BRIEF NOTE

Contrasting DNA methylation responses of inbred fish lines to different rearing environments

Waldir M. Berbel-Filho, Deiene Rodríguez-Barreto, Nikita Berry, Carlos Garcia de Leaniz and Sofia Consuegra*

Department of Biosciences, Swansea University, Swansea SA2 8PP, United Kingdom.

*Corresponding author: email: s.consuegra@swansea.ac.uk

Keywords: Environmental enrichment, epigenetic variation, mangrove killifish, RRBS, genotype by environment interaction

Abstract

Epigenetic mechanisms can generate plastic phenotypes that can become locally adapted across environments. Disentangling genomic from epigenomic variation is challenging in sexual species due to genetic variation among individuals, but it is easier in self-fertilising species. We analysed DNA methylation patterns of two highly inbred strains of a naturally self-fertilising fish reared in two contrasting environments to analyse the obligatory (genotype-dependent), facilitated (partially depend on the genotype) or pure (genotype-independent) nature of the epigenetic variation. We found higher methylation differentiation between genotypes than between environments. Most methylation differences between environments common to both strains followed a pattern where the two genotypes (inbred lines) responded to the same environmental context with contrasting DNA methylation levels (facilitated epialleles). Our findings suggest that, at least in part, DNA methylation could depend on the dynamic interaction between the genotype and the environment, which could explain the plasticity of epigenetically-mediated phenotypes.

Introduction

Epigenetic modifications are one of the potential molecular mechanisms to explain phenotypically plastic responses within genotypes [1, 2]. This is because epigenetic markers can be altered by environmental variation and shape gene expression without changing nucleotide sequences [3], and ultimately affect phenotypic variation [1, 4, 5]. In phenotypic plasticity studies, the genome and epigenome are often experimentally confounded [6] and an implicit assumption is made that they react to environmental variation following similar norms of reaction. However, this may not necessarily be the case and genomes and epigenomes may respond differently to environmental change, thereby generating additional phenotypic variation [3, 7].

To what extent epigenetic modifications act independently from genomic variation is key to understanding the potential role of epigenetics in evolution [3, 7, 8], as epigenetic variation completely under genetic control would not contribute any additional adaptive value [3]. Richards [9] classified epigenetic variation in obligatory, facilitated or pure epialleles, based on their degree of autonomy from the underlying genotype. Obligatory epialleles would be fully dependent on genetic variation and should show no variation across environmental change [10], whereas facilitated and pure epigenetic variation would differ in their degree of autonomy from the genotype (from partially depend to independent) [10], acting as potential intermediaries between environmental conditions and genome responses.

Among the epigenetic modifications, DNA methylation is the best studied, and plays an important role in the pre-transcriptional control of several biological processes, such as cell differentiation and genomic imprinting [11, 12]. While

63 correlations among DNA methylation patterns, environmental conditions and
64 phenotypic traits have been widely investigated [8, 13, 14], the relative contributions
65 of the genetic background and environmental variation to DNA methylation
66 plasticity are still unclear [4, 15]. Studies in humans and model organisms suggest
67 that DNA methylation is influenced by the genotype, the environment and also by
68 their interaction [15, 16], but quantifying their relative influences is particularly
69 challenging in natural populations with high levels of genetic variation [17]. In
70 particular, there is little information on the basis of DNA methylation plasticity
71 beyond model organisms [3], specially in teleost fishes, for which most of the
72 studies are focused on well known organisms such as zebrafish or salmonids [18].

73 The self-fertilising hermaphroditic mangrove killifish (*Kryptolebias*
74 *marmoratus*) [19] has naturally inbred lines [20] which inhabit mangroves with
75 markedly variability in habitat quality subject to tidal variation, ranging from
76 temporary pools to mangrove leaf litter and crab burrows [21, 22].The species
77 displays considerable plasticity in behaviour [23] and reproduction (mixed-mating
78 with different degrees of self-fertilisation and outcrossing) [24], both between and
79 within self-fertilising lines [25], and it has been suggested that regulation of gene
80 expression through DNA methylation could play role in its plastic response to
81 environmental variation [26-28].

82 Inbred organisms provide a uniquely opportunity to detangle genetic from
83 epigenetic variation [29, 30]. Here, we investigated the relative roles of the genotype
84 and the rearing environment (with or without physical enrichment) in DNA
85 methylation plasticity of two genetically different and highly inbred self-fertilising
86 lines of *Krytolebias marmoratus*. We hypothesised that if DNA methylation was
87 mostly autonomous and shaped by environmental change, a higher number of

different epialleles would be found between environments, regardless of the genetic background, than if DNA methylation was mostly under genetic control, where most of the epigenetic differences would occur between genotypes.

Results

We compared DNA methylation patterns in the brain of fish from two highly inbred mangrove killifish lines (DAN and R, originally sampled in Belize mangroves but maintained under laboratory conditions for at least 20 generations) reared under physically enriched (with log and plants) or impoverished conditions (barren) for 10 months. By using Reduced Representation Bisulphite Sequencing (RRBS) we identified 5.5 million cytosine sites, of which 139.908 CpG sites fulfilled the minimum coverage requirement, representing 1.2% of the total number of cytosines of the mangrove killifish genome. This result is similar to recent RRBS studies in other fish (1% in rainbow trout [13]; 1.5-2% in guppies [31]).

The majority of cytosines surveyed mapped gene bodies (71.32%) or intergenic regions (19.10%), while only 2.54% were located on putative promoters. Linear models using the PCA scores for 1064 DMCs and 194 DMRs identified between genotypes and environments revealed that genotypes explained more of the variance for PC1 (54%of overall variation for both DMCs and DMRs) than environment (Figure S2; Table S1). When predictors were analysed individually, differences between genotypes also corresponded to a higher number of DMCs (817 vs 594, four DMCs shared) and DMRs (43 vs 17, no DMR shared) than differences between environments (Figures 1 and S2). Within genotypes, 357 and 3632 DMCs (25 and 373 DMRs) were identified between environments, for DAN and R, respectively. An additional analysis on three different subsets of six randomly selected R individuals (to match the

number of DAN individuals) was carried out to assess possible biases due to differences in sample sizes. This additional analysis identified similar number of DMCs between lines, suggesting that the difference between lines was not due to sampling bias (Table S2). Unsupervised hierarchical clustering revealed distinctive methylation profiles between groups, except for comparisons between environments, where one and two individuals from the poor environment clustered with individuals from the enriched environment for DMCs and DMRs respectively (Figures 1 and S3).

Twenty-five annotated DMCs and four DMRs between environments were shared across genotypes, potentially representing environmentally-affected DMCs, independently of genetic background. Of these, based on the direction of methylation across environments, 22 out 25 DMCs were classified as potentially facilitated, with methylation scores following a genotype-specific pattern under similar environments (Table 1). This pattern was supported by the PCA results based on the DMCs methylation scores, which indicated different methylation profiles between environments (PC1 explaining 55.8% of variation), as well as genotypes (PC2 explaining 22.4% of variation) (Figure 2a). PC1 loadings were significantly influenced by the environment ($t = 1.63$, $df = 1$, $p=0.003$) and the interaction between genotype and environment ($t = -11.25$, $df = 1$, $p<0.001$), while PC2 loadings were only significantly influenced by the genotype ($t =-1.64$, $df = 1$, $p<0.001$) (Table 2a). Methylation differences (with a lower threshold of 20%) for the facilitated DMCs ranged from 20.2% to 48.6% (Table 1). The potentially facilitated DMCs were mostly hypermethylated on enriched environments with respect to poor environments for DAN fish, while the opposite pattern was found for R fish (Table 1; Figures 2c and S4). The four DMRs between environments

and shared by genotypes were also classified as facilitated, following the same methylation pattern found on the facilitated DMCs (Figure S5; Table S3)

Only three of the annotated DMCs within or neighbouring gene bodies were considered pure (Table 1; Figure S6). Average methylation differences for pure DMCs ranged from 25.4% to 34.37% (Table 1). The PCA only using pure DMCs showed a different pattern from the facilitated DMCs, with the PC1 separating environments explaining 72.38% of the variation, and the PC2 partially differentiating genotypes explaining 18.85% of the variation (Figure 2b). PC1 loadings were significantly affected by the environment ($t = -2.81$, $df = 1$, $p < 0.001$) and the genotype ($t = -2.28$, $df = 1$, $p = 0.008$), while PC2 loadings were only significantly influenced by genotype ($t = 0.29$, $df = 1$, $p = 0.003$) (Table 2b, Figures 2d and S6).

Molecular network analysis revealed a highly connected network linked by genetic interactions and co-expression interactions, that was composed by 23 input annotated DMCs (the uncharacterised LOC108245430 and ubald1 with no identified connections were removed) and 20 neighbouring genes (Figure S7). Centrality parameters, such as average degree (mean=10.55; SD ± 5.89), closeness (mean=0.53 ± 0.06), and radiality (mean=0.77 ± 0.06) (Table S4), suggested that any alteration of the expression of the genes contained in the network might have major effects on genetic interactions and gene expression levels.

Twelve of the 15 most connected genes within the network (>10 connections), were input genes (i.e. genes affected by DMCs between environments and shared by genotypes). Gene ontology analysis showed that some of these genes are involved on important cellular and metabolic processes

in zebrafish, such as regulation of transcription by RNA polymerase and gene expression (myc), RNA modification (trit1), intracellular calcium content (ryr3), and lipid metabolism (sorcs2), as well as pathways related to angiogenesis and stress response (ryr3 and myc) (Table S5).

Discussion

The potential adaptive role of epigenetically-mediated plasticity depends on the relationship between the genome, the epigenome and the environment [6, 7]. By using two naturally inbred strains of the mangrove killifish reared under contrasting environmental conditions, we have identified significant methylation differences among genotypes and environments, with different levels of autonomy from the genetic background.

Environmental enrichment in fish affects brain structures [32-34], however few studies have investigated the molecular mechanisms underlying these changes [35, 36] and whether it varies across different genetic backgrounds. *Kryptolebias marmoratus* populations are composed by naturally inbred lines living in highly variable habitats [21, 25], which display remarkable phenotypic variation (e.g. in reproductive output [24, 37], behaviour [23, 38, 39] or sexual differentiation [37]), even under identical environmental conditions [25]. Thus, the strains we used here were previously shown to display different sex-ratios in response to temperature variation [20] as well as differences in gene expression in response to parasitic infection [40], suggesting a potential combination of genetic and non-genetic mechanisms in mediating phenotypic variation [29]. Our results indicate that genotypes have an overriding influence on brain DNA methylation patterns, and that their effect is greater than that caused by environmental enrichment. We only found a few DMCs that could be considered facilitated or pure epialleles,

supporting the idea that environmentally-induced autonomous DNA methylation may be limited [2]. Yet, the DNA methylation patterns of these putative independent epialleles indicated that DNA methylation outcomes could depend on specific combinations of the genotype and environmental conditions, although we cannot fully discard the potential contribution of heritable epigenetic states independent of the genotypes [7] and/or brain cell heterogeneity.

The large differences in number of DMCS we found between lines could be explained by their genetic differences [41] . Studies in model organisms indicate that DNA methylation, and potentially other layers of chromatin organisation, are strongly influenced by genomic variants [42-44]. For example, the spontaneous mutation in a gene related to methyltransferase1 activity, increased in 40% the methylation differences among inbred lines of *Arabidopsis thaliana* [45] and in humans, 25% of variation in neonates' methylomes can be explained by their genotype, while the remaining 75% is related to interactions between the genotype and maternal factors (i.e. smoking, age, intrauterine environment) [15].

Most of the DMCs observed between environments and common to both genotypes were located in gene bodies and were highly integrated within a gene network of genetic interactions and co-expression. Recent evidence in plants [46] indicates that gene body methylation can reduce erroneous transcription, and in oyster [47] and zebrafish [48] there seems to be a positive correlation between gene body methylation, gene expression and transcriptional regulation. Here, some of the genes affected by the DMCs found in gene bodies were related to the regulation of RNA polymerase activity and gene expression patterns (myc and trit1) [47, 48], suggesting that these changes in methylation could be involved in biological and cellular processes.

DNA methylation is a good candidate for mediating phenotypic plasticity, given its responsiveness to environmental change, effects on downstream phenotypes, and transgenerational stability [3, 4, 49]. Our results, suggest that, at least in part, DNA methylation patterns are influenced by a dynamic interaction between genotypes and the environment. Further research to investigate whether the patterns found here might influence transcription is warranted to assess the generality of our results, that might provide a potential mechanistic explanation for the genotype-by-environment patterns often observed in phenotypically plastic responses [6, 50].

Methods

We used hermaphrodite fish from two highly inbred strains (R and DAN) of *Kryptolebias marmoratus* originally collected from Belize [51, 52] and kept in the laboratory conditions (25-27 °C, 16-18‰ ppm salinity under a 12h light:12h dark photoperiod), for at least 20 selfing generations [53]. The R (also called *50.91*) strain was collected in Belize (Twin Cayes) in the early 1990s while the DAN (*Dan06*) strain was also collected from Belize in the early 2000s [51]. These selfing lines had previously shown different DNA methylation responses to environment (temperature) variation [20].

We compared brain methylation of fish reared under enriched and impoverished conditions, as previous studies had shown environmental enrichment can affect behavioural flexibility [54], brain size and cognition [55], and induce epigenetic modifications during early development [56]. We used two different habitats with different levels of environmental enrichment: 1) a physically enriched habitat,

where individual fish were placed in contiguous rectangular tanks (9cm depth x 12cm width x 8cm length) filled with 400ml of brackish water with one perforated artificial log (3cm depth x 4 cm width x 4cm length) and three artificial plants to simulate a complex habitat, and 2) a barren habitat (hereafter called poor) with the same tank conditions but without physical enrichment (Figure S1). Tanks were separated by opaque screens to prevent visual contact between individuals. For both strains, five initial lab-reared hermaphrodite progenitors of similar size (mean=3.8cm, sd= ±0.12) and age (mean=417.3 days' post hatchling, sd= ±13.4) were chosen. Eggs from these progenitors were maintained individually in circular plastic pots containing 100ml of brackish water and checked daily (Figure S1). Upon hatching, individual alevins were randomly assigned to treatment tanks (enriched and poor), with one fish in each tank. Hatching success was of 90%. The initial experimental set up consisted of 29 R fish (18 in enriched habitat, 11 in poor habitat) and 21 DAN fish (10 in enriched, 11 in poor). Fish were maintained under standard laboratory conditions as above and fed three times a week with live brine shrimp (1ml for the first two months post-hatching, and 2ml for the rest of the time). Fish were maintained in the experimental tanks for 10 months before being euthanized for brain methylation analysis. At 7 months post hatching all fish in the experiment had laid at least one egg indicating that they were all sexually mature self-fertilising hermaphrodites.

(a) Genome-wide DNA methylation data

Fish were euthanized using tricaine methane-sulfonate (MS-222) following Home Office Schedule 1 and their brains kept in molecular biology grade ethanol (99%) for DNA extraction. Brain DNA was extracted from 22 individuals for epigenetic analysis (six DANs: three from each environment; 16 Rs: six from poor, ten from

enriched environment) using Qiagen DNeasy Blood and tissue kit (Qiagen). Fish were genotyped for 23 microsatellites [57]. Genetic differences were identified between inbred lines ($F_{ST=}$1.00, Table S6), but not within lines. All individuals tested were homozygotes and identical within each line for all the markers analysed (Table S6).

Bisulphite converted genomic DNA libraries were prepared using Diagenode Premium Reduced Representation Bisulphite Sequencing (RRBS) kit according to manufacturer's indications and sequenced on an Illumina NextSeq 500 platform using a 1x75pb single-end run, with PCR fully methylated and unmethylated spike controls added.

Quality assessment was performed using FastQC [58]. TrimGalore! [59] was used to trim low-quality base calls and adapters. Trimmed reads were aligned to the *Kryptolebias marmoratus* reference genome (ASM164957v1, GCA_00164975.1: source NCBI) prior in-silico bisulphite conversion using Bismark v0.17.0 [60], which was also used for cytosine methylation calls. Only methylation within CpG context [61], with a minimum coverage of 10 reads in each sample across the 22 individuals sequenced [62] was considered for subsequent analysis. Samples were divided into four experimental groups: "DAN enriched", "DAN poor", "R enriched", "R poor". Mapped reads were processed using SeqMonk [63]. After quality filtering, approximately 273 million reads were retained, averaging 12 million reads per sample. Of those ~ 62.9 % were uniquely mapped reads to the reference genome (Table S7). Overall bisulfite conversion was 99.6%.

(b) Differentially methylated cytosines and regions

To identify differentially methylated cytosines (DMCs) across experimental groups, we used logistic regression on quantitated normalised data with $p < 0.01$ after multiple testing correction (Benjamini-Hochberg) and >20% minimal CpG methylation difference (|ΔM|), using R bridge in SeqMonk. We also performed t-tests across experimental group replicates, to generate a more conservative list of DMCs, only considering those shared by both statistical approaches. To identify differently methylated regions (DMRs), we performed a genome-wide unbiased DMR detection using tilling windows of 1000bp on windows with at least five CpGs with ≥10 reads across all individuals.

We used the scores of methylation for DMCs and DMRs between genotypes and environments for principal component analysis (PCA) using ggfortify package [64] in R v. 3. 4. 3 (R Core Team 2014). To test for the effect of the genotype, environment and their interaction on the methylation scores, we used linear models with the scores for the first two PCA axis (>70% of the total variation) as a function of genotype, environment and their interaction. We then individually compared DMCs and DMRs between genotypes, followed by a comparison between environments. Subsequently, a comparison within each genotype between environments was carried out to identify potential environment-dependent DMCs and DMRs. From these comparisons, we identified annotated DMCs and DMRs shared between genotypes, which should represent commonly affected DMCs regardless of the genetic background.

We classified the DMCs and DMRs shared across genotypes between environments as facilitated, when displaying different directions of variation (non-parallel) on methylation scores across genotypes in the same environment (i.e. hypermethylated in an environment for one strain and hypomethylated in the

other), or pure when displaying the same direction of variation (parallel) across genotypes and environments (i. e. hypermethylated or hypomethylated for both genotypes in the same environment) [9].

(c) Molecular network analysis and centrality metrics

To identify potential functional implications of variation in DNA methylation for the annotated DMCs identified across genotypes between environments, we built a functional gene network using GeneMANIA [65]. To identify central genes [66] within the molecular network, we used NetworkAnalyzer [67] plugin into Cytoscape v. 3.7.1 [67]. Panther GO terms [68]) was used to identify biological process and pathways for the most connected genes (>10 connections) within the network.

Ethics

Work was carried out under Swansea University Animal Ethics Committee permit STU_BIOL_30484_110717192024_3.

Data accessibility

Sequences are accessible from https://www.ncbi.nlm.nih.gov/bioproject/506827.

Authors' contributions

SC, WMBF designed the experiment. WMBF, NB, DRB performed the experiment. WMBF, CGL, DRB analysed the data. WMBF, SC wrote the manuscript with participation of all authors.

Competing interests

The authors have no competing interests.

Funding

334 Work supported by the Conselho Nacional de Desenvolvimento Científico e
335 Tecnológico, scholarship 233161/2014-7.

336

References

1. Bossdorf O, Arcuri D, Richards CL, Pigliucci M. Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. Evol Ecol 2010; 24:541-53.

2. Verhoeven KJF, Preite V. Epigenetic variation in asexually reproducing organisms. Evolution 2013:n/a-n/a.

3. Verhoeven KJF, vonHoldt BM, Sork VL. Epigenetics in ecology and evolution: what we know and what we need to know. Mol Ecol 2016; 25:1631-8.

4. Herman JJ, Sultan SE. DNA methylation mediates genetic variation for adaptive transgenerational plasticity. Proc R Soc B 2016; 283:20160988.

5. Vergeer P, Wagemaker N, Ouborg NJ. Evidence for an epigenetic role in inbreeding depression. Biology Letters 2012; 8:798-801.

6. Sultan SE. Organism and environment: ecological development, niche construction, and adaptation. Oxford University Press, USA, 2015.

7. Richards CL, Alonso C, Becker C, Bossdorf O, Bucher E, Colomé-Tatché M, Durka W, Engelhardt J, Gaspar B, Gogol-Döring A. Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. Ecology letters 2017; 20:1576-90.

8. Duncan EJ, Gluckman PD, Dearden PK. Epigenetics, plasticity, and evolution: How do we link epigenetic change to phenotype? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 2014; 322:208-20.

9. Richards EJ. Inherited epigenetic variation—revisiting soft inheritance. Nat Rev Genet 2006; 7:395.

10. Schmitz RJ, He Y, Valdés-López O, Khan SM, Joshi T, Urich MA, Nery JR, Diers B, Xu D, Stacey G. Epigenome-wide inheritance of cytosine methylation variants in a recombinant inbred population. Genome Res 2013; 23:1663-74.

11. Koch IJ, Clark MM, Thompson MJ, Deere-Machemer KA, Wang J, Duarte L, Gnanadesikan GE, McCoy EL, Rubbi L, Stahler DR. The concerted impact of domestication and transposon insertions on methylation patterns between dogs and grey wolves. Mol Ecol 2016; 25:1838-55.

12. Moore LD, Le T, Fan G. DNA methylation and its basic function. Neuropsychopharmacology 2013; 38:23-38.

13. Baerwald MR, Meek MH, Stephens MR, Nagarajan RP, Goodbla AM, Tomalty KM, Thorgaard GH, May B, Nichols KM. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. Mol Ecol 2016; 25:1785-800.

14. Jeremias G, Barbosa J, Marques SM, Asselman J, Gonçalves FJ, Pereira JL. Synthesizing the role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems. Mol Ecol 2018.

15. Teh AL, Pan H, Chen L, Ong M-L, Dogra S, Wong J, MacIsaac JL, Mah SM, McEwen LM, Saw S-M. The effect of genotype and in utero environment on interindividual variation in neonate DNA methylomes. Genome Res 2014; 24:1064-74.

16. Dubin MJ, Zhang P, Meng D, Remigereau M-S, Osborne EJ, Paolo Casale F, Drewe P, Kahles A, Jean G, Vilhjálmsson B, et al. DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. eLife 2015; 4:e05255.

17. Verhoeven KJF, Vonholdt BM, Sork VL. Epigenetics in ecology and evolution: what we know and what we need to know. Mol Ecol 2016; 25:1631-8.
18. Best C, Ikert H, Kostyniuk DJ, Craig PM, Navarro-Martin L, Marandel L, Mennigen JA. Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 2018; 224:210-44.
19. Tatarenkov A, Lima SMQ, Earley RL, Berbel-Filho WM, Vermeulen FBM, Taylor DS, Marson K, Turner BJ, Avise JC. Deep and concordant subdivisions in the self-fertilizing mangrove killifishes (Kryptolebias) revealed by nuclear and mtDNA markers. Biol J Linn Soc 2017:blx103-blx.
20. Ellison A, López CMR, Moran P, Breen J, Swain M, Megias M, Hegarty M, Wilkinson M, Consuegra S. Epigenetic regulation of sex ratios may explain natural variation in self-fertilization rates. Proc Biol Sci 2015:20151900.
21. Ellison A, Wright P, Taylor DS, Cooper C, Regan K, Currie S, Consuegra S. Environmental diel variation, parasite loads, and local population structuring of a mixed-mating mangrove fish. Ecology and evolution 2012; 2:1682-95.
22. Taylor DS. Twenty-four years in the mud: what have we learned about the natural history and ecology of the mangrove rivulus, Kryptolebias marmoratus? : Oxford University Press, 2012.
23. Edenbrow M, Croft DP. Environmental and genetic effects shape the development of personality traits in the mangrove killifish *Kryptolebias marmoratus*. Oikos 2013; 122:667-81.
24. Lin H-C, Dunson WA. An explanation of the high strain diversity of a self-fertilizing hermaphroditic fish. Ecology 1995; 76:593-605.

410 25. Earley RL, Hanninen AF, Fuller A, Garcia MJ, Lee EA. Phenotypic
411 Plasticity and Integration in the Mangrove Rivulus (Kryptolebias marmoratus): A
412 Prospectus. Integrative and Comparative Biology 2012; 52:814-27.
413 26. Ellison A, Lopez CMR, Moran P, Breen J, Swain M, Megias M, Hegarty
414 M, Wilkinson M, Pawluk R, Consuegra S. Epigenetic regulation of sex ratios
415 may explain natural variation in self-fertilization rates. P Roy Soc B-Biol Sci
416 2015; 282.
417 27. Fellous A, Labed-Veydert T, Locrel M, Voisin AS, Earley RL, Silvestre F.
418 DNA methylation in adults and during development of the self-fertilizing
419 mangrove rivulus, Kryptolebias marmoratus. Ecology and evolution 2018;
420 8:6016-33.
421 28. Fellous A, Earley RL, Silvestre F. The Kdm/Kmt gene families in the self-
422 fertilizing mangrove rivulus fish, Kryptolebias marmoratus, suggest involvement
423 of histone methylation machinery in development and reproduction. Gene 2019;
424 687:173-87.
425 29. Verhoeven KJF, Preite V. Epigenetic variation in asexually reproducing
426 organisms. Evolution 2014; 68:644-55.
427 30. Laskowski KL, Doran C, Bierbach D, Krause J, Wolf M. Naturally clonal
428 vertebrates are an untapped resource in ecology and evolution research.
429 Nature ecology & evolution 2019:1.
430 31. Hu J, Pérez-Jvostov F, Blondel L, Barrett RD. Genome-wide DNA
431 methylation signatures of infection status in Trinidadian guppies (Poecilia
432 reticulata). Mol Ecol 2018.

32. Näslund J, Aarestrup K, Thomassen ST, Johnsson JI. Early enrichment effects on brain development in hatchery-reared Atlantic salmon (Salmo salar): no evidence for a critical period. Can J Fish Aquat Sci 2012; 69:1481-90.

33. Kihslinger RL, Lema SC, Nevitt GA. Environmental rearing conditions produce forebrain differences in wild Chinook salmon *Oncorhynchus tshawytscha*. Comparative biochemistry and physiology Part A, Molecular & integrative physiology 2006; 145:145-51.

34. von Krogh K, Sørensen C, Nilsson GE, Øverli Ø. Forebrain cell proliferation, behavior, and physiology of zebrafish, Danio rerio, kept in enriched or barren environments. Physiol Behav 2010; 101:32-9.

35. Lema SC, Hodges MJ, Marchetti MP, Nevitt GA. Proliferation zones in the salmon telencephalon and evidence for environmental influence on proliferation rate. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 2005; 141:327-35.

36. Salvanes AG, Moberg O, Ebbesson LO, Nilsen TO, Jensen KH, Braithwaite VA. Environmental enrichment promotes neural plasticity and cognitive ability in fish. Proc Biol Sci 2013; 280:20131331.

37. Turner BJ, Fisher MT, Taylor DS, Davis WP, Jarrett BL. Evolution of 'maleness' and outcrossing in a population of the self-fertilizing killifish, *Kryptolebias marmoratus*. Evolutionary Ecology Research 2006; 8:1475-86.

38. Wells MW, Wright PA. Do not eat your kids: embryonic kin recognition in an amphibious fish. Behav Ecol Sociobiol 2017; 71:140.

39. Earley RL, Hsu Y. Reciprocity between endocrine state and contest behavior in the killifish, Kryptolebias marmoratus. Horm Behav 2008; 53:442-51.

40. Pawluk RJ, Uren Webster TM, Cable J, Garcia de Leaniz C, Consuegra S. Immune-related transcriptional responses to parasitic infection in a naturally inbred fish: roles of genotype and individual variation. Genome biology and evolution 2018.

41. Gibson G, Dworkin I. Uncovering cryptic genetic variation. Nat Rev Genet 2004; 5:681.

42. Li X, Zhu J, Hu F, Ge S, Ye M, Xiang H, Zhang G, Zheng X, Zhang H, Zhang S. Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. BMC Genomics 2012; 13:300.

43. Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, Bryois J, Giger T, Romano L, Planchon A. Passive and active DNA methylation and the interplay with genetic variation in gene regulation. elife 2013; 2:e00523.

44. van der Graaf A, Wardenaar R, Neumann DA, Taudt A, Shaw RG, Jansen RC, Schmitz RJ, Colomé-Tatché M, Johannes F. Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. Proceedings of the National Academy of Sciences 2015; 112:6676-81.

45. Becker C, Hagmann J, Müller J, Koenig D, Stegle O, Borgwardt K, Weigel D. Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. Nature 2011; 480:245.

46. Horvath R, Laenen B, Takuno S, Slotte T. Single-cell expression noise and gene-body methylation in Arabidopsis thaliana. Heredity 2019:1.

480 47. Riviere G, He Y, Tecchio S, Crowell E, Gras M, Sourdaine P, Guo X,
481 Favrel P. Dynamics of DNA methylomes underlie oyster development. PLoS
482 Genet 2017; 13:e1006807.
483 48. McGaughey DM, Abaan HO, Miller RM, Kropp PA, Brody LC. Genomics
484 of CpG methylation in developing and developed zebrafish. G3 (Bethesda)
485 2014; 4:861-9.
486 49. Eichten SR, Briskine R, Song J, Li Q, Swanson-Wagner R, Hermanson
487 PJ, Waters AJ, Starr E, West PT, Tiffin P. Epigenetic and genetic influences on
488 DNA methylation variation in maize populations. The Plant Cell 2013:tpc.
489 113.114793.
490 50. Des Marais DL, Hernandez KM, Juenger TE. Genotype-by-environment
491 interaction and plasticity: exploring genomic responses of plants to the abiotic
492 environment. Annual Review of Ecology, Evolution, and Systematics 2013;
493 44:5-29.
494 51. Tatarenkov A, Ring BC, Elder JF, Bechler DL, Avise JC. Genetic
495 composition of laboratory stocks of the self-fertilizing fish *Kryptolebias*
496 *marmoratus*: a valuable resource for experimental research. PLoS ONE 2010;
497 5:e12863.
498 52. Lins LSF, Trojahn S, Sockell A, Yee M-C, Tatarenkov A, Bustamante CD,
499 Earley RL, Kelley JL. Whole-genome sequencing reveals the extent of
500 heterozygosity in a preferentially self-fertilizing hermaphroditic vertebrate.
501 Genome 2017.
502 53. Ellison A, Allainguillaume J, Girdwood S, Pachebat J, Peat K, Wright P,
503 Consuegra S. Maintaining functional major histocompatibility complex diversity

504 under inbreeding: the case of a selfing vertebrate. Proceedings of the Royal
505 Society of London B: Biological Sciences 2012; 279:5004-13.
506 54. Roberts L, Taylor J, de Leaniz CG. Environmental enrichment reduces
507 maladaptive risk-taking behavior in salmon reared for conservation. Biol
508 Conserv 2011; 144:1972-9.
509 55. Kihslinger RL, Lema SC, Nevitt GA. Environmental rearing conditions
510 produce forebrain differences in wild Chinook salmon Oncorhynchus
511 tshawytscha. Comparative Biochemistry and Physiology Part A: Molecular &
512 Integrative Physiology 2006; 145:145-51.
513 56. Champagne FA. Epigenetic mechanisms and the transgenerational
514 effects of maternal care. Front Neuroendocrinol 2008; 29:386-97.
515 57. Ellison A, Cable J, Consuegra S. Best of both worlds? Association
516 between outcrossing and parasite loads in a selfing fish. Evolution: International
517 Journal of Organic Evolution 2011; 65:3021-6.
518 58. Andrews S. FastQC: a quality control tool for high throughput sequence
519 data. http://wwwbioinformaticsbabrahamacuk/projects/fastqc/ 2010.
520 59. Krueger F. TrimGalore! A wrapper around cutadapt and FastQC to
521 consistently apply adapter and quality trimming to FastQ files, with extra
522 functionality for RRBS data. .
523 https://wwwbioinformaticsbabrahamacuk/projects/trim_galore/ 2016.
524 60. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation
525 caller for Bisulfite-Seq applications. Bioinformatics 2011; 27:1571-2.
526 61. Feng S, Cokus SJ, Zhang X, Chen P-Y, Bostick M, Goll MG, Hetzel J,
527 Jain J, Strauss SH, Halpern ME. Conservation and divergence of methylation

patterning in plants and animals. Proceedings of the National Academy of Sciences 2010; 107:8689-94.

62. Shafi A, Mitrea C, Nguyen T, Draghici S. A survey of the approaches for identifying differential methylation using bisulfite sequencing data. Brief Bioinform 2017.

63. Andrews S. SeqMonk: A tool to visualise and analyse high throughput mapped sequence data. https://wwwbioinformaticsbabrahamacuk/projects/seqmonk/ 2007.

64. Tang Y, Horikoshi M, Li W. ggfortify: Unified interface to visualize statistical results of popular R Packages. The R Journal 2016; 8:478-89.

65. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C, Kazi F, Lopes CT. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic acids research 2010; 38:W214-W20.

66. del Rio G, Koschützki D, Coello G. How to identify essential genes from molecular networks? BMC systems biology 2009; 3:102.

67. Assenov Y, Ramirez F, Schelhorn SE, Lengauer T, Albrecht M. Computing topological parameters of biological networks. Bioinformatics 2008; 24:282-4.

68. Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic Acids Res 2016; 45:D183-D9.

Figure legends

Figure 1. Heat map illustrating percentage of methylation for all differentially methylated cytosines (DMCs) identified (*a*) between genotypes, (*b*) between environments, (*c*) between environments for DAN strain, and (*d*) between environments for R strain (logistic regression $q < 0.01$ and $|\Delta M| > 20\%$, and t.test $p < 0.01$) using unsupervised hierarchical clustering. Rows represent a unique CpG site and columns individual fish.

Figure 2. Principal component analysis (PCA) and reaction norms of epialleles across genotypes and environments. PCAs were based on individual scores of methylation across either (a) facilitated or (b) pure annotated DMCs. Dark yellow for DAN individuals on enriched environments; light yellow for DAN genotype on poor environments; dark green for R individuals on enriched environments; light green for R genotype on poor environments. Each reaction norm represents the change on averaged methylation scores (in percentage) for (c) facilitated and (d) pure epialelles annotated DMCs across environments. Different colours represent the genotypes (yellow for DAN; green for R). Different shapes (d) represent different annotated DMCs. Epialelles were classified according to Richards (2006). Detailed information for each annotated DMCs methylation score across genotypes is available at Table 1.

574 **Table 1**. Methylation differences averaged (percentage) for differentially methylated cytosines (DM
575 enriched), shared between genotypes (DAN, R) which overlap annotated genes (refe
576 GCA_00164975.1). Epiallele classification (pure or facilitated) followed [9]. Positive and negative
577 decreased methylation in enriched and poor environments, respectively. Q-value is the p-value a
578 (FDR=0.05).

Gene symbol	Entrez gene name	Epiallele classification	Meth diff DAN	*Q*-value
acvr2a	activin A receptor type 2A	P	34.37	0.007
col25a1	collagen type XXV alpha 1 chain	F	43.61	0.005
dmap1	DNA methyltransferase 1 associated protein 1	F	26.99	<0.001
foxp4	forkhead box P4	F	22.50	<0.001
gpc5	glypican 5	F	31.82	0.01
mipol1	mirror-image polydactyly 1	F	35.85	<0.001
necab2	N-terminal EF-hand calcium binding protein 2	F	20.25	0.01
neo1	neogenin 1	F	20.25	<0.001
nudcd1	NudC domain containing 1	F	39.76	<0.001
ramp3	receptor activity-modifying protein 3-like	P	-27.12	0.037
ryr3	ryanodine receptor 3	P	-30.48	0.003
sorcs2	sortilin-related VPS10 domain containing receptor 2	F	36.81	0.008
trit1	tRNA isopentenyltransferase 1	F	20.38	<0.001

trmt44	tRNA methyltransferase 44	F	23.98	<0.001
ubald1	UBA like domain containing 1	F	36.76	0.019
zeb2	zinc finger E-box binding homeobox 2	F	31.83	<0.001
znf516	zinc finger protein 516	F	31.51	<0.001
zranb3	zinc finger RANBP2-type containing 3	F	41.41	<0.001
LOC108234847	adhesion G protein-coupled receptor L3-like	F	48.57	<0.001
LOC108240988	non-muscle caldesmon-like	F	32.49	0.005
LOC108243470	protein-methionine sulfoxide oxidase mical2b-like	F	33.35	<0.001
LOC108243852	receptor-type tyrosine-protein phosphatase N2-like	F	37.97	0.014
LOC108245430	uncharacterized protein	F	42.94	<0.001
LOC108247402	spectrin beta chain, non-erythrocytic 1-like	F	40.73	0.006
LOC108251479	transcriptional regulator Myc-B-like	F	30.49	0.010

579

580

581 **Table 2**. Linear model of principal component scores for mangrove killifish
582 epialleles shared between genotypes (R, DAN) and environments (poor,
583 enriched).

	t-values	Prop. of variance (%)	df	p-value
(*a*) Facilitated epialleles				
PC1 scores				
Genotype	1.63	0.06	1	0.80
Environment	1.58	12.41	1	0.003
Genotype x Environment	-11.25	68.08	1	<0.001
PC2 scores				
Genotype	-1.64	91.35	1	<0.001
Environment	8.28	0.21	1	0.49
Genotype x Environment	10.29	0.35	1	0.38
(*b*) Pure epialleles				
PC1 scores				
Genotype	2.28	13.18	1	0.008
Environment	-2.81	59.28	1	<0.001
Genotype x Environment	0.98	0.09	1	0.80
PC2 scores			1	
Genotype	0.29	37.96	1	0.003
Environment	-1.82	1.14	1	0.56
Genotype x Environment	-2.97	0.67	1	0.65

584