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DNA Methylation Changes in the Sperm of Captive-Reared Fish: A Route to Epigenetic Introgression in Wild Populations

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Abstract

Interbreeding between hatchery-reared and wild fish, through deliberate stocking or escapes from fish farms, can result in rapid phenotypic and gene expression changes in hybrids, but the underlying mechanisms are unknown. We assessed if one generation of captive breeding was sufficient to generate inter- and/or transgenerational epigenetic modifications in Atlantic salmon. We found that the sperm of wild and captive-reared males differed in methylated regions consistent with early epigenetic signatures of domestication. Some of the epigenetic marks that differed between hatchery and wild males affected genes related to transcription, neural development, olfaction, and aggression, and were maintained in the offspring beyond developmental reprogramming. Our findings suggest that rearing in captivity may trigger epigenetic modifications in the sperm of hatchery fish that could explain the rapid phenotypic and genetic changes observed among hybrid fish. Epigenetic introgression via fish sperm represents a previously unappreciated mechanism that could compromise locally adapted fish populations.

Key words: **epigenetic inheritance, DNA methylation, domestication, *Salmo salar*.**

Captive rearing cause rapid phenotypic and genetic changes in fish after just one generation (Araki et al. 2007; Stringwell et al. 2014), and interbreeding between captive-reared and wild fish can lead to maladaptation to natural conditions (McGinnity et al. 2003) and reduced fitness of hybrids (Araki et al. 2007; Araki and Schmid 2010). Genome-wide analyses have explained the molecular basis of phenotypic variation associated with domestication in many species (Rubin et al. 2010; Wilkinson et al. 2013; Carneiro et al. 2014) but have failed to identify common loci or strong signals of selection associated with fish domestication (Ozerov et al. 2013; Mäkinen et al. 2015).

Captive-rearing in fish can result in epigenetic (methylation) changes in immune and stress-related genes (Le Luyer et al. 2017). Such epigenetic changes may respond to environmental stimuli and generate phenotypic variation by modulating gene expression and function. For epigenetic changes to be adaptive and evolutionary relevant, they would need to be transmitted to the offspring (Bossdorf et al. 2008; Youngson and Whitelaw 2008) and persist across generations (Charlesworth et al. 2017) to enable selection to act (Bollati and Baccarelli 2010).

Epigenetic signatures in the sperm of zebrafish are maintained in the embryo until the mid-blastula stage (Jiang et al. 2013). If the same is true for other fish, epigenetic changes in the sperm could facilitate adaptation to captivity. This would be relevant for salmonids which are farmed for food or reared in hatcheries for supportive breeding programmes (Consuegra et al. 2005; Kostow 2009), and for which captive rearing causes epigenetic changes in sperm (Gavery et al. 2018). Wild salmon affected by accidental escapes from fish farms or the deliberate stocking of hatchery fish often display genetic changes (Ciborowski et al. 2007; Glover et al. 2013), altered age and size at maturation (Bolstad et al. 2017), behavioral mismatch (Houde et al. 2010), and lower reproductive success (Theriault et al. 2011). Whether epigenetic changes also arise is not known.

We compared genome-wide DNA methylation profiles in the sperm of wild and hatchery-reared Atlantic salmon males and their offspring to identify potentially heritable hatchery-induced epigenetic modifications. Three groups of wild and hatchery-reared salmon from the River Allier (France) were analyzed (supplementary tables S1–S3, Supplementary Material online). Wild anadromous males (W) were caught in April 2015. Hatchery H1 males were mature parr (0^+) (2014 cohort) produced from reconditioned wild males and females maintained in the hatchery for two consecutive seasons, and hatchery H2 males were mature parr (0^+) (2014 cohort) from crossing females hatched and reared in the hatchery with wild reconditioned males. Both H1 and H2 were reared under identical conditions. Sperm from three males of each group was used to independently fertilize groups of pooled eggs of the same three wild females (fig. 1).

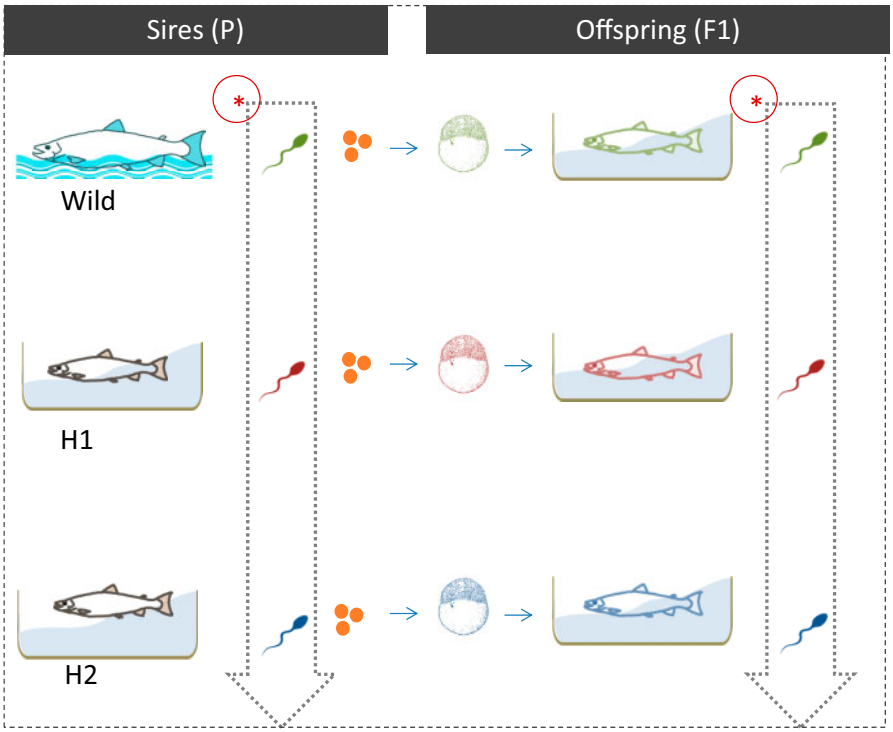

FIG. 1. Outline of the experimental design. Parental origin of wild (W) and hatchery (H1 and H2) groups and their offspring. Wild adult salmon were captured from the river Allier on their return to the spawning grounds, H1 salmon originated from crosses between reconditioned males and females (wild origin fish recovered and maintained in the hatchery for >1 year after spawning) and H2 salmon originated from crosses between reconditioned males and hatchery-born females (details in Supplementary Material online). Sperm of the three groups of parents (W, H1, and H2) was used to fertilize the eggs of three wild females to create the offspring. Sperm sampling points for methylation are indicated by a red asterisk.

Differentially Methylated Regions among Parental Groups

The results from MethylAction and MEDIPS concurred in the identification of differentially methylated regions (DMRs) and the loci affected by them. In total, 165,597 of the DMRs identified among all groups coincided between MethylAction and MEDIPs. Of the loci affected by those DMRs, 19,510 out of the 21,195 identified by MethylAction were also identified using MEDIPS (92.05%).

Pairwise methylome differences using MEDIPS identified 55 significant DMRs between W and H1, 22,563 between H1 and H2, and 298,980 between W and H2 (fig. 2), after applying a q value $\leq$0.05, and merging neighboring significant windows. These DMRs were overlapping or neighboring at 47,11,567 and 38,253 loci between W and H1, H1 and H2, and W and H2, respectively (supplementary fig. S4, Supplementary Material online).

Using MethylAction, DMRs identified from the simultaneous comparison between the three parental groups were classified as frequent if all the samples within each group had a consistent methylation status (hyper- or hypomethylated), or as other if they lacked within-group consistency (Bhasin et al. 2016). Only methylation patterns of frequent and statistically significant DMRs were considered further. Several of those DMRs (46,293) were consistently hypermethylated in H2 compared with W and H1 individuals (fig. 1B). In total, 21,195 loci were affected (overlapping or neighboring) by the +50,000 frequent DMRs identified among all groups.

Of the 55 DMRs identified by MEDIPS between W and H1, 43 (78%) occurred between W and H2. Of these, 35 completely overlapped with DMRs identified by Methylaction, and the rest were between 2 and 1,000 bp distance, all affecting the same loci. These 43 DMRs, shared by both hatchery groups and different from wild individuals, appear to be distinctive signatures of hatchery reared fish.

Methylation Comparison between Parental and Offspring Groups

Parents and offspring (mature male parr) showed significant differences in global sperm methylation enrichment scores only between H2 to W males ($W = 1.14 \pm 0.05$; $H1 = 1.25 \pm 0.10$; $H2 = 1.44 \pm 0.08$; ANOVA $F_{2,6}$=7.683, $P = 0.0221$; Tukey HSD test W-H1; $P = 0.42$; H2-H1; $P = 0.10$; W-H2; $P = 0.02$) (supplementary table S1, Supplementary Material online). Pairwise correlation coefficients of genome-wide coverage were on an average $r\sim0.60$ within groups (supplementary fig. S3, Supplementary Material online).

The first two components of a PCA of normalized total read counts of 1,000-bp sliding windows explained 96.49% of the variance (supplementary fig. S5, Supplementary Material online). PC1 explained 91.32%, and allowed differentiation

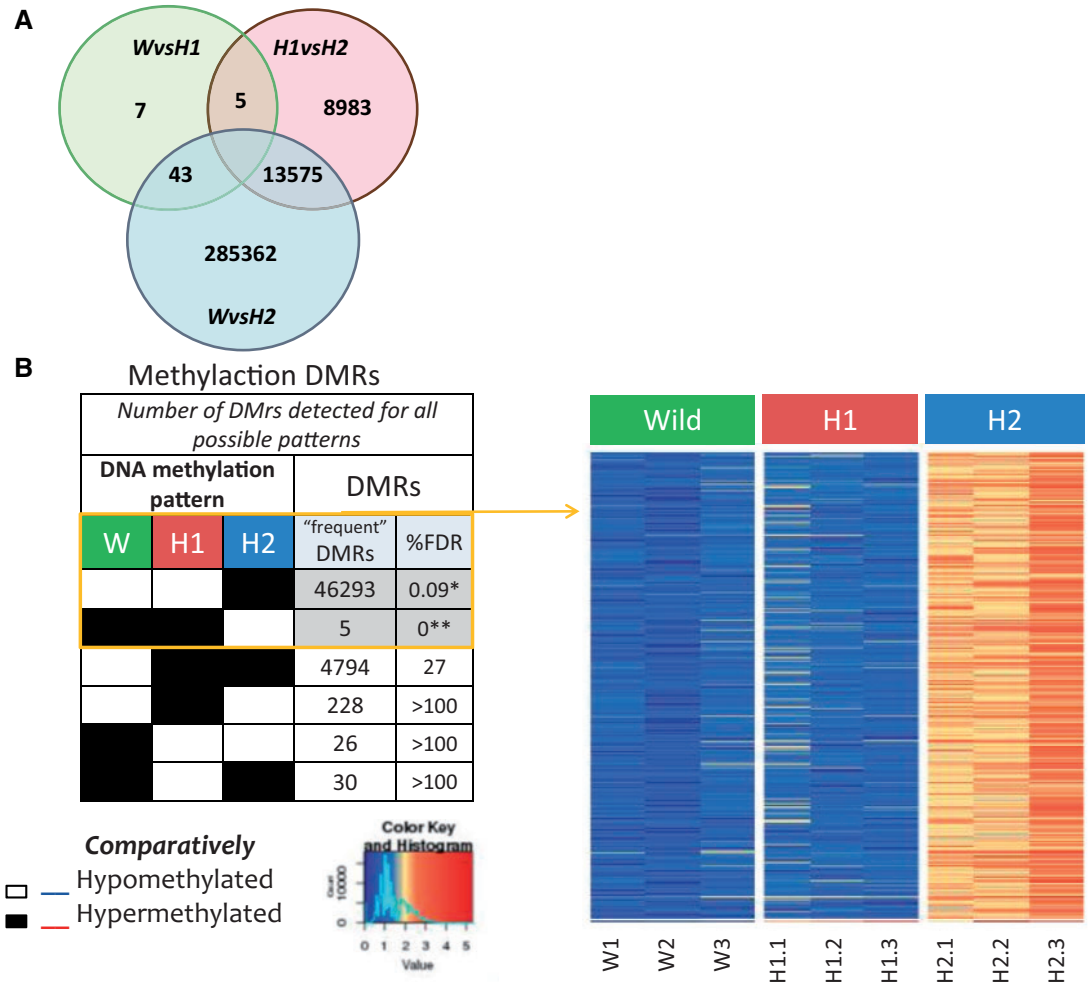

FIG. 2. Differentially methylated regions (DMRs). (*A*) DMRs found using MEDIPS showing unique and shared DMRs among groups comparisons. (*B*) DMRs found using Methylaction. *Table:* Number of DMRs detected for all possible patterns of hyper- (black squares) and hypomethylation (white squares). (**) Patterns with FDR <0.01; (*) Patterns with FDR <0.1. "Frequent" DMRs correspond to those where the methylation status of all the samples within a group is the same (3/3). *Heatmap*: Heatmap of normalized read count distributions for all "frequent" DMRs detected. Columns represent samples, and rows DMRs.

between parents and offspring ($F_{1,12}$=17.258; $P = 0.001$), and groups within generations ($F_{4,12}$=3.576; $P = 0.038$) (supplementary fig. S5, Supplementary Material online). PC1 scores differed significantly between the sperm of wild parents and their offspring (post hoc Tukey HSD test; $P = 0.02$), but not between the sperm of hatchery parents and their offspring (post hoc Tukey HSD test; H1-H1off = 0.98; H2-H2off = 0.19). This suggests that the hatchery environment (e.g., diet, confinement) had an impact on the methylation status of the wild offspring sperm, born and raised under those conditions. The comparisons of genetic diversity among parental groups and between parents and offspring based on 927 SNPs indicated that there were no significant genetic differences among groups (Fisher's exact test, $P = 1.00$), suggesting that differences in the genetic background are not responsible for the methylation differences observed.

For the 43 DMRs between wild and hatchery parents, all hatchery individuals (parents and offspring) clustered together with the wild offspring and separately from the wild parents (fig. 3*A* and *B*) (PC1 score Kruskal–Wallis χ^2=11.99, df = 5; $P = 0.034$). Of the DMRs involved, 12 overlapped with genes or putative promoters, and the remaining with distal intergenic regions (supplementary table S2*a*, Supplementary Material online). Affected genes showing differential methylation between W and H1 included the transcription factor *SOX-13-like* (Pevny and Lovell-Badge 1997), the neuronal migration protein *doublecortin-like*, expressed in fish olfactory bulb and optic tectum (Tozzini et al. 2012) and the small G protein signaling modulator 2-like, related to neural development in human and mice (Yang et al. 2007). Some of the DMRs differentiating parental groups maintained the same methylation pattern in the offspring and may not have been erased during early reprogramming (supplementary table S2*b*, Supplementary Material online). Of these, 2 were maintained between W and H1, 167 DMRs between W and H2 (overlapping genes or promoters of 73 genes), and 105 DMRs between H1 and H2, affecting 24 genes (supplementary fig. S6, Supplementary Material online). These results provide evidence that captive rearing induces

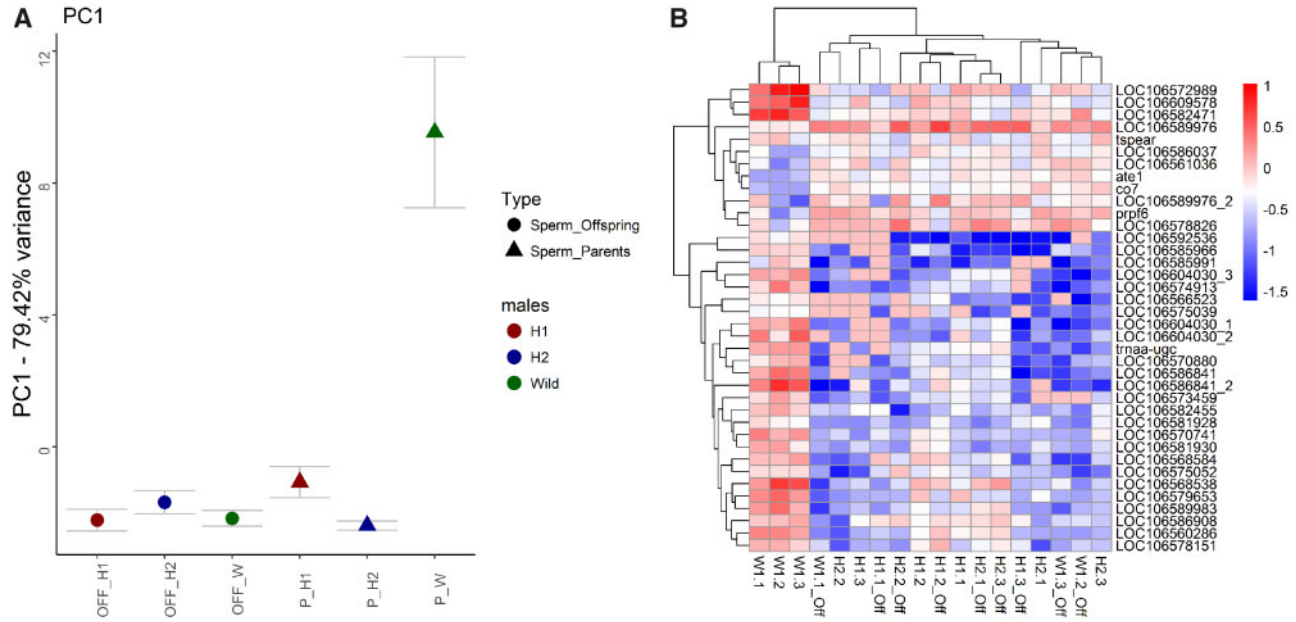

Fig. 3. Clustering of parents and offspring targeting those regions that were differentially methylated (DMRs) between hatchery and wild individuals in the parental group (hatchery reared fish distinctive signatures). (*A*) PCA using normalized total read counts of 1,000-bp sliding windows genome wide for the target regions. (*B*) Clustering and Heatmap of normalized read counts (log transformed) of hatchery reared fish distinctive signatures. Columns represent samples, and rows DMRs (the name of the closest/overlapping loci was assigned to each DMR).

rapid epigenetic (methylation) changes in salmon sperm, some of which can persist for at least one generation.

Variation in life history strategies (anadromous males vs. mature resident males) may account for some observed methylation differences between the sperm of wild and hatchery males (Morán and Pérez-Figueroa 2011). However, some of these DMRs likely characterize hatchery rearing, as methylation signatures among the offspring of wild fish reared under hatchery conditions were more similar to those of hatchery fish than to their wild parents. Furthermore, differences between parental H1 and H2 fish were stronger than those between the wild and H1 groups. The regions affected include genes encoding for coiled coil-type and PH domain proteins that regulate intracellular signaling networks and gene expression (Kutzleb et al. 1998) and changes to the PcG protein L3MBTL4 that regulates transcription and chromatin structure, and could underlie heritable changes in gene expression (Holoch and Margueron 2017). In addition, include the TATA-binding protein like (*tbpl1*), related to spermiogenesis and embryonic development (Akhtar and Veenstra 2011), that displays differential methylation between hatchery and wild coho salmon as well (Le Luyer et al. 2017).

In the parental groups, several regions differentially methylated between the W and H2 parents also differed between H1 and H2, with a high degree of conservation in their functions (i.e., ion transport, metabolic process, methylation; supplementary fig. S7, Supplementary Material online). Even if the hatchery parents (H1 and H2) had been born and raised under the same hatchery conditions, their parents had spent different time in captivity (the mothers of the H2 group were born in the hatchery, whereas both parents of the H1 group had a reconditioned origin, i.e., were born in the wild). Thus, as the main difference between the H1 and H2 groups was the origin of their mothers, the methylation signature shared between W and H1 fish, that differed from H2 salmon, could be the result of their maternal environment (Marshall and Uller 2007). This supports a role for maternal effects, potentially transgenerational, during fish domestication (Christie et al. 2016).

The sperm of parents and offspring displayed distinctive methylation profiles, suggesting that salmon PGCs could undergo a second reprogramming, as in mammals (Hackett and Surani 2013). However, some methylation marks can escape such resetting and result in epigenetic transgenerational inheritance, even if only for a small number of epialleles (Daxinger and Whitelaw 2012). Here, six of the common DMRs shared between W/H2 and H1/H2 were maintained in the next generation, including the transcription factor EB-like, expressed during embryo development (Lister et al. 2011), the SPT20 protein, part of the SAGA complex (Nagy et al. 2009), and the corticotropin-releasing factor receptor 1-like, involved in social stress and aggression (Backström et al. 2015). This indicates a potential mechanism for heritable phenotypic responses to captive rearing, although further confirmation of the functional relevance of these methylation changes, including more populations, is warranted. Given the important contribution that mature male parr make to the reproduction of Atlantic salmon in the wild (Garcia-Vazquez et al. 2001; Garant et al. 2003), interbreeding of hatchery-reared mature parr with wild females could potentially result in epigenetic changes in wild populations.

Our findings suggest that at least part of the sperm epigenetic modifications associated with captive-rearing remain in

the offspring beyond developmental reprogramming and could affect embryo fitness and performance. Hatchery-reared males could cause epigenetic introgression into wild populations after just one generation if they interbred with wild females, potentially disrupting local adaptation (Garcia de Leaniz et al. 2007). The importance of this mechanism in adaptation can be better advanced by further analyses of the candidate genes/DMRs identified and by analyzing the reversibility of these changes following the cessation of hatchery rearing.

Gene expression changes appear associated with captive-rearing (Christie et al. 2016), but the role of epigenetics is only starting to be considered (Nätt et al. 2012). Epigenetic modifications induced by captive-rearing can influence fitness in first-generation hatchery salmonids, but their inter- or trans-generational persistence has not been resolved (Le Luyer et al. 2017). Here, we provide the first evidence of stability of these epigenetic modifications between generations and suggest that sperm-mediated epigenetic introgression could explain the rapid changes experienced by wild fish when they interbreed with hatchery-reared fish (Araki et al. 2009).

Materials and Methods

Sperm from three randomly chosen individuals from each of the male groups (W, H1, and H2) was used to fertilize batches of 300 ova pooled from three wild females (100 ova/female) (supplementary methods, Supplementary Material online). About 125 µl of sperm from each male were pipetted onto Whatman FTA Classic cards for methylation analyses. The remaining sperm was used for sperm quality assessment (Caldeira et al. 2018). Fertilized eggs from each of the parental crosses were reared under identical hatchery conditions for 8 months until maturity, when sperm from eight random juvenile males from each of the offspring groups was analyzed for DNA methylation.

DNA was extracted from 6 mm pieces of each FTA card with a GenSolve kit (GenTegra LLC, Pleasanton), using QIAamp Blood Mini kit (QIAGEN Group) for DNA purification, and the re-extracted to increase DNA recovery.

Methylated DNA Enrichment and Analyses

DNA was fragmented to <1,000 bp by incubating dsDNA with NEBNext dsDNA Fragmentase (New England BioLabs Inc.) for 30 min. Fragmented DNA was cleaned-up using QIAquick spin columns (QIAGEN Group). Methylated DNA was isolated from fragmented whole genomic DNA using MethylMiner kit from Invitrogen (CA). Methylated fragments were eluted using a high salinity elution buffer (2,000 mM NaCl). As a control, gDNA was spiked with 1 pg of synthetic methylated and nonmethylated DNA fragments (Methyl Miner kit, Invitrogen) before MBD-enrichment. Enriched (MBD2-captured) and unbound DNA fractions were amplified using specific primers for each spike-in control (supplementary fig. S1, Supplementary Material online). Additional enrichment quality checks were performed (supplementary fig. S2, Supplementary Material online).

Methylated-enriched DNA was quantified (Qubit), diluted to 0.2 ng ml^{-1}, and used for library preparation using Nextera-XT kit (Illumina Inc., CA). Libraries were indexed for multiplexed paired-end sequencing (2×125 bp read length) on an Illumina HiSeq 2500 platform (Illumina Inc., CA).

After quality check using FastQC/0.11.2. and adaptor trimming (Trimmomatic/0.33, Bolger et al. 2014), reads were aligned to the Atlantic salmon genome (ICSASG_v2) using Bowtie2 (Langmead and Salzberg 2012). MEDIPS (Lienhard et al. 2014) was used for quality control, genomic coverage estimation, and to detect pairwise DMRs among and between groups. We used MethylAction R (Bhasin et al. 2016) to further assess sperm methylome differences among groups. In both cases, a window size of 50 bp and *q* value cutoffs of 0.05 after FDR multitest correction were applied (*P* value [Benjamini–Hochberg] <0.05). BAM files were imported to SeqMonk v1.37.1 (Andrews 2015) for visualization of mapped regions and PCA. To compare the results of MethylAction and MEDIPS, adjacent 50-bp significant windows were merged. BEDTools (Quinlan and Hall 2010) intersect was used to assess overlapping DMRS and enable the comparison between tools. Loci affected consisted of those with DMRs overlapping or neighboring them.

BAM files from the genome-wide MBD enrichment sequencing for a total of nine parental male fish were processed using the AddOrReplaceReadGroups utility in Picard Toolkit (Picard 2018). Indel targets were identified using Target Creator in GATK 4.0.11.0 (DePristo et al. 2011) and variants were exported into Golden Helix SNP & Variation Suite 8.3.3. SNPs were filtered using the LD pruning utility in Golden Helix using default options (Supplementary Material online). Genepop 4.7.0 (Rousset 2008) was used to test for global genotypic differentiation, using Fisher's exact test.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

S.C. and C.G.L. designed the study; D.R.B. carried out the sampling and analyses; E.V., C.G.L., S.C., and M.C. obtained the funding; H.S. carried out the SNPs analyses; all authors contributed to the interpretation of the results; and C.G.L. and E.V. contributed to the writing of the paper which was written by S.C. and D.R.B. and checked and approved by all the authors.

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