6). Eleven genes involved in the GO histone modification were also significantly correlated to the eGST (*SI Appendix,* Table S1). Interestingly, the genes from the GO “histone H3-K27 methylation” and “histone H3-K4 methylation” were negatively correlated with the eGST (thus more expressed in males; *SI Appendix,* Fig. S6).

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

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

The juvenile gonadal transcriptome faithfully reflects the underlying eGST independently of temperature influences

RNA-Seq was performed on total RNA extracted from the gonads of 42 individuals (21 HT and 21 LT) sampled at the juvenile stage (117-124 dph), before the first signs of morphological sex differentiation (*SI Appendix,* Fig. S1). Among the 15724 genes that respected our inclusion criteria, 1297 showed a significant (p-value < 0.01) linear correlation, either positive or negative, between their expression level and the eGST, independently of the initial temperature treatment (HT vs LT). Among those genes, nineteen and six genes (*SI Appendix,* Table S3) were within the gene ontologies (GOs) of sex differentiation and sex determination, respectively, including *cyp19a1a* (gonadal aromatase), *foxl2* (forkhead box l2), *dmrt1* (doublesex and mab-3 related transcription factor 1), *gsdf* (gonadal soma derived factor), *amh* (anti-Müllerian hormone), *sox9a* (sry-related HMG box 9a), and *insr* (insulin 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 *3β-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 °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 hypermethlated (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 ± 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 * interquartile range (IQR), and the lower
331 whisker extends to the minimum value (1.5 * 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^{-1}) 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 Mediterrannean
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 downreguation 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 *jmjd1c*, involved in histone demethylation at the H3K9 site, was upregulated in
504 male gonads compared to female gonads. Additionally, *jmjd1c* 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 dectectable 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 reveral 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 *jmjd1c*), 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 envornmental 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, $DD_{10\ °C}$), 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 $DD_{10\ °C}$: 77 $DD_{10\ °C}$, 242 $DD_{10\ °C}$, and 550 $DD_{10\ °C}$ (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 $DD_{10\ °C}$, 550 $DD_{10\ °C}$, 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 $DD_{10\,°C}$ (n = 192), at 242 $DD_{10\,°C}$ (n = 300) and at 550 $DD_{10\,°C}$ (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 $DD_{10\ °C}$,) and the "all fins" stages (550 $DD_{10\ °C}$,),
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-1 dry weight) using the Given (1986)
681 formula, as recommended by the analyser program:
682
683 Relative energetic content (cal.mg-1) = ((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-1.
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 < 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, eGST < -
709 0.5) or extreme genetic females (eF, eGST > 0.5), respectively, and those in the middle of the
710 distribution were considered neutrals males (nM, -0.5 < eGST < 0) or neutrals females (nF, 0
711 < 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^2) x T) or those where the quadratic term (I(eGST^2)) plus the
729 interaction between the temperature treatment and the quadratic term (I(eGST^2) 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-1) 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 ±
750 2000 bp. DMCs were defined as cytosines with a methylation difference of ≥15% between
751 temperature treatments and a significance threshold of q-value < 0.01.
752

753 **Acknowledgments**

754

755 The study was supported by the European Maritime and Fisheries Fund (3S, Seabass Sex and
756 Stress, grant number 4320175237) allocated to BG. Production of the fish benefited from
757 AQUAEXCEL^{2020} TNA grant "Transsexbass" to FP and NSB. Research at the FP lab
758 supported by Spanish Ministry of Science grant no. PID2019-108888RB-I00. We thank Pierre
759 Lopez (MARBEC) for kindly drawing fish at the different stages of development, Thomas
760 Régnier for providing background on CHNSO analysis, Sandrine Skiba for discussion of the
761 results and Mako Pegart for samplings.
762

763 **References**

764

765 1. B. Capel, Vertebrate sex determination: evolutionary plasticity of a fundamental
766 switch. *Nat. Rev. Genet.* (2017) https:/doi.org/10.1038/nrg.2017.60.

767 2. M. Charnier, [Action of temperature on the sex ratio in the Agama agama (Agamidae,
768 Lacertilia) embryo]. *C. R. Seances Soc. Biol. Fil.* **160**, 620–622 (1966).

769 3. D. O. Conover, B. E. Kynard, Environmental Sex Determination: Interaction of
770 Temperature and Genotype in a Fish. *Science* **213**, 577–579 (1981).

771 4. R. H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: an
772 overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191–364
773 (2002).

774 5. B. Geffroy, A. Bardonnet, Sex differentiation and sex determination in eels:
775 consequences for management. *Fish Fish.* **17**, 375–398 (2016).

776 6. N. Ospina-Álvarez, F. Piferrer, Temperature-Dependent Sex Determination in Fish
777 Revisited: Prevalence, a Single Sex Ratio Response Pattern, and Possible Effects of Climate
778 Change. *PLoS ONE* **3**, e2837 (2008).

779 7. U. Römer, W. Beisenherz, Environmental determination of sex in Apistogrammai
780 (Cichlidae) and two other freshwater fishes (Teleostei). *J. Fish Biol.* **48**, 714–725 (1996).

781 8. R. S. Hattori, D. C. Castañeda-Cortés, L. F. Arias Padilla, P. H. Strobl-Mazzulla, J. I.
782 Fernandino, Activation of stress response axis as a key process in environment-induced sex

783 plasticity in fish. *Cell. Mol. Life Sci.* (2020) https:/doi.org/10.1007/s00018-020-03532-9 (July
784 20, 2020).

785 9. F. Piferrer, *et al.*, The Model of the Conserved Epigenetic Regulation of Sex. *Front.*
786 *Genet.* **10** (2019).

787 10. I. W. Deveson, *et al.*, Differential intron retention in Jumonji chromatin modifier
788 genes is implicated in reptile temperature-dependent sex determination. *Sci. Adv.* **3**, e1700731
789 (2017).

790 11. A. Georges, C. E. Holleley, How does temperature determine sex? *Science* **360**, 601–
791 602 (2018).

792 12. B. Geffroy, M. Douhard, The Adaptive Sex in Stressful Environments. *Trends Ecol.*
793 *Evol.* **34**, 628–640 (2019).

794 13. B. Geffroy, C. Wedekind, Effects of global warming on sex ratios in fishes. *J. Fish*
795 *Biol.* **97**, 596–606 (2020).

796 14. J. F. Baroiller, H. D'Cotta, E. Bezault, S. Wessels, G. Hoerstgen-Schwark, Tilapia sex
797 determination: Where temperature and genetics meet. *Comp. Biochem. Physiol. A. Mol.*
798 *Integr. Physiol.* **153**, 30–38 (2009).

799 15. K. Tabata, Reduction of Female Proportion in Lower Growing Fish Separtated from
800 Normal and Feminized Seedlings of Hirame *Paralichthys olivaceus*. *Fish. Sci.* **61**, 199–201
801 (1995).

802 16. S. Santi, *et al.*, Thermosensitivity of the sex differentiation process in the African
803 catfish, Clarias gariepinus: Determination of the thermosensitive period. *Aquaculture* **455**,
804 73–80 (2016).

805 17. R. S. Hattori, *et al.*, The Duplicated Y-specific amhy Gene Is Conserved and Linked to
806 Maleness in Silversides of the Genus Odontesthes. *Genes* **10**, 679 (2019).

807 18. T. Kitano, Y. Hayashi, E. Shiraishi, Y. Kamei, Estrogen rescues masculinization of
808 genetically female medaka by exposure to cortisol or high temperature. *Mol. Reprod. Dev.* **79**,
809 719–726 (2012).

810 19. K. Valdivia, *et al.*, High Temperature Increases the Masculinization Rate of the All-
811 Female (XX) Rainbow Trout "Mal" Population. *PLOS ONE* **9**, e113355 (2014).

812 20. H. Zhou, *et al.*, Temperature-control-induced masculinization in tiger puffer Takifugu
813 rubripes. *J. Oceanol. Limnol.* **37**, 1125–1135 (2019).

814 21. R. S. Hattori, *et al.*, Cortisol-Induced Masculinization: Does Thermal Stress Affect
815 Gonadal Fate in Pejerrey, a Teleost Fish with Temperature-Dependent Sex Determination?
816 *PLoS ONE* **4**, e6548 (2009).

817 22. Y. Hayashi, *et al.*, High temperature causes masculinization of genetically female
818 medaka by elevation of cortisol. *Mol. Reprod. Dev.* **77**, 679–686 (2010).

819 23. T. Kamiya, *et al.*, A Trans-Species Missense SNP in Amhr2 Is Associated with Sex
820 Determination in the Tiger Pufferfish, Takifugu rubripes (Fugu). *PLoS Genet.* **8** (2012).

821 24. K. Miyoshi, R. S. Hattori, C. A. Strüssmann, M. Yokota, Y. Yamamoto,
822 Phenotypic/genotypic sex mismatches and temperature-dependent sex determination in a wild
823 population of an Old World atherinid, the cobaltcap silverside Hypoatherina tsurugae. *Mol.*
824 *Ecol.* **29**, 2349–2358 (2020).

825 25. A. Yano, *et al.*, The sexually dimorphic on the Y-chromosome gene (sdY) is a
826 conserved male-specific Y-chromosome sequence in many salmonids. *Evol. Appl.* **6**, 486–496
827 (2013).

828 26. J. F. Baroiller, D. Chourrout, A. Fostier, B. Jalabert, Temperature and sex
829 chromosomes govern sex ratios of the mouthbrooding Cichlid fish Oreochromis niloticus. *J.*
830 *Exp. Zool.* **273**, 216–223 (1995).

831 27. X. Wang, *et al.*, High temperature causes masculinization of genetically female olive
832 flounder (Paralichthys olivaceus) accompanied by primordial germ cell proliferation
833 detention. *Aquaculture* **479**, 808–816 (2017).

834 28. M. G. Bulmer, J. J. Bull, Models of Polygenic Sex Determination and Sex Ratio
835 Control. *Evolution* **36**, 13–26 (1982).

836 29. M. L. A. Hujoel, S. Gazal, P.-R. Loh, N. Patterson, A. L. Price, Liability threshold
837 modeling of case–control status and family history of disease increases association power.
838 *Nat. Genet.* **52**, 541–547 (2020).

839 30. F. Piferrer, M. Blázquez, L. Navarro, A. González, Genetic, endocrine, and
840 environmental components of sex determination and differentiation in the European sea bass
841 (Dicentrarchus labrax L.). *Gen. Comp. Endocrinol.* **142**, 102–110 (2005).

842 31. E. Saillant, *et al.*, Temperature effects and genotype-temperature interactions on sex
843 determination in the European sea bass (Dicentrarchus labrax L.). *J. Exp. Zool.* **292**, 494–505
844 (2002).

845 32. M. Vandeputte, M. Dupont-Nivet, H. Chavanne, B. Chatain, A Polygenic Hypothesis
846 for Sex Determination in the European Sea Bass Dicentrarchus labrax. *Genetics* **176**, 1049–
847 1057 (2007).

848 33. M. Vandeputte, *et al.*, Low temperature has opposite effects on sex determination in a
849 marine fish at the larval/postlarval and juvenile stages. *Ecol. Evol.* **10**, 13825–13835 (2020).

850 34. N. Díaz, L. Ribas, F. Piferrer, The relationship between growth and sex differentiation
851 in the European sea bass (Dicentrarchus labrax). *Aquaculture* **408–409**, 191–202 (2013).

852 35. N. Díaz, F. Piferrer, Lasting effects of early exposure to temperature on the gonadal
853 transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed
854 genetic and environmental sex determination. *BMC Genomics* **16**, 679 (2015).

855 36. L. Navarro-Martín, *et al.*, DNA Methylation of the Gonadal Aromatase (cyp19a)
856 Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass.
857 *PLoS Genet* **7**, e1002447 (2011).

858 37. M. Vandeputte, *et al.*, Low temperature has opposite effects on sex determination in a
859 marine fish at the larval/postlarval and juvenile stages. *Ecol. Evol.* **n/a** (2020).

860 38. D. Anastasiadi, M. Vandeputte, N. Sánchez-Baizán, F. Allal, F. Piferrer, Dynamic
861 epimarks in sex-related genes predict gonad phenotype in the European sea bass, a fish with
862 mixed genetic and environmental sex determination. *Epigenetics* **13**, 988–1011 (2018).

863 39. R. Griot, *et al.*, Genome-wide association studies for resistance to viral nervous
864 necrosis in three populations of European sea bass (Dicentrarchus labrax) using a novel 57k
865 SNP array DlabChip. *Aquaculture* **530**, 735930 (2021).

866 40. T. H. Meuwissen, B. J. Hayes, M. E. Goddard, Prediction of total genetic value using
867 genome-wide dense marker maps. *Genetics* **157**, 1819–1829 (2001).

868 41. C. Roblin, J. Bruslé, Ontogenèse gonadique et différenciation sexuelle du Loup
869 Dicentrarchus labrax, en conditions d'élevage. *Reprod. Nutr. Dév.* **23**, 115–127 (1983).

870 42. J. Ødegård, T. H. Meuwissen, Estimation of heritability from limited family data using
871 genome-wide identity-by-descent sharing. *Genet. Sel. Evol.* **44**, 16 (2012).

872 43. L. Ribas, *et al.*, Characterization of the European Sea Bass (Dicentrarchus labrax)
873 Gonadal Transcriptome During Sexual Development. *Mar. Biotechnol.* **21**, 359–373 (2019).

874 44. S. Faggion, M. Vandeputte, B. Chatain, P.-A. Gagnaire, F. Allal, Population-specific
875 variations of the genetic architecture of sex determination in wild European sea bass
876 Dicentrarchus labrax L. *Heredity* **122**, 612–621 (2019).

877 45. C. Palaiokostas, *et al.*, A new SNP-based vision of the genetics of sex determination in
878 European sea bass (Dicentrarchus labrax). *Genet. Sel. Evol.* **47**, 68 (2015).

879 46. M. Bláquez, S. Zanuy, M. Carillo, F. Piferrer, Effects of rearing temperature on sex
880 differentiation in the European sea bass (Dicentrarchus labrax L.). *J. Exp. Zool.* **281**, 207–216
881 (1998).

882 47. M. Pavlidis, *et al.*, Evidence of temperature-dependent sex determination in the
883 European sea bass (Dicentrarchus labrax L.). *J. Exp. Zool.* **287**, 225–232 (2000).

884 48. L. Navarro-Martín, M. Blázquez, J. Viñas, S. Joly, F. Piferrer, Balancing the effects of
885 rearing at low temperature during early development on sex ratios, growth and maturation in
886 the European sea bass (Dicentrarchus labrax).: Limitations and opportunities for the
887 production of highly female-biased stocks. *Aquaculture* **296**, 347–358 (2009).

888 49. C. A. Alper, Z. Awdeh, Incomplete penetrance of MHC susceptibility genes:
889 prospective analysis of polygenic MHC-determined traits. *Tissue Antigens* **56**, 199–206
890 (2000).

891 50. D. N. Cooper, M. Krawczak, C. Polychronakos, C. Tyler-Smith, H. Kehrer-Sawatzki,
892 Where genotype is not predictive of phenotype: towards an understanding of the molecular
893 basis of reduced penetrance in human inherited disease. *Hum. Genet.* **132**, 1077–1130 (2013).

894 51. E. Saillant, *et al.*, Sexual differentiation and juvenile intersexuality in the European sea
895 bass (Dicentrarchus labrax). *J. Zool.* **260**, 53–63 (2003).

896 52. B. Geffroy, Y. Guiguen, A. Fostier, A. Bardonnet, New insights regarding gonad
897 development in European eel: evidence for a direct ovarian differentiation. *Fish Physiol.*
898 *Biochem.* **39**, 1129–1140 (2013).

899 53. J. Kent, S. C. Wheatley, J. E. Andrews, A. H. Sinclair, P. Koopman, A male-specific
900 role for SOX9 in vertebrate sex determination. *Development* **122**, 2813–2822 (1996).

901 54. S. Nef, *et al.*, Testis determination requires insulin receptor family function in mice.
902 *Nature* **426**, 291–295 (2003).

903 55. I. Stévant, S. Nef, Genetic Control of Gonadal Sex Determination and Development.
904 *Trends Genet.* **35**, 346–358 (2019).

905 56. M. Blázquez, L. Navarro-Martín, F. Piferrer, Expression profiles of sex
906 differentiation-related genes during ontogenesis in the European sea bass acclimated to two
907 different temperatures. *J. Exp. Zoolog. B Mol. Dev. Evol.* **312B**, 686–700 (2009).

908 57. N. Moreno-Mendoza, V. R. Harley, H. Merchant-Larios, Temperature Regulates
909 SOX9 Expression in Cultured Gonads of Lepidochelys olivacea, a Species with Temperature
910 Sex Determination. *Dev. Biol.* **229**, 319–326 (2001).

911 58. S. Radhakrishnan, R. Literman, J. Neuwald, A. Severin, N. Valenzuela,
912 Transcriptomic responses to environmental temperature by turtles with temperature-
913 dependent and genotypic sex determination assessed by RNAseq inform the genetic
914 architecture of embryonic gonadal development. *PLOS ONE* **12**, e0172044 (2017).

915 59. C. Shoemaker, M. Ramsey, J. Queen, D. Crews, Expression of Sox9, Mis, and Dmrt1
916 in the gonad of a species with temperature-dependent sex determination. *Dev. Dyn.* **236**,
917 1055–1063 (2007).

918 60. D. Sun, *et al.*, Sox9-related signaling controls zebrafish juvenile ovary–testis
919 transformation. *Cell Death Dis.* **4**, e930–e930 (2013).

920 61. L. D. Moore, T. Le, G. Fan, DNA Methylation and Its Basic Function.
921 *Neuropsychopharmacology* **38**, 23–38 (2013).

922 62. D. Anastasiadi, A. Esteve-Codina, F. Piferrer, Consistent inverse correlation between
923 DNA methylation of the first intron and gene expression across tissues and species.
924 *Epigenetics Chromatin* **11**, 37 (2018).

925 63. S. Urs, *et al.*, Sprouty1 is a critical regulatory switch of mesenchymal stem cell lineage
926 allocation. *FASEB J.* **24**, 3264–3273 (2010).

927 64. S. Urs, T. Henderson, P. Le, C. J. Rosen, L. Liaw, Tissue-specific expression of
928 Sprouty1 in mice protects against high-fat diet-induced fat accumulation, bone loss and
929 metabolic dysfunction. *Br. J. Nutr.* **108**, 1025–1033 (2012).

930 65. P. O. Prada, *et al.*, EGFR Tyrosine Kinase Inhibitor (PD153035) Improves Glucose
931 Tolerance and Insulin Action in High-Fat Diet–Fed Mice. *Diabetes* **58**, 2910–2919 (2009).

932 66. Z. Koledova, *et al.*, SPRY1 regulates mammary epithelial morphogenesis by
933 modulating EGFR-dependent stromal paracrine signaling and ECM remodeling. *Proc. Natl.*
934 *Acad. Sci.* **113**, E5731–E5740 (2016).

935 67. D. Marguet, *et al.*, Enhanced insulin secretion and improved glucose tolerance in mice
936 lacking CD26. *Proc. Natl. Acad. Sci.* **97**, 6874–6879 (2000).

937 68. S. Faggion, *et al.*, Sex dimorphism in European sea bass (Dicentrarchus labrax L.):

938 New insights into sex-related growth patterns during very early life stages. *PLOS ONE* **16**,
939 e0239791 (2021).

940 69. A. Herpin, M. Schartl, Plasticity of gene-regulatory networks controlling sex
941 determination: of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Rep.* **16**,
942 1260–1274 (2015).

943 70. T. Myosho, Y. Takehana, S. Hamaguchi, M. Sakaizumi, Turnover of Sex
944 Chromosomes in Celebensis Group Medaka Fishes. *G3 Bethesda Md* **5**, 2685–2691 (2015).

945 71. E. Sutton, *et al.*, Identification of *SOX3* as an XX male sex reversal gene in mice and
946 humans. *J. Clin. Invest.* **121**, 328–341 (2011).

947 72. Y. Takehana, *et al.*, Co-option of Sox3 as the male-determining factor on the Y
948 chromosome in the fish Oryzias dancena. *Nat. Commun.* **5**, 4157 (2014).

949 73. B. Yao, L. Zhou, Y. Wang, W. Xia, J.-F. Gui, Differential expression and dynamic
950 changes of SOX3 during gametogenesis and sex reversal in protogynous hermaphroditic fish.
951 *J. Exp. Zool. Part Ecol. Genet. Physiol.* **307A**, 207–219 (2007).

952 74. S. Nakamura, *et al.*, Analysis of Medaka sox9 Orthologue Reveals a Conserved Role
953 in Germ Cell Maintenance. *PLOS ONE* **7**, e29982 (2012).

954 75. F. Piferrer, Epigenetics of sex determination and gonadogenesis. *Dev. Dyn.* **242**, 360–
955 370 (2013).

956 76. N. J. Gemmell, E. V. Todd, A. Goikoetxea, O. Ortega-Recalde, T. A. Hore, “Chapter
957 Three - Natural sex change in fish” in *Current Topics in Developmental Biology*, Sex
958 Determination in Vertebrates., B. Capel, Ed. (Academic Press, 2019), pp. 71–117.

959 77. A. Tsakogiannis, *et al.*, The Gene Toolkit Implicated in Functional Sex in Sparidae
960 Hermaphrodites: Inferences From Comparative Transcriptomics. *Front. Genet.* **9** (2019).

961 78. C. Ge, *et al.*, The histone demethylase KDM6B regulates temperature-dependent sex
962 determination in a turtle species. *Science* **360**, 645–648 (2018).

963 79. B. Geffroy, *et al.*, Parental selection for growth and early-life low stocking density
964 increase the female-to-male ratio in European sea bass. *Sci. Rep.* **11**, 13620 (2021).

965 80. E. L. Charnov, J. Bull, When is sex environmentally determined? *Nature* **266**, 828–
966 830 (1977).

967 81. F. Piferrer, D. Anastasiadi, Do the Offspring of Sex Reversals Have Higher Sensitivity
968 to Environmental Perturbations? *Sex. Dev.* **15**, 134–147 (2021).

969 82. U. Mittwoch, The elusive action of sex-determining genes: mitochondria to the
970 rescue? *J. Theor. Biol.* **228**, 359–365 (2004).

971 83. A. Goikoetxea, *et al.*, Genetic pathways underpinning hormonal stress responses in
972 fish exposed to short- and long-term warm ocean temperatures. *Ecol. Indic.* **120**, 106937
973 (2021).

974 84. K. A. Chezik, N. P. Lester, P. A. Venturelli, Fish growth and degree-days I: selecting
975 a base temperature for a within-population study. *Can. J. Fish. Aquat. Sci.* **71**, 47–55 (2014).

976 85. O. Weissbrod, C. Lippert, D. Geiger, D. Heckerman, Accurate liability estimation
977 improves power in ascertained case-control studies. *Nat. Methods* **12**, 332–334 (2015).

978 86. S. Tsuruta, I. Misztal, THRGIBBS1F90 for estimation of variance components with
979 threshold and linear models. *Proc. 8th World Congr. Genet. Appl. Livest. Prod. Belo Horiz.*
980 *Minas Gerais Braz. 13-18 August 2006*, 27–31 (2006).

981 87. M. Blázquez, A. González, M. Papadaki, C. Mylonas, F. Piferrer, Sex-related changes
982 in estrogen receptors and aromatase gene expression and enzymatic activity during early
983 development and sex differentiation in the European sea bass (Dicentrarchus labrax). *Gen.*
984 *Comp. Endocrinol.* **158**, 95–101 (2008).

985 88. R. C. Gentleman, *et al.*, Bioconductor: open software development for computational
986 biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).

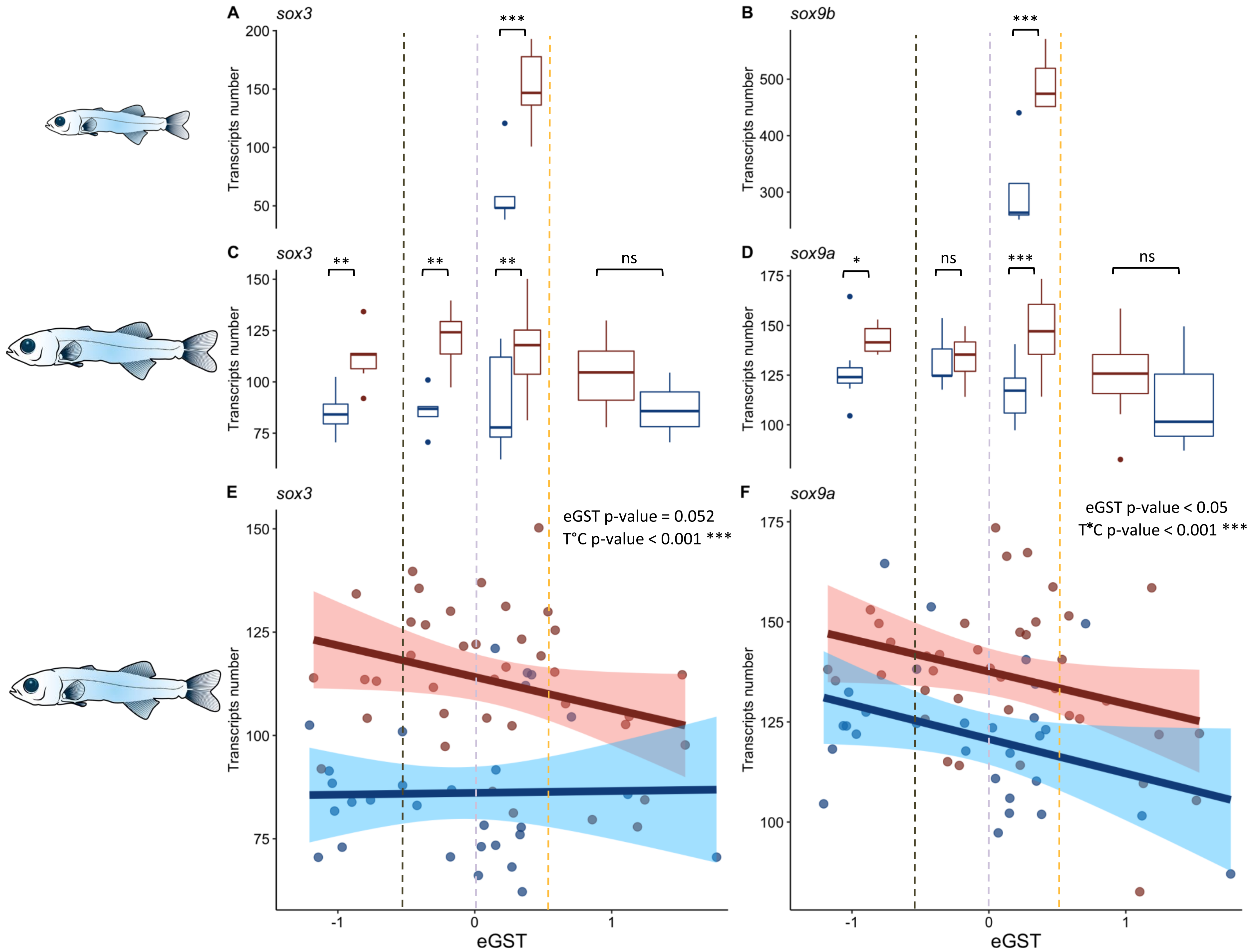
987 89. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion
988 for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

989 90. R Core Team, R: A language and environment for statistical computing. *R Found.*
990 *Stat. Comput. Vienna Austria* (2021).

991 91. G. Yu, L.-G. Wang, Y. Han, Q.-Y. He, clusterProfiler: an R Package for Comparing
992 Biological Themes Among Gene Clusters. *OMICS J. Integr. Biol.* **16**, 284–287 (2012).

993 92. M. Lawrence, *et al.*, Software for Computing and Annotating Genomic Ranges. *PLOS*
994 *Comput. Biol.* **9**, e1003118 (2013).

995


A
sox3
B
sox9b
C
sox3
D
sox9a
E
sox3
F
sox9a
Transcripts number
***
**
*
ns
eGST p-value = 0.052
T°C p-value < 0.001 ***
eGST p-value < 0.05
T°C p-value < 0.001 ***
eGST

A

spry1

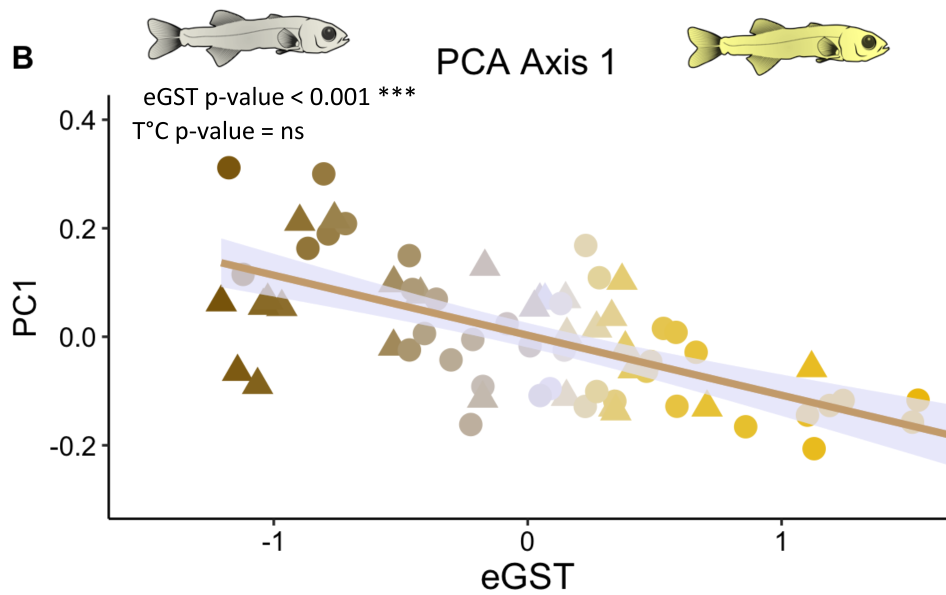
egfr

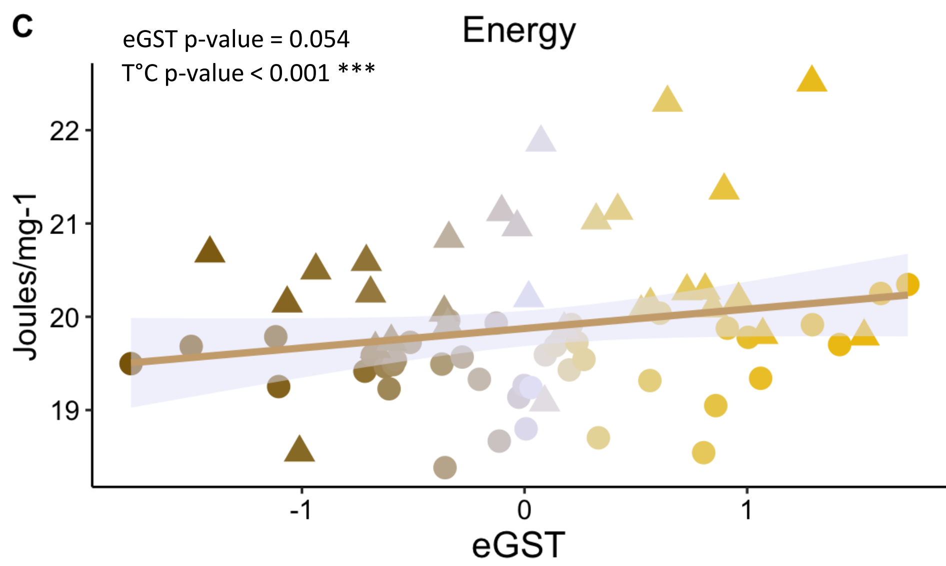
dzip1

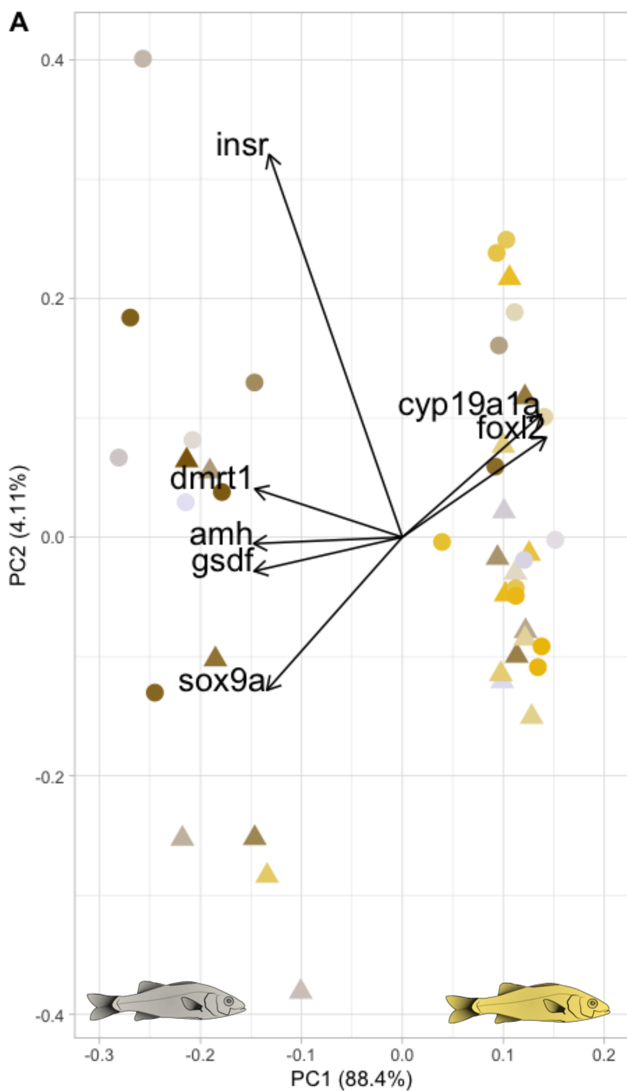
dpp4

PC2 (21.95%)

PC1 (43.58%)







B

cyp19a1a eGST **

foxl2 eGST **

insr eGST ***

sox9a eGST **

dmrt1 eGST **

gsdf eGST **

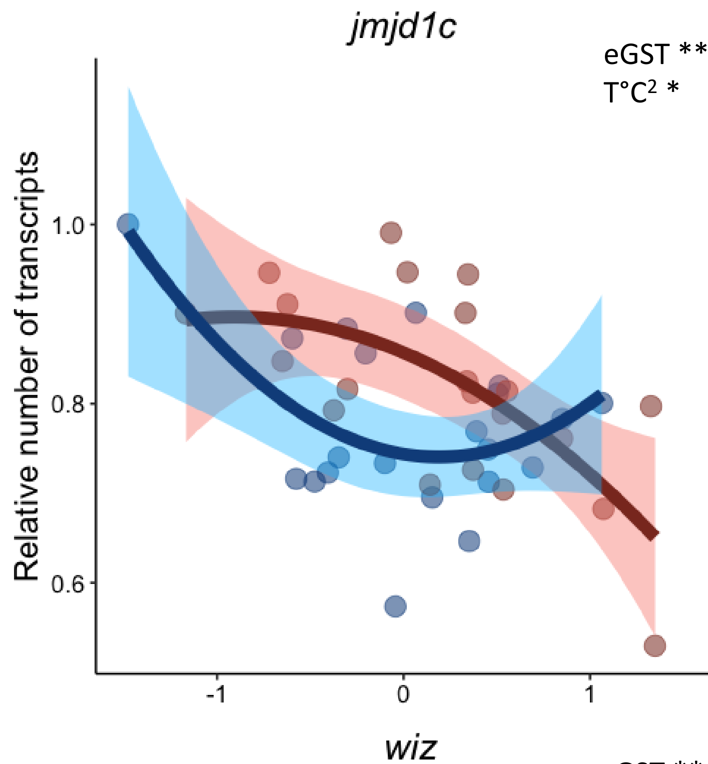
amh eGST **

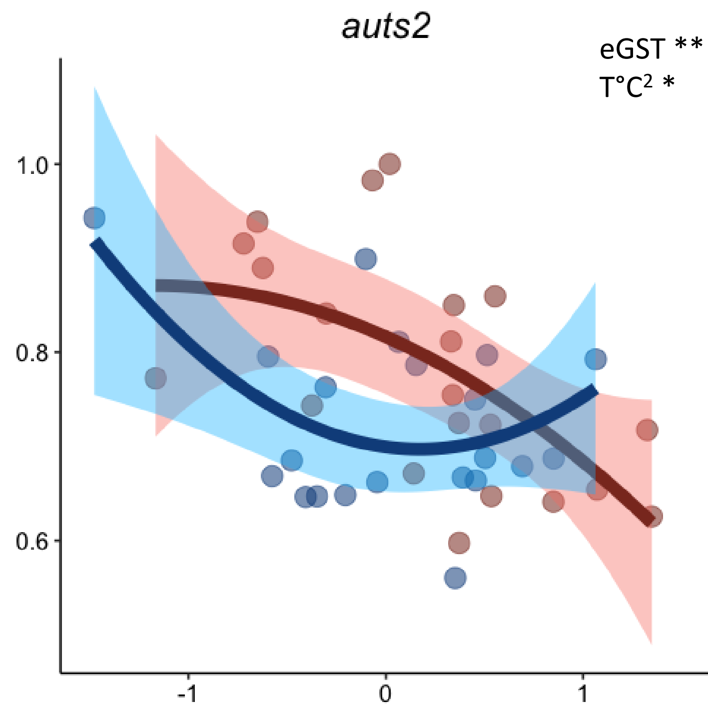
PCA Axis 1 eGST **

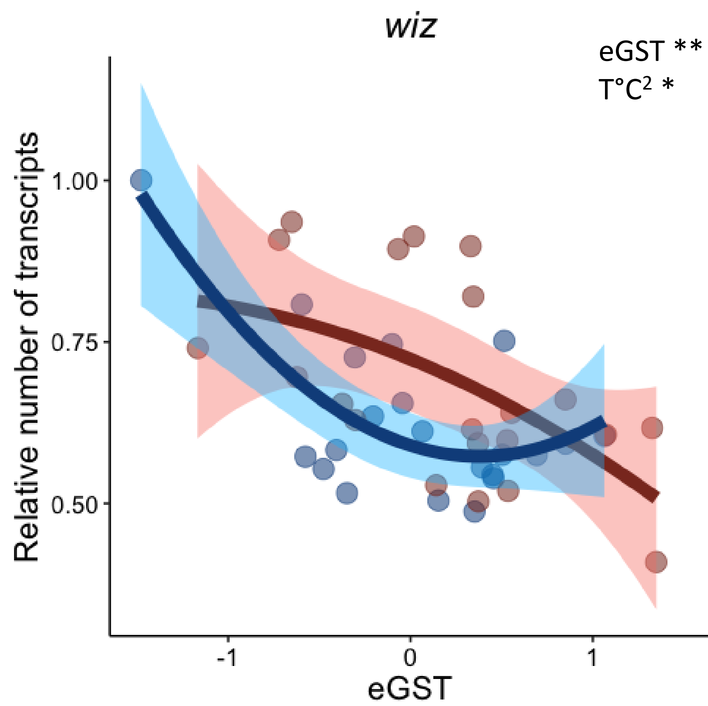
Relative number of transcripts

PC1

eGST


jmjd1c
eGST **
T°C² *
Relative number of transcripts
1.0
0.8
0.6
-1
0
1


auts2
eGST **
T°C² *
1.0
0.8
0.6
-1
0
1


wiz
eGST **
T°C² *
Relative number of transcripts
1.00
0.75
0.50
-1
0
1
eGST

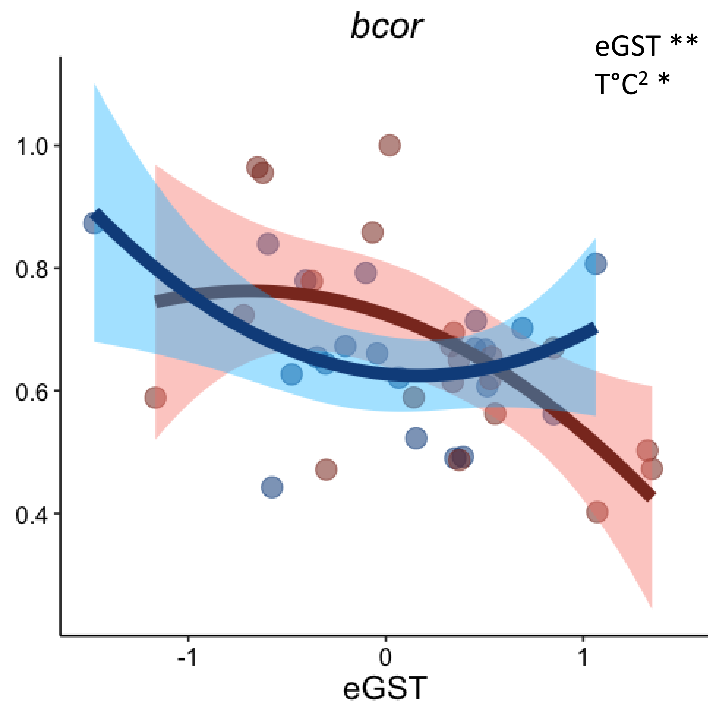

bcor
eGST **
T°C² *
1.0
0.8
0.6
0.4
-1
0
1
eGST

Fig. 5

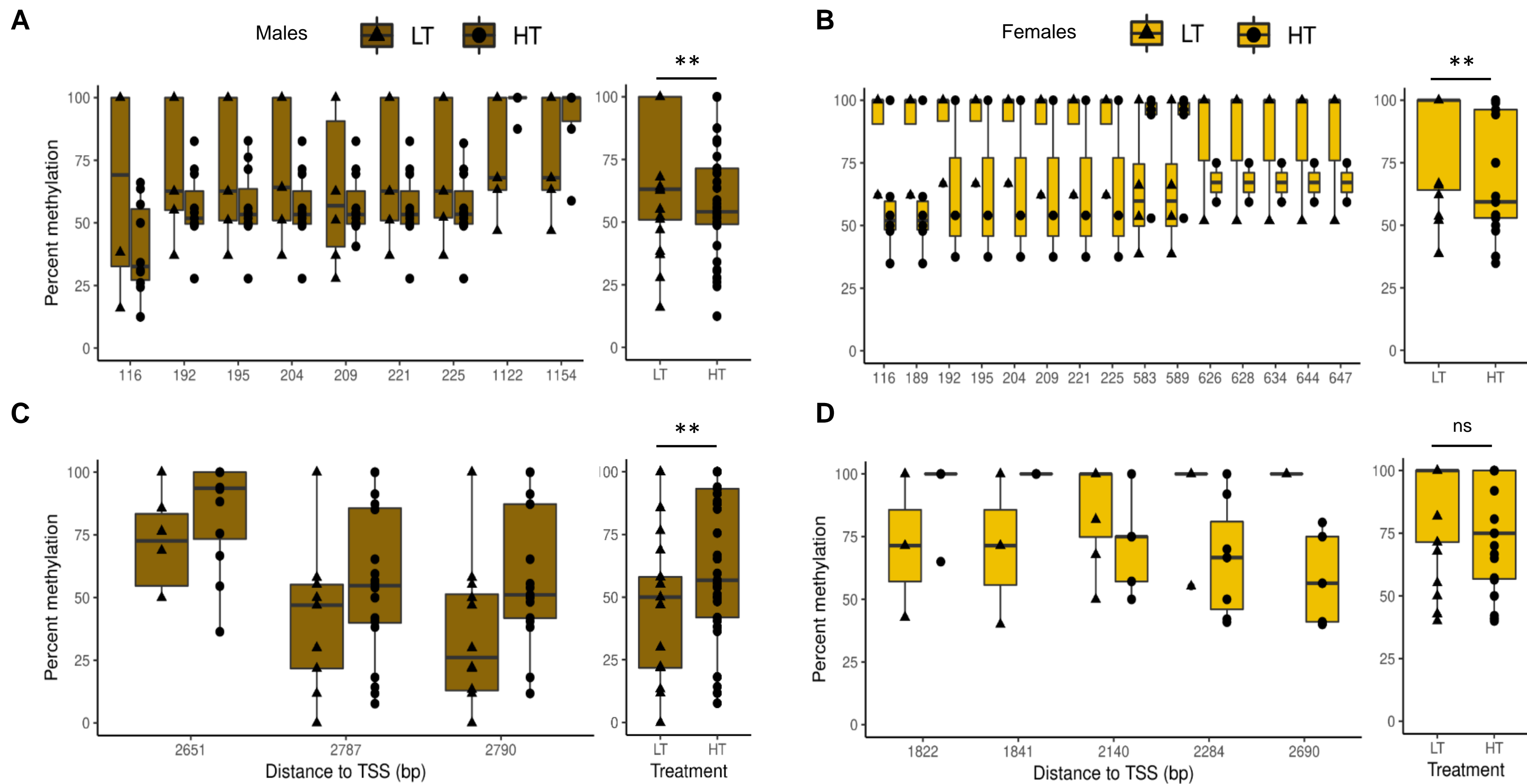


Fig. 6