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Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring

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Abstract

Accumulating evidence shows that environmental changes can affect population sex ratios through epigenetic regulation of gene expression in species where sex depends on both genetic and environmental cues. Sometimes, altered sex ratios persist in the next generation even when the environmental cue is no longer present (a multigenerational effect). However, evidence of transgenerational effects (i.e., beyond the first non-exposed generation), which tend to be paternally transmitted, is scarce and a matter of debate. Here, we used the AB strain of zebrafish, where sex depends on both genetic and environmental influences, to study possible multi- (to the F_1) and transgenerational (to the F_2) effects of elevated temperature during the critical period of sex differentiation. From eight initial different families, five were selected in order to capture sufficient variation between the sex ratio of the control group (28°C) and the group exposed to elevated (35°C) temperature only at the parental (P) generation. Results showed a consistent increase in the proportion of males in the P generation in all five families as a result of heat treatment. Sex ratios were then determined in the F_1 and F_2 offspring derived from both above groups, which were all raised at 28°C . A persisting male-skewed sex ratio in the 35°C -derived, unexposed offspring of the F_1 generation was observed in three families, denoting family-dependent multigenerational effects. However, no transgenerational effects were observed in the F_2 generation of any family. DNA methylation was also assessed in the testis of P, F_1 and F_2 males derived from exposed and non-exposed fathers and grandfathers. DNA methylation was significantly decreased only in the testis of the 35°C -derived males in the F_1 generation but not of the F_2 generation and, surprisingly, neither in the 35°C -exposed males of the P generation. Taken together, our results show great interfamily variation, not only in sex ratio response to elevated temperature, but also on its multigenerational effects, denoting a strong influence of genetics. Alterations in the testicular epigenome in F_1 males calls for attention to possible, previously unnoticed, effects of temperature in the unexposed offspring of heat-exposed parents in a global warming scenario.

Key words: Zebrafish; Epigenetics; DNA methylation; Sex ratio; Transgenerational effects; Global warming

Introduction

Throughout vertebrate evolution, different sex-determining mechanisms have been co-opted (Bachtrog et al., 2014). In vertebrates, sex determination can be controlled by genetic factors (genetic sex determination, GSD), by environmental factors (environmental sex determination, ESD) or a combination of both types (Devlin and Nagahama, 2002; Sarre et al., 2004; Heule et al., 2014). Sex determination produces the sex ratio, defined as the ratio of males and females in a population (Hardy, 2002). Several environmental cues can influence sex determination. The sex ratio is a key demographic parameter in ecology and evolution since it has direct influence on the reproductive capacity of populations and thus is crucial for species survival (Beissinger and McCullough, 2002). Particularly in lower vertebrates, even in GSD species, environmental influences may sometimes override the genetic factors, resulting in a phenotypic sex different from the genotypic sex (Devlin and Nagahama, 2002). Temperature is the most common environmental factor affecting population sex ratio, and exposure to heat in fish usually results in an increase in the number of males (Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009; Ribas et al., 2017a). Actual effects are influenced by developmental stage when abnormal temperature is applied, exposure duration and the genetic background (Dang and Kienzler, 2019). However, there are other environmental factors such as population density, pH, hypoxia and the presence of endocrine disrupting chemicals (EDCs) that can alter the sex ratio as well (Baroiller et al., 2009; Dang and Kienzler, 2019). Thus, prolonged changes in population sex ratio as a result of persistent environmental disturbances can have dramatic demographic consequences, threatening population viability (Le Galliard et al., 2005; Zhou et al., 2010).

Responses to environmental changes are mediated by epigenetic mechanisms, which have the potential to modulate gene expression without changes in the nucleotide sequence (Turner, 2009). Epigenetic changes thus act as organismal responses to cope with new environmental scenarios (Kronholm and Collins, 2016). DNA methylation is the most studied epigenetic mechanism involved in the acquisition of the sexual phenotype during the process of gonadal development, although histone modifications or regulation by microRNAs have been shown to play a role as well (Piferrer, 2019). Evidence regarding the integration of environmental cues such as temperature through

changes in DNA methylation and affecting sexual fate was described, for the first time, in the European sea bass (*Dicentrarchus labrax*). Elevated temperature during early development hypermethylated the promoter region of the *cyp19a1a* gene (gonadal aromatase) resulting in transcriptional silencing (Navarro-Martín et al., 2011). Aromatase is the sole enzyme involved in the synthesis of estrogen such as 17 β -estradiol (E₂), and estrogens are essential for ovarian differentiation in all but eutherian vertebrates (Guiguen et al., 2010). Similar results involving *cyp19a1a* and other key genes for sex differentiation have been found in vertebrates susceptible to the effects of temperature, notably reptiles and fish (Piferrer, 2019). Further, in the European sea bass elevated temperature applied during larval, but not juvenile, development had long-lasting effects on global DNA methylation and affected many key genes involved not only in sex differentiation but also in survival and organ formation (Anastasiadi et al., 2017). However, the extent at which these changes can persist in the exposed individuals as well as their possible transmission to the offspring is currently a matter of debate (Wang et al., 2017).

Intergenerational epigenetic inheritance represents the transmission of epigenetic marks from one generation to the next (Anway et al., 2005; Jirtle and Skinner, 2007; Skinner, 2008; 2014; Pang et al., 2017). Germ cells can transmit not only genetic, but also epigenetic information (Magnúsdóttir and Surani, 2014). During an organism's lifespan, there are critical windows where environmental factors can affect germ cell lineages. Studies in vertebrates, mainly in mammals, have shown that the genome undergoes two main epigenomic reprogramming events, each of which involves waves of DNA demethylation and *de novo* methylation (Cantone and Fisher, 2013; Seisenberger et al., 2013b). The first window occurs in the primordial germ cells (PGCs) which, after fertilization, migrate into the genital ridges to develop future gonads, whereas the second occurs at adult stages during gametogenesis (Jammes et al., 2010; Hales et al., 2011; Seisenberger et al., 2013a). In spite of the extensive DNA demethylation in preimplantation embryonic cells, some of the genomic regions escape this reprogramming process and are transmitted to the newborn individual in methylated form (Borgel et al., 2010; Daxinger and Whitelaw, 2012). This means that DNA methylation changes, induced by environmental stressors in germ cells, may not only have consequences on the exposed individual but can potentially be heritable across generations and cause transgenerational adverse effects (Pacchierotti and Spanò, 2015).

The typology of the environmental exposures which directly induce epigenetic changes in the germinal line can range from temperature, nutrition and stress, to a large number of endocrine disruptors (Jirtle and Skinner, 2007). Consequently, these induced epigenetic marks in the PGCs can be theoretically inherited across generations either via parental, maternal or both (Heard and Martienssen, 2014). Thus, by virtue of inherited epigenetic patterns, offspring are able to manifest the same phenotype without having been exposed to the same environmental cue as their progenitors (Richards, 2006). In fish, due to oviparity and external fertilization, transgenerational effects can be already observed in the F₂ generation, in contrast to what occurs in mammals, in which transgenerational effects are not called as such unless they are observed at least in the F₃ generation if the exposed female happens to be pregnant (Skinner, 2008; Skinner et al., 2010; Nilsson and Skinner, 2015; Hanson and Skinner, 2016). Thus, fish are useful models for studying long-term, transgenerational effects of environmental stressors (Baker et al., 2014; Corrales et al., 2014; Bhandari et al., 2015). In fish, several cases of multigenerational and transgenerational effects due to endocrine disruptors or environmental factors affecting sex development and reproduction have been reported. For example, in medaka (*Oryzias latipes*), exposure to bisphenol A (BPA) and 17 α -ethynylestradiol (EE₂) during embryonic development, induced reproductive impairment and compromised the fertilization rate in offspring of the two following generations (Bhandari et al., 2015). In *Oryzias melastigma*, exposure to hypoxia caused transgenerational impairment of several reproduction traits, delayed gonad development along with a diminished sperm count and motility in the F₁ and F₂ (Wang et al., 2016).

Epigenetic inheritance may contribute to adaptation to new environmental conditions. This is beneficial for the survival of the exposed population and future offspring as they are expected to cope with the changing environment (Burggren, 2016), such as acclimation of poikilothermic animals to current climate changes (Munday, 2014; Veilleux et al., 2015). Nevertheless, some of these inherited epigenetic changes could also constitute what is known as an epigenetic trap, i.e., any epigenetic change that arises in response to novel environmental cues that produce maladaptive phenotypes, with no increase in phenotypic variance (Consuegra and Rodríguez López, 2016). In a climate change scenario, there is an interest in understanding how epigenetic marks are transmitted to subsequent generations and their possible physiological consequences in

the new environment (Consuegra and Rodríguez López, 2016). Recent findings from our laboratory have shown that temperature exposure during sex differentiation was able to alter methylation levels in the promoter regions in a set of reproduction-epigenetic and stress-related genes in adult zebrafish (*Danio rerio*) gonads (Valdivieso et al., unpublished). In the present study, we used the zebrafish model to determine if the masculinization observed when a given generation (generation P) was exposed to elevated temperature during the critical sensitive period could be inherited in subsequent generations. To do so, we analyzed population sex ratios and DNA methylation levels not only in the P generation but also in their offspring up to the F₂ generation.

Materials and methods

Fish and husbandry

Zebrafish (AB strain; ZFIN ID: ZDB-GENO-960809-7) from the European Zebrafish Resource Centre (EZRC, Germany) were housed at the experimental facilities of the Institute of Marine Sciences (ICM-CSIC, Barcelona). The housing conditions, the physicochemical water parameters and the feeding regimes have been described elsewhere in Ribas et al. (2017b). Eggs were reared in sterilized plastic Petri dishes ($\varnothing = 100$ mm) at ~50 eggs per dish filled with embryo medium (pH 7.2 ± 0.5), supplemented with 0.1% methylene blue to suppress growth of mold and fungal infections in the water (Westerfield, 2000) until 6 days post fertilization (dpf) as in Ribas et al. (2017b). The animal facility used for the present study is licensed by the Bioethical Committee of the Government of Catalonia for animal experimentation with reference code no. 9977.

Experimental design

The study involved three generations: parental or the exposed generation (P), and the subsequent first (F₁) and second (F₂) generations (both unexposed; **Fig. 1A**). Eight independent families of the P generation, each consisting of the offspring from a cross between a single male and a single female (i.e., the P₋₁ generation), were used. At 18 dpf, larvae from each family were randomly divided into two groups with equal numbers. In one group, larvae were always raised at control temperature (28°C), while in the other water temperature was gradually increased (i.e., 2°C/day) to 35°C from 18 to 34 dpf, the sensitive period (Ribas et al., 2017b). Thereafter, fish of both groups were

raised under the same conditions until they became sexually mature (> 90 dpf). Although there are sex-related differences between females (round shape and protruding abdomen) and males (slightly more intense yellow coloration) (Hutter et al., 2012; McMillan et al., 2014), to assure the correct identification of sex of adult zebrafish to create the next generation, the presence of a prominent genital papilla zebrafish females was used to identify mature females from males (Yossa et al., 2013). To create the F_1 and F_2 generations, siblings of an unrelated, untreated family (from here onwards external family), raised at 28°C , were mated with fish of the 28°C and 35°C groups. Thus, an external male was mated with a female from the 28°C group and then the same male was mated with a different female from the 35°C group. The same principle was used with external females, which were mated first with a male from the 28°C group and then another male from the 35°C group. Thus, the effect different allelic combinations due to the PSD nature of the AB zebrafish and their contribution to variation in offspring sex ratio was hopefully reduced, as the two groups to be compared consisted of half-sibs.

To obtain the 28°C - and 35°C -derived groups of the F_2 generation the same procedure was applied. All F_1 and F_2 were raised exclusively at 28°C and it was taken that the number of larvae in the tanks, i.e., the rearing density, prior and during the thermal stress and across generations was below the density threshold needed for masculinization, as observed in our previous study (Ribas et al., 2017b). Thus, observed deviations among sex ratios of the 28°C -derived groups was ascribed only to the PSD in zebrafish and deviations of sex ratios in the 35°C -derived groups vs. the 28°C ones was due to primarily temperature, not to rearing density.

Sampling

Fish were euthanized by shock hypothermia using ice and water (**Table S1**). Their phenotypic sex (e.g., size, body shape, color and presence/absence of genital papillae) was visually checked with the aid of a dissecting scope and the number of males and females were counted to determine the sex ratio. Gonads were dissected out and sexed without discrepancy between the visual and gonad-based sexing, snap frozen with liquid nitrogen to finally stored at -80°C until further analysis. We did not use histological analysis because mature ovaries and testes were perfectly distinguishable at > 90 dpf (Fujimoto et al., 2010).

DNA extraction

Samples were digested overnight at 56°C with buffer containing 1 µg of proteinase K (Sigma-Aldrich, St. Louis, Missouri) to eliminate proteins. Then, the standard phenol-chloroform-isoamyl alcohol protocol (PCI 25:24:1) with 0.5 µg ribonuclease A (PureLink RNase A, Life Technologies, Carlsbad, California) was performed to isolate DNA and eliminate RNAs. The quality and quantity of DNA was measured by a NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Isolated DNA samples were stored at -20°C until further analysis.

Global DNA methylation quantification assay

For DNA methylation analysis, testes of fish from the 28°C and 35°C groups of the P generation in family #2 were selected. Both for the F₁ and F₂ generations, testes from offspring of a pair of males (28°C and 35°C) mated with the same external female were used. Global DNA methylation was estimated using the 5m-C DNA ELISA Kit (Zymo Research, D5326, USA) according to the manufacturer's instructions. A total of 100 ng of genomic DNA was used for each biological sample with two technical replicates. To quantify the percentage of 5m-C methylated DNA (%5m-C), a standard curve was built and assayed in parallel with samples. The negative and positive controls (0% and 100% methylation, respectively, from *Escherichia coli* DNA) were premixed to generate a 7-datapoint standard curve (each in duplicate) with known %5m-C DNA methylation concentration. Samples were denatured at 98°C for 5 min in a thermal cycler and then transferred immediately to ice for 10 min. All denatured DNA samples were transferred to plate wells, which were then coated with 5m-C coating buffer by incubation at 37°C for 1 h. After washing three times with 200 µl of 5m-C ELISA buffer, wells were incubated at 37°C for 30 min in 200 µl of 5m-C ELISA buffer. Antibody mix was prepared by diluting anti-5-methylcytosine (1:1000) and horseradish peroxidase Developer (HRP, secondary antibody) (1:1000) in 5m-C ELISA buffer. A total of 100 µl antibody mix was added to each well and incubated at 37°C for 1 h. After washing three times with 200 µl of 5m-C ELISA buffer, 100 µl of HRP Developer was added to each well and then incubated at room temperature for 60 min. Absorbance at 405 nm was measured using an Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland). To determine %5m-C of the biological samples, a logarithmic second-order regression was generated with the relation of %5m-C and absorbance from the

standard curve using the function $e^{\frac{(Absorbance - y_{intercept})}{Slope}}$. Percent 5m-C values were corrected with the zebrafish CpG density according to the manufacturer instructions. To obtain the most accurate values, the latest zebrafish genome was downloaded (GRCz11; Assembly accessions: GCA_000002035.4) from Ensembl (www.ensembl.org). We extracted the length of the genome (L) = 1,674,207,132 bp, and we calculated the total numbers of cytosines (C) and the number of CpG dinucleotides (CG) using the command: “grep -v '>' zebrafish_genome.fa | grep -o -i 'X' | wc -l”, (where X is for 'C' and then for 'CG', respectively) in Linux operative system (Ubuntu v. 16.04.6 LTS). Once we obtained the total number of C = 306,412,859 and CG = 29,220,867 from the zebrafish genome, we calculated the fold difference of CpG density (total CG genome / L) between the genomes of (*E. coli* / *D. rerio*) = (0.07472 / 0.0175) = 4.2811. Finally, to obtain the global methylation values, the % 5m-C/CpG density values were multiplied by the value obtained from the total number of C / L zebrafish = 0.1830.

Statistical analysis

The sex ratio at 28°C and the sex ratio response to temperature in the P generation was analyzed in each one of the eight tested families with the χ^2 test with Yates' correction (Yates, 1934). A factorial ANOVA was used with family, generation and temperature as independent factors and the sex ratio as the dependent variable. In case of significant differences, a post-hoc Tukey test was used to detect pairwise differences between the three different generations. The Student's *t*-test was used to compare the global DNA methylation levels in the testes of males exposed at 28°C and 35°C in each generation. Values were logit transformed prior analyses in order to avoid problems with parametric assumptions. Two outliers, as assessed with a 95% confidence test, were removed (one from the F₁ and another from the F₂ generation data). Relationship between quantitative variables was measured using correlation and linear regression, and the coefficient of determination R² was computed. All statistical analyses were carried out using Statgraphics Centurion XVI (v. 16.1.18) and R software (v. 3.6.1) (R Core Team, 2013). Significant differences were considered when *P* < 0.05. All graphs were generated using the *ggplot2* package (v. 3.2.1) (Wickham, 2011) in R software.

Results

Sex ratio and sex ratio response to temperature in the P generation was family dependent

Sex ratio in the P generation of families #1 through #8 varied greatly, ranging from 18 to 72% males when raised at 28°C, in accordance to PSD in the zebrafish AB strain (Ribas et al., 2017b). When siblings from the same families were raised at 35°C, the number of males increased in all families but only in five showed a significant increase (χ^2 -test: families #2 and #7 $P < 0.05$; families #3 and #8 $P < 0.01$ and family #5 $P < 0.001$) (**Fig. S1A**). Thus, when considering the eight families together, a temperature-dependent significant increase (χ^2 -test; $P < 0.01$) in the number of males was observed, as expected (**Fig. S1B**).

There was a significant overall relationship (Adjusted $R^2 = 0.7419$; P -value = 0.00371) between the number of males at 28°C and at 35°C across the eight families, meaning that the higher number of males at 28°C also meant a higher number of males at 35°C (**Fig. S2A**). However, the actual increase in the number of males due to temperature varied between ~15% and ~45%, but was independent of the number of males at 28°C, as evidenced by the lack of correlation, (Adjusted $R^2 = -0.1652$; P -value = 0.9327) and with an average increase between 25 and 30% (**Fig. S2B**).

In order to capture sufficient biological variation in sex ratio at 28°C, sex ratio response to elevated temperature and, importantly, also considering the number of available fish for experimentation, we selected five families out of the initial eight: family #1 (female-skewed initial sex ratio), families #2, #4 and #6 (balanced initial sex ratio) and family #7 (male-skewed initial sex ratio; **Fig. S1C**; **Table S1**) to study possible transgenerational effects. Family #5, for example, despite being highly influenced by temperature could not be selected because there were no females at 35°C and thus no female-derived F_1 generation could be created. The five selected families together reflected the variability typically associated with sex ratio in the AB laboratory strain of zebrafish, and, as such, it was suitable for the study of possible transgenerational effects (up to the F_2 generation) of elevated temperature in the P generation.

Sex ratio in the F_1 and F_2 generations was also family-dependent

The sex ratios of the five selected families in the F₁ and F₂ generations, in addition to the P generation, varied considerably (**Fig. 2**). Except for family #1, which was highly female-biased at 28°C (**Fig. 2A**), and regardless of the number of males in the 35°C groups of the different families in the P generation, the sex ratio of the different families tended to equilibrium (~50% males) along the F₁ and F₂ generations (**Fig. 2B, C, D and E**). This, without exception, was particularly evident in all 28°C-derived groups, since the sex ratio was close to 1:1 in the F₂ (blue lines in **Fig. 2**). However, when analyzing the contribution of generation and temperature in the sex ratio of the P, F₁ and F₂ generations in these five families a multi-factorial ANOVA showed no significant effects of temperature and generation and, importantly, neither family (**Table 1**), suggesting that, despite the increase in the number of males at the P generation, male-skewed sex ratios were not globally maintained in the subsequent generations.

However, these overall results were likely influenced by the great variability of the sex ratios in the 28°C groups of the P generation (18–72% males). These extreme values coincided with family #1 (18% males at 28°C in the P generation), which also showed a difference in the behavior of the 35°C-derived groups in the subsequent generations compared to other families, since sex ratios kept departing away from 1:1 (**Fig. 2A**). The other extreme value (72% males at 28°C in the P generation) corresponded to family #7, in which we had no data in the 35°C-derived groups in the F₂ generation due to poor viability of the offspring (**Fig. 2E**). Thus, in order to determine which one of the three possibilities (namely, no effect, multigenerational effect or transgenerational effect; **Fig 1B**) was actually taking place, we excluded the two extreme families (#1 and #7). Then, using the three “central” families (#2, #4 and #6), i.e., with sex ratios at 28°C in the P generation in the range of ~40–60%, which were not different from the Fisherian 1:1 sex ratio (χ^2 -test; $P = 0.5374$), we calculated the contribution of family, temperature and generation to sex ratio. Results of the multifactorial ANOVA showed, as expected because the sex ratios across generation of three families showed similar behavior (**Fig. 2F**), no effect of family but significant effects not only of temperature but also of the generation (**Table 2**). Further, the *post hoc* Tukey test showed that the sex ratio of the F₁ was similar to that of the P generation but different from the F₂ (**Table 2**), indicating multigenerational effects but not transgenerational effects.

Global DNA methylation

Of the three families with balanced sex ratios at the P generation, family #2 clearly maintained male-skewed sex ratios in the F₁ generation (χ^2 -test: 7.7115; *P*-value: 0.005487) (**Fig. 2B**), denoting multigenerational effects. When testicular global DNA methylation levels across generations for the 28°C- and 35°C-derived groups in family #2 were tested, we found no significant differences in global methylation levels in the P generation (**Fig. 3**). In contrast, significant differences (*t*-test, *P* < 0.001) in methylation levels in the F₁ in testes of the 35°C-derived group were found when compared to testes of the 28°C-derived group. Differences were lost in the F₂ generation.

Discussion

Our results confirm the wide variation in sex ratio when zebrafish of the AB strain were reared at the control temperature of 28°C, as previously described (Liew et al., 2012; Ribas et al., 2017a). Our results also confirm that heat-induced masculinization in zebrafish is family-dependent (Ribas et al., 2017a). Thus, population sex ratio in the laboratory AB strain of zebrafish, which lost the sex-determining gene during the process of domestication (Wilson et al., 2014), reflects the polygenic nature of sex determination, where sex depends primarily on genetic factors presumably distributed across the genome, but can be overridden by environmental effects.

To the best of our knowledge, this is the first study in zebrafish examining possible multi- or transgenerational effects of elevated temperature on population sex ratio. Analysis of the sex ratios across the generations (F₁ and F₂) derived from the 35°C-exposed P generation showed that population sex ratio tended towards 1:1 (male:female), with the exception of family #1, as one would expect if frequency-dependent selection was taking place. Our results regarding the male-biased sex ratios of the 35°C-derived groups in the F₁ are consistent with the presence of multigenerational effects when only the three tested families (#2, #4 and #6) that initially did not depart too far from 1:1 at 28°C in the P generation are considered, but not when all five families are taken into account, i.e., including also families #1 and #7, which had the most skewed sex ratios at control temperature in the P generation (**Fig. 2**). Our results with the male-skewed sex ratio in the F₁ of 35°C-derived groups of families #2, #4 and #6 are in agreement with results obtained in the half-smooth tongue sole (*Cynoglossus semilaevis*), also with a ZZ/ZW system, where male-skewed sex

ratios when compared to control populations was found in the F_1 generation derived from heat-exposed parents (Shao et al., 2014). These observations were taken as evidence for the presence of multigenerational inheritance of the masculinizing effect induced by high temperatures (Shao et al., 2014). Our results also resemble those obtained with the Atlantic silverside (*Menidia menidia*), a GSD-ESD species, in the sense that progressively balanced sex ratios were obtained in the subsequent generation after the environmental effect ceased (Conover et al., 1992). Masculinizing effects of high temperature were gradually reduced from one generation until Fisherian sex ratios were obtained after 8–10 generations (Conover et al., 1992), suggesting that balanced sex ratios evolve by frequency-dependent selection of the minority sex (Fisher, 1958), and thus a balanced sex ratio constitutes an evolutionary stable strategy (Smith and Maynard-Smith, 1978).

In fish, evidences of transgenerational effects caused by elevated temperature mostly concern metabolic scope rates (Donelson et al., 2012), aerobic capacity and acclimation (Veilleux et al., 2015; Ryu et al., 2018), and growth (Salinas and Munch, 2012; Shama et al., 2014) but not the resulting sex ratio. In fact, transgenerational effects on sex ratios and reproductive capacity have been mostly investigated after exposure to EDCs, not temperature. This is the case of BPA exposure of zebrafish that led to epigenetic changes affecting expression of genes involved in reproduction and caused a reduction of fertilization rates (Laing et al., 2016; Santangeli et al., 2019). Similar results were observed in the F_2 and F_3 generations in medaka and in fathead minnows (*Pimephales promelas*) exposed to EDCs (Schwindt et al., 2014; Bhandari et al., 2015), although possible mutagenic effects of BPA and not strictly transgenerational effects should not be discarded (Jalal et al., 2018). When fertilized zebrafish embryos were exposed to 5-azacytidine (5AC) until 6 dpf, a significant shift towards male bias in the F_1 generation was found when compared to the exposed F_0 population (Kamstra et al., 2017). However, female-biased sex ratios were obtained after treatment of young zebrafish with a another DNA-methyltransferase inhibitor, 5-aza-2-deoxycytidine (5-aza-dC) (Ribas et al., 2017c).

Although we did not find transgenerational effects on sex ratios, we tested whether exposure to elevated temperature could lead to DNA methylation changes. Since previous studies showed that epigenetic changes are transmitted mostly through the

sperm (Bogdanović et al., 2011; Jiang et al., 2013; Potok et al., 2013) —but see also (Santangeli et al., 2019)— we examined the testes of family #2 males of the P, F₁ and F₂ generations for both the 28°C- and the 35°C-derived groups through the father. Interestingly, no global DNA methylation differences were observed in fish from the P generation. These results are similar to those found in three spine stickleback (*Gasterosteus aculeatus*), where exposure to elevated temperature during embryonic development did not change global methylation in adult testes from males of the same exposed generation (Metzger and Schulte, 2017). It should be noted that germ cells of the testes in the exposed P generation are diluted among somatic cells and this could explain why there were no differences in global DNA methylation in the testes. In contrast, the testes of the F₁ males from the 35°C-derived groups exhibited significantly lower DNA methylation levels when compared to 28°C-derived groups. Albeit these fish never experienced any thermal episode, it appears that they inherited the effects from the exposed father. In the present study, the exposure period was chosen because we knew it actually represents a sensitive period for the effects of temperature on zebrafish sex ratios (Ribas et al., 2017). However, we cannot rule out differences in the results obtained if a wider period of exposure had been tested. The importance of sensitive windows in zebrafish has been discussed elsewhere (Dang and Kienzler, 2019). In contrast, what could be ruled out with more confidence is the possibility of sex reversal after the effects of temperature fade away, i.e., that fish that would be masculinized by temperature reversed to females. Again, with our previous experience (Liew et al., 2012; Ribas et al., 2017a), this is unlikely to occur, although the possibility cannot be completely ignored. In any case, the interplay of genetic and epigenetic factors related to reproduction needs further investigation. For instance, it has been shown that direct exposure of gamma ionizing radiation in adult zebrafish during gametogenesis results in transgenerational effects that can be linked with DNA methylation changes (Hurem et al., 2018; Kamstra et al., 2018).

In mammals, epigenetic reprogramming of PGCs occurs in two sequential steps, the first during PGC expansion and migration, and the second upon entry of PGCs into the gonads (Sasaki and Matsui, 2008; Seisenberger et al., 2013a). During reprogramming, there is an state for totipotency in which most of the environmentally-induced epigenetic changes are erased (Santos et al., 2002; Surani et al., 2007; Seisenberger et al., 2012). Unlike mammals, and in contrast to what occurs in medaka (Wang and

Bhandari, 2019), studies in zebrafish post-fertilization embryos suggested that their genome does not undergo strong genome-wide DNA demethylation and, consequently, a global erasure is absent (Bogdanović et al., 2011; Jiang et al., 2013; Potok et al., 2013). In addition, in zebrafish embryos, global DNA methylation is largely preserved during germline differentiation, where the paternal methylome is inherited (Ortega-Recalde et al., 2019; Skvortsova et al., 2019). Thus, along the zebrafish lifespan, global methylation levels in PGCs at early stages showed similar values as the somatic cells, suggesting that the putative epigenetic changes acquired are maintained (Ortega-Recalde et al., 2019). In our experiments, we exposed zebrafish larvae to high temperature during gonadal development (18 to 34 dpf), i.e., past the period when the highest number of PGCs are found (6–14 dpf) (Uchida et al., 2002; Orban et al., 2009; Tzung et al., 2015). Consequently, it is safe to assume that only a few PGCs were still present in the differentiating gonads at the time of heat exposure. Therefore, rather than PGCs, spermatogonia would have been mostly affected by temperature. Thus, global hypomethylation found in the testes of 35°C-derived males in the F₁ generation could be explained by taking into account that F₁ fish are in fact derived from the presumably affected germ cells of the P generation. In rodents exposed to stress, partial inheritance of affected phenotypes was observed in a given generation without being observed in the previous one (Franklin et al., 2010). Taken together, these studies indicate not only a sex-specific but also a complex mode of transmission since certain traits are expressed in the progeny despite not being clearly expressed by parents. This suggests that these are “silent” or asymptomatic carriers of specific alterations (Franklin et al. 2010), although mechanisms are not well understood.

Conclusions

In this study carried out using the laboratory AB strain of zebrafish, we confirm a genetic and environmental component in sex ratios and great inter-family variation in response to elevated temperature. We provide evidence suggestive of multigenerational effects of elevated temperature in sex ratios. However, we could not demonstrate effects beyond the first non-exposed generation, i.e., in the F₂ generation. Interestingly, although no changes in global DNA methylation in the testis of exposed fish was observed, a clear hypomethylation in the testes of the offspring of exposed fathers was recorded. This calls attention to possible effects of high temperature in fishes that was

previously unnoticed and implying the presence of asymptomatic carriers of temperature effects. These aspects deserve further study for a better understanding of the effect of global changes in natural populations. In addition, further analyses, targeting different periods, e.g., germ cell differentiation, gonad formation, sexual differentiation and gametogenesis, should be carried out to determine sensitive windows in which external perturbations affect the germline with potential transmission to the following generations.

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Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. The contribution of family, generation and temperature and their interactions in the sex ratio of the five initially tested families (families #1, #2, #4, #6 and #7) used in this study

<i>Source</i>	<i>Sum of Squares</i>	<i>D.f.</i>	<i>Mean Square</i>	<i>F-Ratio</i>	<i>P-Value</i>
MAIN EFFECTS					
A:Family	19.5878	4	4.89694	2.43	0.1443
B:Generation	5.90938	2	2.95469	1.47	0.2941
C:Temp. (°C)	5.06099	1	5.06099	2.51	0.1572
INTERACTIONS					
AB	22.7996	8	2.84995	1.41	0.3308
AC	8.55343	4	2.13836	1.06	0.4422
BC	10.2322	2	5.11608	2.54	0.1484
RESIDUAL	14.1179	7	2.01685		
TOTAL (CORRECTED)	95.8113	28			

A multi-factorial ANOVA was used with family, generation and temperature as independent factors and the sex ratio (proportion of males) as the dependent variable. (Abbreviations: D.f., degrees of freedom).

Table 2. The contribution of family, generation and temperature and their interactions in the sex ratio of the P, F₁ and F₂ generations in families #2, #4 and #6, whose sex ratios did not depart from 1:1 at the control temperature of 28°C in the P generation

<i>Source</i>	<i>Sum of Squares</i>	<i>D.f.</i>	<i>Mean Square</i>	<i>F-Ratio</i>	<i>P-Value</i>
MAIN EFFECTS					
Family	0.773694	2	0.386847	3.75	0.1208
Generation	2.29148	2	1.14574	11.12	0.0232
Temp. (°C)	1.41849	1	1.41849	13.77	0.0206
INTERACTIONS					
Family x Generation	1.9836	4	0.495899	4.81	0.0786
Family x Temp. (°C)	0.458138	2	0.229069	2.22	0.2243
Generation x Temp. (°C)	1.33316	2	0.666579	6.47	0.0558
RESIDUAL	0.412149	4	0.103037		
TOTAL (CORRECTED)	8.67071	17			

Method: 95,0 percent Tukey HSD

<i>Generation</i>	<i>Count</i>	<i>LS Mean</i>	<i>LS Sigma</i>	<i>Homogeneous Groups</i>
P	6	0.663333	0.131045	X
F ₁	6	0.617333	0.131045	X
F ₂	6	-0.1155	0.131045	X

A multi-factorial ANOVA followed by a *post-hoc* test was used with family, generation and temperature as independent factors and the sex ratio (proportion of males) as the dependent variable. Statistically significant differences are shown in bold. (Abbreviations: D.f., degrees of freedom)

Figure legends

Figure 1. Study of possible transgenerational effects of exposure to elevated temperature during sex differentiation on resulting zebrafish adult sex ratios. **(A)** Experimental design applied in each one of the five families used. An untreated male and female (defined as P₁) were crossed and larvae were divided in two groups. Coinciding with gonadal development of zebrafish (18–34 days post fertilization) one group was exposed to 28°C (control) and the other to 35°C (treated), and constituted the parental (P) generation. Populations of first generation (F₁) and second generation (F₂) were obtained from each temperature treatment using both males and females from the previous generation, which were outcrossed with unrelated, untreated females and males, respectively. Sex ratio were determined when fish were sexually mature (> 90 days post fertilization). Zebrafish images by Lizzy Griffiths. **(B)** In families with sex ratio response to temperature as evidenced by an increase in the proportion of males, the following scenarios could be expected, as depicted by lines of different colors: control (CTRL), with no significant departures from the 1:1 sex ratio along the different generations; transgenerational effects (TGE), effects of elevated temperature persist at least until the F₂; multigenerational effects (MGE), with effects seen only at the F₁ but not at the F₂; and no effect (NE). **(C)** Observed sex ratios along the P, F₁ and F₂ generations in the control and treated lines. Each datapoint is the weighted mean $\pm$ SEM of percent males of families #2, #4 and #6.

Figure 2. Sex ratios in the parental (P), first (F₁) and second (F₂) generations according to temperature experienced in the P generation in the five selected families: **(A)** family #1; **(B)** family #2; **(C)** family #4; **(D)** family #6; **(E)** family #7; **(F)** combined data from families #2, #4 and #6; **(G)** Observed sex ratios along the P, F₁ and F₂ generations in the control and treated lines. Each datapoint is the weighted mean $\pm$ SEM of percent males of families #2, #4 and #6. Families are arranged according to increasing proportion of males at 28°C. Data as mean $\pm$ S.E.M.

Figure 3. Global DNA methylation in testis of adult (> 90 days post fertilization) zebrafish (*Danio rerio*) in the parental (P), first (F₁) and second (F₂) generations of family #2. Half of the P generation was kept at control temperature (28°C), whereas the other half was exposed to elevated temperature (35°C). Controls and their offspring

(LT-derived) are shown on the left (light blue), whereas heat-exposed and their offspring (HT-derived) are on the right (pink) at every generation. In the F_1 and F_2 generations all fish were grown at 28°C. Means were compared with the Student's t -test. In the boxplot, each datapoint corresponds to an individual sample, and the thick line represents the median. Abbreviations: n = sample size; n.s. = not significant; *** = $P < 0.001$.

Figure 1

Figure 2

Figure 3

Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring

Alejandro Valdivieso, Laia Ribas, Antonio Monleón-Getino, László Orbán and Francesc Piferrer

Highlights

- Temperature effects on sex ratio in the unexposed F_1 are family-dependent
- Temperature effects on sex ratio in the unexposed F_2 were not observed
- Testicular epigenome hypomethylation was observed in the F_1 but not in the F_2
- Thus, temperature can affect the unexposed offspring of heat-exposed parents
- Epigenetic inheritance can play a crucial role in a global warming scenario

Credit author statement

AV – Alejandro Valdivieso

LR – Laia Ribas

AMG – Antonio Monleón-Getino

LO – László Orbán

FP- Francesc Piferrer

FP, LR and AV conceived and designed the study; AV carried out the experiments; AV and LR sampled the fish; AV did the DNA methylation analysis; FP, LR, AV, AMG and LO analyzed the data; FP provided resources; AV, FP and LR wrote the paper; AV, LR, AMG, LO, FP revised the paper and approved the final version.

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Barcelona, April 23, 2020

Dr. José Domingo, Editor-in-Chief
Environmental Research

Dear Dr. Domingo,

Regarding, the manuscript entitled “Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring”, by A. Valdivieso, L. Ribas, A. Monleón, L. Orbán and F. Piferrer, submitted for consideration for publication as a research paper in *Environmental Research*, on behalf of the rest of authors, we declare that we have not any conflict of interest whatsoever,

Yours sincerely,

F. Piferrer