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# Dynamic epimarks in sex-related genes predict gonad phenotype in seabass, a fish with mixed genetic and environmental sex determination

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**Abstract :**

The integration of genomic and environmental influences into methylation patterns to bring about a phenotype is of central interest in developmental epigenetics, but many details are still unclear. The sex ratios of the species used here, the European sea bass, are determined by genetic and temperature influences. We created four families from parents known to produce offspring with different sex ratios, exposed larvae to masculinizing temperatures and examined, in juvenile gonads, the DNA methylation of seven genes related to sexual development by a targeted sequencing approach. The genes most affected by both genetics and environment were *cyp19a1a* and *dmrt1*, with contrasting sex-specific methylation and temperature responses. The relationship between *cyp19a1a* methylation and expression is relevant to the epigenetic regulation of sex, and we report the evidence of such relationship only below a methylation threshold, ~80%, and that it was sex-specific: negatively correlated in females but positively correlated in males. From parents to offspring, the methylation in gonads was midway between oocytes and sperm, with bias towards oocytes for *amh-r2, er-β2, fsh-r* and *cyp19a1a*. In contrast, *dmrt1* levels resembled those of sperm. The methylation of individual CpGs from *foxl2, er-β2* and *nr3c1* were conserved from parents to offspring, whereas those of *cyp19a1a, dmrt1* and *amh-r2* were affected by temperature. Utilizing a machine-learning procedure based on the methylation levels of a selected set of CpGs, we present the first, to our knowledge, system based on epigenetic marks capable of predicting sex in an animal with ~90% accuracy and discuss possible applications.

**Keywords** : early development, environmental temperature, DNA methylation, sex determination, aromatase, cyp19a1a, dmrt1, epigenetic marks, DNA methylation threshold, epigenetic inheritance

## Background

The integration of genomic and environmental information to bring about a given phenotype is a central area of current research on developmental epigenetics [1,2]. Species where sex determination is dependent on both genetic and environmental influences provide excellent systems to address important questions such as how the environment shapes the epigenome during critical stages of early development with lifelong lasting consequences, and what is the contribution of epigenetic regulatory mechanisms into these processes [3]. Thus, many poikilothermic vertebrates, i.e., species whose body temperature varies depending on the environment, exhibit environmental sex determination (ESD) [4,5]. This occurs in contrast to homoeothermic vertebrates, where sex is determined by a chromosomal system of genetic sex determination (GSD), either with male (XX/XY) or female (ZW/ZZ) heterogamety, as in mammals and birds, respectively. In species with ESD, sex is determined according to the magnitude of an environmental variable, of which temperature is the most relevant [6]. ESD is present in many reptiles [7] and in some fish [8]. Fish, in particular, are known for having a remarkably plastic sex, and this applies not only to sequential hermaphrodites, which naturally undergo sex change as adults in response to external stimuli [9] but, importantly, also to gonochoristic species, i.e., species where sex is separated in different individuals, exposed to abnormal conditions [10] or to pollution [11]. In fish, GSD includes species with sex chromosomes, where sex depends on the action of a "master" gene, not conserved even in closely related species [12,13], species with a major sex locus plus secondary loci [14,15], and species with polygenic sex determination (PSD), where many autosomal loci contribute with minor additive effects to sex determination [16,17]. Even "pure" GSD species may contain populations where sex is also environmentally influenced if the function of a lability trait,

for example, a transcription factor or an enzyme, becomes under the influence of temperature. Consequently, GSD and ESD, rather than two mutually exclusive types of sex determination, are nowadays regarded as the two ends of a continuum, where the sex of an individual can also be the result of both genetic and environmental influences, with transitions from one system to another occurring frequently [18,19]. This is the case of Nile tilapia (*Oreochromis niloticus*), with a XX/XY system but where quantitative trait loci (QTL) have been identified between families that respond differently, indicating a genetic basis to the response to temperature [20]. It is also the case of the pejerrey, *Odontesthes bonariensis* [21]. However, the underlying molecular mechanism linking temperature to sex ratios in poikilothermic vertebrates, including fish, but also reptiles, has been the subject of much debate [22]. In this regard, PSD species offer a particularly attractive situation because, in a given batch of fertilized eggs, sex ratio is naturally dependent on both maternal and paternal influences (i.e., the "sex tendency" of each progenitor) and thus there will be variations in sex ratios across different families, although successive batches of the same parents will tend to exhibit similar sex ratios [23,24]. Hence, inter-family variation in offspring sex ratios facilitates the quantification of the genetic contribution. As PSD is midway between the two extremes, GSD and ESD, it also facilitates the quantification of the environmental contribution [25].

The European sea bass, *Dicentrarchus labrax*, is one of those gonochoristic teleosts with PSD [17]. At least three QTLs associated with sex determination have been identified in this species [26]. Temperatures 13-16°C constitute the common range for spawning and early development. Variations in offspring sex ratios among families are observed [17,27]. However, temperatures >17ºC masculinize fish that under lower temperatures would develop as females [28,29]. The thermosensitive period is 0-60 days post-fertilization (dpf)

[28]. Like in other species, masculinization by elevated temperature involves downregulation of aromatase (*cyp19a1*) expression levels [30]. Aromatase is the steroidogenic enzyme that irreversibly catalyzes the conversion of androgens to estrogens [31]. *cyp19a1* expression and Cyp19a1 enzymatic activity are necessary to produce adequate amounts of estrogen, which are required for ovarian development in all vertebrates, except therian mammals. Thus, *cyp19a1* is a key gene for sexual development in vertebrates [31–33]. It was in the European sea bass where evidence of an epigenetic mechanism linking environmental temperature during early development and sex ratio was first described [34]. Using bisulfite sequencing and a single gene approach targeting *cyp19a1a*, the gonadal isoform of aromatase, an inverse relationship between methylation of the *cyp19a1a* promoter and gene expression in females was found. Males have constitutively higher levels of *cyp19a1a1* methylation than females. Masculinization results from high temperature-induced hypermethylation of *cyp19a1a* in females during early development, which prevents the binding of the transcription factor forkhead box L2 (*foxl2*) necessary for its transcriptional activation [34]. This leads to a decrease of aromatase expression and the effect is carried out throughout adulthood [34]. Similar results using a targeted single gene approach concerning the hypermethylation of the *cyp19a1* promoter in male-producing temperatures were later reported in other sensitive vertebrates, including turtles [35], alligators [36] and other fish, e.g., the olive flounder [37,38], so the role of aromatase as key actor in sexual development is well established. However, DNA methylation may also be positively correlated with gene expression under some situations [39]. Thus, whether the relationship between *cyp19a1* promoter methylation and gene expression holds across a wide range of methylation levels and is similar for both sexes is not clear. Furthermore, the methylation of other genes involved in sexual development might as well be affected by temperature. In this regard, in the European sea

bass, even small temperature increases during early development are able to affect the DNA methylation of many genomic loci [40], as assessed by methylation-sensitive AFLP, a technique that does not allow single nucleotide resolution. Further, recent studies using whole genome bisulfite sequencing (WGBS) have shown that temperature can indeed affect many loci, as evidenced in Nile tilapia [41]. In the half-smooth tongue sole, *Cynoglossus semilaevis*, a species with a ZW/ZZ chromosomal system with *dmrt1* as the sex determining gene [42], WGBS revealed that the effects of elevated temperature on DNA methylation involved several genes related to sexual development such as *dmrt1*, *amh-r2* and *foxl2* [43]. In this species, fish with a ZW genotype (females) were sex-reversed into phenotypic males and their offspring even when reared at normal temperature still exhibited altered patterns of DNA methylation in the Z chromosome, suggesting an epigenetic transmission of altered DNA methylation patterns [43]. These studies, however, did not consider possible differences between families (genetic variation) nor did they attempt to determine the relative contribution of genetic vs. environmental influences.

Taking advantage of the European sea bass PSD system with mixed genetic and environmental influences, the objective of this study was to further investigate the role of *cyp19a1a* promoter methylation on the regulation of *cyp19a1a* expression and sex determination. We also aimed to determine the parental vs. the environmental influences on the DNA methylation. To this end, four sires known to produce progeny with different proportions of females were crossed with two dams and the resulting offspring from each cross was subjected to either control or masculinizing temperatures. About one year later, when the gonads were fully differentiated, we sampled ~800 fish, genotyped them, calculated the resulting sex ratios and took individual gonads samples in a subsample of 200 randomly selected fish. WGBS, apart from being beyond our possibilities for so many

152 samples, is also considered generally inefficient, with ~80% of reads being uninformative
153 [44]. We, thus, specifically developed a cost-efficient multiplex bisulfite sequencing (MBS)
154 approach for many samples, easily customizable for any species and any genomic region
155 of interest and applicable to any model or non-model species. We used targeted MBS to
156 determine not only the methylation levels of *cyp19a1a* but also of six additional genes
157 (*foxl2, dmrt1*, *amh-r2*, *er-β2*, *fsh-r* and *nr3c1*) at single nucleotide resolution and high
158 coverage. We also measured *cyp19a1a1* expression levels by qPCR. Most important of
159 all, this study allowed the identification of CpGs spread in key sex-related genes the
160 methylation of which is faithfully transmitted from parents to offspring, as well as the
161 identification of CpGs most affected by temperature. Finally, we were able to develop the
162 first, to our knowledge, system capable of predicting sex in a vertebrate based on the
163 analysis of a carefully selected set of epigenetic marks.
164

165 # Results

166

167 **Genotyping and sex ratios**

168 Four European sea bass sires (sires a-d) with different sex tendencies, known from
169 previous experiments to produce more or less females in the offspring, were individually
170 crossed with two dams (Fig 1A). During the thermosensitive period, from 13 to 65 dpf,
171 half of the offspring was reared at low (16.5ºC) and the other half at high temperature
172 (21ºC) in replicate treatments. From the end of this period and onwards, all fish were kept
173 at 20-22ºC until sampling. In total we had 790 European sea bass sampled at ~11 months
174 available for genotyping and sex ratio analysis. Genotyping unambiguously assigned 764
175 fish to the parents but showed that most (99%) offspring came from dam b, meaning that
176 the rate of fertilization of the eggs of dam a was low, that the viability of the offspring of

177 dam a was low, or both, something that is frequent in fish[45–51]. Thus, the few offspring of
178 dam a were no longer considered and we only report results related to the four families
179 derived from dam b. Considering only the offspring of one dam and increasing sample
180 size allowed us in fact to unambiguously disentangle the sire-specific effects. Sex ratio
181 analysis showed that the offspring of dam b crossed with sires a, b, c or d had different
182 percentages of females: 11.9, 25.2, 35.0 and 48.3%, respectively (Fig 1B). Thus, the
183 progeny of sires a and b, previously shown to give less female offspring, had a lower
184 percent of females than the progeny of sires c and d, previously shown to give more
185 female offspring (Fisher's exact test for count data; $p<0.0001$). The sex ratios were
186 independent of the replicate tank in which fish were raised (Fig S1) and those of the
187 progeny of sires a, b and c significantly departed from the Fisherian sex ratio (Table S1).
188 High temperature masculinized a subset of the females (Fig 1B). This masculinization
189 was similar to that observed in the internal control group of albino European sea bass,
190 which were added in each tank (see Materials and Methods) in order to check that the
191 observed effects were indeed due to the temperature treatments and not to spurious tank
192 effects (Fig S2). Masculinization by high temperature was sire-dependent: 72, 49, 21 and
193 45% of the females were masculinized in the offspring of sires a–d, respectively (percent
194 between the dotted lines inside the LT offspring; Fig 1B) and the effect was significant in
195 the progeny of sires b and d, but not in the progeny of sires a and c (Table S1). Therefore,
196 these results clearly show sire-specific differences not only in sex ratios at control
197 temperature but also in the degree of masculinization as a response to elevated
198 temperature.
199

As usual in European sea bass, females were bigger than males at the time of sampling, with small differences among the offspring of the different sires. Early exposure to temperature slightly increased growth in both sexes (Fig S3 and Table S2 for statistics).

**DNA methylation levels of genes related to sexual development in the gametes of the parents and the gonads of the offspring**

The overall DNA methylation profiles of the seven genes related to sexual development were gene-specific and showed a wide range of values (Fig 2). The most extreme values were present in *foxl2* (<1%) and *er-β2* (>95%). Of the seven CpGs measured in the European sea bass *cyp19a1a* promoter, DNA methylation was essentially 100% in CpGs at positions -431 (Fig S4A and S5) and +60 (Fig S4B and S5) relative to the transcription start site (TSS) in all fish. Thus, these two CpGs were excluded from further analysis. Among the remaining five CpGs, CpG at position 9 showed a strong positive correlation with the rest of the CpGs at positions -56, -49, -33 and -13 ($\rho$=0.77, $p$<2.2e-16; Fig S4C and S5). Even stronger was the positive correlation of the CpGs at positions -56, -49, -33 and -13 (an example of the four possible combinations is shown in Fig S4D and all of them in Fig S5).

In the offspring gonads, and regardless of sire, sex and temperature significantly influenced DNA methylation of *cyp19a1a* (2-way ANOVA using robust M-estimators and 5000 bootstraps; sex: $p$=0.027 and temperature: $p$=0.025). Further, the interaction of both factors was also significant (Sex x Temperature: 0.018). DNA methylation of *dmrt1* was also influenced by sex and temperature (2-way ANOVA; sex: $p$<0.001 and temperature: $p$=0.016). For *cyp19a1a*, higher DNA methylation levels were observed in

females reared at high temperature, while in *dmrt1*, the inverse pattern was present, with lower DNA methylation in females reared at high temperature and even lower in males (Fig 2). For the rest of the examined genes (*foxl2, amh-r2, er-β2* and *fsh-r*), non-significant effects of sex and temperature were observed, except from a significant interaction of sex and temperature in *nr3c1* (2-way ANOVA; $p$=0.030; Fig. 2). However, when individual sires were taken into account, with the exception of *nr3c1*, sire-specific tendencies of increase or decrease of DNA methylation according to sex and/or temperature were evident. For example, in *cyp19a1a*, in the offspring of sires b and c, there was higher DNA methylation in females reared at high temperature and in males, and the levels of DNA methylation were similar to the ones of the progeny of all sires combined. In the offspring of sire a, however, although the trends were the same, the actual DNA methylation levels were lower in the females reared at low temperature. On the contrary, in sire d, the opposite pattern was evident, with a tendency of lower DNA methylation in females reared at high temperature and in males. These results not only show that there are important differences in absolute methylation levels depending on the gene considered but also the existence of sex-specific differences in methylation levels for some genes, the existence of gene- and sex-specific responses to temperature and, finally, that there also are sire-specific differences. Together, they clearly illustrate that both the genetic and environmental contribution to DNA methylation of specific genes are important for sexual development in this fish.

We also measured DNA methylation in the oocytes of dam b, the one from which all analyzed offspring were derived, as well as the sperm of the four sires. For five out of the seven analyzed genes (*cyp19a1a, foxl2, amh-r2, er-β2* and *fsh-r*), DNA methylation in oocytes was lower than in sperm. In the case of *dmrt1* it was the other way round:

250 methylation in the oocytes was higher than in the sperm, whereas *nr3c1* showed very
251 stable methylation levels across parents, resembling the situation described above
252 concerning the offspring. Focusing on the sperm, sire a departed from the rest in the sense
253 that DNA methylation of some genes (*cyp19a1a*, *dmrt1, fsh-r*) was clearly lower when
254 compared to values found in the other sires; for *foxl2* sire a, in contrast, exhibited the
255 highest DNA methylation levels. These results also show clear gene- and sire-specific
256 differences in methylation but, in general, methylation in sperm was higher than in
257 oocytes, although this should be taken with caution since only one dam was analyzed.
258
259 **DNA methylation and gene expression relationships in *cyp19a1a***
260
261 Sufficient estrogen levels, resulting from aromatase enzyme activity, are essential for
262 ovarian development in all egg-laying vertebrates. Thus, we examined the factors
263 affecting *cyp19a1a* expression. The most influential factor on *cyp19a1a* expression was
264 sex (F=9.11, $p$=0.003), with higher levels in females, followed by sire (F=3.21, $p$=0.025;
265 Table 1 and Fig S6) and with a significant interaction between them (F=5.18, $p$=0.002;
266 Table 1). However, the effect of temperature was not significant (F=0.11, $p$=0.741; Table
267 1).
268
269 We also found a significant interaction between DNA methylation and sex on *cypa19a1a*
270 expression (F=10.34; $p$<0.001; Table 1). We further investigated this relationship and
271 found that when the promoter of *cypa19a1a* was hypermethylated (~80% and above) in
272 both sexes, there was no sex-dependent difference in neither mean methylation nor in
273 expression of *cypa19a1a* (W=286, $p$=0.065 and W=468, $p$=0.304 respectively; Fig 3, red
274 data points). However, when there was intermediate methylation (Fig 3, green data points)

or hypomethylation (Fig 3, blue data points) there was significantly more methylation in males than in females (W=198, $p$=0.00059 and W=188, $p$=0.00048 respectively), as well as significantly less expression in males than in females (W=624, $p$=0.00087 and W=699, $p$=4.717$^{e-07}$ respectively). Thus, although in general the correlation between *cypa19a1a* promoter methylation and expression was weakly negative (Spearman's rank correlation ρ=-0.023, $p$=0.76) this inverse relationship holds well only below a certain methylation threshold. However, when we considered the sexes separately we found that this correlation was indeed negative for females but actually positive for males. Further, in both sexes correlation coefficients varied according to sire and temperature (Fig 4), suggesting again the existence of genetic x environment interactions.

**Stable and dynamic CpGs from parents to offspring**

Next, to identify stable methylation patterns from parents to offspring, we calculated, for each gene, the methylation difference between levels in parents (specifically, the sperm of the different sires) minus levels in offspring for each analyzed CpG. To do this, we contemplated both sexes but we only used fish reared at low temperature to exclude possible environmental influences. Methylation differences close to zero were considered indicative of stable methylation levels, whereas differences above and below zero indicated hypomethylation and hypermethylation, respectively, in offspring vs. parents. Methylation differences depended on individual CpGs within a given gene, the sex and the sire (Fig 5A). However, regardless of sex and sire, the more stable levels of methylation were observed in the CpGs of *er-β2*, *foxl2* and *nr3c1* (Fig 5B). These low differences were independent from the actual methylation levels in the sires, whether close to 0 or close to 100%, although there were no intermediate values to compare,

suggesting that low difference values had not their origin in low or very low methylation levels. Thus, genes were grouped into two categories, stable (*er-β2*, *foxl2* and *nr3c1*) or dynamic (*cyp19a1a, dmrt1, fsh-r* and *amh-r2*) independently of sex (Fig 6).

The same approach based on the analysis of differences was used to identify the CpGs most responsive to temperature. In this case, we searched for the CpGs with the highest methylation differences between fish exposed to HT vs LT. Again, changes were both gene- and sire-dependent (Fig 7A). Nevertheless, and regardless of sire and even sex, the methylation of CpGs of *dmrt1* and *cyp19a1a* showed the biggest differences between offspring exposed to high and low temperature. Additionally, the CpGs of *amh-r2* were responsive to temperature, but only in male offspring (Fig 7B). As before, these temperature-dependent differences in methylation in specific CpGs were independent of the actual methylation levels (Fig S7).

**Prediction of phenotypic sex by analysis of epigenetic profiles: epigenetic biomarkers**

Based on the above, we hypothesized that the methylation of the CpGs of the genes most influenced by temperature in this species with mixed genetic and environmental sex determination might be indicative of the sex. Therefore, we performed Principal Component Analysis (PCA) with 5-fold cross validation with 87 fish which represented the offspring for which information on all these genes was available, using the CpGs (n=23) of these genes as variables. The five independent validations gave similar results and one of them is shown as representative (Fig 8A). The first two dimensions of the PCA explained 61.9% of the total variation and the 72 individuals of the training set were

clearly separated according to their sex (blue and red dots, Fig 8A). For the remaining 15 individuals (the test set), the sex was predicted using the information of the training set (green dots, Fig 8A). Only for two male individuals (pink arrows, Fig. 8A) out of 15 in total, the sex was incorrectly predicted. The average success rate for this method of predicting sex, using 5-fold cross validation, based only on methylation information of the CpGs of these genes was 88.09%. To our knowledge, this constitutes the first prediction of phenotypic sex using methylation status in an animal. Confirmation of the prediction power of this method was evidenced by the lack of differences in *cyp19a1a* expression levels between fish of the test set vs. the training set in each sex. The levels of the two males wrongly classified coincided with the extremes of the distribution of *cyp19a1a* expression levels for males (Fig 8B).

## Discussion

In this study, we show that the methylation levels of the promoters of one of the most important genes for male sexual development, *dmrt1*, and one of the most important genes for female sexual development, *cyp19a1a*, are affected by both genetic background, sex and temperature in a fish with mixed genetic and environmental sex determination. As for *cyp19a1a*, understanding how its expression is regulated by DNA methylation is of relevance across vertebrates, and here we also show a negative association between *cyp19a1a* promoter methylation and expression levels in females, as expected, but, surprisingly, a positive association is seen in males. The individual CpGs of some of the studied gene promoters, namely *er-β2*, *foxl2* and *nr3c1*, exhibit stable methylation levels between sperm and gonads at control temperature and regardless of sex, suggesting parent-to-offspring transmission of the methylation state. Lastly, we found that the

methylation levels of the CpGs of the genes most responsive to temperature were sufficient to group the fish by sex and predict it with close to 90% accuracy.

For the specific needs of the study, we successfully developed and applied a low-cost version of targeted MBS to simultaneously interrogate the methylation status of several genes in a large sample size at single nucleotide resolution and with very high coverage. The objective was to study specific questions related to sexual development, therefore, a candidate gene approach was ideal. In addition, by targeting only 7 genes, we were able to identify CpGs sufficient for sex prediction; our approach was thus not only reasonable but also valid. In comparison with other similar approaches [52,53], we have considerably decreased the cost of the protocol by reducing to the minimum the use of proprietary kits at all steps and especially during size-selection and normalization by using the BeNUS protocol [54] with home-made version of magnetic beads and 3D-printed magnetic stands. This renders our method an accessible approach to more researchers that aim to analyze a considerable amount of samples to address specific questions.

The sex ratio results of this study (Fig. 1), where European sea bass larvae were exposed to control or elevated temperature during early development, provide several insights worth mentioning. First, the eggs of the same dam, when separately fertilized with the sperm of four different sires produced offspring with different proportions of males. This disentangles the sire-specific effects on sex ratios and indicates inter-family variation [30,55]. Second, sires a and b, who produced more males in a previous cross with a different dam, also produced the highest proportion of males. Conversely, sires c and d previously gave the highest percent of females, and this was also the case in this study. Together, these results reflect the quantitative nature of the sex tendency in the European sea bass [56].

375 Third, the response to high temperature was also family-dependent and with different
376 proportions of sex-reversals, an observation that evidences the existence of genotype-
377 environment interactions [27]. In general 22–72% (average ~50%) of fish that would
378 develop as females at low temperature, differentiated as males when exposed to elevated
379 temperature, a standard figure for sea bass [28]. Taken together, these observations confirm
380 the polygenic nature of sex determination in the European sea bass [17], where phenotypic
381 sex emerges out of a combination of heritable and temperature influences [29]. Within this
382 framework, it is pertinent to ask how genetics and environment shape the methylation
383 status of key genes related to sexual development. Here, to disentangle the genetic from
384 the environmental effects on DNA methylation, we used 100-200 fish from the offspring
385 of the 4 sires and the dam and bisulfite sequenced at 7 genes related to sex determination
386 and differentiation, among them *cyp19a1a* for which gene expression was also measured.
387
388 Of the network of genes involved in vertebrate gonadal differentiation, *cyp19a1a* and
389 *dmrt1* exhibit a clear sex-dimorphic expression across a wide range of species including
390 fish, with higher expression in ovaries and testis, respectively [57–60]. In accordance with
391 this, when we compared the methylation of these genes in juvenile, sexually differentiated
392 gonads, we found *cyp19a1a* hypomethylated in ovaries with respect to testis and *dmrt1*
393 hypomethylated in testis with respect to ovaries. These results are in accordance with
394 results in other species where the methylation of these two genes has been measured
395 [34,38,43], and support the Conserved Sexual Development (CSD) model in fish, which is
396 based on the assumption that there are “pro-male” and “pro-female” genes and that
397 predicts that a given set of epigenetic marks and gene expression patterns are associated
398 with the male or the female sexual phenotype [37]. However, in contrast with past studies,
399 ours is the first that takes into consideration genetic variation (Fig. 2), showing the clear

existence of inter-family variation in methylation levels of some of the genes examined. It is known that genetic variation may influence epigenetic variation by the presence of single nucleotide polymorphisms (SNP), which may influence the methylation of CpG sites by acting in *cis* [61,62]. In this regard, the European sea bass *cyp19a1a* promoter exhibits three polymorphisms [63], but we could not find any particular association with these polymorphisms and methylation levels in the present study. Apart from *cyp19a1a* and *dmrt1*, and, to a lesser extent, *amh-r2*, the rest of genes did not show clear sex-specific methylation levels and, further, did not either show a clear response to temperature.

Thus, there was variation in the methylation levels of *cyp19a1a* and *dmrt1* dependent on the family and on the response to temperature. In particular, the female offspring of sire a had lower methylation levels for both genes than the female offspring derived from sires b-d. The overall methylation levels of *cyp19a1a*, i.e., lower in females than in males and increased, particularly in females, after exposure to elevated temperature confirm previous results in the European sea bass [34]. However, with respect to our previous study, here we show that *dmrt1* has exactly the opposite behavior of *cyp19a1a,* hence supporting the CSD model mentioned above [37]. Besides, we show the importance of taking variation into account. Further, we show that other genes the expression of which may be dimorphic during sex differentiation —for example, *foxl2* is more expressed in developing ovaries [64] whereas *amh-r2* is more expressed in developing testes [65]— did not really show sex-specific differences in methylation. These findings suggest that either not all differences in expression are regulated via DNA methylation changes or that differences in expression regulated by the recruitment of transcription factors may precede the changes in DNA methylation, that in addition are very likely to occur in distal genomic elements, e.g., in enhancers [66].

The relationship between *cyp19a1a* methylation and gene expression is of relevance to understand the epigenetic regulation of this important gene for vertebrate sexual development. In this study, we first found that such a relationship was seen only for methylation levels below ~80% (Fig. 3). This suggests the existence of an effective range of DNA methylation available for gene expression regulation that should be considered in future studies in this and probably also in other species. Second, whereas there was a clear inverse relationship between methylation and expression in females, surprisingly, this relationship was positive in males (Fig. 4). In juvenile testis, *cyp19a1a* is expressed at varying but in general low levels. Several recent studies have revealed that a positive correlation of DNA methylation with gene expression is present at the genome level [61,67]. Importantly, there are *cis* acting genetic loci associated with DNA methylation of CpGs, especially outside CpG islands, as well as with the expression of nearby genes in which positive correlation of DNA methylation and expression is evident [68]. The positive correlation observed in males, therefore, could be resulting from the genetic component of the sex determination system. This would be the case of male-specific genetic variants in genomic proximity to *cyp19a1a* that influence the methylation and the expression of the gene. In any case, our results call for taking into account sex-dependent differences in the relation between DNA methylation and gene expression in future studies. Further, a recent study has shown that the first intron is the gene region that, along the promoter and the first exon is the most informative as regards to the inverse relationship between DNA methylation and gene expression, and that this applies across tissues and species [69]. Thus, in future similar studies, the first intron should be considered.

We also examined the methylation levels of the target genes of this study in the oocytes and sperm of the progenitors. The aim of this was to identify possible relationships between the methylation of the gametes of the progenitors and that of the juvenile gonads. Given that the offspring of only one dam was taken into account for analysis, we were able to decipher the effects of the sires without confounding maternal effects. In zebrafish, global methylation levels of the sperm are higher (~91%) than those of the oocytes (~80%). Right after fertilization, global zygotic methylation is midway between sperm and oocyte levels, but by mid-blastula transition and gastrulation levels increase [70,71]. Global methylation levels in the adult muscle are also midway between sperm and oocyte [71]. To our knowledge, there is no similar information for other fishes. Our data cannot be directly compared to that of zebrafish because rather than global levels we measured levels of seven genes at single nucleotide resolution in gametes and gonads. However, this approach provides some interesting insights. For example, in all examined genes except *dmrt1*, methylation levels of the sperm tended to be higher than those of oocytes, thus resembling the situation in zebrafish [70,71]. Also, methylation levels of the offspring tended to be similar compared to the dam and tended to be lower compared to the sires, although there were sire- and gene-specific tendencies. *foxl2* showed a distinct profile, but this should be considered with care, since this gene always showed extreme hypomethylation (0-1%). Furthermore, *nr3c1* was the only analyzed gene where methylation levels were essentially the same, around 10%, in sperm, oocytes and gonads, regardless of family and temperature conditions. Changes in the methylation status of *nr3c1* have been associated with different illnesses in humans [72] and in the European sea bass *nr3c1* expression in the liver is downregulated following exposure to stress [73]. *nr3c1* has different alternative promoters [72] and the stable methylation levels of *nr3c1* may

473 indicate that epigenetic regulation is done in a different promoter than the one analyzed
474 here, the one adjacent to the TSS.
475
476 Different CpGs from the same gene promoter may exhibit differences in methylation
477 levels and in the response to environmental cues, changes that may affect gene expression
478 [74]. Consequently, we focused on the methylation differences of individual CpGs between
479 sires and offspring (Figs. 5 and 6) and between fish reared at high vs. low temperature
480 (Fig. 7). We observed that stable methylation levels between sires and offspring (defined
481 here as sire-offspring differences <5%) of three genes, *er-β2*, *foxl2* and *nr3c1*, were
482 independent of actual methylation levels. We cannot exclude the possibility that this lack
483 of differences in *foxl2* and *er-β2* is due to fixed hypo- (<2%) or hyper- (>95%)
484 methylation, respectively, in these genomic regions. In *nr3c1* though, the actual levels in
485 all offspring were around 10%. In contrast, the rest of the examined genes, *amh-r2*, *fsh-*
486 *r*, *dmrt1* and *cyp19a1a,* showed differences of approximately ± 30% when individual
487 CpGs were considered (Fig. 5A). It is interesting to note that methylation levels in male
488 and female offspring of *cyp19a1a, amh-r2, fsh-r* and *er-β2* were roughly similar to levels
489 in the oocytes of the dam. On the other hand, methylation levels of *dmrt1* in the offspring
490 were similar to levels in sperm, but not oocytes, and, further, roughly matched levels of
491 each corresponding sire. These findings are novel in the sense that, to our knowledge, no
492 similar data is available for other vertebrates. Nevertheless, it seems that there are gene
493 promoters in which CpG methylation is conserved, while in other genes the methylation
494 varies, at least from sperm to gonads. It could be argued that stable CpGs constitute
495 transmissible epigenetic marks but they also could be regarded as uninformative if it was
496 confirmed in other species that they lack variation. However, they could also be regarded
497 as a sort of "essential epigenetic marks", as defined elsewhere [37], with respect to dynamic

498 CpGs, which when properly combined can help to predict phenotypic sex (see below).
499 Also, in a recent study in the European sea bass we have shown that the sperm, in addition
500 to its DNA, carries a complex population of chromatin-associated proteins [75], providing
501 further possibilities for the epigenetic transmission of information across generations.
502
503 The CpGs in the promoter of three genes, *amh-r2*, *dmrt1* and *cyp19a1a*, not only showed
504 the highest variation from sire to offspring at low temperature (Fig. 5B) but also were the
505 most responsive to elevated temperature (Fig. 7A). The response of specific CpGs were
506 sire-dependent but, at the same time, there was an overall pattern evident in the female
507 and male offspring when all sires were combined (Fig. 7B). It is interesting to note
508 differences in the methylation changes between different CpGs within the same gene
509 promoter. This was evident in *dmrt1* and *cyp19a1a*. Also, there was a sex-specific
510 component in the case of *amh-r2* especially, which responded to temperature in the males
511 but not in the females.
512
513 In species without external sexual dimorphism and with late sex differentiation, the
514 phenotypic sex of an individual remains unknown unless it is sacrificed. Being able to
515 predict the sex of an undifferentiated individual based on epimarks is, therefore, of great
516 interest. We selected the CpGs of these three genes, because they captured well both
517 components of the sex determination system, the genetic and the environmental, since
518 they showed variation between sires and they responded to temperature (Fig. 7). The
519 combination of the CpGs which were more sensitive to temperature collectively
520 contributed to a robust outcome variable that allowed prediction of phenotypic sex
521 independently of sire and temperature (Fig. 8). Since DNA methylation levels are
522 established during development and are stable throughout adulthood, the methylation of

523 the panel of these CpGs is most likely to be established during the sex determination
524 period. Identifying a defined panel of 23 CpGs located in only three genes that are enough
525 for phenotypic sex prediction, especially before the period of sex differentiation, is of
526 high importance for a species with a polygenic system of sex determination and without
527 sex chromosomes, in which until now sex could only be identified after gonad formation.
528 In addition, considering that the methylation levels of *cyp19a1a* and *dmrt1* seem to follow
529 the same pattern in unrelated species [37], the same panel of CpGs from the corresponding
530 gene regions might work to predict the sex of other species. Prediction of sex based on
531 epigenetic marks has recently been achieved in the balsam poplar, *Populus balsamifera*
532 [76]. Thus, to the best of our knowledge, our study is the first to identify CpGs enabling the
533 prediction of sex in an animal.
534
535 Epigenetically mediated responses of phenotypically plastic traits, such as sex, can be
536 adaptive or maladaptive depending on the speed of environmental change. It has been
537 suggested that these responses may have implications on population sex ratio in species
538 responding to climate change, as well as for farmed species [77,78]. In sea bass aquaculture,
539 male-biased stocks are often still present despite of thermal protocols applied to control
540 sex. This could be due to an epigenetic maladaptive response or epigenetic trap leading
541 to male-biased offspring of male-biased stocks. Thus, in a sort of "reproductive epigenetic
542 programming" applied to fish farming [79], the identification of broodstock fish with a
543 particular methylation profile holds promise because these animals may pass to their
544 offspring specific DNA methylation marks. These epigenetically inherited DNA
545 methylation profiles would provide offspring with desired features. For example, by
546 conferring them resistance to the masculinizing effect of elevated temperature.
547

### Conclusions

The changes in DNA methylation of the promoters of key genes related to sexual development in response to temperature are influenced by parents. There are CpGs with a stable parent-to-offspring pattern, suggestive of inheritance of epigenetic marks, although this aspect needs confirmation. Taking these into account, a complex epigenetic layer contributing to sex determination and differentiation is revealed, adding to the better understanding of the shaping of population sex ratios. The European sea bass is a vertebrate where a major plastic phenotypic trait, sex, is under the control of genetic and environmental influences with approximate equal strength of each. This study clearly illustrates how the epigenome integrates environmental information to the genome. It also shows variation in this epigenetic component and calls for further studies to gain a better picture of the interplay between these different regulatory components that bring, as Conrad Waddington said, "the phenotype into being". Importantly, we came up with a combination of CpGs belonging to key genes for sexual development and sensitive to temperature that jointly contributed to an efficient prediction of phenotypic sex for the first time in an animal.

## Materials and Methods

### Fish and general rearing conditions

Four West Mediterranean European sea bass sires (sires a–d) were selected based on their tendency to produce more or less females in the offspring, as assessed in two preliminary crossings. Thus, sire a had given 0% and 20.5% females (mean: 10.25%); sire b, 7.5 and 8.8% females (mean: 8.15); sire c, 25.0% and 43.2% females (mean: 34.1); and sire d, 25.9% and 58.6% females (mean: 42.25%). We crossed each sire with two randomly

chosen West Mediterranean dams, thus producing 8 families. Cryopreserved sperm from the four sires and eggs from the two dams were kept for evaluation of the DNA methylation analysis. After fertilization, the batches corresponding to the four sires were incubated separately at ~14.5°C, then equalized in volume of floating eggs at 48 hours post fertilization and mixed in two groups, a "male-prone" group, containing the offspring of sires a and b, and a "female-prone" group, containing the offspring of sires c and d (Fig 1A). A second crossing of eleven dams and twenty albino sea bass sires was done the same day of the experimental crossing. These fish were used as "spike-ins" to control for possible tank effects on sex ratio (see below). All procedures performed were in accordance with the ethical standards of the institution and followed the European Directive 2010/63 UE. This study was conducted under the official national license of Dr. Marc Vandeputte (B34-437) in the premises of Ifremer in Palavas-les-Flots (France), registered as an authorized structure for animal experimentation (agreement C34-192-6). All fish handling procedures were conducted under anesthesia (40 ppm benzocaine) to minimize animal stress and suffering. Terminal sampling was performed after euthanasia by an overdose of benzocaine (150 ppm).

**Temperature treatments**

The hatched larvae from the male- and female-prone groups were split in two groups at the age of 13 days-post-fertilization (dpf). One group was reared at 16.5°C (low temperature, LT), the control group, and the other group at 20°C (high temperature, HT), the latter to induce environmentally-mediated masculinization. Thus, four combinations were available: male-prone (offspring from sires a and b) at LT, male-prone at HT, female-prone (offspring from sires c and d) at LT and female-prone at HT. Each combination was replicated in two experimental tanks, so that 8 tanks were used during

598 this period (Fig 1A). In each tank, an equal number of albino fish was also included, in
599 order to be able to ascertain that the expected distorted sex ratios at HT were indeed due
600 to the temperature treatments and also to identify any possible tank effect. Temperature
601 treatments lasted until 65 dpf, the end of the thermosensitive period, at which point
602 temperature was raised to 21°C to allow sufficient growth of the LT groups in order to
603 facilitate sexing of the fish at the end of the experiment. From day 135 onwards,
604 temperature was maintained at 20-22°C in all tanks until sampling.
605
606 **Tagging and Samplings**
607 After anesthetic sedation by immersion in 40 ppm of benzocaine, fish were individually
608 tagged with nano-tags (Nonatec) as soon as it was feasible, i.e., when they reached an
609 average weight of ~1.5 g (85 dpf for HT and 105 dpf for LT) to follow their growth rate,
610 which is highly linked to sex determination in the juvenile sea bass [80].
611
612 At 323 dpf, fish were sedated in their rearing tank by adding a mild dose of anesthetic,
613 captured and then euthanized with excess anesthesia. A total of 790 experimental and 868
614 albino fish were measured for body weight and length and were sexed by visual
615 inspection, which allows unambiguous sex identification at this age. Gonad samples were
616 preserved in liquid nitrogen for 200 fish in total: 10 females and 10 males from each one
617 of the four LT tanks (80 fish) and 10 females and 20 males from each one of the four HT
618 tanks (120 fish). The higher number of males was used to take into account for
619 masculinized females in the HT groups. For a summary of the experimental design see
620 Fig 1A. All related data are available in Supplementary Data 1.
621
622 **Genotyping**

The 790 experimental fish were genotyped for 12 microsatellite markers by Labogena-DNA (Jouy-en-Josas, France). Seven hundred sixty eight of those (97.2%) gave adequate markers' amplification, and 764 (96.7%) were traced back to a single parent pair, using VITASSIGN [81] with 1 mismatch tolerated. It turned out that almost all fish were derived from only one dam, so the remaining fish from the second dam (only 9 in total) were not included in the analyses, which then comprised 755 fish with known pedigree. The number of fish available for analysis of sex ratios from the LT groups was: 42 from sire a, 147 from sire b, 100 from sire c, and 87 from sire d. The number of fish available for analysis from the HT groups was: 59 from sire a, 132 from sire b, 76 from sire c, and 112 from sire d.

**Quantitative real-time PCR (qRT-PCR)**

RNA was extracted from the 200 fish gonad samples using the TRIzol® Reagent (ThermoFisher Scientific) according to manufacturer's instructions after homogenization of the tissues using a pistil immersed in TRIzol® solution. RNA was quantified using a ND-100 spectrophotometer (NanoDrop Technologies). Five hundred nanograms of RNA were reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase (ThermoFisher Scientific) and 100 µM of random hexamers (ThermoFisher Scientific). Primers for cytochrome p450 aromatase (*cyp19a1a*) were previously validated in sea bass [34,82], as well as primers for the two reference genes used, the elongation factor-1 (*ef-1a*) and the 40S ribosomal protein S30 [83]. All primers targeted regions between two exons to avoid amplification of possible traces of genomic DNA. Primers efficiency was estimated using serial dilutions (1, 1:5, 1:10, 1:50, 1:100, 1:500) of a pool of 1 µl from each sample (200 µl in total) as $E=10^{(-1/slope)}$, with slope derived from the log-linear regression of the calibration curve. qRT-PCR reactions were carried out in triplicate including negative

controls without cDNA in a total volume of 10 µl using the EvaGreen dye (Biotium) under the following conditions: 50ºC for 2 min, 95ºC for 10 min and 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The specificity of the amplification was evaluated using melting curve with the following conditions: 95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s. qRT-PCR reactions were performed on an ABI 7900HT machine (Applied Biosystems).

**Quantitative real-time PCR (qRT-PCR) data analysis**

Cq values were exported from a multiple plate analysis, which included five 384-well plates using the RQ Manager 1.2.1 (Applied Biosystems). The mean Cq values and standard deviations (<0.3) were calculated for each technical triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method [84]. The geometric mean of the two reference genes was subtracted from the *cyp19a1a* Cq values to obtain the normalized dCq for statistical analysis. The efficiency of the *cyp19a1a* primers was estimated to be 2.15. All related data are available in Supplementary Data 2.

**Multiplex bisulfite sequencing (MBS) library preparation and bioinformatics**

Two separate MBS libraries were constructed: the first library (MBS1) contained *cyp19a1a* amplicons from 183 valid samples (out of 200 original ones) from the offspring gonads as well as the sperm samples of the four sires and the oocytes of the two dams. The second library (MBS2) contained *amh-r2*, *dmrt1*, *er-β2*, *foxl2*, *fsh-r* and *nr3c1* amplicons in a representative subset of 94 samples and in the 5 parents. An average of 6 individual samples per sire, sex, and temperature treatment was ensured for all groups. For the HT males, the maximum available of 12 fish were included.

673 ***DNA extraction and bisulfite conversion.*** DNA was extracted by the standard phenol-
674 chloroform-isoamyl alcohol (PCI; 25:24:1) protocol. Treatment with 1 μg of proteinase
675 K (Sigma-Aldrich) and 0.5 μg of ribonuclease A (PureLink RNase A; Life Technologies)
676 were used to eliminate the presence of proteins and RNA, respectively. For cryopreserved
677 sperm samples, two PBS washings followed by a 1:5 dilution in PBS preceded the
678 incubation with proteinase K. Five hundred nanograms of DNA per sample were bisulfite
679 converted using the EZ DNA Methylation-Direct™ Kit (Zymo Research; D5023) in two
680 batches of 96-well plates, following the manufacturer's instructions with extended
681 desulphonation time to 30 min. Elution of bisulfite converted DNA was performed with
682 20 μl of Milli-Q autoclaved $H_2O$ passing the same volume twice through the column by
683 centrifugation.
684
685 ***Primers design.*** Primers were designed for bisulfite converted DNA using MethPrimer
686 (Table S3 [85]). Primers were further validated using Primer3Plus [86] after *in silico* bisulfite
687 conversion of the target sequence using Bisulfite Primer Seeker (Zymo Research).
688 Amplicons were designed so that they never exceeded 550 bp in length in order to
689 ascertain the acquisition of overlapping paired-end reads using the 300 bp paired-end
690 Illumina sequencing protocol. An ideal amplicon's range was considered between 450
691 and 500 bp encompassing as many CpGs as possible. The target regions included as much
692 as possible from the first exon and the promoter, in this order of priority due to the
693 importance of the first exon in the regulation of gene expression [87], of each target gene.
694 At this point it should be recalled that our own recent studies in the European sea bass
695 show that not only the first exon but specially the first intron is very informative of the
696 inverse relationship between DNA methylation and gene expression [69], so the first intron
697 should be considered in future similar studies. Adapters were added to the 5' ends of the

region-specific primers as in Illumina's protocol for 16S metagenomic library preparation (Table S3). The target region of *cyp19a1a* included the 7 CpGs studied previously in sea bass [34] at positions -431, -56, -49, -33, -13, 9 and 60 relative to the TSS, encompassing parts of the promoter, 5' UTR and first exon.

***Amplicons PCR.*** Amplifications of targeted regions were performed in a total volume of 25 µl containing: 25 ng of DNA (2 µl), 4 mM $MgCl_2$, 0.8 mM dNTPs, primers at 0.8 µM (Life Technologies), 2.5 U of GoTaq G2 Hot Start polymerase (Promega) and its corresponding 5X Green GoTaq Flexi Buffer (Promega). PCR conditions were as follows: 7 min at 95ºC, followed by 40 cycles of 95ºC for 1 min, annealing temperature between 55 and 60ºC depending on each primer pair (Table S3) for 2 min and 65ºC for 2 min, with a final step at 65ºC for 10 min. The primers were validated by Sanger sequencing of amplicons from a pool of two samples. The presence and size of bands were confirmed by agarose gel electrophoresis in all samples.

***Size-selection and normalization.*** After PCR amplification of the target regions, we performed size-selection (MBS1 and MBS2) and normalization of DNA quantities (MBS2) across PCR products following a customized version of the bead-based normalization of Hosomichi et al. [54]. The working solution of serapure magnetic beads was prepared by washing 2 ml of Sera-mag SpeedBeads (Fisher 09981123) with Tris-EDTA (TE; 10 mM Tris; 1 mM EDTA) and adding the beads in a total volume of 50 ml containing 20% PEG-8000, a concentration of 2.5 M NaCl, 500 mM of Tris-HCl, 1 mM EDTA and 0.00055% Tween 20 (Adapted from: [88]). In brief, 8 µl of PCR product and 42 µl of Milli-Q autoclaved $H_2O$ were incubated for 5 min at room temperature with 20 µl of beads. Following 2 min incubation on the magnetic stand (3D-printed 96-well magnetic

723 rack designed by http://www.thingiverse.com/acadey/ and realized by MAKE Creative
724 Spaces), supernatants were transferred to new wells and incubated for 5 min with 0.8x of
725 magnetic beads for MBS1 and with 0.6x of magnetic beads for MBS2. After discarding
726 the supernatant, a single wash with 70% freshly prepared ethanol was performed and
727 DNA was eluted in 20 µl Milli-Q autoclaved $H_2O$. For MBS2, size-selected PCR products
728 were incubated in equal volumes with 20-fold diluted magnetic beads (PEG 20% and 2.5
729 M NaCl) and isopropanol. After incubation for 5 min at room temperature and washing
730 with 70% freshly prepared ethanol, PCR products were eluted in 20 µl Milli-Q autoclaved
731 $H_2O$. Since each amplicon contained theoretically equal DNA amounts, identical volumes
732 of each amplicon were pooled for each biological sample, resulting in 99 wells containing
733 6 amplicons each.
734
735 ***Index PCR and size-selection.*** Sample-specific indices were incorporated into the
736 amplicons following a dual-index strategy with i7 indices from Nextera XT index Kit
737 SetA and i5 indices from Nextera XT index Kit SetD (Illumina; FC-131–2001 and FC-
738 131–2004). For MBS1, the Nextera XT index Kit SetA for 94 samples and the Nextera
739 XT index Kit SetD (Illumina; FC-131-2001 and FC-131-2004 accordingly) for 95
740 samples were used. For MBS2, a combination of indices from the same Nextera XT index
741 kits was used. PCR reactions were performed using the 2x KAPA HiFi HotStart
742 ReadyMix, 5 µl of each primer and 5 µl of template pooled amplicons DNA in a total
743 volume of 50 µl with the following conditions: 95ºC for 3 min, 8 cycles of 95ºC for 30 s,
744 55ºC for 30s and 72ºC for 30s and a final step at 72ºC for 5 min, according to Illumina's
745 protocol for 16S metagenomic library preparation. Size-selection and normalization of
746 DNA quantities across samples was carried out after the index PCR according to the
747 customized bead-based normalization using 0.6x of magnetic beads. PCR products were

748 eluted in 15 µl Milli-Q autoclaved $H_2O$ and 2 µl of each sample were pooled together.
749 Therefore, we obtained a single multiplexed library with *cyp19a1a* for 189 samples and
750 another single multiplexed library with 6 genes (*amh-r2, dmrt1*, *er-β2*, *foxl2*, *fsh-r* and
751 *nr3c1*) for 99 samples. After pooling, extra clean-up steps were performed using 0.5x
752 magnetic beads in order to ensure the absence of primers. DNA quantity of final libraries
753 was measured trice by the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and the
754 Agilent DNA 1000 chip and DNA High Sensitivity (Agilent) by which the size of bands
755 were also visualized. The multiplexed final libraries were additionally quantified by real-
756 time qPCR using the Kapa system prior to sequencing on a MiSeq (Illumina) using the
757 paired-end 300 bp protocol.
758
759 ***Multiplex bisulfite sequencing library bioinformatics.*** Samples were demultiplexed
760 based on the dual-indices by the instrument's software. Adapters and linker sequences
761 were trimmed for paired-end reads by Trim Galore! (Babraham Bioinformatics), while
762 filtering for low quality bases (Phred score < 20). Quality controls of the data were carried
763 out before and after trimming using FastQC (Babraham Bioinformatics). Trimmed reads
764 were aligned against the *in silico* bisulfite converted sea bass genome (dicLab1 v1.0c, Jul.
765 2012) [89] using Bismark (v. 0.14.4) [90] for both *in silico* bisulfite conversion and alignments.
766 Mappings were done in three steps in a non-directional way: 1) paired reads were aligned,
767 2) unmapped reads from the first step were aligned as single reads, and 3) unpaired reads
768 from the first step of trimming were aligned like the unmapped reads. An alignment was
769 considered valid if the score attributed was above f(x) = 0 + -0.6 * read length. The
770 alignments of unmapped (2) and unpaired (3) reads were merged using samtools (v. 1.5)
771 and treated as single-end reads. Alignments were visually inspected using the Integrated
772 Genome Browser [91] and the genomic boundaries and amplicon sizes were confirmed.

Methylation calling was performed by the bismark_methylation_extractor of Bismark separately for paired-end and for single-end reads. Paired and single reads were merged for each sample in a single file and the rest of the analysis were carried out using R, Rstudio [92,93] and Bioconductor [94]. Only CpGs with coverage more than 5 were retained for further analysis. For each CpG per sample, counted cytosines and thymines were summed up from paired-end and single-end reads and percent of methylation was calculated as 100*(Cs/Cs+Ts). The bisulfite conversion ratio was calculated as 100 minus the percent of Cs methylated in CHH context from the report of Bismark for paired-end reads. The mean bisulfite conversion ratio was 99.55%. The libraries' statistics can be found in Supplementary Data 2 and all related data are available in Supplementary Data 2.

**General statistical analysis**

All statistical analysis were performed using R [92,93]. The tank effect on resulting sex ratios was evaluated by Fisher's exact test for count data. Departures from Fisherian sex ratios and effects of temperature on sex ratios were assessed by Fisher's exact test for count data. The effects of sex, sire and temperature were evaluated on body weight, fork length and condition factor (K), the latter defined as $K = 100*(W/L^{3.02})$, where W = weight in g and L = length in cm, of the offspring by multifactorial ANOVA for unbalanced data separately. The data were previously tested for homogeneity of variances by Levene's test and the normality of the residuals of the linear regression was assessed by the Shapiro-Wilk normality test using $\log_2$-transformed values for body weight and fork length and sine-transformed values for condition factor (K). Methylation data was evaluated by 2-way ANOVAs and the normality of the residuals was tested by the Shapiro-Wilk test. In the cases where the assumption of normality of the residuals was violated, the values were

798 logit-transformed. In the cases where the normality of residuals was violated even with
799 transformed values, 2-way ANOVAs with modified M-estimators and 5000 bootstraps
800 were performed.
801
802 **Analysis of DNA methylation data**
803 ***Mean DNA methylation levels.*** Association between DNA methylation levels of the
804 CpGs was estimated using Pearson's product-moment correlation coefficients. Mean
805 DNA methylation levels were calculated by averaging the methylation percentages of
806 each CpG per gene per sample and subsequently averaging the overall methylation per
807 grouping factor depending on the comparison. For *cyp19a1a*, mean methylation levels
808 were calculated using only the 5 central CpGs (-56, -49, -33, -13 and +9) per sample
809 because the two extreme CpGs (-431 and +60) turned out to be always 100% methylated.
810 Two-way ANOVA was used to assess the effects of sex and temperature on mean DNA
811 methylation levels for each gene. The normality of the residuals was tested by the
812 Shapiro-Wilk test. For three genes (*er-β2*, *fsh-r* and *nr3c1*), logit-transformed values were
813 used for the two-way ANOVAs since the assumption of normality of the residuals was
814 otherwise violated. In the case of *cyp19a1a* and *amh-r2*, we used two-way ANOVAs with
815 modified M-estimators and 5000 bootstraps (function pbad2way of the WRS2 package
816 v.0.9-2).
817
818 ***Transmission of DNA methylation patterns.*** Next, we focused on individual CpGs,
819 which were 82 in total distributed in 7 genes, in order to investigate, on one hand, the
820 possible transmission of the methylation status from sire to offspring, and, on the other
821 hand, to identify the CpGs that were most responsive to temperature. To study sire-to-
822 offspring transmission, only the offspring reared at low temperature was used to exclude

823 the possible distorting effects of high temperature on methylation levels. For each CpG,
824 the methylation in each offspring was subtracted from the methylation of the
825 corresponding sire and the mean of these differences was calculated per sex and per sire.
826 Thus, positive values indicate hypermethylation in sire and negative values indicate
827 hypermethylation in offspring. The idea was to identify those CpG with the differences,
828 positive or negative, as close as possible to zero. On the other hand, to study the effects
829 of high temperature, for each CpG the mean methylation per sex and sire in offspring
830 reared at low temperature was subtracted from the mean methylation in offspring reared
831 at high temperature. In this case, positive values indicate hypermethylation at high
832 temperature and negative values indicate hypermethylation at low temperature. Here, the
833 idea was to identify those CpGs with the highest differences. The latter approach allowed
834 us to identify the genes that exhibited the most dynamic changes with temperature.
835
836 ***Epigenetic biomarkers to predict sex***. We used the 26 CpGs of the above genes in 93
837 fish. We filtered the data as follows: 1) we removed 6 samples for which there was no
838 information on the methylation of 15 or more of the CpGs, and 2) we removed 3 CpGs
839 for which there was no information in more than 12 fish. Therefore, for the rest of the
840 analysis we used 23 CpGs in 87 fish. The rest of missing data were imputed by the MICE
841 algorithm that generates multivariate imputations by chained equations using the mice
842 package (v. 2.46.0) [95]. We performed Principal Component Analysis (PCA) with 5-fold
843 cross validation. The offspring was divided into 5 groups by the function createFolds of
844 the caret package (v. 6.0-78). Each of the 5 groups was used as test set, the coordinates
845 of which were predicted based on the PCAs performed on the remaining individuals
846 (training sets). In this way, each fish was used as both training and test. The average
847 success of the method was calculated based on the average percent of individuals for

which the prediction failed. The results of the PCA and the predicted individuals were visualized using the package factoextra (v. 1.0.5).

**Statistical analysis of DNA methylation and gene expression data for *cyp19a1a***

A multifactorial ANOVA was used to assess the effects of sex, sire parent, temperature and methylation levels, as well as their possible interactions, on the expression of *cyp19a1a* using $\log_2$-transformed $2^{\Delta Cq}$ values. The sum of squares for ANOVA was calculated by the Type III approach since the design was unbalanced and interactions between factors were expected. The Shapiro-Wilk normality test was used to confirm normality of the residuals' distribution. The methylation levels in this ANOVA model were integrated as categorical variables per sex, hypomethylation being the first 33.3% of the total distribution of values, intermediate methylation the values between 33.3% and 66.6% of the total distribution and hypermethylation values above the 66.6% of the total distribution. Differences in *cypa19a1a* expression were tested using Wilcoxon signed rank test applying a continuity correction when needed on $2^{\Delta Cq}$ values. Associations between DNA methylation levels and *cypa19a1a* expression were estimated using Spearman's rank correlation coefficients. The offspring of sire a was excluded from the analysis of correlations since very few individuals were present.

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## Availability of materials and data

All data are available as Supplementary Files.

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**Disclosure statement**

The authors declare that they have no conflict of interest.

**Authors' contributions**

DA designed and carried out the MBS protocol, performed data analysis, interpreted results and wrote the article. MV and FA performed the temperature experiments and genotyping. MV conceived the study, provided reagents, interpreted results and revised the article. NSB constructed the MBS2 library and revised the article. FA interpreted results and revised the article; FP conceived the study, designed experiments, provided reagents, interpreted results and wrote the article. All authors read and approved the final manuscript.

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## Figure legends

**Figure 1**. **Experimental set-up and sex ratios of the offspring.** A) Experimental set-up of crossings and temperature treatments. Eggs were obtained from crossing sires known from previous trials to produce offspring with lower (sires a and b) or higher (sires c and d) percentage of females. The sires were crossed with two females, however the offspring of one dam only represented 0.9% of the total offspring and was thus excluded from further analysis. Two days post fertilization (dpf) eggs were mixed according to the male prone and female prone groups. On day 13, larvae from the two groups were divided into four with half of the fish being raised at low temperature (LT; 16.5ºC) and the other half at high temperature (HT; 21ºC) until 65 dpf, the end of the thermosensitive period. In each tank, an equal number of albino fish was added as a control for tank effects. Fish were sampled at one year of age (323 dpf) and gonad samples were taken from 10 females and 10 males for the LT groups and 10 females and 20 males from the HT groups from each tank. B) Paternal effects on European sea bass sex ratio and effects of elevated temperature. Percent of female (red) and male (blue) offspring of each sire (a, b, c and d) raised at low (LT) or high (HT) temperature. Absolute numbers of analyzed fish are shown in the bottom of each bar. The dotted lines indicate the differences of sex ratios between the LT and the HT offspring of each sire. The numbers between the dotted lines inside the LT bars indicate the percent of sex-reversed females among the LT females, while the numbers inside the HT bars indicate the percent of presumed neomales (sex-reversed females into males) among the HT males. The effects of temperature on sex ratios were assessed by Fisher's exact test for count data and shown with the following equivalence: ** = $p<0.01$.

**Figure 2. DNA methylation levels of the genes examined in the parents and the offspring.** In the left part of the figure, the DNA methylation in the oocytes of the dam (grey), the sperm of sire a (deep pink), b (violet), c (orange) and d (green blue) is shown. The central and right part of the figure illustrate the DNA methylation levels in the offspring, separately by each sire with the background color indicating the corresponding sire and also, in the offspring of the four sires combined. The offspring is divided in four groups according to sex and temperature experienced during early development and DNA methylation values are shown in the ovaries of low (yellow) and high (red) temperature females and in the testis of low (light blue) and high (blue) temperature males. The far-right data indicate p-values for the effects of sex (S), temperature (T) or their interaction (SxT). The absolute numbers of fish analyzed in each case are shown inside the bars. Data as mean ± SEM.

**Figure 3. Expression of *cyp19a1a* in gonads of females and males with low, intermediate or high DNA methylation levels**. In the left side, the distribution of DNA methylation values is shown by individual points for females (F) and males (M). Datapoints in blue (low), green (intermediate) and red (high) correspond to the first, second and third terciles of the distribution, respectively. The central boxplots represent low (blue), intermediate (green) and high (red) DNA methylation levels in females and males. The boxplots on the right side display the distribution of *cyp19a1a* expression depending on the level of DNA methylation in females and males. The boxes include the values distributed between the lower and upper quartiles, the upper whisker = min(max(x), Q3 + 1.5 * IQR), the lower whisker = max(min(x), Q1 – 1.5 * IQR), where IQR= third quartile (Q3) – first quartile (Q1). The black dots inside the boxplots indicate the mean and the line the median. Asterisks represent the level of significance of Wilcox

rank sum test between females and males: ns=not significant; *** =$p$<0.001. Notice the increase in inverse relationship between DNA methylation and gene expression with lower methylation levels.

**Figure 4. Correlations of expression of *cyp19a1a* and mean DNA methylation of the five central CpGs of its promoter**. Correlations between *cypa19a1a* expression and promoter DNA methylation are shown by Spearman's rank correlation coefficient (ρ) in the gonads of female and male offspring of sires b, c and d reared at low (LT) or high temperature (HT). There was insufficient data for offspring of sire a. The direction of the long axis of the ellipses and the color indicate the type of correlation, with negative shown in shades of red and positive shown in blue. The short axis of the ellipse and the color intensity are proportional to the correlation coefficients.

**Figure 5. Methylation differences from sire to offspring in individual CpGs.** Mean methylation difference in individual CpGs of the seven genes analyzed between the sperm and the gonads of their male and female offspring reared at low (control) temperature. Information is provided individually for sires a–d (A) and independently of sire (B). Data are shown as the mean of methylation differences of the corresponding sire to the individual fish in each group ± SEM.

**Figure 6. Relationship of methylation in the sires and in the offspring.** Scatterplot of the mean methylation differences per gene calculated as levels in the sires minus levels in their corresponding offspring reared at low temperature (female offspring: circles; male offspring: squares). Methylation differences close to zero indicate stable methylation levels, whereas differences above and below zero indicate hypomethylation and

hypermethylation, respectively, in offspring vs. parents. The regression lines correspond to genes with low methylation differences between sires and offspring (*foxl2*, *nr3c1* and *er-β2*) and genes with higher methylation differences between sires and offspring (*cyp19a1a*, *dmrt1*, *fsh-r* and *amh-r2*).

**Figure 7. Methylation differences between high and low temperature in individual CpGs.** Mean methylation difference in individual CpGs of the seven genes analyzed between the offspring reared at high vs. low temperature according to sex. Information is provided individually for sires a–d (A) and independently of sire (B). Data are shown as the difference of the mean methylation of individual fish reared at high temperature minus the mean methylation of individual fish reared at low temperature.

**Figure 8. Prediction of offspring phenotypic sex using the methylation of selected CpGs as epigenetic biomarkers.** A) The DNA methylation levels of individual CpGs from the three genes that presented the highest differences between fish reared at low and at high temperature were the multiple variables used in the PCA. The individual fish are plotted as dots in the space of the two principal components. The percentage of variance explained by the two first components is shown in parenthesis. Of the total sample size available (n=87), 83% (n=72) were used as training set and are colored according to sex (female, red; male, blue) and 17% (n=15) of the individual fish are colored in green for which the coordinates and hence the sex was predicted based on the training set (F, females; M, males). Confidence ellipses are drawn for the two groups and colored according to sex. The pink arrows point to the two predicted individuals for which prediction of sex failed. The names of the variables (Bn and Cn; n = 1, 2, …) correspond to the CpGs of informative genes. Of the total CpGs used for the PCA only the 10 with

1289 the highest contribution to the principal components are shown for clarity. B) Distribution
1290 of the expression of *cyp19a1a* in females and males. Fish are divided in three groups
1291 based on the PCA analysis: training set, test set with success and test set with fail. The
1292 expression is shown by boxplots as $2^{\Delta Cq}$ values for the first two groups and individual
1293 points for the third group. The boxes include the values distributed between the lower and
1294 upper quartiles, the upper whisker = min(max(x), Q3 + 1.5 * IQR), the lower whisker =
1295 max(min(x), Q1 – 1.5 * IQR), where IQR= third quartile (Q3) – first quartile (Q1), the
1296 black triangle indicates the mean, the tick line the median and the points outside the boxes
1297 represent values higher than the upper whisker. Statistical significance is shown as
1298 follows: ns=not significant; *** = $p$<0.001.
1299

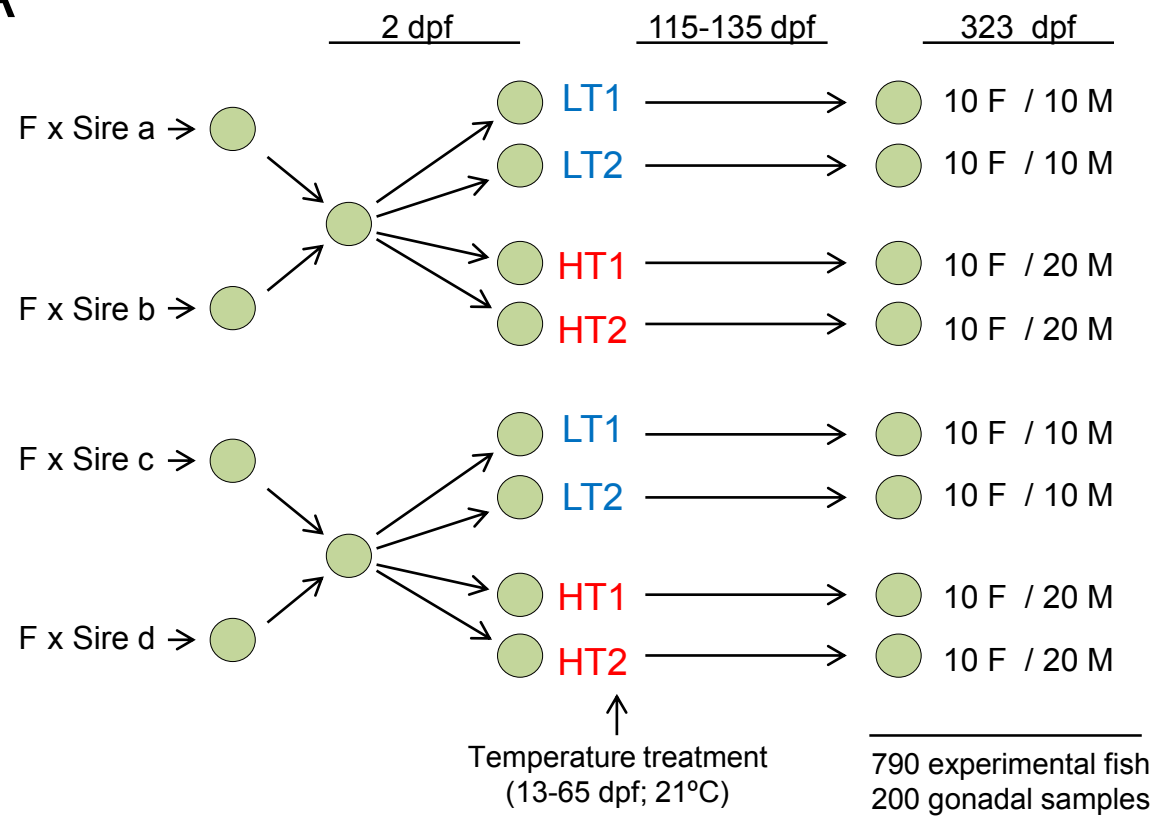
1300 **Table 1**. Contribution of genetic (sire), epigenetic (promoter methylation), physiological
1301 (sex) and environmental (temperature) factors to the expression of *cyp19a1a* in the
1302 European sea bass

| | | SS | d.f. | F value | p |
|---|---|---|---|---|---|
| Factors | Intercept | 2792.04 | 1 | 4516.30 | $<2.2^{e-16}$ |
| | Sex | 5.63 | 1 | 9.11 | **0.003** |
| | Sire | 5.96 | 3 | 3.21 | **0.025** |
| | Methylation level | 1.06 | 2 | 0.86 | 0.426 |
| | Temperature | 0.07 | 1 | 0.11 | 0.741 |
| Interactions | Sex:Temperature | 1.44 | 1 | 2.33 | 0.129 |
| | Sex:Sire | 9.61 | 3 | 5.18 | **0.002** |
| | Sex:Methylation level | 12.78 | 2 | 10.34 | $\mathbf{6.224^{e-05}}$ |
| | Sire:Temperature | 2.00 | 3 | 1.08 | 0.360 |
| | Methylation level:Temperature | 0.23 | 2 | 0.19 | 0.830 |
| | Sire:Methylation level | 2.02 | 6 | 0.54 | 0.774 |
| | Residuals | 92.73 | 150 | | |

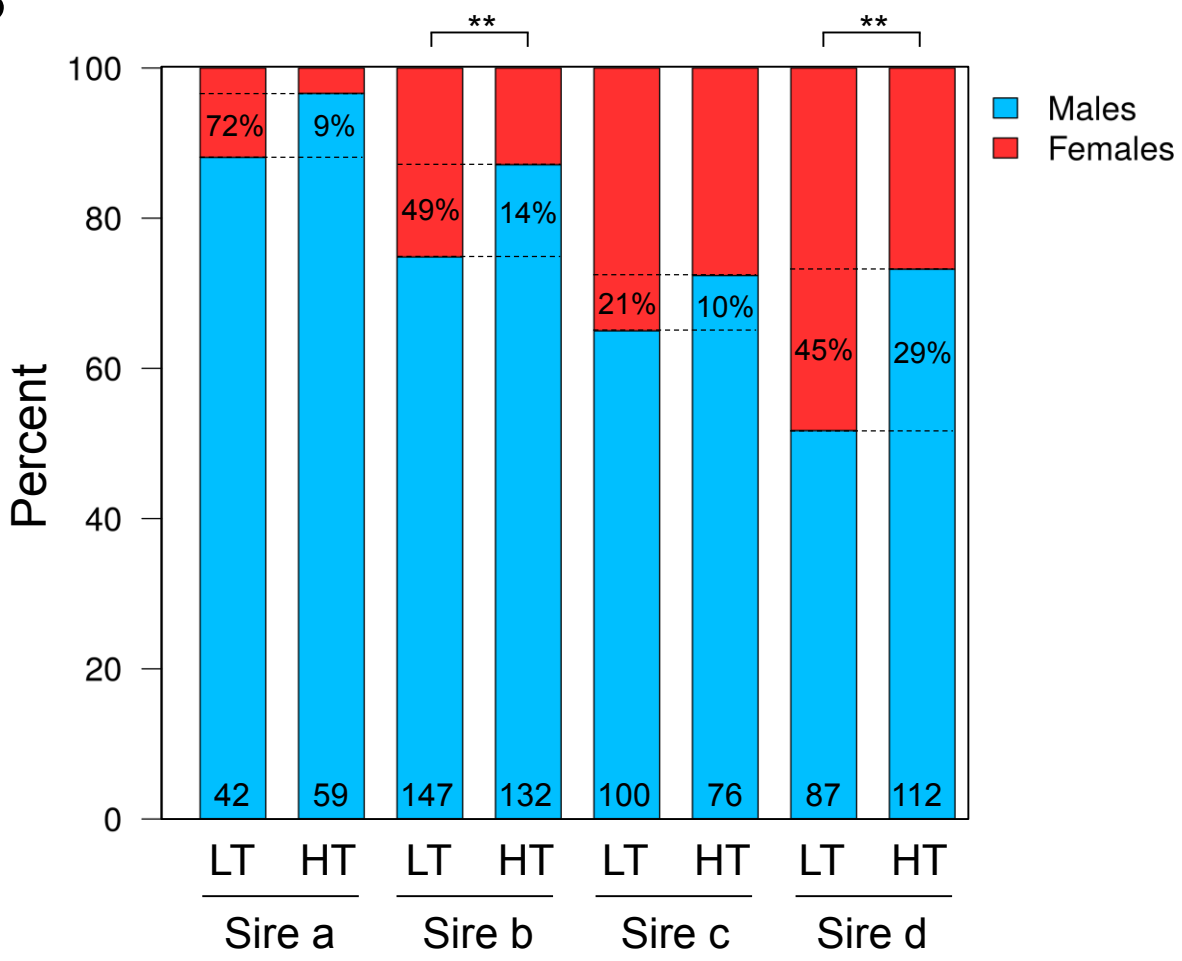
1303 The effects were tested by multifactorial ANOVA and statistically significant factors are
1304 shown in bold.
1305 Abbreviations: d.f., degrees of freedom; SS, Sums of Squares
1306

1307

A


2 dpf
115-135 dpf
323 dpf
F x Sire a
F x Sire b
F x Sire c
F x Sire d
LT1
LT2
HT1
HT2
10 F / 10 M
10 F / 20 M
Temperature treatment
(13-65 dpf; 21ºC)
790 experimental fish
200 gonadal samples

B


**
**
Percent
100
80
60
40
20
0
72%
9%
49%
14%
21%
10%
45%
29%
42
59
147
132
100
76
87
112
LT
HT
Sire a
Sire b
Sire c
Sire d
Males
Females

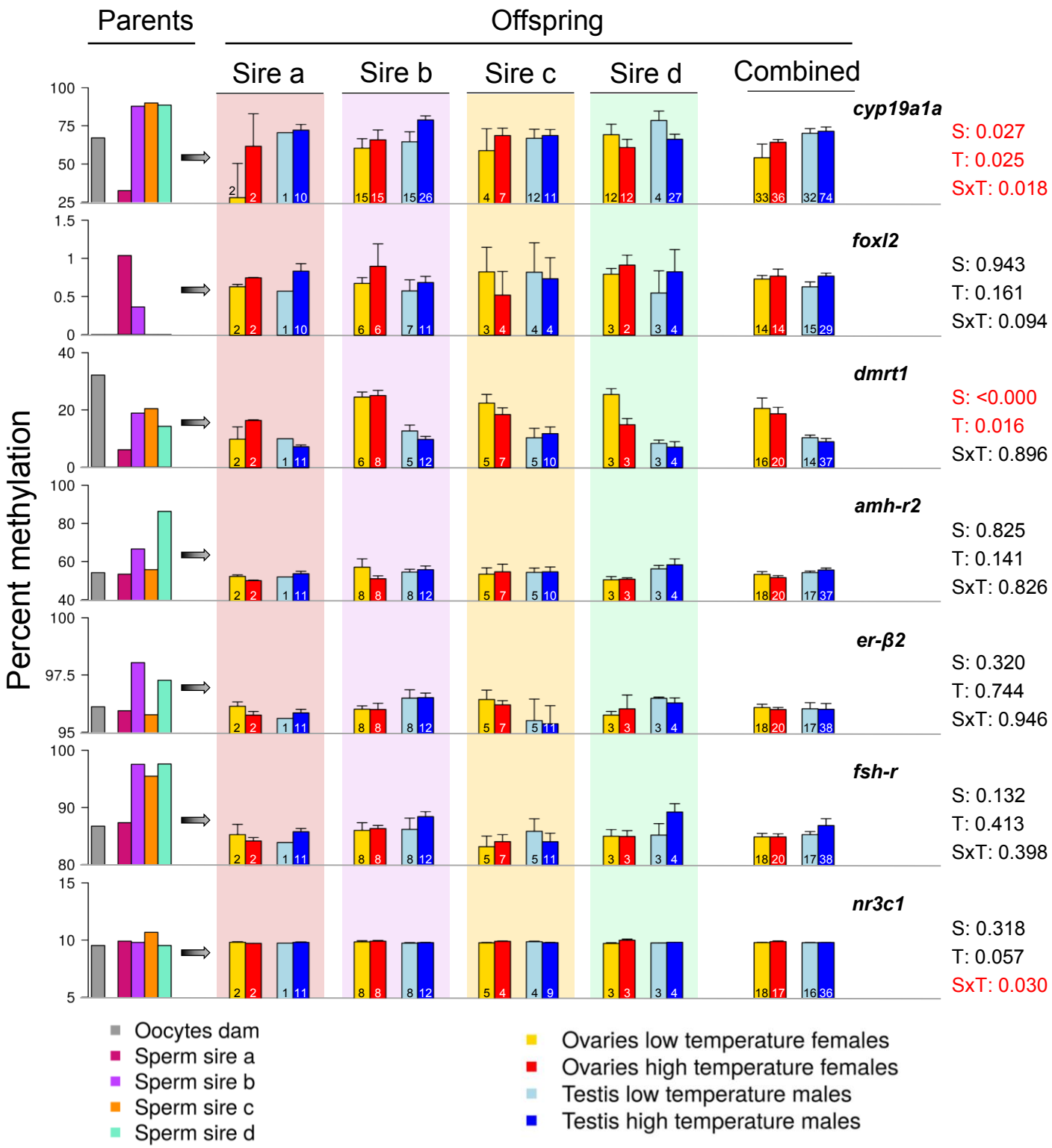

Parents
Offspring
Sire a
Sire b
Sire c
Sire d
Combined
Percent methylation
cyp19a1a
S: 0.027
T: 0.025
SxT: 0.018
foxl2
S: 0.943
T: 0.161
SxT: 0.094
dmrt1
S: <0.000
T: 0.016
SxT: 0.896
amh-r2
S: 0.825
T: 0.141
SxT: 0.826
er-β2
S: 0.320
T: 0.744
SxT: 0.946
fsh-r
S: 0.132
T: 0.413
SxT: 0.398
nr3c1
S: 0.318
T: 0.057
SxT: 0.030
Oocytes dam
Sperm sire a
Sperm sire b
Sperm sire c
Sperm sire d
Ovaries low temperature females
Ovaries high temperature females
Testis low temperature males
Testis high temperature males

Figure 2

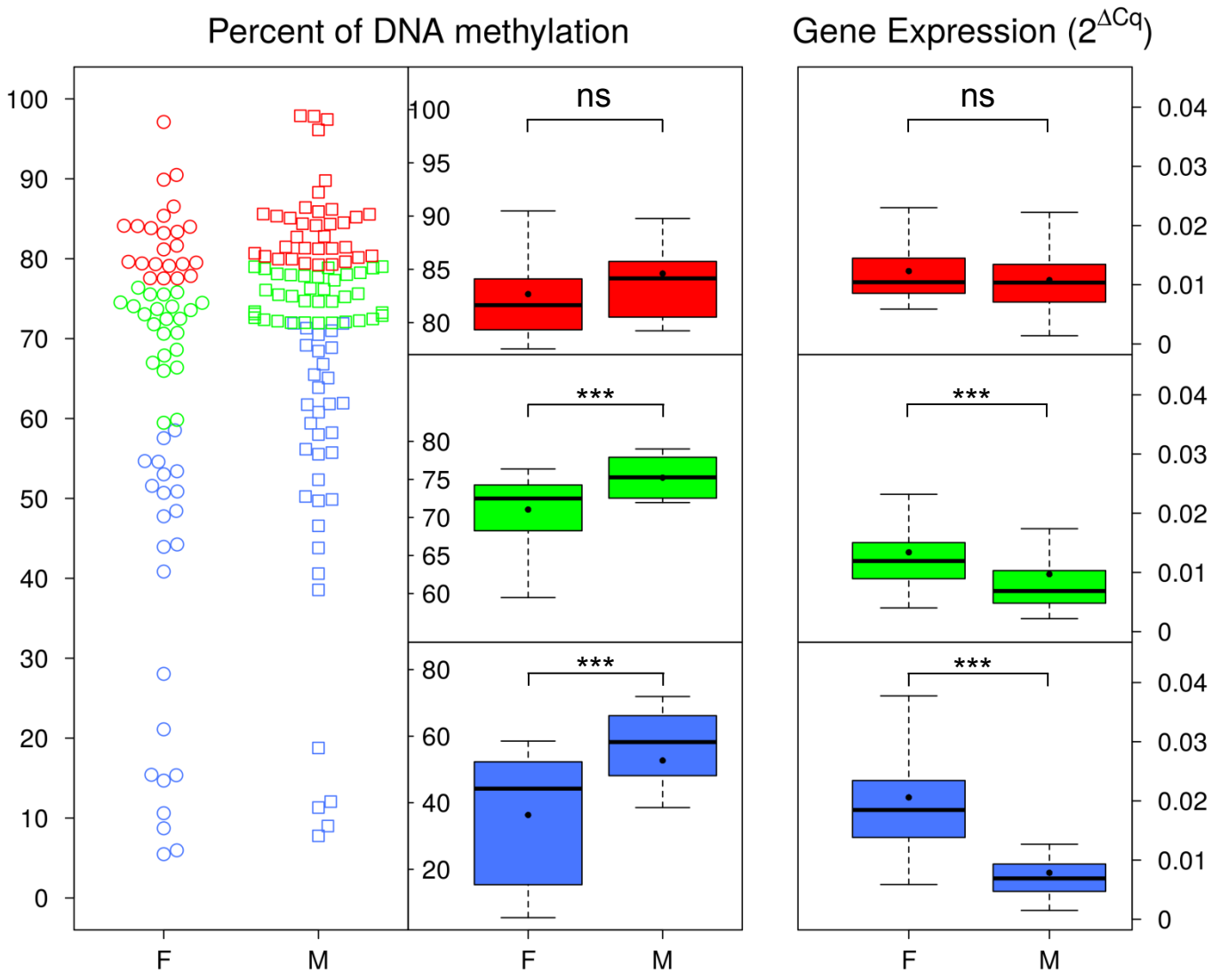

Percent of DNA methylation
Gene Expression ($2^{\Delta Cq}$)
ns
***
***
ns
***
***
F
M
F
M
F
M

Figure 3

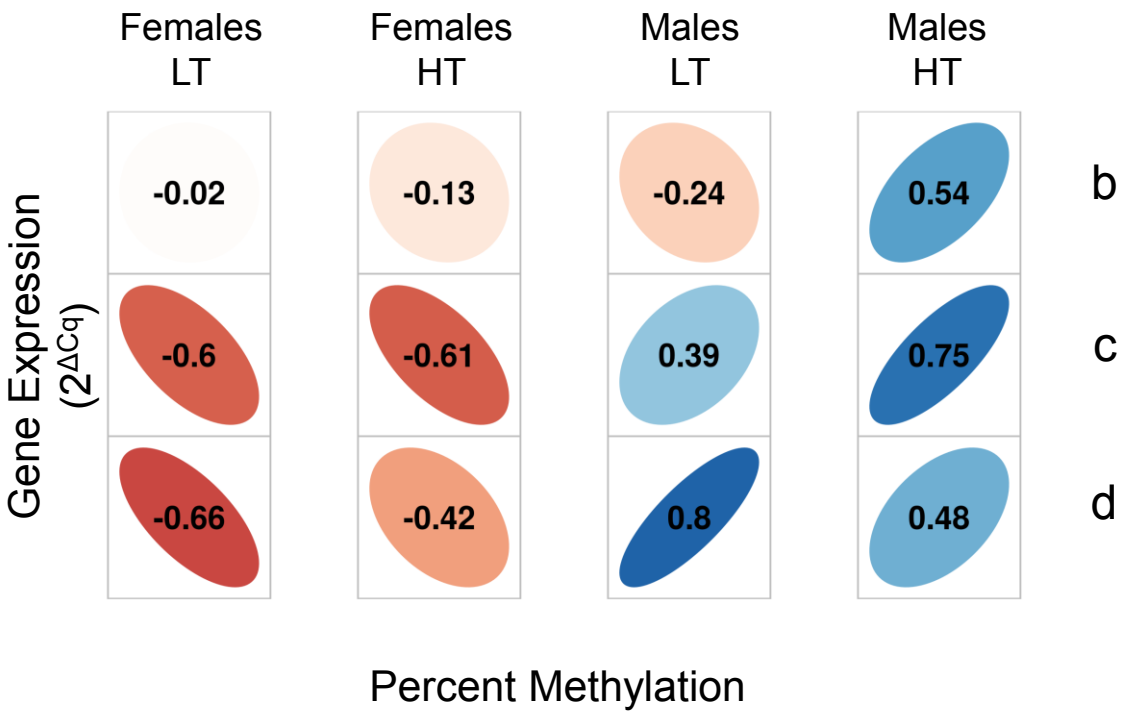

Females LT
Females HT
Males LT
Males HT
-0.02
-0.13
-0.24
0.54
-0.6
-0.61
0.39
0.75
-0.66
-0.42
0.8
0.48
b
c
d
Gene Expression ($2^{\Delta Cq}$)
Percent Methylation

Figure 4

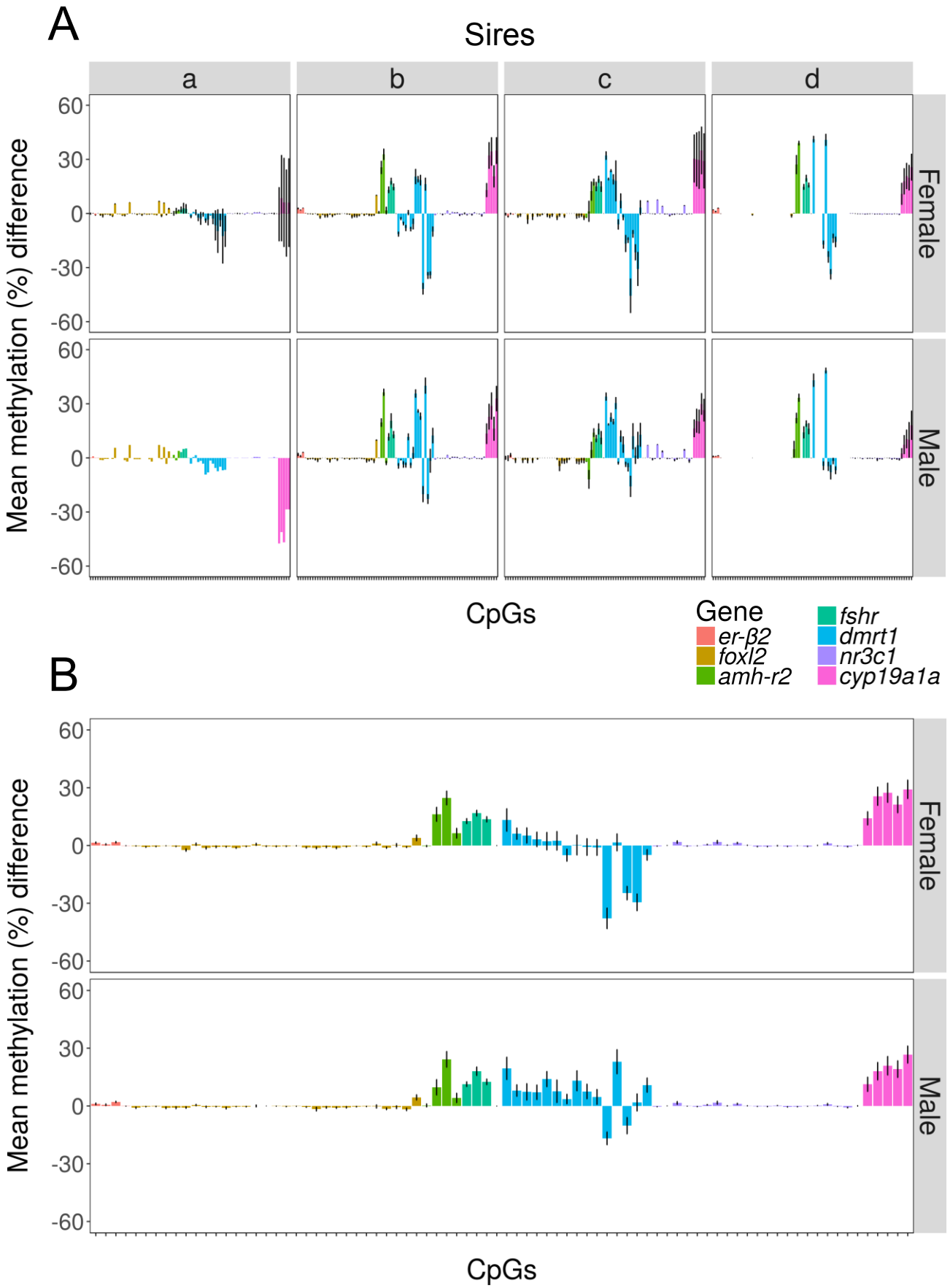

A
Sires
a
b
c
d
Female
Male
Mean methylation (%) difference
60
30
0
-30
-60
CpGs
Gene
er-β2
foxl2
amh-r2
fshr
dmrt1
nr3c1
cyp19a1a
B
Mean methylation (%) difference
Female
Male
CpGs

Figure 5

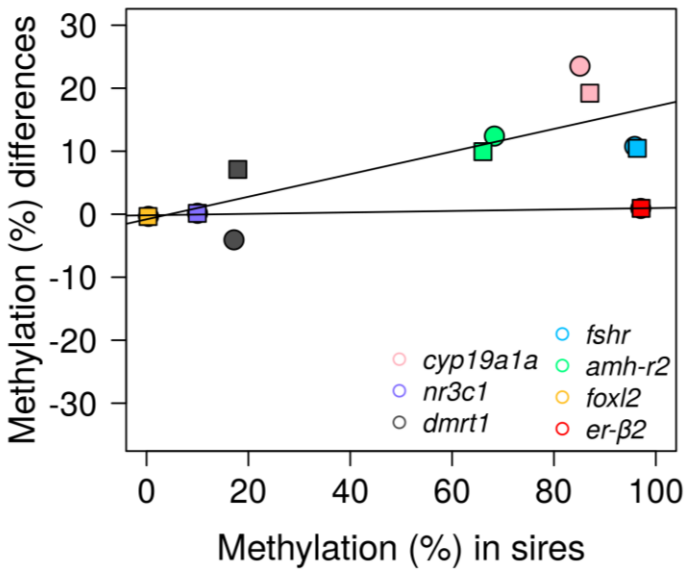

Methylation (%) differences
30
20
10
0
-10
-20
-30
0
20
40
60
80
100
Methylation (%) in sires
cyp19a1a
nr3c1
dmrt1
fshr
amh-r2
foxl2
er-β2

Figure 6

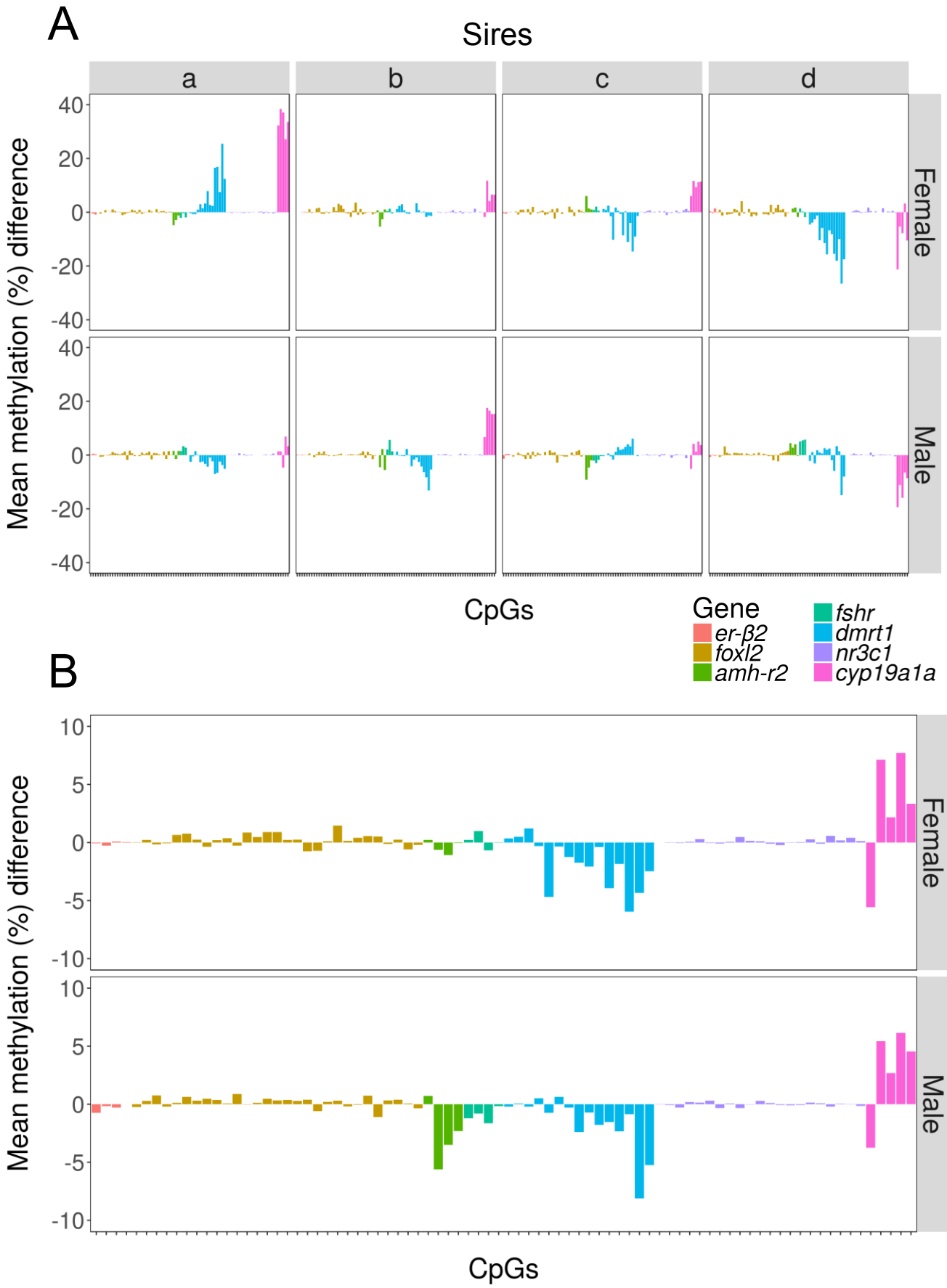

A
Sires
a
b
c
d
Female
Male
Mean methylation (%) difference
40
20
0
-20
-40
CpGs
Gene
er-β2
foxl2
amh-r2
fshr
dmrt1
nr3c1
cyp19a1a
B
10
5
0
-5
-10

Figure 7

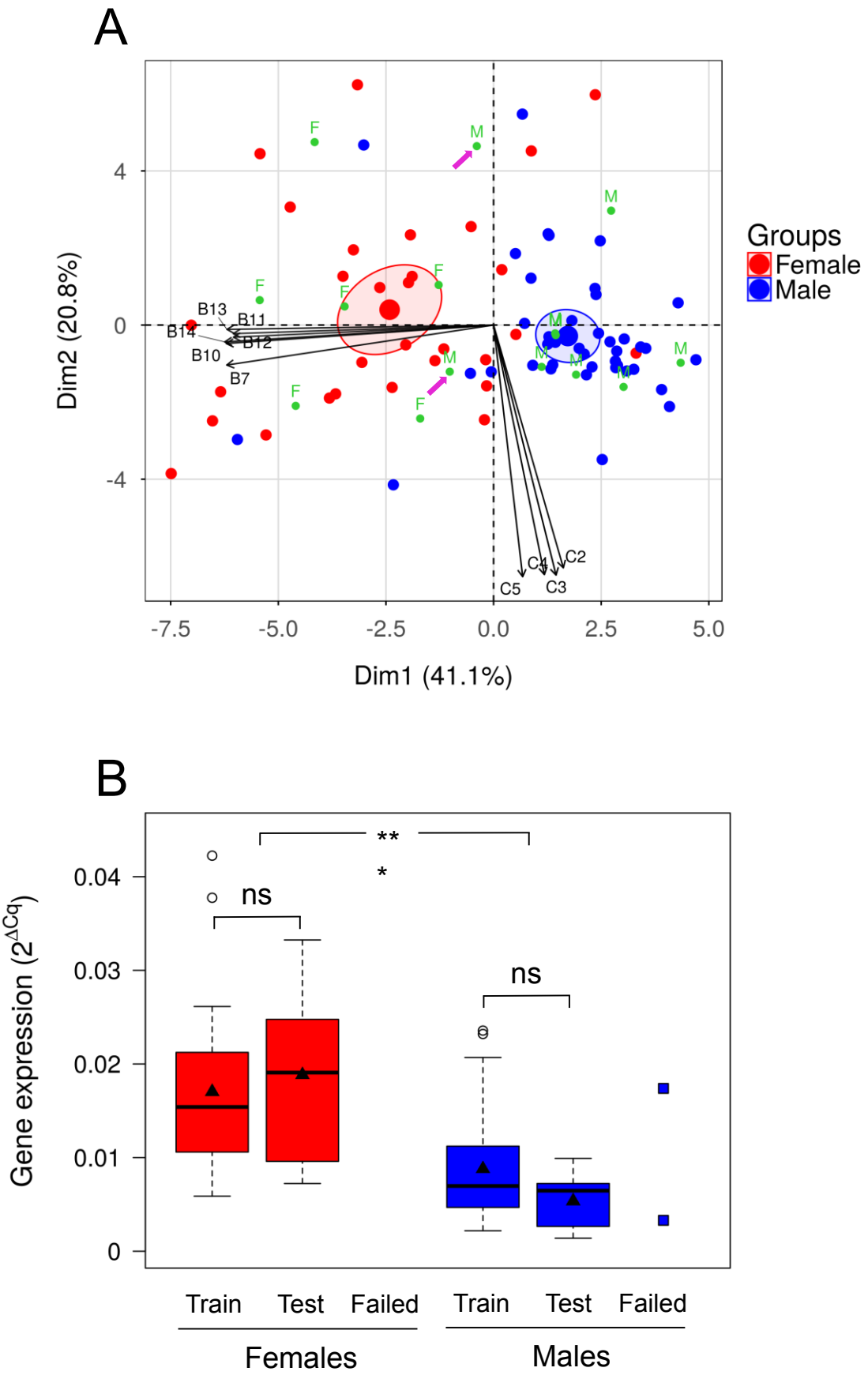

A
Dim2 (20.8%)
Dim1 (41.1%)
Groups
Female
Male
B13
B11
B14
B12
B10
B7
C5
C3
C4
C2
B
Gene expression ($2^{\Delta Cq}$)
ns
**
*
ns
Train
Test
Failed
Train
Test
Failed
Females
Males

Figure 8