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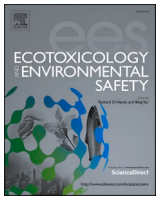

Gamma irradiation-induced offspring masculinization is associated with epigenetic changes in female zebrafish

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ABSTRACT

Sex ratio variation is a key topic in ecology, because of its direct effects on population dynamics and thus, on animal conservation strategies. Among factors affecting sex ratio, types of sex determination systems have a central role, since some species could have a sex determined by genetic factors, environmental factors or a mix of those two. Yet, most studies on the factors affecting sex determination have focused on temperature or endocrine-disrupting chemicals (EDCs), and much less is known regarding other factors. Exposure to gamma irradiation was found to trigger offspring masculinization in zebrafish. Here we aimed at deciphering the potential mechanisms involved, by focusing on stress (i.e. cortisol) and epigenetic regulation of key genes involved in sex differentiation in fish. Cortisol levels in exposed and control (F0) zebrafish females' gonads were similar. However, irradiation increased the DNA methylation level of *foxl2a* and *cyp19a1a* in females of the F0 and F1 generation, respectively, while no effects were detected in testis. Overall, our results suggest that parental exposure could alter offspring sex ratio, at least in part by inducing methylation changes in ovaries.

1. Introduction

Adult sex ratio is often viewed as a key demographic parameter, as it largely influences population dynamics through variance in reproductive success (Nunney, 1993; Schacht et al., 2022). Any disequilibrium of the adult sex ratio might increase negative consequences of the Allee effect (Stephens et al., 1999) leading to potential extinction of the species (Valenzuela et al., 2019).

Multiples evidences coming from studies on reptiles and fishes have found that environmental factors could affect the sex determination period, and later adult sex ratio (Valdivieso et al., 2022, 2020). Such a pattern has been detected in species displaying environmental sex determination (ESD), where various abiotic (e.g. temperature, pH) or biotic factors (e.g. social interactions) could influence the sex of an individual. This, by opposition to species with genetic sex determination (GSD) – under genetic control that supports sexual differences (Capel, 2017), includes chromosomal sex determination (CSD), as in mammalians and birds with a major sex locus. Some species also display polygenic sex determination, with an influence of the environment (GSD + EE). This is the case of the European seabass, *Dicentrarchus labrax*, (Geffroy et al., 2021a, 2021b) and of most laboratory-reared zebrafish strains (Liew et al., 2012; Ribas et al., 2017a; Valdivieso et al., 2022; Wilson et al., 2014).

Yet, two main routes by which the environment can affect the sex of fish have been identified, one involving the major stress hormone, cortisol (Geffroy and Douhard, 2019; Hattori et al., 2020) and another one involving epigenetic mechanisms (Piferrer, 2013; Piferrer et al., 2019). The former has been pinpointed as a possible transducer between the environment and sex determination in some (Adolfi et al., 2019; Geffroy and Bardonnet, 2016; Hattori et al., 2009; Ribas et al., 2017b), but not all (Goikoetxea et al., 2022), phylogenetically diverse fish species. The latter - epigenetic mechanisms - could also play a crucial role since they regulate gene transcription and respond to environmental factors (Best et al., 2018). Over the three major epigenetic mechanisms (DNA methylation, histone modification, and noncoding RNA action), DNA methylation is undoubtedly the most studied, and previous works highlighted its potential role in sex determination in diverse fish species (Navarro-Martín et al., 2011; Shao et al., 2014), including zebrafish

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(Pierron et al., 2021; Ribas et al., 2017c).

Yet, while most of the studies on ESD have focused on the effects of temperature on sex in fish (Geffroy and Wedekind, 2020) or in endocrine-disrupting chemicals (EDCs) (Dang and Kienzler, 2019; Santos et al., 2017), only a paucity of studies has investigated the effects of contaminant on sex determination or sex differentiation. This is surprising as it appears as a straightforward hypothesis, since exposure to contaminant likely triggers a stress at the cellular and the organism level (Geffroy, 2022). In this sense, transgenerational feminization of a zebrafish population exposed to cadmium was recently detected (Pierron et al., 2021). The progressive feminization was associated with changes in the promoter methylation levels of genes involved in sex differentiation. Consistent with those transgenerational effects, we also previously reported alteration of sex ratio in zebrafish for which the parents were irradiated with gamma rays at moderate dose rate (5 mGy.h^{-1}), but with more males produced (Guirandy et al., 2022). In this context, studying molecular events that underpin adverse effects at the individual or population scales, represents an emerging concept in ecotoxicology, especially for the definition of Adverse Outcome Pathway (AOP).

Hence, to explore the underlying mechanisms responsible to irradiation-induced offspring masculinization, we tested here whether parental exposure to irradiation can trigger (i) changes in the cortisol level of female gonads and/or (ii) changes in the promoter methylation level of key genes involved in sex differentiation. Cytochrome P450 19a1A (*Cyp19a1a)*, also known as ovarian aromatase, is involved in androgen to oestrogen conversion. Altered methylation of this transcript was associated with male-biased sex ratio in a GSD + EE species (Navarro-Martín et al., 2011). Doublesex and mad-3 related transcription factor 1 (*dmrt1*) allows testis development (Webster et al., 2017). Forkhead box L2 (*foxl2*) are involved in early ovarian differentiation and maintenance (Yang et al., 2017). The platelet-derived growth factor receptor b (*pdgfrb*) is involved in steroidogenesis control (Schmahl et al., 2008).

Here, we first assessed the concentration of cortisol in female gonads. Second, the promoter methylation and transcription levels of the five above-mentioned genes (*cyp19a1a*, *dmrt1*, *foxl2a*, *foxl2b*, *pdgfrb*) were measured in F0 and F1 gonads. Since Cadmium-induced biased sex ratio in offspring was previously associated with DNA methylation changes in female zebrafish (Pierron et al., 2021), analyses were performed principally in female gonads. In another way, maternal effect was suspected to be at the origin of altered sex ratio in offspring via epigenetic modifications (Pierron et al., 2021).

2. Materials and methods

2.1. Experimental design, sampling and sex ratio

This study followed that of Guirandy et al. (2022), using same fish. Project #20,995 was authorized by the "Institut de Radioprotection et de Sûreté Nucléaire" (IRSN) ethics committee No. 81 (EU 0520, C13–013–07) and complied with French regulations on performing experiments on animals in application of directive 2010/63//UE relating to animal protection. Briefly, F0 adult zebrafish (AB strain with GSD + EE) were exposed to 5 mGy.h^{-1} gamma irradiation and control condition for 30 days (F0 control and F0 I5) (Fig. 1). F1 offspring were then obtained from 15 spawning F0 couples. F1 from irradiated F0 (F0 I5) were separated into two groups: (i) F1 were continuously irradiated at 5 mGy.h^{-1} until 131 days post fertilization (dpf), that corresponds to F1 I5; (ii) F1 were placed in non-irradiated condition until 131 dpf, that corresponds to F1 recovery. Dissections were done on sexually mature adults at 30 days post irradiation and 131 dpf for F0 and F1 generations, respectively. Different methods for determining fish sex were used. First, we proceeded in visual inspection to make an early classification in aquarium, based on coloration, body shape and visibility of urogenital papilla. Finally, we determined sex fish by examining the macroscopic appearance of the gonads during dissections, as explained by Dang and Kienzler (2019). Sex ratio was calculated after dissections of all individuals. Female and male gonads from each generation were sampled and fixed into RNALater or flash-frozen in liquid nitrogen and kept at −80 °C until extraction.

2.2. DNA and RNA extraction

Eight ovaries and testis from individuals at each generation and from each condition were sampled. DNA and RNA extractions were performed as described by Guirandy et al., 2023 using the AllPrep DNA/RNA kit (Qiagen) according to manufactures' guidelines.

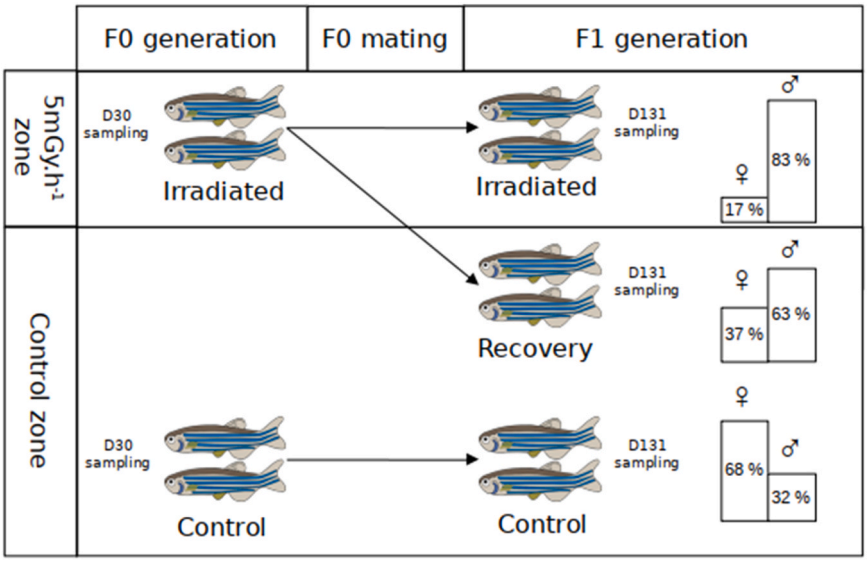

Fig. 1. Experimental design and exposure conditions (duration and dose rate (mGy.h−1). D corresponds to exposure time in days with gonad sampling at the end. F0 adults were exposed over 30 days until reproduction. F1 progenies were then placed in irradiated condition and control condition (recovery) for 131 days until sexual maturity, when the gonads were sampled. Male and female percent for F1 generation are indicated on the right and were presented in details in our previous study (Guirandy et al., 2022).

2.3. Promoter methylation levels and RT-qPCR analysis

Quantification of single cytosine percent methylation at specific CpG sites in five genes (*cyp19a1a*, *foxl2a*, *foxl2b*, *dmrt1*, *pdgfrb*) was performed using targeted bisulfite-pyrosequencing method, as described in Pierron et al. (2019). Primers were designed from the Pyromark assay design software (Table 1). For *cyp19a1a*, *foxl2a* and *dmrt1*, we used the already published method (Pierron et al., 2021). For *foxl2b* and *pdgfrb*, the mean methylation percent of all CpG sites was used, corresponding to three or five sites respectively. The average methylation level of one CpA was measured to confirm great conversion after bisulfite treatment (Supplementary data 1).

Gene transcription was measured by RT-qPCR using the ΔΔCt method (Livak and Schmittgen, 2001). Relative quantification of gene expression was achieved by amplification of endogenous control genes chosen from the RNAseq dataset of our previous study (Guirandy et al., 2023), i.e. genes for which their transcription level was not impacted by irradiation (*aspa*, *pdia6*). Primers used are available in Table 1.

2.4. Steroid extraction and cortisol measurement

Steroid extraction on female gonads (n = 14 and n = 8 for control and irradiation groups, respectively) was done following recommendation in Sadoul and Geffroy, 2019. After sample evaporation under nitrogen at 40 °C, cortisol measurement was performed with the Cortisol ELISA kit (Neogen Lexington, KY, USA). According to the supplier, the cross-reactivity of the antibody with other steroids is as follows: prednisolone 47.5%, cortisone 15.7%, 11-deoxycortisol 15.0%, prednisone 7.83%, corticosterone 4.81%, 6β-hydroxycortisol 1.37%, 17-hydroxyprogesterone 1.36%. Following manufacturer's instructions, samples or standard (Cortisol standard solution) were added in each well in duplicate and supplemented with the conjugated cortisol enzyme. After one hour of incubation, each well was washed and filled with the substrate. Absorbance was read at 650 nm with a microplate reader (Synergy HT, BioTek Instrument, VT, USA) after 30 min of incubation in the dark. To confirm the repeatability of the experiment, one sample was placed on the three different plates. Parallel displacement curves were obtained for plasma by comparing serial dilutions of pooled plasma (1:1 – 1:250) and the cortisol standard preparation (0.04 – 10 ng/ml).

2.5. Statistical analysis

For all measured parameters data are presented as mean values ± SE, with significance taken as $P < 0.05$. Irradiation effect on cortisol and gene transcription levels were assessed using Anova and with BoxCox transformation when normality and homoscedasticity of the error terms were not verified. When assumptions were not met after transformation, a non-parametric test was used (kruskal wallis). For parametric and non-parametric test, Tukey HSD and Dunnet test with Bonferroni correction were used respectively as post hoc test. Irradiation effect on F0 methylation and sex differentiation for F0 and F1 methylation were assessed using GLM with binomial distribution. Irradiation effect on F1 methylation was assessed using a GLMM (Generalized Linear Mixed Model, glmm function in R), with binomial distribution and with spawn as a random effect as explained by Guirandy et al. (2022). The analyses were performed using R software (Team, 2010) with the following packages: "tidyverse", "here", "knitr", "lme4″, "MASS","car". Figures were produced using the package "ggplot2″.

3. Results

3.1. F0 female gonads cortisol

Cortisol concentration in F0 female gonads after exposure to 5 mGy. h^{-1} gamma irradiation (Fig. 2) reached 0.021 ng/mg ± 0.003 and 0.017 ng/mg ± 0.003 for control and irradiated groups, respectively. No statistical difference was observed between conditions.

3.2. Evidence of sex-specific DNA methylation levels in control individuals

A significant difference was observed between females and males for the three genes studied, considering control individuals of the two generations (Fig. 3). Indeed, a higher average methylation percent was found in males in comparison to females for *cyp19a1a* (♀:41.0% ± 1.8; ♂: 96.7% ± 0.3). Conversely, average methylation percent of males was significantly lower for *foxl2a* (♀: 17.3% ± 1.4; ♂: 6.6% ± 0.6) and *dmrt1* (♀: 27.6% ± 1.6; ♂: 4.5% ± 0.4) in comparison to males.

3.3. Female gonad promoter methylation and gene transcription levels after gamma irradiation

Analysis were also performed on F0 and F1 male gonads but no significant difference was observed (data not shown) between controls and irradiated fish.

In ovaries, no differences were observed regarding *cyp19a1a* promoter methylation after gamma irradiation at the F0 generation (black

Table 1
primer list for methylation and transcriptomic analyses. F, R, S and * correspond to forward, reverse, sequencing and biotinylated sequences, respectively.

Gene	Coordinates GRCz11	Sequencing primers	RT-qPCR primers
cyp19a1a	NC_007129.7 39636332–39636181	F: ATGTAGTTGTTGGGATATAAAAGTG R*: CTTTAAATAAAAAAATCCACCACAACC S: TGTTGGGATATAAAAGTGT	F: AAAGGGCTCATAACGGCACT R: TGTGTGGTCGATGGTGTCTG
foxl2a	NC_007126.7 7054447–7054695	F: TTTGTTTTGTTTTGAATGGGGATT R*: TCTCCCACCAACTCTAAAATACACAAATT S: TGTTTTGAATGGGGATTA	F: CATCGCTAAGCCAAGTAGCC R: AGGGCCCAATTATTCACTCC
dmrt1	NC_007116.7 44944800–44944918	F: AGGAGAGAGGGGAAAGATAG R*: AATTTTTTCCCTCTTCAAATATAATTTCAC S: GAGGGGAAAGATAGG	F: AAGTCCTGCAATGTCCAGCA R: GGAGGAGTATGGAGTGGGCT
foxl2b	NC_007113.7 58257650–58257750	F: GGATTGTTTTGGATTGTTGTAAGG R*: CTTTCCTCACTTCTCTCTTCTATTTTACC S: GGAAAAATATAAATATAAAGTGTTA	F: GCCTGCGAGGATATGTTCGA R: GTTCATGAAGCCCGACTGGA
pdgfrb	NC_007125.7 3229650–3229800	F*: GGGAAATGAGATGGATTTTGGTAG R: ACATTTTCCATTTTTCCCCAATT S: CCATTTTTCCCCAATTTA	F: ATCCCCATCCCTGATCCCAA R: TTCCAGAGTGTTCGGTCAGC
aspa			F: CCGCTATGAGTTCGCGTACT R: GTGTCGATGTACCTGGTGCA
pdia6			F: CTCTCCTGTGGATGAGGGGA R: GTGTGCGTGTGTGAGAGAGA

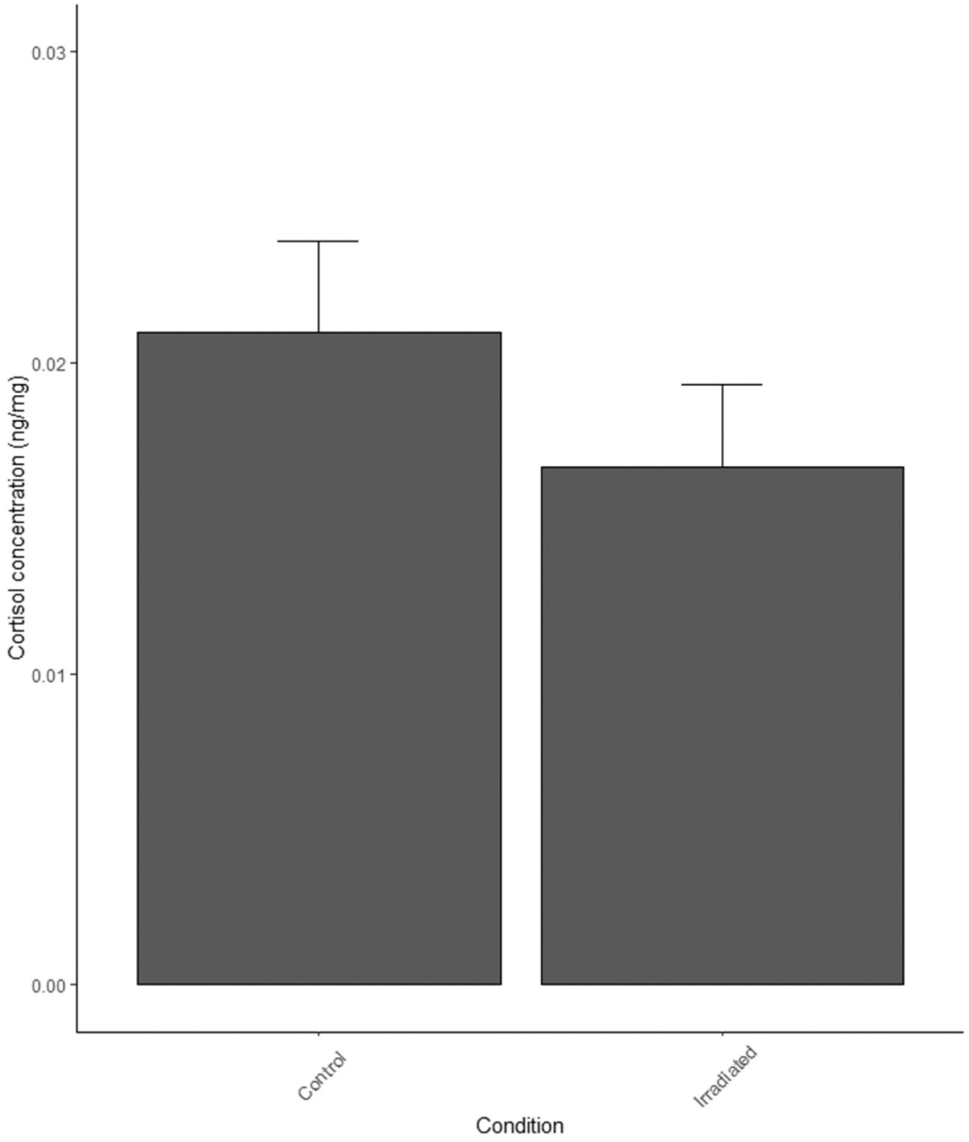

Fig. 2. Barplot of F0 ovaries cortisol concentration after control and exposure to 5 mGy.h-1 (irradiated). n = 14 and n = 8 for control and irradiation groups, respectively.

bar, control = 34.80% ± 1.14; irradiated = 38.01% ± 1.97) (Fig. 4 A). However, a significant hypermethylation was detected in the F1 generation following gamma irradiation (grey bar control = 41.38% ± 1.33; irradiated = 49.67% ± 1.73). This effect was not observed for F1 recovery (44.88% ± 1.61). Nevertheless, no difference was observed between F1 irradiated and recovery groups, indicating that F1 recovery presented intermediate values between control and F1 irradiated fish. This notion of intermediate values was also observed for sex ratio of the recovery group which was between control and F1 irradiated sex ratio (Fig. 1). For *dmrt1* and *pdgfrb* genes, no significant variation was observed whatever the generation. For the two *foxl2* genes, an increase in their methylation levels was observed at the F0 generation (black bar, *foxl2a*: control = 14.92% ± 0.82; irradiated = 21.52% ± 3.55; *foxl2b*: control = 7.41% ± 1.80; irradiated = 10.88% ± 3.28). However, this variation was only statistically significant for *foxl2a*. Conversely, a trend towards a decrease in methylation was observed for these two genes in the next generation (F1, grey bar *foxl2a*: control = 18.21% ± 1.81; irradiated =13.02% ± 2.24, recovery = 15.66% ± 1.05; *foxl2b*: control = 7.37% ± 0.82; irradiated = 5.95% ± 1.26; recovery = 4.22% ± 0.52.). However, this hypomethylation was only significant in the recovery group for the *foxl2b* gene. Fig. 4B presents the RNA transcription levels of these genes. An effect of irradiation was observed only for *pdgfrb* at the F0 generation, showing an overexpression (control: 1.55 ± 0.65; irradiated: 3.03 ± 0.52).

4. Discussion

There is emerging evidence that stressful environment can induce skewed sex ratio. While temperature is by far the most studied environmental factor, recent studies also showed that other factors such as EDCs, hypoxia, population density and photoperiod can induce biases in sex ratio, through direct effects on sex determination/differentiation on the animal undergoing these stress (Corona-Herrera et al., 2018; Geffroy and Bardonnet, 2016; Ribas et al., 2017b). In agreement with these finding, we recently detected that zebrafish exposed to gamma irradiation predominantly develops testis (Guirandy et al., 2022). But somehow intriguingly, we also detected that those effects could be maternally transmitted, since unexposed offspring from exposed parents also showed a biased sex ratio in favour of males (Guirandy et al., 2022), advocating for the existence of transgenerational plasticity. This suggests that biased sex ratio did not only occur when animals were exposed during the sex determination window (at early stages of development,

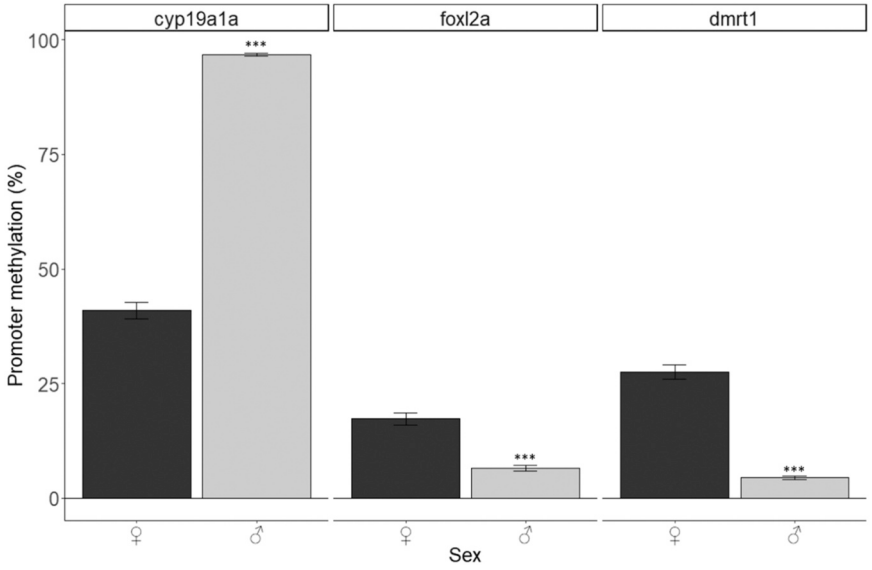

Fig. 3. Sex-specific promoter DNA methylation pattern of genes involved in sex differentiation. For each sex, all individuals from F0 and F1 control groups. N = 22. * ($p < 0.05$), ** ($p < 0.01$), ***($p < 0.001$).

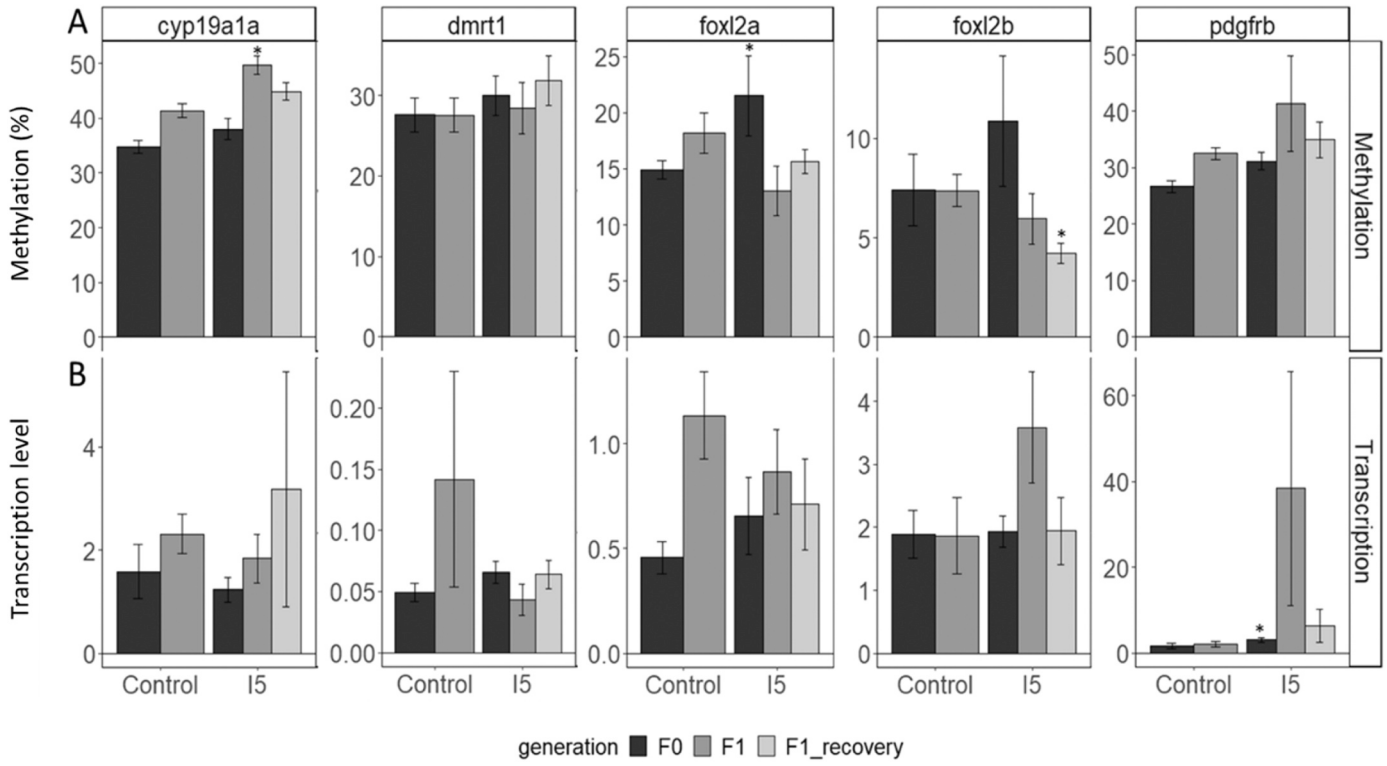

Fig. 4. DNA methylation and RNA transcription levels after irradiation at 5 mGy.h-1 and control. (A): Barplot of average DNA methylation (%) of genes for control and irradiated female fish. N = 6, 4, 16, 6 and 8 for F0-ontrol, F0-I5, F1-control, F1-I5 and F1-I5 recovery, respectively. (B) Barplot of RNA transcription level (a.u.) for control and irradiated female fish. N = 8, 7 6, 4 and 9 for F0-control, F0-I5, F1-control, F1-I5 and F1-I5 recovery, respectively. Colours correspond to the generation (F0 in black and F1 in grey). * ($p < 0.05$), * * ($p < 0.01$), * ** ($p < 0.001$), compared to control group.

around 11–45 dpf in the case of zebrafish) as previously observed (Piferrer, 2013; Piferrer and Anastasiadi, 2021; Santos et al., 2017) but also when the exposure occurs during the gonad maturation stage of the parents (Pierron et al., 2021). In other words, gamete irradiation, i.e., future F1, in parental gonads could have effects on F1 sex ratio. Offspring (F1) effects from exposed genitors raise real questions about wild irradiated population fate. Prompted by a similar study on the effects of cadmium (Pierron et al., 2021), we also investigated epigenetic factors as possible transducer of the information. Interestingly, we observed similar results at the molecular scale since no difference was observed here between the recovery and irradiated group. This suggest that male-biased sex ratio is induced by adult genitor irradiation rather than offspring irradiation.

4.1. 5 mGy.h^{-1} irradiation did not induce change in cortisol production in F0 female gonad

Cortisol, can be transmitted from mother to embryos and is involved in the proper development of the offspring (Faught and Vijayan, 2018; Nesan and Vijayan, 2016, 2012). Cortisol can also be actively excreted from the eggs by ATP-binding cassette to avoid negative effects of a too high exposure (Paitz et al., 2016). Here, cortisol accumulated in ovaries was not impacted after gamma irradiation at 5 mGy.h^{-1}. Until now, all studies reporting an effect of cortisol on sex were based on a direct exposure of the animal itself, where cortisol bind to its receptor and this complex then bind to specific promoter regions (i.e. glucocorticoid response element, GRE) of genes involved in sex differentiation (e.g. dmrt1 and cyp19a1a) (Geffroy and Wedekind, 2020). Here we envisioned possible transgenerational effects for the first time, and the fact that the cortisol accumulated did not differs significantly between conditions, does not totally rule out possible long-term effects on sex. First, studies suggest that cortisol levels after acute exposure is relevant but is attenuated after chronic exposure (as we did; Cohen et al., 2012; Ribas et al., 2017b; Van Weerd and Komen, 1998). Secondly, the transcription level of the 11-beta-hydroxysteroid dehydrogenase (*hsd11b2*) gene, which is involved in cortisol regulation by converting cortisol into its physiologically inert metabolite (cortisone) was found to be up regulated (log2FoldChange = 1.22, p.adj <0.01) in F0 female gonad (RNA-sequencing data, not shown).

4.2. Evidence of sex-specific DNA methylation levels in control zebrafish

As observed in Fig. 3, *cyp19a1a* and *dmrt1* methylation levels were different between females and males as previously reported by Navarro-Martín et al. (2011) in the European sea bass or by Pierron et al., 2021 in the zebrafish. These differences were also observed on gene transcription levels (Anastasiadi et al., 2018; Pierron et al., 2021). Several studies did not show sex-specific methylation for *foxl2a* (Anastasiadi et al., 2018; Piferrer et al., 2019). Here, as in Pierron et al. (2021) we detected significant differences in *foxl2a* methylation levels between male and female zebrafish. Moreover, *dmrt1* and *foxl2a* were highly methylated in female gonads compared to male gonads. Pierron et al., 2021 highlighted that the relationship between methylation and transcription pattern of *foxl2a* was different from what is generally expected (i.e, hypomethylation leads to down-regulation). Taking this into account, our results on females likely confirm the antagonistic role of these two genes in maintaining sexual phenotype of gonads (Huang et al., 2017; Li et al., 2018). Independently of the underlying mechanisms, the sex specific pattern of DNA methylation observed in our study reinforces the role of DNA methylation in zebrafish sex differentiation.

4.3. Irradiation induced changes in methylation marks in female gonads

As early embryonic development is principally dependent on maternal transcripts, only female gonads were analysed (Laing et al., 2018). Moreover, for all genes under study, no effect of irradiation was observed on methylation marks in males. In addition, no effect was observed on the "male" genes, i.e. *dmrt1* and *pdgfrb*. Interestingly, similar results were observed in the zebrafish (wild-type) exposed to Cd (Pierron et al., 2021) or the European sea bass exposed to a high temperature (Navarro-Martín et al., 2011). In both works, environmentally-induced skewed sex ratio was associated with DNA methylation changes in females, but not in males. However, we cannot totally exclude paternal involvement in gamma irradiation-induced masculinization.

Thus, in our study, no effect of irradiation was observed on the methylation level of *dmrt1* or *pdgfrb* for all generations. In contrast, a hyper-methylation of *foxl2a* was observed after exposure to irradiation in females of the F0 generation. This hyper-methylation was associated with a masculinisation of the population at the next generation. Pierron et al. (2021) showed that *foxl2a* methylation level in F0 female gonads after Cd exposure was correlated with the sex ratio at the next generation. More precisely, Cd exposure induced a hypo-methylation of *foxl2a* in female gonads, associated to an increased proportion of females at the next generation. As in our study, no effect of Cd was observed in males and despite a significant effect of Cd was observed on the methylation level of *foxl2a* in female gonads at the (exposed) F0 generation, an effect of Cd on the sex ratio was observed only from the (unexposed) F1 generation. In our case, irradiation induced a hyper-methylation of *foxl2a* in F0 female gonads. This hyper-methylation was associated with a masculinisation of the population at the next generation. Our results seemed to confirm the relationship between the methylation level of *foxl2a* in female gonads and the sex ratio of their offspring. A similar trend was observed for *foxl2b*, without significant difference, probably due to high inter-individual variability. Thus, these results suggested transmitted epigenetic effects from genitors to offspring. Epigenetic mark inheritance had been already observed after exposure to gamma irradiation (Kamstra et al., 2018). This is further supported by the fact that epigenetic marks are not erased in zebrafish and could be inherited via germ cells (Ortega-Recalde et al., 2019; Valdivieso et al., 2020). We also observed a *cyp19a1a* hyper-methylation in F1 female gonad after irradiation. Since this enzyme converts androgen to oestrogen, altered steroid synthesis in favour of males, as explain above, could explain masculinization (Pierron et al., 2021; Piferrer, 2013). In both the zebrafish (Pierron et al., 2021) and European sea bass (Navarro-Martín et al., 2011), a significant hypermethylation of *cyp19a1a* was observed in adult female gonads after exposure to heat, i.e. a masculinizing factor. Thus, a similar pattern was observed between two strains of zebrafish (wild-type in Pierron et al., 2021 and AB in our case) and two fish species (Zebrafish and European sea bass), reinforcing the role of *cyp19a1a* DNA methylation in species with GSD + EE. As a matter of fact, several studies suggested that aromatase (*cyp19a1a*) plays a key role in fish sex determination (Guiguen et al., 2010). Finally, *foxl2a* is required for oogenesis maintenance rather than its initiation (Kossack and Draper, 2019) which suggests that *cyp19a1a* methylation and its transcription is the initiating event.

While a direct or intergenerational effect of irradiation was observed on the methylation level of *foxl2a* and *cyp19a1a*, no effect of irradiation was observed on their transcription levels in F0 and F1 generations. Non-impacted transcription could be explained by stage of dissection. Indeed, analyses were carried out in adult mature fish; i.e. when the gonads were already fully developed. Thus, it would be necessary to measure both transcription and methylation levels in primordial germ cells (PGCs) during the sex determination window in order to strengthen the link between DNA methylation and sex differentiation (Ortega-Recalde et al., 2019; Yang et al., 2017).

However, even if increasing evidences suggest that DNA methylation is involved in ESD, cautions must be taken. Notably, previous works reported inter-family variations in the sex ratio response to environmental cues, highlighting the role of parental genes, i.e. genetic influence (Anastasiadi et al., 2018; Ribas et al., 2017a; Valdivieso et al., 2020). For instance, it is well known that maternally provided mRNA are necessary to offspring development, including germline development (Dosch, 2015; Lehmann and Ephrussi, 1994). However, we previously reported irradiation-induced effects of the maternal transcriptome including genes involved in germline development (Guirandy et al., 2023). Such dysregulations could alter the proper proliferation and migration of PGCs or induce their apoptosis. In our case, PGC number could thus be affected by maternal exposure to gamma irradiation, participating to masculinization since depletion of PGCs promotes male differentiation in zebrafish (Tzung et al., 2015).

While further investigations are needed, this study joins other regarding epigenetic events at the origin of environmentally skewed sex ratio. However, it is the first time that such results were observed after exposure to gamma irradiation. Since similar results were obtained in different studies, involving different stressors and species, such

epigenetic events could represent relevant biomarkers in ecotoxicology. Indeed, there is a growing interest in linking molecular events to adverse effects at the individual or population scale.

5. Conclusions

This study aimed to identify mechanisms involved in male biased sex ratio after exposure to gamma irradiation on F0 and F1 generations. Two hypotheses were tested in order to explain offspring male-biased sex ratio. Cortisol levels were not impacted in F0 female gonad after irradiation, but further investigations are needed before to discard its role. In contrast, irradiation effects were observed in the promoter methylation level of three genes involved in sex determination (*cyp19a1a*, *foxl2a*, *foxl2b*) which presented sex-specific methylation levels. Specifically, we detected that (i) irradiation-induced changes in the *foxl2a* methylation level in mother gonads was associated with change in the sex ratio of their offspring and (ii) irradiation-induced masculinization of the population was associated to an increased methylation of *cyp19a1a* in female gonads. Our results reinforce the role of DNA methylation as a key transducer between environment and sex. Moreover, since epigenetic marks are inherited in zebrafish, effects could be carried over to subsequent generations via the germ line. Our results underline the fact that, a simple (only one generation), short-term exposure at population scale is sufficient to affect sex ratio and thus, the eco-evolutionary dynamic of the population.

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CRediT authorship contribution statement

Guirandy Noemie: Conceptualization, methodology, Validation, Investigation, Formal analysis, Writing - Original Draft **Olivier Simon**: Conceptualization, Investigation, Writing - Original Draft, Supervision **Benjamin Geffroy**: Methodology, Investigation, Writing - Review & Editing **Guillemine Daffe**: Investigation **Flore Daramy**: Investigation **Camille Houdelet**: Investigation **Patrice Gonzalez**: Writing - Review & Editing **Fabien Pierron**: Conceptualization, Methodology, Investigation, Writing - Original Draft, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Guirandy reports financial support was provided by Institute of Radiation Protection and Nuclear Safety Cadarache.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115790.

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