Environmental enrichment induces intergenerational behavioural and epigenetic effects on fish

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Abstract

Parental effects influence offspring phenotypes through pre- and post-natal routes but little is known about their molecular basis, and therefore their adaptive significance. Epigenetic modifications, which control gene expression without changes in the DNA sequence and are influenced by the environment, may contribute to parental effects. Taking advantage of the self-fertilising and inbred nature of the mangrove killifish Kryptolebias marmoratus, we investigated the effects of the rearing environment on parents and offspring by comparing neophobia, metabolic rate and brain epigenetic (DNA methylation) patterns of genetically identical fish reared in enriched or barren environments. Parental fish reared in enriched environments had lower cortisol levels, lower metabolic rates and were more active and neophobic than those reared in barren environments. They also differed in 1,854 methylated cytosines (DMCs). Offspring activity and neophobia were determined by the parental environment and we also found evidence of, limited but significant, parental influence on the DNA methylation patterns of the offspring. Among the DMCs of the parents, 98 followed the same methylation patterns in the offspring, three of which were significantly influenced by parental environments irrespective of their own rearing environment. Our results suggest that the environment experienced by the parents influences the behaviour and, to some extent, brain DNA methylation patterns of the offspring in an environment-specific manner.

Introduction

Parental effects occur when maternal, paternal or both parental phenotypes affect offspring phenotypes (Bonduriansky & Day 2018; Uller *et al.* 2013). Such effects occur in a wide range of taxa (Uller 2008) via different pre- and post-natal routes (e. g. microhabitat selection for eggs, reproductive investment, intrauterine environment, parental care). Parental experiences can affect offspring fitness (Burton & Metcalfe 2014), although are not necessarily adaptive (Bonduriansky & Day 2018). For example, maternal undernourishment is associated with the development of diabetes and obesity in the progeny (Hales & Barker 2001), while paternal undernutrition in mice results in altered glucose metabolism and growth in the offspring (Anderson *et al.*2006).

The knowledge about the molecular mechanisms of the pre-natal parental effects is still limited (Gluckman*et al.* 2005; Jensen *et al.*2014). The transmission of some parental effects via germline has been related to genetic mechanisms, such as the association between the frequency of some deleterious mutations in sperm and increasing male's age (Wyrobek *et al.* 2006). However, it is likely that non-genetic mechanisms also play a major role in parent-offspring information transfer (Danchin *et al.* 2011; Jablonka & Raz 2009), as genetic-based inheritance solely cannot fully explain the variation of offspring phenotypes (Danchin *et al.* 2011). Epigenetic modifications, such as DNA methylation, histone modifications and microRNAs, mediate rapid changes in transcription influenced by environmental changes (Richards 2006) that can affect phenotypes (Richards*et al.* 2017; Verhoeven *et al.* 2016). Among the epigenetic mechanisms, DNA methylation

is the best characterized, being important on several biological processes, from genomic imprinting to cell differentiation (Jones 2012; Lea *et al.* 2017). DNA methylation on regulatory regions generally supresses gene expression (Moore *et al.* 2013), whereas methylation in gene bodies contributes to reducing transcriptional noise (Huh *et al.* 2013). Thus, differential methylation can affect gene expression and result in phenotypic plasticity (Baerwald *et al.* 2016; Herman & Sultan 2016). However, while the transmission of environmentally-induced epialleles via DNA methylation from parents to offspring has been identified in plants, whether epigenetic mechanisms can provide a heritable memory of environmental influence in animals remains controversial (Heard & Martienssen 2014), as well as the potential adaptive value of this type of transmission (Perez & Lehner 2019).

The parental rearing environment can induce phenotypic modifications during early development which can be long-lasting and potentially intergenerational (Burton & Metcalfe 2014). A well known example is the effect of structural environmental complexity on behaviour (Braithwaite & Salvanes 2005; Roberts *et al.*2011), physiology (Näslund *et al.*2013), cognitive capacity (Salvanes*et al.* 2013) and brain structure (Kihslinger *et al.* 2006) in fish. Physical structures are critical for most fish at different points of their life cycle (e. g. for spawning, sheltering, foraging), suggesting that structural complexity is an important ecological factor of their natural environment (Näslund & Johnsson 2016). Captive fish reared in enriched environments have shown increased survival in the wild compared to those reared in impoverished environments (D'Anna *et al.* 2012; Roberts *et al.* 2014), as well as enhanced cognitive capacity and behavioural flexibility (Salvanes *et al.* 2013; Spence *et al.* 2011; Strand *et al.* 2010). However, little is known about the molecular mechanisms underlying plastic responses to environmental enrichment, or whether these changes could be transmitted across generations (Näslund*et al.* 2012; Näslund & Johnsson 2016).

Kyrptolebias marmoratus (Poey 1880) is a predominantly self-fertilising fish living in mangrove forests in North and Central America (Tatarenkov *et al.* 2017), occupying a varied range of mangrove fossorial microhabitats influenced by periodical tide variation (Ellison*et al.* 2012b). Its naturally inbred nature makes *K. marmoratus* populations particularly suited to assess the influence of the environment on behaviour (Ellison*et al.* 2013; Ellison *et al.*2012b), phenotypic plasticity (Earley*et al.* 2012) and epigenetics (Ellison *et al.* 2015). In their natural environment, the species inhabits inherently heterogenous mangrove habitats, with different selfing lineages coexisting in the same microhabitat (Ellison *et al.*2012b), and displays aggression towards conspecifics (Taylor 2000) that vary depending on kinship relationship (Edenbrow & Croft 2012; Ellison *et al.* 2013). These fish ermerse to forage or in response to intraspecific aggression or poor water quality (Turko *et al.*2011), suggesting that environmental complexity may play an important role on their ecology and behaviour.

We reared two generations of genetically-identical *K. marmoratus* in matched and mismatched environments with different levels of structural complexity to examine the intergenerational influence of environmental enrichment on individual physiology and behaviour, and the potential role of epigenetic mechanisms (brain DNA methylation) to mediate environmentally-induced parental effects.

Materials and Methods

Experimental design

We used a highly inbred (at least 20 generations of inbreeding) strain of *K. marmoratus* (R (Ellison*et al.* 2012a)) kept under standard laboratory conditions (25-27 °C, 16-18photoperiod). For the parental generation, eggs from five fish of similar size and age were reared individually until hatching, when larvae were transferred to tanks with either enriched environment (shelter and plants, n=14) or poor environment (identical except no enrichment, n=13), where they were kept for 10 months (Berbel-Filho *et al.*2019).

To standardise potential age-related parental effects, eggs were only collected from parents of similar age (7-10 months). The offspring of five genetically inbred parents (three from enriched and two from poor environments) were set up following a factorial design with matched or mismatched parent-offspring environments (Fig. S1; Table S1). The offspring consisted of 15 mismatched individuals (seven poor to enriched and eight enriched to poor), and 13 matched individuals (eight enriched to enriched and seven poor to poor).

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Metabolic rate and cortisol measurements

We measured basal metabolic rate (oxygen consumption) of 55 adults (14 parental enriched; 13 parental poor, eight offspring from enriched parents reared in enriched environment; ten offspring from enriched parents reared in poor environment; five offspring from poor parents reared in poor environment; five offspring from poor parents reared in enriched environment), 30 of which were also analysed for epigenetic variation (Table S1). Basal metabolic rates were measured at eight months of age using four identical close respirometers consisting of 120 ml sealed dark chambers filled with oxygen saturated autoclaved water. Two blank trials were carried out to confirm no leakage of oxygen. Oxygen levels were calibrated in trials using saturated oxygenated water (100% dissolved oxygen) and anoxic water (2% dissolved oxygen). Fish were fastened for 48 hrs prior to acclimation for 20h. Oxygen consumption was measured once for each fish for 40 minutes after acclimation, with oxygen levels always above 60%. Chambers were drained and cleaned between runs. Basal metabolic rate was calculated taking into account the rate of oxygen decrease in the chamber, mass of the individual, volume of water and time of measurement (mg O_2 g^{-1} min^{-1}). Averaged background respiration levels across runs was 12.34% (sd=+-9.71).

We used ultrasensitive graphene immunosensors (Barton *et al.* 2018) for measuring waterborne cortisol non-invasively from parents reared in both enriched and poor environments. For this, 120ml of water were taken from the respirometer after each individual measurement of metabolic rate and kept at -80 degC until the analysis. A total of 10ml were centrifuged at 1000rpm for 5 minutes, and 10ul of the supernatant were pipetted onto the modified sensor surface. Electrochemical measurements were conducted with a potentiostat/ galvanostat (Autolab), controlled with NOVA software as in (Barton *et al.*2018).

Behavioural analyses

Neophobia (number of contacts and inspections of a novel object) and exploratory behaviour were assessed using a plastic test arena (7 cm depth x 7 cm width x 30 cm length) filled with 0.7 L of water. The arena was divided into six equally spaced zones: a covered acclimatisation section (zone 0) with a sliding opaque door, and five open test zones (5 cm each) without cover (zones 1, 2, 3, 4 and 5) delineated by marks at the top margins of the arena walls. A coloured toy block (0.5 cm depth x 0.5 cm width x 3.5 cm height) was glued at the middle of zone 3 to serve as a novel object (Fig. S2). Nine-month old fish were placed individually into zone 0 for 15 min acclimatisation, after which the removable gate was slowly lifted, and the fish behaviour recorded for 20 min with an overhead camera fixed 0.5m above the arena. After the test period, tanks were drained, rinsed with ethanol and distilled water. Videos were analysed by the same person using BORIS v. 7. 1. 4 (Friard & Gamba 2016) to ensure consistency. The following four behaviours were quantified for both parents and offspring: (1) latency (s) to exit the acclimatisation zone, (2) number of inspections within 3 cm of the novel object (i. e. individual facing towards the novel for more than three seconds), (3) number of contacts with the novel object, and (4) number of movements between zones (activity).

Statistical analysis

All statistical analyses were ran in R v. 3. 4. 3. Cortisol levels and basal metabolic rate were analysed for the parents using a linear model with environment (poor vs enriched), and body weight as predictors. We used GLM with a quassipoisson link to account for overdispersion for the parental behavioural count data (no. contacts, no. inspections and activity) and a gaussian link for latency as a function of environment and body weight.

To test for parental effects on the offspring phenotype, we only analysed those phenotypes significantly different between parental environments, using the same model structure as described above but including also the parental values and environment as predictors. We used the multi-model approach implemented in the R package glmulti v 1.0.7 (Calcagno & de Mazancourt 2010) for model selection, which tests all possible models and all interactions, and considered models within 2 AIC units as being equivalent. To take into account potential parentage effects, we first selected the best-fit model (highest Akaike weight) using glmulti and then ran generalized mixed-models including parent of origin as a random factor using mlmRev v.1.0-7. Models were tested for overdispersion and individual observations (fish ID) were also taken into account

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when models displayed overdispersion. Outliers were identified using the function aout.pois in the package alphaOutlier.

Genome-wide DNA methylation data

All individuals were analysed at the same age (10 months old). Fish were euthanized using an overdose of methane-sulfonate (MS-222) following UK Home Office Schedule 1, their brains were dissected and stored in molecular grade ethanol before DNA extraction using Qiagen DNeasy Blood and tissue kit (Qiagen Ltd, Crawley, UK).

Bisulphite converted genomic DNA libraries were prepared using the Diagenode Premium Reduced Representation Bisulphite Sequencing (RRBS) kit (Diagenode, Liege, Belgium) according manufacturer's indications. For the first generation, 16 individuals (ten from enriched, six from poor) were multiplexed into a single library, pooled samples were bisulphite-converted, amplified by enrichment PCR and their quality assessed using Agilent D1000 ScreenTape System (Agilent Technologies, Inc. 2014). The library was then sequenced on an Illumina NextSeq 500 platform using a 1x75pb single-end run (Cardiff University, Genomics Research Hub). Standard PCR fully methylated and unmethylated spike controls were used to monitor bisulphite conversion efficiency.

A second library was created using fourteen individuals from the offspring (five from enriched to enriched environments, three from enriched to poor, three from poor to poor, and three from poor to enriched). The library followed the same procedures and sequencing conditions as the first library.

Sequence quality and alignment

We assessed the quality of the sequences using FastQC (Andrews 2010), trimming of adaptors and low-quality reads was done using the RRBS default parameters (function: –rrbs) in TrimGalore! (Krueger 2016). Reads were aligned to the *Kryptolebias marmoratus* reference genome (NCBI ASM164957v1) (Rhee *et al.* 2017) prior to in-silico bisulphite conversion using Bismark v0.17.0 (Krueger & Andrews 2011), which was also used to perform cytosine methylation calls. We only considered methylation within CpG context for the downstream analysis (Feng *et al.* 2010) and included CpGs with a minimum coverage of [?]10 reads in each sample across the 30 individuals sequenced. Individuals were grouped into generations (parents/offspring) and environments (own/parental) (Table S2). Mapped reads were processed and compared using the R package methylKit v. 1. 10 (Akalin *et al.* 2012). All analyses were conducted on a local server running NEBC Bio-Linux 8.

Differentially methylated cytosines and methylation patterns

We first assessed differentially methylated cytosines (DMCs) between parental environments (enriched vs poor), using logistic regression on quantitated normalised data with q-value < 0.01 after multiple testing correction and >20% minimal CpG methylation difference ($|\Delta M|$), using methylKit. To test whether the number of DMCs between environments were different from the expected by random, we generated with 4,000 random combinations of 16 parental individuals and tested for the number of DMCs for each combination following the same parameters as the ones described for the original grouping.

We then analysed whether the DNA methylation patterns (hypomethylated or hypermethylated) in the parents were maintained in the offspring. For this, we classified DMCs in two categories (i) environmentally-induced (differences in methylation patterns between the parents changed in the offspring depending on the offspring rearing environment) and (ii) intergenerational (differences in methylation patterns between parental environments were maintained in the offspring regardless of their rearing environment) (Fig. S3). We set up a threshold of $\pm$10% average methylation score value in the offspring relatively to its parents to consider whether an individual epiallele methylation pattern maintained the parental methylation state. For DMCs classified as intergenerational we identified the genomic location (within gene body, promoter region ([?]2 kb upstream of the transcription start site (TSS)), or intergenic region ([?]2kb upstream of TSS or downstream the gene bodies).

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To test whether the methylation patterns of the offspring on the DMCs classified as potentially intergenerational were significantly influenced by the parental environment, we analysed the methylation score of the offspring for each DMC (as a proportion index) as a function of the parental environment (enriched or poor), the offspring environment and their interactions using a generalized linear model with a binomial link, with multiple testing correction.

The annotated regions affected by these DMCs were used for the gene ontology enrichment analysis using zebrafish (*Danio rerio*) gene orthologs in PANTHER v. 11 (Mi *et al.* 2016). We searched for enrichments across biological process and pathways ontologies curated for zebrafish. Only genes which matched with the genes names annotated for zebrafish were included in the gene ontology analysis.

Results

Parental physiology and behaviour

For the parental individuals, the multi-model selection approach identified two models within 2 ΔAIC, one for which cortisol levels were only affected by body weight (estimate: -3.39; t-value: 4.22, df=1, p=0.001) and a second model (ΔAICc=1.87) which included both body weight (estimate=-3.03; t-value: 2.86, df=1, p<0.001) and environment (enriched or poor) (t-value: 7.38, df=1, p=0.03) as significant factors affecting cortisol levels, with individuals from enriched environments having lower cortisol levels (Fig. 1a-b; Table S3). Basal metabolic rate decreased with body weight (estimate: -2.40; t-value:-2.35, df=1, p=0.01) but was not affected by the parental environment (Table S3b). A linear regression analysis between cortisol levels and basal metabolic rate showed strong correlation between them (adjusted $R^2 = 0.41$; F-value=16.73, df=1, p<0.001) (Fig. 1c).

Parental activity significantly decreased with body weight (estimate: -2.88; z-value: 31.58, df=1, p=0.01), was lower in individuals reared in poor environments (estimate: -0.25; z-value: 34.98, df=1, p<0.001) and was influenced by with the interaction between environment and body weight (estimate: 2.45; z-value: 38.76, df=1, p=0.02) (Fig. 1d; Table S3). The number of inspections of the novel object was significantly explained by body weight (estimate: -9.50; z-value: -2.21, df=1, p=0.02; Fig. 1e; Table S3). The number of contacts with novel object was significantly affected by environment, with individuals from poor environments having higher number of contacts (estimate:0.92; z-value: 2.98, df=1, p=0.001) (Fig. 1f; Table S3e). No other equivalent model was found for activity, number of inspections and number of contacts. Two of the individuals, one form the enriched and one from the poor environment, were identified as outliers (P<0.01) for the number of contacts (7 and 14 respectively) but re-running the analyses without these two individuals did not change the significance or direction of the difference in number of contacts between groups. The same individual from the poor environment was identified as an outlier for the inspections (10) and after its removal from the analysis, neither body weight (estimate: -2.82; z-value: -0.51, df=1; p=0.89) or the environment (estimate: 0.05; z-value: 0.12; df=1; p=0.60) significantly influenced the number of inspections. Parental latency to leave acclimatisation zone variation was not affected by body weight or rearing environment (estimate: 0.12; z-value: 1.08, df=1, p=0.10).

Parental effect on offspring behaviour

For the offspring, the best-fit model after correcting for overdispersion indicated that offspring activity was only significantly influenced by parental activity, increasing with increased parental activity (estimate= 5.28, z-value: 2.61, df=1, p=0.009) (Fig. 2a-b; Table S4a). No other equivalent model was found (Table S4a).

The number of contacts with the novel object was significantly affected by the offspring environment (estimate= 0.48, z-value: 2.44, df=1, p= 0.008), parental environment (estimate= -0.31, z-value: -2.54, df=1, p= 0.01) and increased with the increased number of contacts by the parents (estimate=1.30, z-value: 7.16.57, df=1, p= 0.03) (Fig. 2c). No other equivalent model was found (Table S4b).

Differential methylation between parental environments

After quality filtering, approximately 378 million reads were retained (range: 6-25 million), averaging 12.5

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million reads per sample. Of those, approximately 61.1% were uniquely mapped reads to the reference genome. Overall bisulfite conversion efficiency was 99.5% (Table S1). In total, we identified 5.5 million cytosine sites of which 39,205 CpG sites matched the coverage requirements. The majority of the cytosines surveyed mapped gene bodies (71.12%) or intergenic regions (12.61%), while 2.61% were located on putative promoters.

Parental methylation profiles significantly differed in 1,854 methylated cytosines (DMCs) between environments. Unsupervised hierarchical clustering of these DMCs revealed clear distinctive methylation profiles between environments (Fig. S3). Most of these DMCs were overlapping gene bodies (69.69%), followed by intergenic regions (7.19%) and putative promoters (2.56%). The number of DMCs identified in 4,000 randomly generated parental groups was in all cases lower than 1,854 with an average of 247.3 (s.d. $\pm$158.5), indicating that the DMCs identified between environments was significantly higher than expected by chance ($P<0.001$).

Methylation patterns for parents and offspring

Of the 1,854 DMCs identified between parental environments, 724 (39.05%) maintained the same methylation profile (either hyper or hypomethylated relatively to the other environment) in the offspring reared in an environment matching their parent, but changed in the offspring reared in a mismatching environment and were classified as environmentally-induced epialleles. Of the remaining 1,130 DMCs, 98 (5.28% of the total) maintained the parental methylation patterns in the offspring regardless of their own rearing environment, of which five (scaffold: NW_016094248.1, position: 1049469; scaffold: NW_016094269.1., position: 1135514; scaffold: NW_016094316.1, position: 636543; scaffold: NW_016094324.1, position: 879262; scaffold: NW_016094376.1, position: 917192) had less than 10% change in methylation score across all experimental groups (classified as potentially intergenerational) (Table 1). Three of the five DMCs which maintained the parental methylation patterns on the offspring were significantly influenced by parental environment (Table 2; Fig. 3).

When analysed separately by environment-specific context, 30 DMCs in the offspring originated from enriched environment, and 19 in the offspring originated from poor environment maintained their methylation score relatively to its parents regardless of the offspring environment within less than 10% change.

Gene ontology

From the 1,854 DMCs found between parental environments (Fig. S3), 1,449 cytosines (78.15%) were neighbouring or overlapping a total of 728 genes. The most common biological processes affected were cellular process (84 genes, GO:0009987), metabolic process (39 genes, GO:0008152) and biological regulation (31 genes, GO:0065007) (Table S5a) and the main pathways were represented by 3-5 genes each (Table S5b).

Discussion

The transmission of environmentally-induced epigenetic modifications to the offspring could have important implications for evolution (Richards *et al.* 2017; Verhoeven *et al.* 2016) but has proven challenging to study in natural populations, due to the confounding effects of genotype-by-environment interactions (Berbel-Filho *et al.* 2019; Herman & Sultan 2016) and also to the unequal paternal and maternal contributions to epigenetic states (Soubry *et al.* 2014). By rearing the self-fertilising mangrove killifish *K. marmoratus* under controlled environmental conditions, we identified significant physiological (basal metabolic rate and cortisol levels), behavioural (neophobia, activity) and epigenetic differences among parents reared under two different levels of environmental enrichment, which influenced the offspring phenotypes.

Environmental enrichment influence on physiology and behaviour

Structural environmental enrichment has been used in captive fish as an attenuator of maladaptive or aberrant traits (Näslund & Johnsson 2016; Roberts *et al.* 2014; Roberts *et al.* 2011). Shelter-like structures (e. g. perforated logs, pipes) have generally beneficial effects, such as decreased metabolic rates (Fischer 2000; Millidine *et al.* 2006) and reduced plasma cortisol levels (Näslund *et al.* 2013), particularly in aggressive species

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(Näslund *et al.* 2013). Our results indicate that parental fish reared on enriched environments have lower basal metabolic rates and waterborne cortisol levels. While metabolic rate did not appear related to the rearing environment of the parents, the tight correlation between waterborne cortisol levels and basal metabolic rates in parental fish, suggests that parental individuals reared in enriched environments were less stressed and spent less energy to maintain basal metabolic rate than individuals reared in barren environments.

In fish, structural environmental enrichment tends to decrease activity, mainly due to increased sheltering (Moberg *et al.* 2011; Roberts *et al.* 2011; von Krogh *et al.* 2010), and exploratory activity and boldness tend to be positively correlated (Champneys *et al.* 2018; Mazué *et al.* 2015). Here, parents reared on enriched environments were slightly more active, but also more neophobic, than individuals reared in poor environments, suggesting no clear boldness-exploratory relationship in response to environmental enrichment in *K. mamoratus* . In this sense, plastic behavioural responses during the ontogeny of this species have been previously suggested, based on its variable responses to conspecific presence and simulated predation risk (2013).

Although behavioural effects of environmental enrichment on fish are well known (Jonsson & Jonsson 2014; Näslund & Johnsson 2016), the understanding of their potential inter- or transgenerational effects is limited, as most studies have focused on one generation (Näslund & Johnsson 2016). Our results indicate that offspring activity and neophobia were influenced by the parental rearing environment. In general, offspring from parents reared in enriched environments had higher activity levels, regardless of their own environment, suggesting a sustained parental effect on activity levels. Previous studies in this species suggested that life-history traits (offspring size), but not behaviour (exploration, boldness and aggression), were affected by the parental environment (2013). However, in mammals there is ample evidence of parental effects caused by environmental enrichment, where the offspring from enriched environments tend to be more exploratory (Dell & Rose 1987; Mychasiuk *et al.* 2012), have increased learning capacity and memory formation (Bygren 2013), than those reared in non-enriched environments. While in fish increased cognitive capacity due to environmental enrichment was known (Roberts *et al.* 2011; Salvanes *et al.* 2013), this is the first evidence of behavioural intergenerational (parental) effects.

Environmental enrichment effect on DNA methylation

Our results revealed strong effect of environmental enrichment on brain DNA methylation patterns, with 1,854 differentially methylated cytosines (DMCs) (neighbouring or on gene bodies of 728 genes) between genetically uniform parents reared on enriched and poor environments. Several studies have reported effects of environmental enrichment on brain growth (Näslund *et al.* 2012), cell proliferation (von Krogh *et al.*2010), cognitive capacity (Salvanes*et al.* 2013; Spence *et al.*2011), and gene expression levels (Evans*et al.* 2015) in fish. The functional analysis of the genes affected by DMCs in the parents showed that among the most relevant biological regulation processes affected by environmental enrichment, 33 annotated genes were involved on signalling transduction, which involves transmembrane signal reception and regulation of downstream cellular processes. Among metabolic processes, 14 genes affected by DMCs were involved on the transcription by RNA polymerase II, suggesting that those could regulate differential gene expression levels in the brain of individuals reared in enriched and poor environments. Regarding pathways, Wnt and cadherin signalling pathways were shown to be the most representative. Wnts are growth factors signalling proteins which interact cadherin-mediated cell adhesion through β-catenin (Nelson & Nusse 2004). These two signalling pathways are known to converge and have crucial roles on gene expression, cell migration, proliferation, adhesion, differentiation and renewal (Cadigan & Nusse 1997). In the vertebrate brain, Wnt/cadherin signalling pathways regulate the patterns of axes (anterior-posterior and dorsal ventral) of neural regions, promote size expansion and brain morphogenesis (Ille & Sommer 2005; Nyholm *et al.* 2007). Therefore, the differential methylation patterns between rearing environments could be related to differential transcription levels (or transcriptional noise reduction, depending of the genetic context) (Evans *et al.* 2015) in pathways of the brain development that could be the basis of the physiological and behavioural differences observed between enriched and poor reared individuals. Further research targeting specific epigenetic variants (e. g. using CRISPR/Cas9 (2017)), would expand the information how the specific epigenetic variants found here

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may be affecting gene expression, and the consequent effects for downstream phenotypes.

Due to epigenetic reprogramming during embryogenesis, only a small subset of epigenetic variants on the parents are likely to be transmitted to the offspring (Burggren 2016; Illum *et al.* 2018). DNA methylation changes during embryogenesis in *K . marmoratus* has a longer and later DNA methylation reprogramming period when compared to other fish and mammals (Fellous *et al.* 2018), which might represent an epigenetic window of environmental sensitivity. In the offspring, most of our results indicated a stronger effect of their own rearing environment than that of their parents on DNA methylation patterns. Thus, although there were clear effects of environmental enrichment on the brain DNA methylation patterns of the parents, these changes may not have influenced the germline to the same extent, suggesting limited potential for epigenetically-mediated parental effects being transmitted trans-generationally and/or scape epigenetic reprogramming. Yet, three DMCs maintained the same methylation patterns in both parents and offspring while additional DMCs maintained the parental methylation patterns in the offspring in a more environment-specific manner. To our knowledge, this is the first evidence of parental effects on the offspring epigenetic patterns caused by environmental enrichment in fish, extending previous results in mice, in which parental enrichment has shown to affect offspring brain weight, global methylation levels (Mychasiuk*et al.* 2012) and learning capacity (Arai & Feig 2011).

In summary, our results reveal behavioural and, limited but significant, epigenetic parental effects in the offspring caused by environmental enrichment which, if maintained, could have long-term evolutionary implications.

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Data accessibility

Sequence data (RRBS) that supports this study have been deposited in GenBank under accession code https://www.ncbi.nlm.nih.gov/bioproject/PRJNA506827. Raw data for figures 1-3 will be submitted to Dryad upon acceptance.

Authors' contributions

SC, WMBF designed the experiment. WMBF, NB performed the experiment. WMBF, analysed the data with contributions from CGL, SRT and DRB. WMBF, SC wrote the manuscript with participation of all authors. The authors declare no competing interests.

Ethics

All experiments were approved by Swansea University Ethics Committee (permit STU_BIOL_30484_-110717192024_3).

Table 1. Average methylation score differences (for parents) and for the offspring for methylated cytosines (DMCs) which maintained o methylation patterns (hyper or hypomethylated) relatively to the parental patterns (within +-10% change) Positive and negative values on methylation differences represent hyper or hypomethylation in relation to the enriched environment. "G", and "U" refer to gene bodies and unnanotated regions, respectively. Q-value is the p-value adjusted for the False Discovery Rate (FDR=0.05). Asterisks represent DMCs significantly affected by parental environment. Asterisks represent DMCs which were significantly influenced by parental environment.

Scaffold	Position	Genomic context	Meth diff 1st (E-P)	*Q*-value	Meth mean E>E	Meth me
NW_016094248.1	1049469	G	20.79	<0.001	94.89	84.46
NW_016094269.1	1135514	G	-22.81	<0.001	78.56	82.11
NW_016094316.1*	636543	U	-22.17	<0.001	29.96	47.85

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Scaffold	Position	Genomic context	Meth diff 1st (E-P)	Q-value	Meth mean E>E	Meth me
NW_016094324.1*	879262	G	26.35	<0.001	84.73	59.00
NW_016094376.1*	917192	G	-20.81	<0.001	22.36	34.30

Table 2. Results of most plausible binomial generalised models obtained by multi-model averaging approach for offspring methylation scores for the DMCs which maintained the parental methylation score (within 10% difference) across all offspring groups. Models are ranked according to the corrected Akaike Information Criterion (AICc), the difference with the most plausible fitting model (ΔAICc), and the Akaike weight (Wi), which represents the ratio between the weights of the best and competing models. Only models within two AICc units are shown.

	Df	t-value	P-value	AICc
NW_016094316.1.636543	*NW_016094316.1.636543*	*NW_016094316.1.636543*	*NW_016094316.1.636543*	*NW_016*
Parental environment	1	2.70	0.002	-6.81
NW_016094324.1.879262	*NW_016094324.1.879262*	*NW_016094324.1.879262*	*NW_016094324.1.879262*	*NW_016*
Parental environment	1	-1.83	0.01	2.66
NW_016094376.1. 917192	*NW_016094376.1. 917192*	*NW_016094376.1. 917192*	*NW_016094376.1. 917192*	*NW_016*
Parental environment	1	1.99	0.01	-10.78

FIGURE 1. Raw data for (a) cortisol levels, and environment and (b) cortisol levels and body weight (g); (c) basal metabolic rate and cortisol levels; (d) activity and environment; (e) number of inspections of novel object and body weight; (f) number of contacts with novel object and environment; for parental individuals. Green and orange represent parents reared in enriched and poor environments.

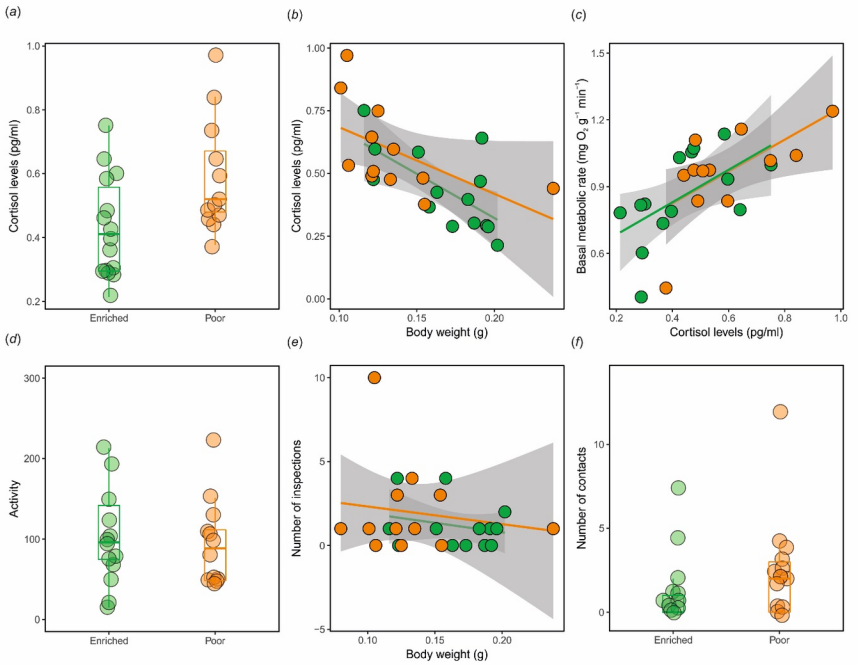

FIGURE 2. Relationships for behavioural metrics in the offspring. (a) Offspring and parental activity; (b) activity across experimental groups; (c) number of contacts with novel object and number of contacts

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by parent. Light green and orange represent offspring which matched environment relative to their parents (green enriched, orange poor). Dark green (enriched) and orange (poor) represent individuals which mismatched environment relative to their parents. Solid lines in (c) offspring environments.

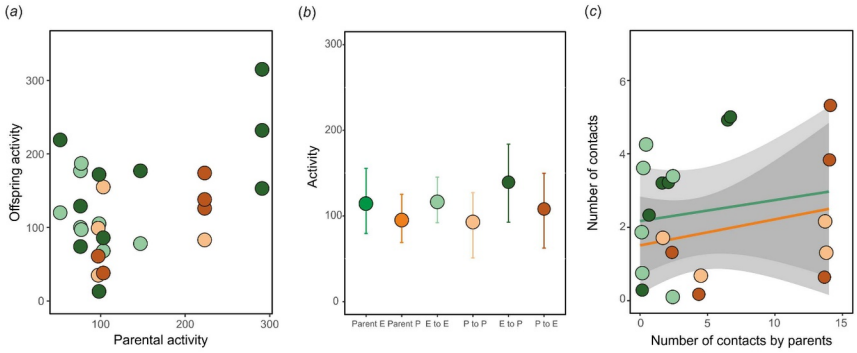
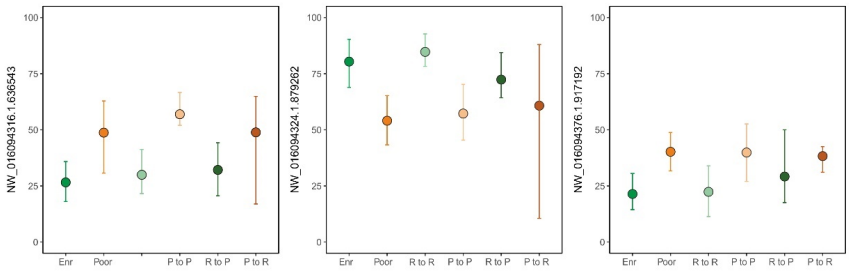


FIGURE 3 . Average methylation scores for DMCs which maintained parental methylation patterns and score (within 10% change) across all offspring groups. Light-coloured circles indicate the mean of individuals which mismatched environment relative to their parental environment. And dark-coloured circles indicate means of individuals which matched the environment of their parents.



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