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1 **Main Manuscript for**

2 Transgenerational epigenetic sex determination: environment experienced by female zebrafish
3 affects offspring sex ratio

4
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27 F.P., J.C., B.M., S.D. and P.G. designed the research; F.P., S.L., D.H., G.D. and B.E.
28 performed research; F.P. and S.L. analyzed data; F.P. wrote the paper.

29
30 **This PDF file includes:**

31 Main Text

32 Figures 1 to 6

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34
35 **Abstract (250 words)**

36
37 Sex determination is a complex process that can be influenced by environment in
38 various taxa. Disturbed environments can affect population sex ratios and thus threaten their
39 viability. Emerging evidences support a role of epigenetic mechanisms, notably DNA
40 methylation, in environmental sex determination (ESD). In this work, using zebrafish as
41 model and a transgenerational experiment comprising 4 successive generations, we report a
42 strength link between the promotor methylation level of three genes in female gonads and
43 population sex ratio. One generation of zebrafish was exposed throughout its lifetime to
44 cadmium (Cd), a non-essential metal. The subsequent generations were not exposed. At the
45 first and the third generation a subset of individuals was exposed to an elevated temperature, a
46 well-known masculinizing factor in zebrafish. While heat was associated to an increase in the
47 methylation level of *cyp19a1a* gene and population masculinization, *foxl2a/dmrt1*
48 methylation levels appeared to be influenced by cadmium (Cd) and density leading to
49 offspring feminization. Ancestral Cd exposure indeed leads to a progressive feminization of
50 the population across generations and affected the sex plastic response of zebrafish in

51 response to heat. The effect of Cd on the methylation level of *foxl2a* was observed until the
52 third generation, supporting potential transgenerational inheritance. Our results support the
53 fact that the environment experienced by parents, namely mothers in the present case, can
54 affect their offspring sex ratio via environment-induced DNA methylation changes in gonads.
55 Our results also support a key role of *cyp19a1a* methylation in ESD in zebrafish.
56

57 **Significance Statement (120 words,** Explain the significance of the research at a
58 level understandable to an undergraduate-educated scientist outside their field of specialty)

59 In zebrafish, as in other various species, the sex (male or female) is not solely under
60 the dependence of genetic components but can also be influenced by environmental cues.
61 Disturbed environments can induce shift in population sex ratios. Underlying mechanisms
62 remain elusive. Our results support a key role of DNA methylation, i.e. an epigenetic
63 mechanism, in environmental sex determination in zebrafish. The DNA methylation levels of
64 three genes known to be involved in gonadal sex differentiation and maintenance were found
65 to be influenced by direct or ancestral exposure to cadmium, elevated temperature or fish
66 density. Our results support the fact that the environment experienced by mother,
67 grandmother or even great grandmother can affect the offspring sex ratio via environment-
68 induced DNA methylation changes in gonads.

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71 Main Text

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74 Introduction

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76 Sex ratio (SR) represents a critical demographic parameter that affects the growth rate
77 as well as the evolutionary trajectories of wild populations (1-3 West et al., 2002; Wedekind,
78 2012 and 2017). This parameter is directly under the dependence of sex determination (SD)
79 mechanisms; i.e. the processes by which an organism initiates differentiation as a male or
80 female. These processes are unexpectedly diverse among species and were traditionally and
81 basically divided into two main categories: genetic sex determination (GSD) and
82 environmental sex determination (ESD) (4 Capel, 2017). In organisms with GSD, the sex is
83 determined by heritable genetic components whereas in organism with ESD, the sex is
84 induced by environmental factors during early steps of development. Moreover, even if the
85 sex of GSD species is normally inherited and fixed for life, the primary genetic sex can be
86 challenged during early development in response to environmental factors in a such way that
87 animal develop into the opposite sex of its genetic background. This phenomenon is relatively
88 common in insects, fish, amphibians and reptiles (4 Capel, 2017). The most striking example
89 of such phenomenon is temperature-dependent sex determination (TSD) where the thermal
90 conditions experienced during the early stages of development of an organism influence its
91 sex differentiation and development, determining irreversibly its gonadal sex at adulthood.
92 Despite the mechanisms responsible for such plasticity, particularly in vertebrates, have long
93 been unknown and remain a matter of debate, recent works, notably in fish (5-6 Shao et al.,
94 2014, Navarro-Martin et al., 2011), would support a key role of epigenetic mechanisms,
95 notably DNA methylation, in germline differentiation in response to high temperature (HT).

96 Epigenetics is the study of changes in gene function that are mitotically and/or
97 meiotically heritable and that do not entail a change in the DNA code (7 Feil and Fraga,
98 2012). Epigenetic mechanisms by controlling the activity of genes play a key role in cell

99 differentiation during development. One of the most studied epigenetic process, DNA
100 methylation, is commonly associated with “gene silencing”, i.e. transcriptional repression
101 (with few exceptions; 8-9 Harris et al. 2018, Hammoud et al., 2014). During development cell
102 differentiate, acquire and maintain identity by expressing a particular transcriptome (10
103 Piferrer, 2013). It is not surprising that such mechanisms could play a key role in SD. In
104 gonochoristic species, SD indeed relies on the activation of one of the two developmental
105 pathways (male or female) and shut down the other (4 Capel, 2017). There is increasing
106 evidences that environmental factors such as nutrition, social interactions or temperature can
107 strongly affect the epigenetic status of cells (7, 11-12 Feil and Fraga, 2012, Head et al., 2014;
108 Jaenisch and Bird, 2003). By modulating the establishment and maintenance of epigenetics
109 marks, environmental factors can influence the phenotype of organisms, a phenomenon by far
110 the most documented in invertebrates (13 Kucharski et al., 2008). There is still a controversy
111 in whether such environmental-induced changes in epigenetic marks can be inherited from
112 one generation to the next, especially in vertebrates. The epigenome do not persist throughout
113 life but undergo precise and coordinated changes at defined stage of development (7 Feil and
114 Fraga, 2012). In mammals, both maternal and paternal genomes undergo extensive
115 demethylation to reset the epigenome for totipotency during early embryonic development (14
116 Heard and Martienssen, 2014). However, in stark contrast to mammals, recent discoveries
117 (15-16 Ortega-Recalde et al. 2019; Skvortsova et al. 2019) have reported that the germline of
118 zebrafish does not undergo genome-wide erasure of DNA methylation at any stage of
119 development, which could lead to transgenerational inheritance of environmental-induced
120 epigenetic modifications in such non-mammalian species. In the half tongue, HT-induced
121 methylation modifications in pseudomales (i.e. individuals with a female genetic background
122 (ZW chromosomes) and a male phenotype) is globally inherited in their ZW offspring, which
123 develop into pseudomales without further HT exposure (5 Shao et al., 2014). However, this
124 does not represent a true epigenetic transgenerational inheritance as the developing germ
125 cells that gave rise to this generation were already exposed to HT as gametes in their parents.

126 There is increasing evidences that anthropogenic factors such as pollution can also
127 lead to changes in the methylome of organisms. The defects or unintended changes in DNA
128 methylation marks induced by pollutants can be associated with negative health outcomes
129 such as embryonic lethality, tumor genesis, diabetes and infertility (7,11-12 Feil and Fraga,
130 2012, Head et al., 2014; Jaenisch and Bird, 2003). As epigenetic mechanisms play a key role
131 in phenotypic plasticity, it could appear highly relevant to investigate whether pollutant-
132 induced epigenetic modifications can impede the plasticity of an organism face to another
133 stress, i.e. the establishment of an epigenetic response that aims to plastically adapt the
134 organism to an environmental cue. Phenotypic plasticity represents the ability of a genotype
135 to yield different phenotypes in response to an environmental stimulus. In other words, it
136 represents the capability of an organism to change its physiology, morphology, behavior and
137 even its sex to cope with variations in its environment. Phenotypic plasticity plays thus a
138 central role in the persistence of populations (17 Fusco and Minelli, 2010). This appears
139 especially true when the plastic trait is the sex of animals, linking individual phenotype to
140 population structure. In this view, we must note that the theoretical advantage of TSD is to
141 allow individuals to convert to the sex that offers the best fitness and reproductive success
142 under prevailing thermal regimes (18 Spencer and Janzen, 2014). Moreover, past studies,
143 even on taxa with GSD, have reported shifts in offspring SRs consistent with plastic
144 adaptation (1 West et al., 2002). SR is not always random and parents can adjust the sex of
145 their offspring to strategically maximize the fitness of their progeny according to the
146 environmental conditions they experienced (1-2, 19-20 West, 2002; Komdeur et al., 1997;
147 Wedekind, 2012, Shino, 2004).

148 In this context, the aims of the present study were (i) to gain insight into the
149 contribution of DNA methylation in ESD and (ii) to test whether direct or ancestral exposure
150 to a pollutant could affect the plastic ESD response of organisms face to another stress via
151 pollutant-induced epigenetic modifications. To test this hypothesis, we used wild-type
152 zebrafish (*Danio rerio*). Despite the zebrafish is a popular model in many disciplines of
153 biology, little is known about the primary mechanisms involved in SD and the subject still
154 remain a matter of debate. Domesticated zebrafish lack identifiable heteromorphic sex
155 chromosomes and sex is certainly polygenic (for review see 21-22 Liew and Organ, 2013;
156 Nagabhushana and Mishra, 2016). Some influences from the environment were reported (23
157 Santos et al., 2017). Multiple factors like hypoxia, density, exposure to hormones and
158 temperature were found to affect the SR. In all-genetic female zebrafish, exposure to HT at
159 early stages of development leads to a complete masculinization of fish (24 Uchida et al.,
160 2004). Ribas et al. (2017 25) also reported feminization of zebrafish treated with an inhibitor
161 of DNA methyltransferase. One generation of zebrafish were exposed throughout their life,
162 from fertilization until sexual maturity, to dissolved cadmium (Cd). Cd is a non-essential
163 metal with estrogenic effects (26-27 Kortenkamp, 2011; Pierron et al., 2008) and that was
164 previously found to affect the methylome of animals, including fish (28 Pierron et al. 2014).
165 Rather than to expose fish at a defined stage of development, from far the most common
166 approach, zebrafish were life-long exposed to Cd to encompass the remodeling events of
167 DNA methylation that occur in germline during the embryolarval stage but also later in adult
168 gonads at the onset of “puberty” (15-16, 29 Kesley and Feil, 2013; Ortega-Recalde et al.
169 2019; Skvortsova et al. 2019). At the first (direct exposure) and the third (transgenerational
170 inheritance) generation, a subset of individuals was raised at an elevated temperature. The
171 promoter methylation level of three genes (*cyp19a1a*, *foxl2a*, *dmrt1*) involved in gonadal sex
172 differentiation and maintenance were measured in the gonads of zebrafish. The gene
173 *cyp19a1a*, also called aromatase, encodes for the enzyme that converts androgens into
174 estrogens. Previous works evidenced that aromatase could be a key player in the SR response
175 to temperature in organisms with TSD (6 Navarro-Martin et al., 2011). Among the sex-
176 determining genes identified in zebrafish, forkhead box L2a (*foxl2a*) is involved in ovary
177 maintenance (30 Yang et al. 2017) while doublesex and mad-3 related transcription factor 1
178 (*dmrt1*) was found necessary for testis development (31 Webster et al., 2017).

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181 Results and Discussion

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183 As the aim of this study was to investigate the transgenerational action of Cd, only
184 zebrafish from the first generation (F0) were exposed to dissolved Cd at an environmentally
185 realistic concentration of 1 $\mu\text{g}\cdot\text{L}^{-1}$ (32 Couture et al. 2008) throughout their life, i.e. from the
186 gamete to the adult stage. The next generations (F1, F2, F3) were not directly exposed to Cd
187 (Fig. 1). DNA methylation analyses by bisulfite sequencing and transcription analyses by RT-
188 qPCR were carried out on gonads of 124-dpf-old fish. The SR of the population was
189 determined at 170 dpf (days post-fertilization). At the F0 and the F2 generation, a subset of
190 individuals from different mating pairs was raised from 14 dpf at an elevated temperature
191 (34°C). All these high temperature-treated fish (HT) were analyzed at 130 dpf and were not
192 reproduced.

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195 *Masculinization of zebrafish in response to heat and transgenerational feminization in*
196 *response to Cd*

197

198 At the first generation (F0), Cd exposure had no effect on the SR (Fig. 2). The SR of
199 control (C) and Cd-exposed (Cd) fish reached 59.3 and 57.2 %, respectively. Such SR values
200 are typically observed under experimental conditions. Indeed, the SR is generally around
201 60:40 (male to female) under normal laboratory conditions (23 Santos et al., 2017). The SR
202 was then found to progressively and regularly decrease (6.9% per generation) across
203 generations in the case of fish transgenerationally exposed to Cd, reaching 36.4 % at the last
204 generation (F3). In parallel the SR of C fish was constant until the F2 generation before to
205 dramatically decrease at the F3 generation, reaching 39.1%. This unexpected decrease will be
206 further described. Thus, despite direct Cd exposure had no effect on sex determination in
207 zebrafish, Cd affected transgenerationally the SR of zebrafish, leading to a progressive
208 feminization of the population. Such an effect of Cd on SD was further supported by data
209 obtained at HT.

210 At the F0 and F2 generations, a subset of individuals was raised at an elevated
211 temperature (34°C) from 14 to 130 dpf. As expected, HT was found to significantly increase
212 the proportion of males in the case of C fish. The SR reached 88.6 and 91.0% at the F0 and F2
213 generations, respectively. Cd exposure or transgenerational Cd exposure was found to
214 minimize the masculinizing effect of HT. At F0, SR values of CdHT fish were not statistically
215 different from both fish raised at 27.7°C (C or Cd) and CHT fish. At the F2 generation, Cd
216 fish raised at 34°C presented a SR that was equivalent to C fish raised at 27.7°C, i.e. 62.7 and
217 61.2 %, respectively. Thus, both direct or transgenerational exposure to Cd affected the
218 plasticity of fish and their SD in response to HT.

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221 *Sex-specific DNA methylation and RNA transcription profiles*

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223 Gender-specific DNA methylation and transcription profiles were observed for the 3
224 genes studied (Fig. 3A and 3B). A highly significant difference ($P < 0.0001$) was observed
225 between males and females (Fig. 3A). This appears in agreement with previous works carried
226 on diverse fish species that reported sex dimorphic gonad expression of *cyp19a1a*, *foxl2a* and
227 *dmrt1* between sexes, including in danio (see 23 Santos et al., 2017 for review). In a lesser
228 extent, previous works also reported sex dimorphic methylation levels of *cyp19a1a* and *dmrt1*
229 in fish gonads (33-34 Laing et al., 2018, Domingos et al., 2018). While *cyp19a1a* is
230 hypermethylated in males, *dmrt1* is hypermethylated in females. Despite *foxl2* was found to
231 be play a key role in fish ovarian differentiation and/or maintenance, the situation is less clear
232 in literature and no gender-specific methylation patterns were reported to our knowledge (34-
233 35 Domingos et al., 2018., Anastasiadi et al., 2018). In our case, the methylation level of
234 *foxl2a* was found to be significantly higher in females in comparison to males, whatever the
235 generation or the condition (i.e. C or Cd) considered.

236 A significant effect of temperature (Fig. 3A) was also observed on the three genes
237 studied. However, this effect was mainly restricted to the DNA methylation data and to
238 females. Concerning RNA, only the transcription level of *foxl2a* was found to be significantly
239 influenced by HT in both males and females. The most important effect of temperature was
240 observed on the DNA methylation level of *cyp19a1a*. HT triggered a significant increase of
241 *cyp19a1a* methylation levels in females and on the contrary, a significant decrease in the case
242 of males.

243 A significant effect of Cd was also observed (Fig. 3A). This effect was limited to the
244 methylation levels of *foxl2a* in females of the first three generations and on the transcription
245 level of *cyp19a1a* in both males and females of the first three generations. This could appear
246 in agreement with the fact that *foxl2* was identified as a transcriptional activator of *cyp19a*
247 expression in diverse fish species (36-38 Fan et al., 2019; Zhang et al., 2017; Si et al. 2016).

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The relationship between the methylation state of *cyp19a1a* and its transcription level was in agreement with the consensus view that DNA methylation leads to “gene silencing”, i.e. transcriptional repression (Fig. 3A; 7 Feil and Fraga, 2012). This is also in agreement with previous results obtained in the European sea bass (6 Navarro-Martin et al. 2011). An inverse relationship was observed between the promoter methylation state of the aromatase gene and its transcription level. A similar relationship was observed in the case of *dmrt1*. However, this relationship was not encountered in the case of *foxl2a*. The higher methylation levels of *foxl2a* observed in females in comparison to males were associated with higher transcription levels of *foxl2a* in females. Whereas up-regulation from methylated promoters is not common, there is increasing evidences that such a phenomenon is more widespread than expected, notably in gonads (16 Skvortsova et al., 2019). It was notably observed in mammals that gametogenesis involves transcription of many genes with DNA-methylated promoters (9 Hammoud et al., 2014). In addition, a high correlation was observed between the DNA methylation levels of *foxl2a* and *dmrt1* (Fig. 4A). At the opposite, such a correlation was not observed at the transcriptional level (Fig. 4B). This reflects the fact that while *foxl2a* and *dmrt1* present comparable levels of methylation within an individual, their transcription levels are very different. It is intriguing to speculate that a common mechanism regulates the DNA methylation levels of the two genes or at least, that the DNA methylation levels of these two genes influence each other. As DNA methylation has opposite effect on their respective transcript levels, this leads to noticeable sex difference in transcription levels. Interestingly, several authors proposed that the couple of *foxl2a* and *dmrt1* genes function in a Yin and Yang relationship to determine and maintain the fates of testis and ovaries in both mammals and mollusks (39-40 Huang et al., 2017; Li et al., 2018).

DNA methylation of cyp19a1a promotor as the key element of environmental sex determination

To get more insight into the distinct role of the three genes in ESD, we also investigated the distribution of DNA methylation levels of these genes by pooling data from all generations. To more clearness, we used the normal density distribution of the data (Fig. 3B). However, raw data are available in Fig. S1. As we can observe in the Fig. 3B, a bimodal distribution clearly discriminating and separating males and females was observed only in the case of the *cyp19a1a* gene. A threshold value of ~66% was observed. Below this threshold, only females were observed and above, only males were observed. In the case of *foxl2a* or *dmrt1*, in response to HT, males and females can present similar levels. We must note however that data from males and females raised at 27.7°C were clearly separated and a threshold around 15% and 12% for *foxl2a* and *dmrt1* respectively could also be determined.

Aromatase appeared to be thus the main discriminating factor between the two sexes. This is consistent with the role of the *cyp19a1a* gene that encodes for an enzyme responsible for the transformation of testosterone into estradiol. This suggests that *cyp19a1a* could be the main effector of sex differentiation, at least in response to HT. This is also in agreement with the results obtained by 6 Navarro-Martin et al. (2011) in the European Seabass. Interestingly, as in our study, authors reported both (i) a significant increase in the methylation level of the gonadal *cyp19a* promoter in female European sea bass previously exposed to HT during early development (0-60 dpf) and (ii) clearly distinct frequency distributions between males and females with a threshold value of 67% (66% in the present study). As the European sea bass, zebrafish are also classified as a gonochoristic species. Despite the mechanisms that control sex determination are still poorly understood in wild-type or laboratory strains of zebrafish, it

298 is generally accepted that the ovarian development is a default pathway and that larvae and
299 juveniles (from 0 to 21 dpf) initially develop an immature ovarian tissue independently of
300 their genetic background (21-23). Afterwards, the ovarian tissue will be either maintained or
301 undergo apoptosis, leading to the development of testis. In general, gonad differentiation
302 begins from around 21 dpf to be completed at 60 dpf. Thereafter, no sex reversal is observed.
303 Moreover, intersex does not normally occur in danios that have already completed the sex
304 differentiation process. In the same way, it has been proposed that exposure to endocrine
305 disrupting chemicals (ECDs) have no effect on SR once phenotypic sex is established (with
306 exceptions, see 23 Santos et al., 2017). Despite this subject is still a matter of debate, there is
307 increasing evidences that the period from 0 to 60 dpf constitutes a critical window of exposure
308 during which the genetic sex of zebrafish can be challenged by environmental factors, notably
309 by HT. In our case, zebrafish were exposed to HT from 0 to 124 dpf. However, in a previous
310 work, 24 Uchida et al. (2003) reared wild-type zebrafish genetic females from 15 to 25 days
311 post-hatching (dph) at 3 temperatures 28.5, 35 and 37°C. Before and after this sensitive
312 period, all fish were reared at 28.5°C. The percentage of masculinization in the genetic all-
313 females at 40 dph at water temperatures of 28.5, 35 and 37°C were 0, 68.8 and 100%,
314 respectively. Dietary administration of a cytochrome P450 aromatase inhibitor to genetic
315 females during the same sensitive period (15-25 dph) also resulted to a masculinization of fish
316 at 40 dph. This study highlighted both the importance of the timing of exposure as well as the
317 key role of *cyp19a1a* in TSD in zebrafish.

318 In contrast to the study of Navarro-Martin et al. (2011 6), HT was not only associated
319 with an increase of *cyp19a1a* methylation levels in females but also and, at the opposite, with
320 a decrease in the case of males. *Cyp19a1a* methylation levels in C females increased
321 significantly from 41.3 ± 10.2 % (mean \pm SE, n = 24) to 51.0 ± 6.6 % (mean \pm SE, n = 10)
322 between 27.7 and 34°C, respectively (Fig. S2). In parallel, the methylation level of *cyp19a1a*
323 significantly decreased in males exposed to HT. Methylation levels of *cyp19a1a* in C males
324 raised at 27.7°C ranged from 90.0 to 97.4 % (94.1 ± 2.0 %, mean \pm SE, n = 24) and from 75.0
325 to 94.6 % in males raised at 34°C (88.3 ± 5.5 %, mean \pm SE, n = 16). While mean decreased
326 significantly with increasing temperature, at the opposite, the variation among phenotypic
327 males increased significantly (Levene test p = 0.002) with increasing temperature. Such a
328 decrease in mean associated to an increase in variance could be imputed to the contribution of
329 pseudomales. Indeed, in response to HT, a significant proportion of genotypic females
330 became phenotypic males (i.e. pseudomales). For recall, in response to HT, the SR of controls
331 significantly increased from 59.3 to 88.6 % at the F0 generation and from 61.2 to 91.0 % at
332 the F2 generation. The methylation levels of these pseudomales can be responsible to the
333 decrease in the mean value of *cyp19a1a* methylation levels measured in all-HT males in
334 comparison to control males (27.7°C). Thus, our results suggest that HT would be responsible
335 for an increase in the methylation levels of *cyp19a1a* during the thermosensitive period.
336 Genotypic females that reach a methylation level of *cyp19a1a* above a critical threshold (66%
337 at the adult stage in gonad) would become pseudomales. Data obtained from individuals
338 directly or transgenerationally exposed to Cd can further support this hypothesis. Indeed, as
339 previously described, Cd exposure minimized the masculinizing effect of HT at the SR level.
340 No significant increase in *cyp19a1a* methylation levels was observed in Cd females in
341 response to HT. Moreover, the methylation levels reached by CdHT females was not
342 significantly different from C females raised at 27.7 or 34°C (Fig. S2). Thus, if Cd minimized
343 the effect of HT at the phenotype level, it also minimized the effect of HT-induced changes in
344 the methylation levels of *cyp19a1a*. As all larvae and juveniles (from 0 to 21 dpf) initially
345 develop an immature ovarian tissue, it is intriguing to speculate that the methylation state of
346 *cyp19a1a* during early stage of development plays a key role in sex determination. The
347 apoptosis of the ovarian tissue during the period of gonad differentiation (21-60 dpf) would be

348 linked to an increase in the methylation level of *cyp19a1a*. This appears in agreement with a
349 previous work that reported how the use of an inhibitor of DNA methyltransferase (5-aza-dC)
350 induces feminization of zebrafish when the treatment coincides with the period of gonadal
351 development (10-30 dpf, 25 Ribas et al., 2017).

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354 *DNA methylation of foxl2a/dmrt1 in oocytes is a sensor of environmental cues affecting the* 355 *offspring SR*

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357 In order to get more insight into the role of the three genes and sex determination at
358 the population level, we also tested for a potential correlation between DNA methylation or
359 transcription levels and the SR among generations. A very high correlation was observed
360 between the DNA methylation level of *cyp19a1a* in female gonads and the SR (Fig. 5A). This
361 appears quite surprising as such a correlation was established between the methylation level
362 of one gene measured in 5-8 biological replicates per condition and the total number of adult
363 fish produced at each generation (n = 303-663 per condition and generation at 27.7°C and n =
364 44-68 at 34°C). No significant correlations were found with males, neither with transcript
365 levels nor with the methylation levels of *foxl2a* and *dmrt1*. This again supports a key role of
366 *cyp19a1a* methylation in sex determination. The question is: how is such relation possible?
367 Subsequently, we also tested for a potential correlation between the same parameters at the
368 generation F and the SR at the next generation (F+1). Unexpectedly, we also found a highly
369 significant relationship between the DNA methylation of *foxl2a* in mother gonads and the SR
370 of the offspring (Fig. 5B). We must note that fish raised at HT were not taken into account in
371 this relation as they were not reproduced. Despite surprising, these results could appear in
372 agreement with data obtained in species where shifts in offspring SR in response to the
373 environmental conditions experienced by their parents were reported. Parents, especially
374 mothers, are able to manipulate the SR of their offspring in response to ecological or social
375 factors prevailing in their rearing environments (2, 18-20 Wedekind, 2012).

376 This latter result could explain, at least in part, why a transgenerational feminization of
377 Cd-zebrafish was observed across generations. In fact, a significant effect of Cd was observed
378 on the methylation level of *foxl2a* across the F0, F1 and F2 generations. It appears intriguing
379 to speculate that Cd by affecting the methylation levels of *foxl2a* in mothers affected the
380 offspring SR. We must note that, despite the methylation level of *foxl2a* was significantly
381 affected by Cd at the F0 generation, no effect of Cd was observed on the SR at this
382 generation. Considering that sex is determined during the early stages of development, this
383 could suggest that Cd mainly affected the methylation level of *foxl2a* in fully differentiated
384 females of the F0 generation, during the maturation stage (23 Santos et al., 2017). In the same
385 view and as the methylation level of *cyp19a1a* plays a key and early role in SD, we then
386 measured the methylation level of *cyp19a1a* in the whole larvae (5 dpf) of each generation.
387 A highly significant correlation was observed between the DNA methylation level of *foxl2a* in
388 female gonads of the generation F and the methylation levels of *cyp19a1a* in the larvae of the
389 generation F+1 (Fig. 5C). This could explain how changes in the methylation level of *foxl2a*
390 in mother gonads can affect the SR of the offspring. In addition, a significant correlation was
391 observed between the methylation level of *cyp19a1a* in larvae and in female gonads of the
392 same generation (i.e. from larval to adult stage; $r = 0.904$, $P = 0.002$, data not shown). This
393 latter result could explain why a highly significant correlation was observed between the
394 DNA methylation level of *cyp19a1a* in female gonads and the SR at the same generation.

395 To get more insight, we then searched why the SR of controls dramatically drops at
396 the F3 generation (Fig. 2). The only factor that was not similar among generations was fish
397 density. Despite no significant difference in adult fish density was observed between control

398 and Cd-exposed fish over the four generations, the density of fish was significantly two-fold
399 higher in fish of the generation F2 (Tukey's HSD test, $P < 0.05$, Fig. S3) in comparison to the
400 other generations. Highly significant correlations were observed between fish density and the
401 methylation levels of *foxl2a* or *dmrt1* in females (Fig. 6). We must note that despite all data,
402 i.e. from both C and Cd fish, were considered in the case of *dmrt1*, only data from C females
403 were used for *foxl2a*. Indeed, as previously described, while Cd did not affect the methylation
404 level of *dmrt1*, Cd significantly decreased the methylation levels of *foxl2a* from the F0 to the
405 F2 generation. At the generation F3, this variable was found to be no further affected by Cd.
406 This effect is also observable in Fig. 6A. Such an effect of density on the methylation level of
407 *foxl2a* and *dmrt1* undoubtedly reinforces the role of DNA methylation marks in mother
408 gonads on the SD of their offspring. This appears especially true for *dmrt1*. Indeed, the
409 promoter methylation level of this gene was found to be significantly (Tukey's HSD test, $P <$
410 0.05) lower in the female C fish of the generation F2 in comparison to C females of the other
411 three generations (F0, F1 and F3).

412 Taken together, our results would suggest a role of ovarian promoter methylation state,
413 notably of the couple *foxl2a/dmrt1*, as a sensor of environmental cues (Cd or density) and as a
414 transgenerational "inducer" of SD in zebrafish. In response to Cd or density (i.e. social
415 interactions), the methylation level of *foxl2a/dmrt1* was significantly affected in female
416 gonads. This tends to affect the methylation level of *cyp19a1a* in the offspring as suggested
417 by the significant correlation observed between the methylation state of *foxl2a* in mother
418 gonads and the methylation level of *cyp19a1a* in the larvae of the next generation. In both
419 cases, i.e. in response to Cd exposure or to high density, feminization of the offspring can
420 appear as an adaptive response. Indeed, at the end of the exposure period (170 dpf), a
421 significant effect of Cd was observed on the Fulton condition factor (K, an index frequently
422 used in ecology to assess the relative plumpness of fish) of male fish of the generation F0.
423 While Cd did not affect significantly the Fulton of females ($K = 0.737 \pm 0.08$ and $0.712 \pm$
424 0.08 for C and Cd females respectively, $P = 0.194$, Tukey's HSD test), Cd triggered a
425 significant decrease of this index in males ($K = 0.745 \pm 0.08$ and 0.659 ± 0.08 for C and Cd
426 males respectively, $n = 111-115$, $P < 0.001$, Tukey's HSD test). This suggests a higher
427 sensitivity of males to Cd. In the same view, high density can represent stressing conditions,
428 producing more females at the next generation, i.e. individuals that were found to be less
429 sensitive to stress in comparison to males in zebrafish (40 Rambo et al., 2017), can represent a
430 strategy to maximize the fitness of their offspring. Indeed, Rambo et al. (40 2017) have
431 reported gender difference on behavioral parameters and cortisol levels in response to
432 unpredictable chronic stress (UCS) in zebrafish. While UCS triggered an increase in both the
433 aggressive behavior and in cortisol levels in males, no significant effects were observed in
434 females. More generally, feminization is usually expected to boost population growth (3
435 Wedekind, 2017).

436 Another interesting point of our results relies on the fact that the effect of Cd on the
437 methylation level of *foxl2a* was observed on three successive generations (F0, F1 and F2). For
438 recall, only zebrafish of the F0 generation were exposed to Cd. If a direct effect of Cd at the
439 F1 generation cannot be excluded due to a potential transfer of metal via the gametes (notably
440 via oocytes and vitellogenin, 27 Pierron et al. 2008), such an effect appears difficult to
441 conceive at the generation F2 (notably due to growth dilution). Recent discoveries (15-16
442 Ortega-Recalde et al. (2019), Skvortsova et al. (2019)) have reported that, in stark contrast to
443 mammals, germline of zebrafish does not undergo genome-wide erasure of DNA methylation
444 at any stage of development, from 4 hpf until sexual maturity. Such results could be
445 explained, at least in part by the fact that, in contrast to mammals, zebrafish have a
446 'preformed' germline whereby primordial germ cells (PGCs) are specified by cytoplasmic
447 determinants called germplasm that were produced during oogenesis; i.e. these determinants

448 are maternally provided. These recent studies can thus support transgenerational inheritance
449 of stress-induced changes in DNA methylation marks. In our case, the effect of Cd on the
450 methylation levels of *foxl2a* in female gonads was no more observed at the generation F3,
451 suggesting a potential reset of Cd-induced epigenetic effect at the fourth generation. However,
452 this reset coincided with the effect of density on the methylation level of the couple
453 *foxl2a/dmrt1* at the F2 generation. Thus, a potential transfer of Cd-induced changes in the
454 methylation level of *foxl2a* beyond the third generation cannot be ruled out. In this view, we
455 must add that the significant effect of density on the methylation level of *dmrt1* observed in
456 control females of the generation F2 was no more observed at the generation F3. The question
457 of transgenerational inheritance of stress-induced DNA methylation changes appears to be far
458 from simple, appearing to be sex-dependent and involving undoubtedly other genes and
459 mechanisms.

460 Finally, as our conclusions were mainly drawn from correlation analyses, cautions
461 must obviously be taken. In this view, we must note that our results were obtained from a
462 gonad sample containing several oocytes. A way to better understand the link between female
463 gonad methylation and the offspring SR could be to analyze the methylation level of
464 individual eggs. Oocytes/eggs from a single female could indeed present very different levels
465 of methylation. In other words, each egg could contain not only the genetic but also the
466 epigenetic information that would induce the phenotypic sex of the individual to which it will
467 give birth after fertilization. This could explain why very strong correlations were observed
468 between the methylation state of female gonads and the SR of the entire population while
469 weak or moderate effects were recorded among fish groups (i.e. when mean values are
470 compared). More generally, in a context of global change, it appears urgent to better
471 characterize stress-induced changes in epigenetic marks and their phenotypic impacts as our
472 results tend to show that such modifications can not only affect transgenerationally the
473 plasticity of individuals but also the sex ratio of populations. Such stress-induced changes and
474 genotype-phenotype mismatches can indeed have long-lasting effects on population
475 demography, genetics and viability (3 Wedekind, 2017).

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478 **Material and Methods**

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481 ***General rearing and breeding program***

482

483 Wild-type adult mature danios were purchased from a commercial vendor (Hâpyfish,
484 France). Wild-type (domesticated) zebrafish were preferred to laboratory-bred lines as a
485 previous work reported an effect of inbreeding and loss of genetic polymorphism on the
486 transcriptomic response of zebrafish to metal exposure (42-43 Gentès et al., 2015; Gurvey et
487 al., 2006). After 3 months of acclimation under rearing conditions, one male and one female
488 (one pair) were randomly placed in a small breeding tank (2 L) in the afternoon. A total of 40
489 pairs were used, 20 pairs for the C condition and 20 for the Cd condition. Indeed, at this time
490 Cd exposure began. As reproduction occurs the next morning, genitors (F-1) were exposed
491 only one night to Cd. Eggs and larvae were raised in breeding tanks until 14 dpf. From the 5th
492 day, larvae were fed *ad libitum* 2 times daily with live *Artemia nauplii*, infusoria and dry
493 flakes (Tetramin baby). Water was changed 2 times daily by means of a drip system adding
494 clean or Cd-contaminated water. At 14 dpf, juveniles were placed in 4 large tanks (75 L) per
495 condition permanently supplied with water (flow rate 6.5 ml.min⁻¹). In the case of Cd-
496 contaminated fish, this water was contaminated by means of a peristaltic pump which added
497 Cd at the desired concentration. The use of a flow-through system aimed maintaining water

498 quality throughout the experiment among tanks. Indeed, all tanks were supplied with water
499 providing from a single reservoir. Water in the reservoir was treated (filtered, aerated, heated
500 and pH was regulated by adding CO₂) before to be distributed to tanks. Fish were fed two
501 times daily with live *Artemia nauplii* and one time with dry granules (Tetramin). At 124 dpf,
502 20 individuals per sex and condition were sampled and dissected. Samples for DNA
503 methylation and transcription analyses were fixed in RNAlater solution before to be stored at
504 -20°C. At 170 dpf, 20 pairs per condition were constituted to produce the next generation. We
505 must note that pairs were not fully randomly constituted. As all individuals from one clutch
506 (i.e. from one pair) were raised in the same large tank, one male from one tank was mated
507 with a female from another tank. The aim was to limit consanguinity, genetic drift and
508 inbreeding depression. All remaining individuals after mating were measured and weighted to
509 estimate the Fulton condition factor [$K = (\text{total weight (g)}) / (\text{total length (cm)})^3 \times 10^5$] and
510 sexed by dissection and visual inspection under binocular. Cd concentration in water was
511 determined as described in 28 Pierron et al. 2014. The average concentrations were $0.049 \pm$
512 $0.004 \mu\text{g.L}^{-1}$ and $0.987 \pm 0.025 \mu\text{g.L}^{-1}$ (mean \pm SE, n = 260) in control and contaminated
513 tanks, respectively. The same protocol as previously described was used for mating and
514 sample collection for the F1, F2 and F3 generations. However, for both groups of animals
515 (controls and Cd), spawning was carried out in Cd-free water. Throughout the experiment, the
516 light-dark cycle was controlled and fixed at 14-hour light and 10-hour dark. Temperature and
517 pH in tanks were recorded daily. Temperature in C and Cd tanks reached 27.72 ± 0.60 and
518 27.70 ± 0.64 (mean \pm SD), respectively. Mean values of pH in control and Cd tanks were 7.91
519 ± 0.16 and 7.92 ± 0.15 (mean \pm SD), respectively. Total duration of the experience was 643
520 days.

521 For the F0 and F2 generation, at 14 dpf, 75 juveniles were randomly chosen from
522 different mating pairs and were placed in large tank (75 L) to be raised at a nominal
523 temperature of 34°C. With exception to the temperature, the same rearing conditions as
524 previously described were used. All these fish were dissected for analysis at 130 dpf.
525 Temperature in control and Cd tanks reached 34.44 ± 0.47 and 34.48 ± 0.47 (mean \pm SD),
526 respectively.

527 No significant differences were observed regarding the survival rates between the
528 control and the treatment groups. Mortality at the adult stage was less than 1% at 27.7°C and
529 ranged from 4.0 to 7.5% at 34°C. Cannibalism was observed until 60 dpf (personal
530 observation).

531 The SR was calculated as follow: (number of males/total number of individuals) x
532 100.

533 All procedures used in this experiment were approved by the Aquitaine fish-birds ethic
534 committee (APAFIS#7535-2016111009351504).

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536

537 ***Quantitative analysis of methylation levels***

538 Genomic sequences were obtained from the Genome Reference Consortium Zebrafish
539 (GRCz11). Specific primer used for PCR amplification and sequencing were designed in the
540 promoter region of genes by means of the Pyromark assay design software (Table S1).

541 A total of 6 control or Cd fish at 124 dpf were used per sex and generation. For the HT
542 condition, due to higher variability, 8 fish at 130 dpf were used per condition (C or Cd) and
543 generation. However, due to masculinization of the population, only 5 CHT females could be
544 obtained and used. For each fish, DNA and RNA were extracted using the AllPrep
545 DNA/RNA kit (Qiagen) as previously described in 44 Pierron et al. (2019). Quantification of
546 single cytosine percent methylation at specific sites was then performed using the bisulfite-

547 pyrosequencing method. First, 500 ng of DNA were bisulfite converted using the MethylEdge
548 Bisulfite Conversion System (Promega) according to manufactures' guidelines. Then, 1 µl of
549 bisulfite converted DNA was amplified by PCR using the PyroMark PCR kit (Qiagen)
550 according to manufactures' instructions. Cycling conditions were as follows: 10-min initial
551 denaturation step at 95 °C, followed by 45 cycles of 94 °C for 30 s, 56°C for 30 s, 72 °C for
552 30, 10 min of final elongation at 72 °C. Pyrosequencing of biotinylated PCR products was
553 performed by means of the PyroMark Q48 Autoprep (Qiagen). Average DNA methylation
554 levels of CpG sites were quantified using the PyroMark Q48 autoprep software (Qiagen). As a
555 similar pattern was observed for all CpG analyzed and as the fluorescence progressively
556 decreases during the sequencing run, only the methylation percent of two first CpG sites were
557 used in subsequent analyses. For example, for *cyp19a1a*, the mean methylation levels
558 determined in the first two CpG sites was highly correlated with the mean methylation levels
559 determined using all the six CpG analyzed ($r = 0.998$, $P < 0.0001$; Fig. S4). The aim was to
560 use the most accurate results.

561 In addition, for *cyp19a1a*, in order to assay the conversion efficiency of the bisulfite
562 treatment the average methylation level of one CpA site was measured. The methylation level
563 of this control site was always undetectable, indicating very good conversion efficiency.
564 Examples of pyrograms as well as further details are available in Fig. S5.

565 The same protocol was used for larvae (5 dpf, $n = 6$ per condition and generation).
566 DNA was extracted from a single whole larva. Twenty µl of DNA were bisulfite converted.

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569 *Quantitative analysis of transcription levels*

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571 RT-qPCR analyses were carried out as previously described in 44 Pierron et al. (2019)
572 with few modifications. First-strand cDNA was synthesized from 250 and 500 ng of total
573 RNA for male and female respectively. Following the reverse transcriptase reaction, cDNA
574 was diluted 8- and 16-fold from male and female respectively. Relative quantification of gene
575 transcription was achieved by concurrent amplification of the *actb1*, *eef1a1a* and *rpl13a*
576 endogenous controls (45 McCurley and Callard, 2008). Hence, during our experiment, total
577 RNAs were quantified and the same quantity was used to be reverse-transcribed. During the
578 subsequent qPCR amplifications, the output cycle of the endogenous controls were examined.
579 Contrary to *actb1* (sex-specific), no significant difference was observed on the output cycle of
580 *eef1a1a* and *rpl13a* among fish. The mean Ct value of the two latter genes was used as
581 reference. Primers used are available in Table S1.

582
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584 *Statistical analyses*

585

586 Due to the bimodal distribution of data from females and males, the effect of sex on
587 DNA methylation or transcription levels was analyzed by means of the nonparametric Mann-
588 Whitney test. Then, data from each sex were analyzed separately. Comparisons among fish
589 groups of the same sex were performed by two (Generation and Cd) or three-ways (including
590 HT for F0 and F2 generations only) analysis of variance (ANOVA), after checking
591 assumptions of normality (Kolmogorov-Smirnov) and homoscedasticity of the error terms
592 (Levene). If significant effects were detected, the Bonferroni test was used to determine the
593 effect of each individual factor. When the assumptions were not met as deduced from ad-hoc
594 tests, we used box-cox data transformations or nonparametric Kruskal–Wallis test. The
595 Tukey's HSD test or the Dunn test with Bonferroni correction were used to determine
whether means between pairs of samples were significantly different from one another.

596 Comparisons of proportions (SR) were performed using the Chi squared test and the Monte
597 Carlo method using 5000 permutations followed by the Marascuilo procedure to compare
598 proportions among them. Computations were performed using STATISTICA version 6.1
599 software (StatSoft) and XLSTAT (Addinsoft version 2020.1.1). Numerical results are given
600 as means \pm SE.

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602

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604

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739 **Figures**

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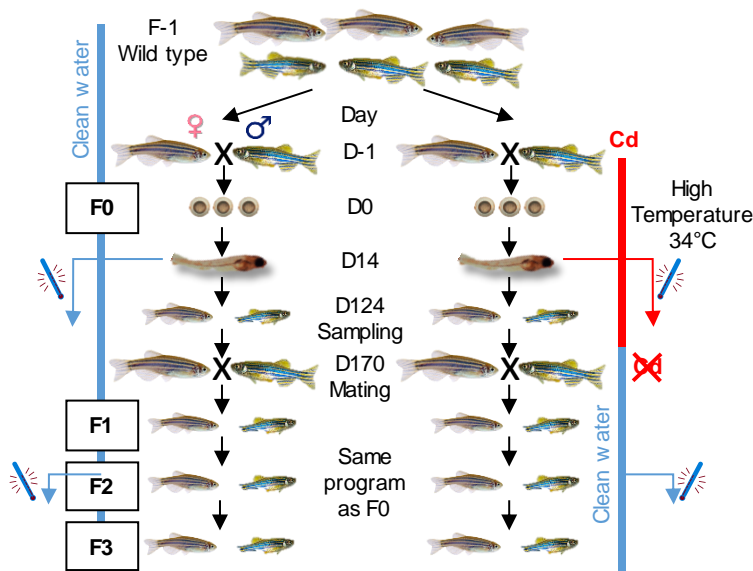
741

742 **Figure 1.** Synoptic of the experimental design.

743 Only fish of the F0 generation were exposed to cadmium (Cd) throughout their life (0 to 169
744 dpf). Their parents (F-1) were exposed only one night to Cd. At each generation, 20 mating
745 pairs and 4 experimental units were used per condition (C, control; Cd, cadmium). At the F0
746 and F2 generations, a subset of individuals was raised at an elevated temperature (34°C, 14 to
747 130 dpf). See Material and Methods for more details.

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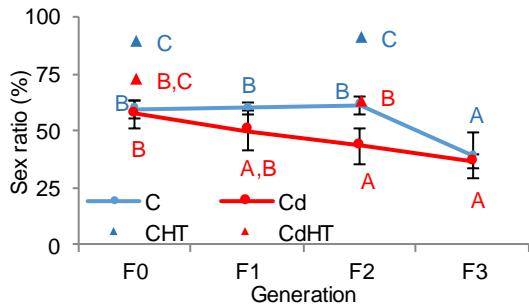
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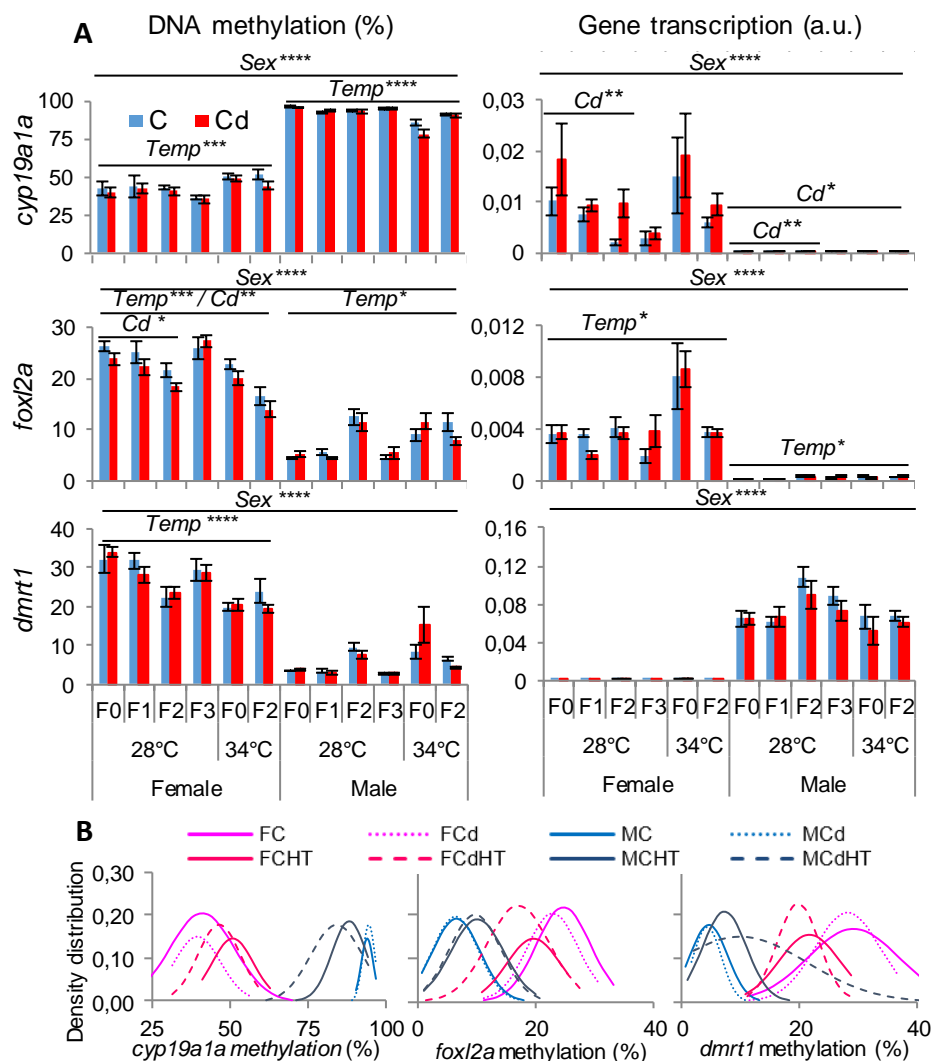
751

752 **Figure 2.** Direct or ancestral exposure to Cd affects the sex ratio.
 753 Change in the sex ratio across generations (F0 to F3) in response to cadmium (Cd) and/or
 754 high temperature (HT) exposure. Means (\pm SE, n = 4) designated with different letters are
 755 significantly different (P < 0.05, Marascuilo procedure). Sex ratio was calculated as follow:
 756 (number of males/total number of individuals) x 100.
 757



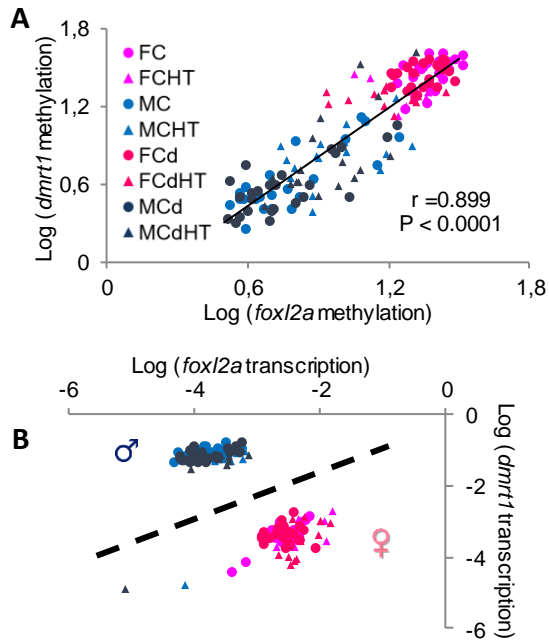
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759 **Figure 3.** Sex-specific DNA methylation and RNA transcription profiles.
 760 (A) DNA methylation and transcription levels (mean \pm SE, n = 5-8) of *cyp19a1a*, *foxl2a* and
 761 *dmrt1* in gonads of female and male zebrafish from each generation (F0 to F3) and from the
 762 different experimental conditions: C, control (blue); Cd, cadmium (red); 28°C and 34°C (i.e.
 763 high temperature). For more clarity, only the significant effect of sex (Mann-Whitney test)
 764 and of Cd and high temperature (Bonferroni test) are indicated: *P < 0.05; **P < 0.01;
 765 ***P < 0.001 and ****P < 0.0001. (B) Normal density distribution of the promoter methylation
 766 levels of *cyp19a1a*, *foxl2a* and *dmrt1* in gonads of female and male zebrafish from the
 767 different experimental conditions: F, female; M, male; C, control; Cd, cadmium; HT, high
 768 temperature. Raw data are available in figure S1.
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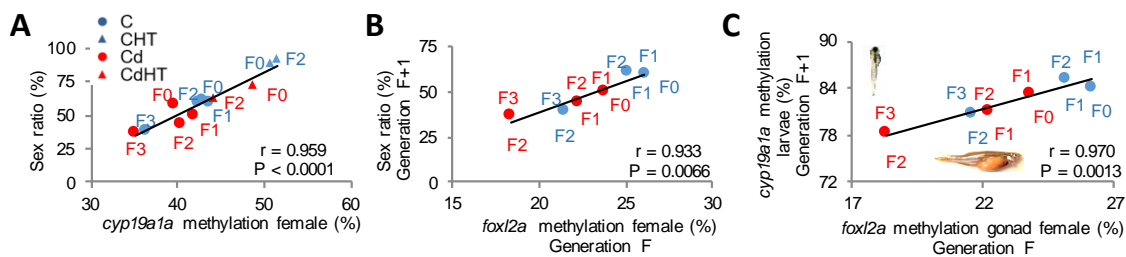
771

772 **Figure 4.** *foxl2a/dmrt1* function in a Yin and Yang relationship
 773 Relationships between *foxl2a* and *dmrt1* at (A) the promoter DNA methylation level (%) and
 774 at (B) the transcriptional level (a.u.). The Pearson correlation coefficient (r) and the associated
 775 P value are reported, n=154. F, female; M, male; C, control; Cd, cadmium; HT, high
 776 temperature.
 777



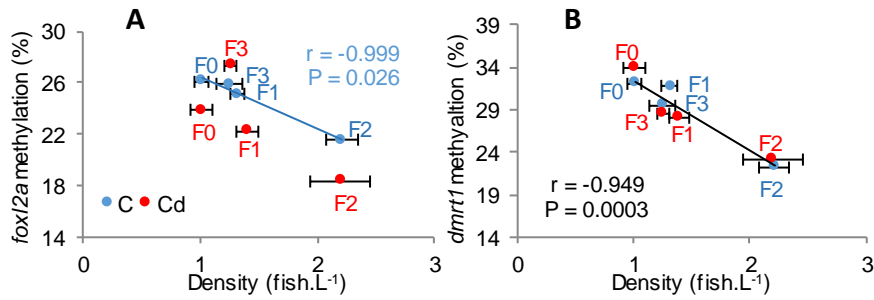
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779 **Figure 5.** Female gonad methylation influences the sex ratio of the population.
 780 (A) Relationship between the mean methylation level of *cyp19a1a* in females (n = 5-8) of
 781 each generation (F) and the sex ratio. (B) Relationship between the mean methylation level of
 782 *foxl2a* in females (n = 6) of each generation (F) and the sex ratio at the next generation (F+1).
 783 (C) Relationship between the mean methylation level (n = 6) of *foxl2a* in female gonads of
 784 each generation (F) and the mean methylation level of *cyp19a1a* in whole larvae (n = 6) of the
 785 next generation (F+1). (A)(B)(C) The Pearson correlation coefficient (r) and the associated P
 786 value are reported. For (B) and (C) while the generation (F) indicated under the regression
 787 line refers to the variable of the x axis, the generation indicated above refers to the variable of
 788 the y axis. C, control; Cd, cadmium; HT, high temperature
 789



790

791 **Figure 6.** Influence of fish density on the methylation level of the couple *foxl2a/dmrt1*.
 792 Relationship between the mean methylation level (n = 6) of (A) *foxl2a* or (B) *dmrt1* in female
 793 gonads of each generation with fish density (mean ± SE, n = 4). The Pearson correlation
 794 coefficients (r) and the associated P values are indicated.



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