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No evidence of evolutionary impact from size-selective harvesting on epigenetic and reproductive molecular markers in zebrafish (*Danio rerio*) gonads

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Abstract

This study investigates whether size-selective harvesting induced heritable changes reflected in epigenetic and reproductive molecular markers in zebrafish (*Danio rerio*), thereby indicating potential evolutionary responses. We used an experimental harvest model where zebrafish populations were subjected to five generations of size-selective harvesting, followed by eight generations without harvesting in a controlled environment to examine evolutionarily fixed outcomes in response to harvest selection. We assumed the evolutionary adaptations to size-selection to have left a molecular legacy related to sexual development, as previous studies have shown that evolution of reproductive timing is a common response to size-selection. To that end, we examined the expression of specific genes related to sexual development, such as *dmrt1* and *cyp19a1a*, and epigenetic markers, including *dnmt1* and *dnmt3b*, in the gonads in those experimental lines selected for this study. Additionally, global DNA methylation patterns were analysed to explore potential long-term epigenetic changes associated with size-selection. The results revealed no significant differences in gene expression related to sexual development or epigenetics between the size-selected and control zebrafish lines in the gonads in the F13 generation, eight generations after size-selection stopped. Also, global DNA methylation patterns were similar across selection lines and sexes. These findings suggest that five generations of size-selective harvesting, followed by eight generations of maintenance reproduction without further selection, did not induce lasting epigenetic or molecular changes related to the target molecular markers of sexual development in the gonads of zebrafish. The no significant molecular responses to size-selective harvesting observed here, based on specific reproductive and epigenetic markers, differ from previous studies targeting other tissues such as brain and liver, highlighting that not all genes

or processes necessarily respond to size-selection and underscoring the importance of marker-specific comparisons.

KEYWORDS
aquaculture, artificial evolution, domestication, fisheries-induced evolution, methylation, reproduction, transgenerational

1 | INTRODUCTION

Fishing differs from many other forms of predation by primarily targeting adult individuals who typically experience limited natural mortality (Jørgensen et al., 2007). Intensive and trait-selective harvesting can shift the fitness landscape and foster evolutionary adaptations at a rate and speed that is rarely experienced in the evolutionary history of many animal populations (Allendorf & Hard, 2009; Hendry et al., 2008; Palumbi, 2001; Sih et al., 2011). The potential for harvest-induced evolutionary adaptations in fisheries was mainly inferred from phenotypic time series (e.g. changes in maturation timing and size) and modelling (Heino et al., 2015), but several studies in the wild and in experimental harvest models have shown that size-selective harvest can cause genetic changes (e.g. Czorlich et al., 2022; Reid et al., 2023; Therkildsen et al., 2019; Uusi-Heikkilä et al., 2015). Experimental evolution studies serving as a proof-of-concept that fisheries-induced evolution is plausible have been used to simulate fishing patterns of size-selective mortality in common garden experiments in the laboratory to understand the evolutionary outcomes of harvest on life history, and physiological and behavioural traits (e.g. Conover & Munch, 2002; Diaz Pauli et al., 2015; Le Rouzic et al., 2020; Sbragaglia, Klamser, et al., 2022; Uusi-Heikkilä et al., 2015; van Wijk et al., 2013). These experiments showed that intensive and positively size-selective mortality has the potential to change the allele frequencies of genes and expression of genes that control life history, physiology and behaviour (Le Rouzic et al., 2020; Sadler et al., 2024; Sbragaglia et al., 2021; Uusi-Heikkilä et al., 2015). Surprisingly, although the role of epigenetic mechanisms has been explored in domestication processes (Turner, 2009; Vogt, 2017), including farmed fish species (Anastasiadi & Piferrer, 2019), it has been largely overlooked in the context of studies in fisheries-induced evolution. Epigenetic mechanisms, the study of heritable changes in gene expression and resulting phenotype without alterations in the DNA sequence (Bird, 1986), play a crucial role in shaping the ecology and evolution of organisms, including fishes (Herrel et al., 2020; Turner, 2009). Therefore, it is conceivable that intensive and selective fishing could influence epigenetic regulation, leading to rapid changes in gene expression, which in turn may impact life-history traits over shorter timeframes compared to evolution of DNA sequence after many generations.

Evolutionary and epigenetic pathways are intricately linked, as epigenetic modifications can influence gene expression (Jablonka & Lamb, 2014). Epigenetic modifications, such as DNA methylation and histone modification, can be heritable, allowing organisms to increase fitness more rapidly than through evolution alone (Skinner et al., 2010). Exploring epigenetics in evolutionary history involves tracing a pathway by which epigenetic processes may have initially evolved through exaptation (i.e. a trait that originally evolved for one function is co-opted for a new use), subsequently playing a significant role in the development and evolution of various phenotypes (Moore, 2023). Among many epigenetic mechanisms, DNA methylation, governed by DNA methyltransferases (dnmts), stands out as a pivotal regulatory process (Turek-Plewa & Jagodzinski, 2005). Because of their significant involvement in DNA methylation across the genome, dnmts are extensively studied, not just in mammals but also in fish (Cavalieri & Spinelli, 2017). For example, *dnmt1* is primarily responsible for maintaining DNA methylation patterns during cell division, while *dnmt3b* is involved in de novo DNA methylation (Okano et al., 1999; Pradhan et al., 1999).

Since the first evidence of crosstalk between the environment and epigenetics in fish, wherein high temperatures were able to masculinize domesticated fish by altering DNA methylation of a promoter of key reproduction genes (Navarro-Martín et al., 2011), interest has flourished in this direction in fish biology. Reproduction in fish is intricately linked to epigenetic regulation, with DNA methylation patterns governing the expression of key genes involved in the fish reproduction system (Piferrer et al., 2019). In this context, it is conceivable that epigenetic pathways are also altered by fisheries selection by means of alterations in methylation profiles, affecting traits related to reproductive fitness and behaviour similar to farmed fish (Anastasiadi & Piferrer, 2019; Bélteky et al., 2018). Epigenetic modifications induced by selective pressures can be transmitted across generations, influencing phenotypic plasticity and adaptive responses to environmental changes (Skvortsova et al., 2018). Two molecular markers, doublesex and mab-3 related transcription factor 1 (*dmrt1*) and cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), are of particular interest. In domesticated zebrafish (*Danio rerio*), as most of fish species without a sex determining gene, *dmrt1* plays a crucial role in male sex differentiation, while *cyp19a1a* is involved in the conversion of androgens to oestrogens, influencing female reproductive development (Guiguen et al., 2010; Webster et al., 2017). Understanding the epigenetic regulation of these genes provides insights into the mechanisms underlying reproductive strategies in fish.

Zebrafish is a recognized model organism across various research disciplines (Piferrer & Ribas, 2020; Ribas & Piferrer, 2014), including in fisheries-induced evolution and evolution to temperature to study responses in terms of life history, and molecular,

physiological and behavioural traits (e.g. Crespel et al., 2021; Morgan et al., 2022; Sbragaglia et al., 2021; Uusi-Heikkilä et al., 2015; Wootton et al., 2021). One important finding of size-selection studies on zebrafish in the laboratory was that zebrafish populations exposed to large-size selective harvesting evolved increased reproductive investment relative to controls (Uusi-Heikkilä et al., 2015). There were also significant changes in sex-specific reproductive behaviour, and males of large- and small-size selected lines increased intersexual aggression during spawning (Sbragaglia, Gliese, et al., 2019). Uusi-Heikkilä et al., (2017) revealed that thousands of genes were differentially expressed in response to size-selection in both brain and liver of zebrafish, some of which were involved with maturation pathways. Despite the crucial importance of reproductive investment in the context of fisheries-induced evolution (Heino et al., 2015; Jørgensen et al., 2007), previous studies aiming to assess the molecular impact of size-selective mortality on zebrafish molecular markers focused on gene expressing in the brain (Sbragaglia et al., 2021; Uusi-Heikkilä et al., 2017) and liver (Sbragaglia et al., 2021), but not on gonads. Understanding how size-selective mortality affects molecular markers of sexual development and epigenetics mechanisms in the gonads, the tissue responsible to transfer the epigenetic memory to the progeny, in a well-established model such as zebrafish, can provide new insights into the role of molecular and epigenetic mechanisms in fisheries-induced evolution.

We took advantage of an ongoing experimental system where size-selective harvesting has been imposed on wild-collected zebrafish populations (Uusi-Heikkilä et al., 2015). The experimental lines were subjected to five generations of opposing size-selective harvesting (Uusi-Heikkilä et al., 2015) consisting of a large-harvested line (largest individuals harvested, a common scenario in many fisheries worldwide and in the presence of predators where large individuals are selectively preyed on, leading to selection for small fish, 'small line' from now on), a small-harvested line (smallest individuals harvested, a possible scenario in specific fisheries or in the presence of gape-limited predators that preferentially feed on the smaller size classes, leading to selection for large fish, 'large line' from now on) and a random-harvested line with respect to length (control). Previous results showed substantial molecular changes in the brain and liver of the selected lines (large- and small-harvested lines) compared to the control (Uusi-Heikkilä et al., 2017; Sbragaglia et al., 2021), and substantial evolutionary adaptations in life-history and behavioural traits (Sbragaglia, Alós, et al., 2019; Sbragaglia, Gliese, et al., 2019; Uusi-Heikkilä et al., 2015). To advance knowledge on the role of the key reproduction- and epigenetic-related markers in the gonads in the context of fisheries-induced evolution, we asked the following research questions: (i) did size-selective mortality affect gonadal molecular indicators related to reproduction such as *dmrt1* and *cyp19a1a*, (ii) did size-selective mortality affect gonadal molecular markers related to epigenetic processes such as *dnmt1* and *dnmt3b*; and (iii) did size-selective mortality affect gonadal global DNA methylation patterns?

2 | MATERIALS AND METHODS

2.1 | Ethics approval

All applicable international (2010/63/EU), national and/or institutional guidelines for the use of animals were followed. The experimental protocols were approved by a committee on animal welfare (reference number A13191003).

2.2 | Experimental approach with selected lines and housing

The experimental system consisted of wild-collected zebrafish from West Bengal in India, sampled with a range of fishing gears (seine, cast nets and dip nets) as previously described (Uusi-Heikkilä et al., 2015). The wild-collected zebrafish were randomly subdivided in six selection lines and then exposed to strong directional harvest selection (a 75% per-generation harvest rate) acting on either large (two large-harvested lines, hereafter small line) or small (two small-harvested lines, hereafter large line) fish, relative to two control lines harvested randomly with respect to body size (Uusi-Heikkilä et al., 2015). Zebrafish were exposed to size-selection during the first five generations (F1–F5), after which harvesting was halted for eight generations (until F13) to remove potential maternal effects and thus allow the study of possibly fixed evolutionary outcomes in a common-garden setting through persistent changes across generations. Age at harvest varied from generation to generation associated with potential changes in age at 50% maturation of the random line (which was used as a time period when size selection was imposed). Each selected parental fish was only able to spawn once, i.e. the evolving fish could not reap benefits of repeated spawning (Uusi-Heikkilä et al., 2015). Therefore, the selection was directed to size at age and underlying physiological and behavioural traits, and could have affected all growth-related processes, including reproductive investment, and energy acquisition, including foraging. In terms of previously reported phenotypic changes, the small line showed evolution of a smaller adult length and weight and higher relative fecundity compared with the control line (Uusi-Heikkilä et al., 2015), and it also formed less cohesive shoals and was shyer than controls (Sbragaglia, Klamser, et al., 2022). By contrast, the large line showed reduced reproductive investment and no change in adult length compared with the control line (Uusi-Heikkilä et al., 2015), as well as more cohesive shoals and increased risk-taking behaviour (boldness) (Sbragaglia et al., 2021; Sbragaglia, Klamser, et al., 2022). Both lines evolved maturation at smaller and younger age than the control line (for more details, see Uusi-Heikkilä et al., 2015, Sbragaglia, Alós, et al., 2019, Sbragaglia, Gliese, et al., 2019), and both size-selected lines showed increased aggression by males during mating (Sbragaglia, Gliese, et al., 2019). In summary, the small line evolved a fast life history and the large line a slow life history, with substantial changes in individual and collective behaviour related to food acquisition and spawning.

The six selection lines were reared in six different tanks in a common garden set-up under the following conditions: water temperature at 26 ± 0.5°C, photoperiod of 12:12 h light/dark and ad libitum feeding (TetraMin, Tetra) three times per day. At F12, we randomly selected groups composed of two females and four males to create the next generation used in the experiments here. Spawning was carried out as previously described for these selection lines (Sbragaglia, Gliese, et al., 2019; Uusi-Heikkilä et al., 2015). After hatching, we maintained the larval fish in 3-L boxes and fed them with dry food (TetraMin; Tetra) and artemia. At 30 days post fertilization (dpf), we randomly sorted zebrafish from F13 into experimental groups. Specifically, we randomly stocked eight juveniles into 3-L rearing boxes, using 36 groups (six replicates per each of the six selection lines, 12 groups per treatment). Fish were maintained under the same environmental conditions as the common garden set up. The gonads used for molecular analysis were dissected from zebrafish adult individuals (at about 280 dpf) at F13 at the end of a controlled ontogeny experiment (Sbragaglia, Roy, et al., 2022).

2.3 | Gonadal gene expression of epigenetic- and reproduction-related markers

To test whether size-selective harvesting left a legacy in molecular markers related to sexual development (*dmrt1* and *cyp19a1a*) and epigenetics (*dnmt1* and *dnmt3b*), RNA was individually extracted from six gonads (ovary and testis) in each of the three experimental lines at F13 ($N = 18$ each sex). TRIzol reagent (T9424; Sigma-Aldrich) was used for the extraction, following the manufacturer's recommended procedures. The obtained RNA pellets were then dissolved in 25 μL of Diethyl pyrocarbonate treated water and stored at −80°C. To assess RNA concentration, a ND-1000 spectrophotometer (NanoDrop Technologies) was utilized, and the quality of RNA was verified by electrophoresis on a 1% agarose/formaldehyde gel. Following the supplier's protocols, 100 ng of total RNA from each sample underwent DNAse I treatment using Amplification Grade DNAse I (Thermo Fisher Scientific Inc.). The quality of the samples was assessed by Nanodrop (Agilent Technologies) with ratios 260/280 = 2.1 ± 0.05 and 260/230 = 1.8 ± 0.51, and the RNA quality was measured by the RNA Integrity Number (RIN; Bioanalyser; Agilent Technologies). For all the samples it was >8 and absorbances were between 1.80 and 2.10. Subsequently, cDNA was synthesized with SuperScript III RNase Transcriptase (Invitrogen) using random hexamers (Invitrogen). For the quantitative polymerase chain reaction (qPCR), the synthesized cDNA was first diluted 1:10 with DNase-free water. Each qPCR reaction mixture contained 5 μL of 2× qPCRBIO SYBR Green Mix Lo-ROX (PCR Biosystems), 0.5 μL of both forward and reverse primers, and 2 μL of DNase-free water. qPCR was conducted in triplicate for each sample. The thermocycler conditions included an initial denaturation step at 95°C for 3 min, followed by 39 cycles consisting of 10 s at 95°C and 30 s at the annealing temperature. A melt curve analysis was then performed by gradually increasing the temperature from 65 to 95°C at a rate of 0.5°C every 5 s to verify the amplification of a single product. The specificity of each primer pair was confirmed through a dissociation step, primer efficiency curves and PCR product sequencing, with all primer pairs demonstrating efficiencies between 95% and 104%. Primer sequences were designed using Primer3web v4.1.0 and additional information can be found in Table 1.

2.4 | Global DNA methylation

Gonadal DNA was extracted following the commercial kit protocol (DNeasy Qiagen). Global DNA methylation analysis was carried out on genomic DNA using a 5-mC DNA ELISA kit (Zymo Research) in accordance with the manufacturer's protocol and as described by Valdivieso et al. (2020). To summarize, 100 ng of each DNA sample was used for analysis. A standard curve was generated by combining negative and positive controls in varying proportions, resulting in standard methylation concentrations of 0%, 5%, 10%, 25%, 50%, 75% and 100%, respectively. Absorbance was measured at 405 nm using an ELISA plate reader (Infinite® 200 PRO, Tecan™), with all samples analysed in duplicate. The percentage of 5-mC in unknown DNA samples was determined using the formula %5-mC = e(absorbance − *y* intercept)/slope. The % 5-mC values were adjusted for zebrafish CpG density as per the manufacturer's instructions. The percentage of CpG was calculated based on the formula described by Valdivieso et al. (2020), using the latest zebrafish genome from Ensembl (www.ensembl.org). The genome's length (*L*) was determined to be 1,674,207,132 bp, and the total number of cytosines (C) and CpG dinucleotides (CG) were computed from the zebrafish genome, yielding C = 306,412,859 and CG = 29,220,867. To assess the fold difference in CpG density between the genomes of *E. coli* and *D. rerio*, the ratio (0.07472/0.0175) = 4.2811 was calculated. Finally, to obtain global methylation values, the % 5 m-C/CpG density values were multiplied by the CpG density value obtained from the total number of C in the zebrafish genome (0.1830).

2.5 | Statistical analysis

We measured the body mass and standard length of each fish at about 280 days post fertilization before dissecting the tissues for molecular analysis. Body mass and standard length were modelled with linear mixed-effects models with a Gaussian distribution to assess possible differences with respect to selection lines (fixed factor with three levels) and sex (fixed factor with two levels). The two replicates of each selection lines were used as a random intercept.

As regarding gene expression, data obtained from qPCR were collected using SDS 2.3 and RQ Manager 1.2 software. For each sample, the relative quantity (RQ) values of the genes of interest were normalized against the geometric mean of two reference genes (EFα and RPL3A), validated for zebrafish (Tang et al., 2007). The fold change was calculated using the 2ΔΔCt method (Schmittgen & Livak, 2008). We modelled markers related to sexual development (*dmrt1* and

TABLE 1 Molecular markers used to assess the impact of size-selective mortality on sexual development and epigenetics together with the primers for each marker and their accession number on Gen Bank.

Tissue	Function	Gene name	Primer sequence forward (5′-3′)	Primer sequence reverse (5′-3′)	Accession number (Gene Bank)
Testis	Male gonad development	doublesex and mab-3 related transcription factor 1 *(dmrt1)*	TGCCCAGGTGGCGTTACGG	CGGGTGATGGCGGTCCTGAG	NM 205628
Ovary	Female gonadal development	cytochrome P450, family 19, subfamily A, polypeptide 1a *(cyp19a1a)*	GATATTTGCTCAGAGCCATGGA	GCTCTGGCCAGCTAAAACACT	NM 131154
Testis and ovary	Epigenetics	DNA (cytosine-5-)-methyltransferase 1 *(dnmt1)*	TCTTCAGCACTACAGTTACCAATCCT	CGTGCACATTCCCTGACACT	NM_131189
	Epigenetics	DNA (cytosine-5-)-methyltransferase 3 beta *(dnmt3b)*	AAGATTTAGGCGTCGGTTTCG	GTGTCACCCCCTTCAATTAACTG	NM_131386

cyp19a1a) and epigenetics (*dnmt1* and *dnmt3b*) with generalized linear mixed-effects model with a log-normal distribution for the response variable. The log-normal distribution was chosen because the response variable was continuous and positively skewed. We created a total of four models. The models related to *dmrt1* and *cyp19a1a* had gene expression fold change as response variable, selection lines as fixed factors (three levels) and the two replicates of each selection lines as random intercept. The models related to *dnmt1* and *dnmt3b* had gene expression fold change as response variable, selection lines (three levels) and sex (two levels) as fixed interacting factors, and the two replicates of each selection lines as random intercept. Regarding global methylation values, we modelled it with a generalized linear mixed-effects model with a beta distribution for the response variable. The percentage values of global methylation were treated as response variable, selection lines (three levels) and sex (two levels) as fixed interacting factors, and the two replicates of each selection lines as random intercept.

We selected the best model by comparing all possible combinations of fixed effects (Johnson & Omland, 2004; Symonds & Moussalli, 2011). To identify the most parsimonious model explaining the variation of the response variables (for each one of the four models), we generated all possible subsets of each model. We used two different metrics to identify the best model. First, we used the Delta Akaike Information Criterion corrected for small sample sizes (ΔAICc) to compare how close each model is to the best model based on the AICc values (only models with ΔAICc <2 were kept). Second, we used the Akaike weights to provide a relative measure of the likelihood of a model among the set previously selected (weights >1). Both metrics are useful for model comparison and selection, with ΔAICc helping to identify a subset of competitive models and weights aiding in model averaging and understanding the relative evidence for each model (Richards et al., 2011). We ran all analyses using r version 3.4.3 (www.R-project.org/) with the additional package 'MuMIn' (Bartoń, 2014) for model selection.

3 | RESULTS

As expected, given the past selection history and despite controlled rearing at similar densities, the body mass of fish at 280 dpf was significantly different among selections lines ($\chi_{2,279} = 19.3$, $p < 0.001$). Specifically, the body mass of the large line (0.32 ± 0.06 g, $N = 96$) was significantly ($p < 0.001$) greater compared to control line (0.29 ± 0.06 g, $N = 96$), while no significant differences ($p = 0.401$) were found between the small line (0.27 ± 0.07 g, $N = 96$) and the control line. The body mass of females and males did not significantly (interaction term, $\chi_{2279} = 2.5$, $p = 0.280$) differ within each selection lines (small line females = 0.29 ± 0.07 g, $N = 33$; small line males = 0.26 ± 0.06 g, $N = 60$; control line females = 0.31 ± 0.06 g, $N = 52$; control line males = 0.26 ± 0.03 g, $N = 44$; large line females = 0.34 ± 0.06 g, $N = 50$; large line males = 0.29 ± 0.05 g, $N = 46$). The standard length of fish at 280 dpf was significantly different among selections lines ($\chi_{2279} = 7.5$, $p < 0.05$), however the only significant ($p < 0.01$) difference was between the small (2.54 ± 0.19 cm) and large (2.62 ± 0.17) lines. We did not find significant differences between the small ($p = 0.083$) and large ($p = 0.399$) lines with respect to control fish in standard length. The standard length of females and males did not significantly (i.e. interaction term, $\chi_{2279} = 2.6$, $p = 0.278$) differ within the three selection lines (small line females = 2.52 ± 0.21 cm, small line males = 2.55 ± 0.19 cm, control line females = 2.61 ± 0.17 cm, control line males = 2.57 ± 0.14 cm, large line females = 2.61 ± 0.17 cm, large line males = 2.64 ± 0.18 cm).

Gene expression of the gonadal molecular markers related to sexual development and epigenetics (*dmrt1*, *cyp19a1a*, *dnmt1*, *dnmt3b*) were not significantly affected by the selection lines, sex or their interaction (Table 2). In all cases, model selection favoured the null models, with the null models for *dmrt1* and *cyp19a1a* having strong support (ΔAIC = 0, weights of 0.86 and 0.88, respectively; Table 2 and Figure 1). For *dnmt1* and *dnmt3b*, while the null models remained the best (ΔAIC = 0, weights of 0.49 and 0.59,

TABLE 2 The statistical models implemented together with the family distribution and transformation used to model the dependent variables, the difference in second-order quasi-Akaike (AIC) between the best model and the other models (*D*) and weight (*W*).

Target	Family	Model	*df*	*D*	*W*
dmrt1	Log normal	Mod1: Line	5	3.71	0.14
		Mod2: Null model	**3**	**0.00**	**0.86**
cyp19a1a	Log normal	Mod3: Line	5	4.00	0.12
		Mod4: Null model	**3**	**0.00**	**0.88**
dnmt1	Log normal	Mod5: Line × Sex	8	3.63	0.08
		Mod6: Line + Sex	6	3.89	0.07
		Mod7: Line	5	2.02	0.18
		Mod8: Sex	4	2.00	0.18
		Mod9: Null model	**3**	**0.00**	**0.49**
dnmt3b	Log normal	Mod10: Line × Sex	8	8.79	0.01
		Mod11: Line + Sex	6	5.03	0.05
		Mod12: Line	5	3.14	0.12
		Mod13: Sex	4	1.84	0.23
		Mod14: Null model	**3**	**0.00**	**0.59**
DNA methylation	Betareg	Mod15: Line × Sex	8	5.14	0.03
		Mod16: Line + Sex	6	2.27	0.12
		Mod17: Line	5	0.56	0.28
		Mod18: Sex	4	1.42	0.19
		Mod19: Null model	**3**	**0.00**	**0.38**

Note: Bold indicates the model used to interpret the results.

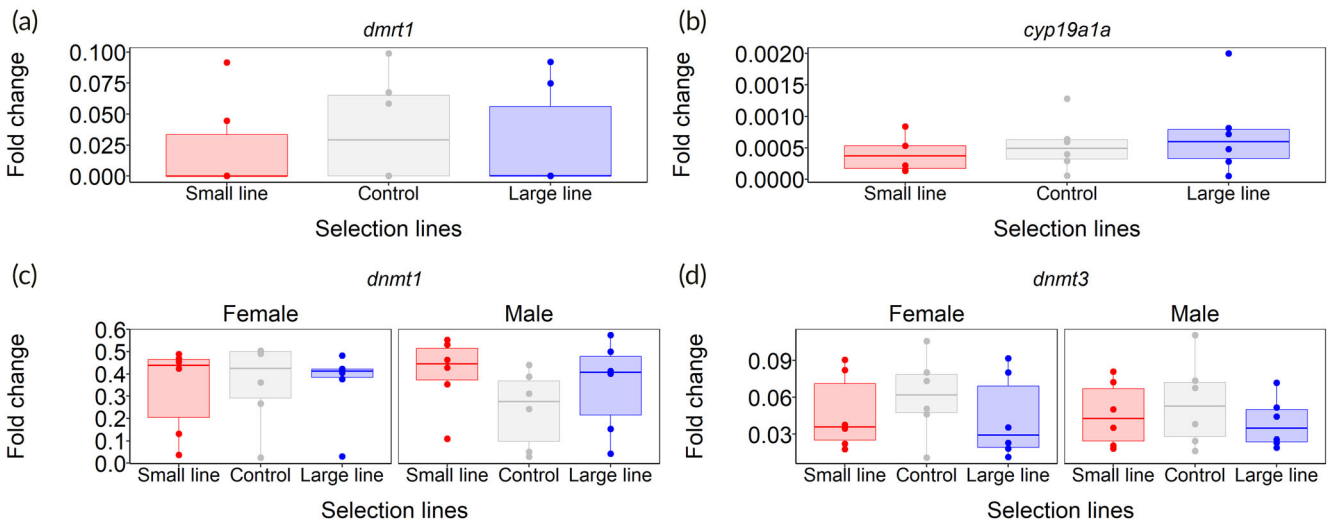

FIGURE 1 Gene expression of the gonadal molecular markers related to sexual development ((a) *Dmrt1* in males, $N = 6$; (b) *Cyp19a1a* in females, $N = 6$) and epigenetics ((c) *Dnmt1* in males and females, $N = 6$; (d) *Dnmt3* in males and females, $N = 6$) in gonads of zebrafish selections lines (small line, large line and control; see text for more details on the selection treatment).

respectively), there was some support for the models with sex and selection lines as fixed effects, but these effects were minimal (Table 2). Similarly, global DNA methylation levels did not differ significantly between selection lines, sex or their interaction, with the null model providing the best fit (ΔAIC = 0, weight = 0.38), although the model with selection lines as fixed effect also had some support (ΔAIC = 0.56, weight = 0.28; Table 2 and Figure 2). These results suggest that the selection lines and sex had no detectable influence on gene expression or DNA methylation under the conditions tested.

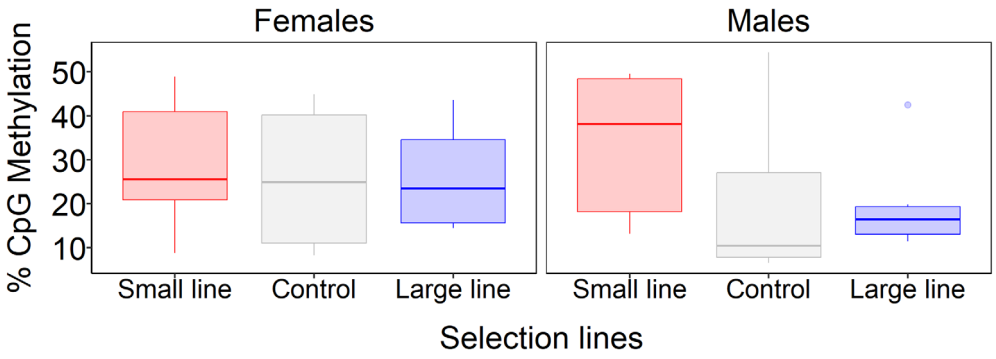

FIGURE 2 Global DNA methylation in zebrafish gonads. Data refer to $N = 6$ ovaries or testes per group of zebrafish selection lines (small line, large line and control) of the F13 generation.

4 | DISCUSSION

We showed that five generations of size-selective harvesting, followed by eight generations during which selective harvesting halted, did not impact the gonadal gene expression levels of molecular markers related to sexual development and epigenetics. Further, global methylation patterns were not significantly different between selection lines and controls in zebrafish. We first discuss the results in the context of epigenetics studies in fishes, then we interpret the results according to previous findings related to maturation patterns and reproductive behaviour. We also provide possible interpretations on the suitability of the molecular markers related to epigenetics and the global methylation patterns. Finally, we critically analyse the possibility that evolutionary rebound occurred to justify the lack of impact of size-selective harvesting on the molecular markers studied here.

DNA methylation plays a pivotal role as a driver of evolution in fish populations because the interaction between the environment and epigenetics defines the final phenotype (Piferrer et al., 2012). Environmental factors, such as temperature fluctuations, chemical exposures, rearing density or domestication—and possibly intensive and selective harvesting—can all induce changes in DNA methylation patterns, thereby influencing the expression of genes involved in phenotypic variation in behaviour and life history within a species (Jensen, 2015). Our key finding—no statistically significant gene expression responses to size-selective harvesting in zebrafish, based on specific reproductive and epigenetic markers—differs from previous studies targeting other tissues of the size-selected zebrafish, such as brain and liver (e.g. Sbragaglia et al., 2021; Uusi-Heikkilä et al., 2017), highlighting that not all tissues necessarily respond to size-selection and underscoring the importance of marker-specific comparisons. In fact, distinct epimutations associated with the domestication process from wild to farmed fish were found in tissues of different embryonic origins, with differentially methylated regions (DMRs) varying across the brain, muscle, testis and liver (Anastasiadi & Piferrer, 2019). Interestingly, testis showed a lower absolute number of DMRs when compared to other tissues (Anastasiadi & Piferrer, 2019). Further, DNA methylation of the age-prediction model based on muscle tissue epigenetics proved reliable for testis samples but not for ovarian tissues, likely due to distinct aspects of fish reproductive physiology (Anastasiadi & Piferrer, 2020).

Previous work has shown that size-selective mortality can alter gene expression related to circadian systems and other biological processes (Sadler et al., 2024; Sbragaglia et al., 2021; Uusi-Heikkilä et al., 2017). The recent genetic study by Sadler et al. (2024) used whole-genome sequencing, revealing that the genes being selected for as a result of size selection in zebrafish were related to nervous system functions, phosphorylation, morphogenesis and locomotion, but epigenetic or transcriptomic markers were not tackled in detail. Importantly, in agreement with our findings previous work, they also did not find differential genetic impacts of genes related to sexual development in zebrafish size selection lines (Sadler et al., 2024). Similarly, three-spined stickleback (*Gasterosteus aculeatus*) exposed to elevated temperatures during embryonic development also did not show changes in global methylation levels in the adult testes of males (Metzger & Schulte, 2017). In zebrafish treated with high temperature during early development, masculinization effects together with global DNA methylation were observed only at the F1 generation, but not onward (Valdivieso et al., 2020). When viewed in relation to our present work, the published record suggests that size-selective harvesting in zebrafish may not have influenced the molecular mechanisms related to sexual development and epigenetic regulation in gonads, or the effects might have been too weak to be detected in our study (see also limitations section below). A further possibility is the loss of epigenetic modifications over successive generations, a phenomenon referred to as 'epigenetic washout' (Burggren, 2015). If the observed absence of effects is true, a possible interpretation is that molecular pathways in gonads are less sensitive to size-selective mortality than processes controlled in the brain and liver that determine feeding and other important behaviours that relate to size at age (Roy & Arlinghaus, 2021; Sbragaglia et al., 2021), the trait on which selection acted. Another possible interpretation is that reproductive traits might have recovered faster from the F5 when further harvest selection stopped than size-at-age or other adaptive traits (van Dijk et al. 2024). The molecular, and above all epigenetic, pathways of fisheries-induced evolution are still not well understood and deserve further targeted experiments in both laboratory and wild situations.

Previous studies with the same zebrafish model system that we used showed that the probabilistic maturation reaction norms (i.e. the 50% probability of maturation as a function of age and size while controlling for the effect of growth on maturation) showed a similar evolutionary response in both size-selection treatment compared to the control (i.e. age and size at maturation shifted to younger ages and smaller size at maturation; Uusi-Heikkilä et al., 2015). Theory would have suggested evolution of younger age and size at maturation only in the small line, consistent with an evolutionary response towards a faster pace of life (Heino et al., 2015). This counterintuitive finding may be related to the design of the selection experiment, where harvesting only happened once per generation, which prevented evolutionary benefits of repeated spawning within the same generation (Uusi-Heikkilä et al., 2015). Consistent with a similar trajectory in evolution of maturation in the two zebrafish size-selection lines, Sbragaglia, Gliese, et al. (2019) showed significant changes in sex-specific reproductive behaviours in the same zebrafish lines, where both large and small lines increased aggression by males during spawning. Additionally, Uusi-Heikkilä et al. (2015) showed that the small line evolved higher reproductive investment compared to controls, consistent with a fast pace of life and heavy investment early in reproduction as opposed to growth. These findings indicate that phenotypes related to reproduction were evolutionarily altered in the zebrafish model. Despite these phenotypic changes, we did not detect significant effects of size-selection on the gonadal molecular markers investigated. We cannot rule out that such effects were present in earlier generations, but we are relatively sure they were absent at F13, 13 generations after size-selection stopped.

There are several limitations in our study. First, the results reflected the size-selection consequences after generation F13 (eight generations after selection was halted). There are no data from previous generations, therefore we are not able to say whether gene expression differences in the selected molecular markers were present right after selection at F5 and then were eroded overtime, for example due to fecundity selection in the small selection line (Conover et al., 2009; Salinas et al., 2012), or they were not present at all. Second, a wider array of targets and pathways could have provided a different result with evidence that size-selective harvesting can indeed affect molecular markers related to sexual development and epigenetic in gonads. Third, other more sensitive DNA methylation analyses, such as whole-genome methylation sequencing (WGMS), might have identified differences related to size-selection at F13. Finally, genomic coverage and sample size were relatively low in our work, which could have resulted in an underpowered design to detect effects.

We tentatively conclude that the specific molecular markers related to sexual development and epigenetic in the gonads that we examined did not respond to past size-selective mortality in zebrafish or if they responded differences were no longer visible at F13. These findings suggest that the studied markers may either be evolutionarily resilient or that any past effects have rebounded after eight generations without selection. However, this conclusion is limited to the specific molecular targets examined here and should not be generalized to all gonad-related molecular processes. Additionally, while zebrafish is indeed a well-established model for genomic studies, our study focused on a targeted set of candidate genes known to be involved in reproductive and epigenetic processes. Broader genomic approaches, such as RNA-sequencing or WGMS, might uncover additional effects of size-selection on gonadal function and epigenetic regulation. Future studies should therefore aim for broader genomic coverage and include earlier generations to clarify the persistence and dynamics of molecular changes associated with size-selective harvesting in the gonads of fish.

AUTHOR CONTRIBUTIONS

L.B., R.A. and V.S. conceived the idea. V.S. ran the experiments. L.B. and M.S. ran the molecular analysis. V.S. analysed the results. L.R. and V.S. wrote the manuscript with input from all the other coauthors.

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