Aquaculture Reports 39 (2024) 102405

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Methylation profile of the testes of the flatfish *Solea senegalensis*

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ARTICLE INFO

Keywords:
Solea senegalensis
Flatfish
Aquaculture
Epigenetics
Testes

ABSTRACT

In aquaculture production, fish phenotypes are influenced by a combination of external and internal factors, while the underlying mechanisms often involve distinct DNA methylation patterns. These play a fundamental role in regulating gene expression in response to environmental shifts. The Senegalese sole (*Solea senegalensis*) holds significant potential in aquaculture due to its high commercial importance. However, its production faces several challenges, including the inability of F1 soles to reproduce. In our study, we analysed the methylation patterns in the testicular DNA of 12 male *S. senegalensis* individuals from four distinct groups, which differed in rearing origins (wild and F1 soles) and sexual maturity stages (mature and immature). We employed reduced representation bisulfite sequencing to identify significant methylation differences among studied groups. The differential methylation analysis showed a substantial disparity between the F1 and wild groups. This difference was evident in both the number of different methylated CpGs (DMCpGs) and methylation levels, highlighting a distinction in groups reared under different conditions. The comparison between immature and mature wild groups revealed notable differences, suggesting that the testes methylation profile undergoes few changes in wild individuals during sexual maturity. These differences in methylation between wild and hatchery-born and reared offspring imply epigenetic modifications triggered by rearing in captivity and by the absence of natural *stimuli* such as temperature fluctuations. We further annotated the DMCpGs based on their position and co-localization with genes in the Senegalese sole genome. Our analysis revealed that the methylation differences observed in DMCpGs were more pronounced in intron regions, with higher methylation levels. Conversely, the methylation differences in exon and promoter areas were more subtle. Upon annotation, we discovered that these DMCpG sites were located near or within various transcription factors. Our comprehensive analysis sheds light on the intricate methylation profile of *S. senegalensis*, focusing on genes associated with reproductive traits, particularly those related to sex determination (SD) systems and spermatogenesis. Overall, we observe a trend toward higher methylation levels in DMCpGs when comparing F1 individuals to wild groups, as well as immature to mature wild groups. Hence, sexual maturity and place of origin resulted in marked methylation differences, which could potentially affect the expressions of genes involved in both development and reproduction of farmed males. This analysis explores the gene-regulating effects of this epigenetic mechanism providing valuable insights into the variations in methylation found in the testes of Senegalese sole.

Abbreviations: CpGs, CpG sites; DMCpGs, Different methylated CpGs; F1, Filial 1; RRBS, Reduced Representation Bisulfite Sequencing; DNMTs, DNA methyltransferases; SD, Sex determination; S.D., Standard deviation; PCR, Polymerase chain reaction; QPCR, Quantitative polymerase chain reaction; W, Wild; MF, Mature F1; MW, Mature Wild; IF, Immature F1; IW, Immature Wild; TSS, Transcription Start Site; TTS, Transcription Termination Sites; MDS, Multi-dimensional scaling; LogFC, Log_2 Fold Change; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene ontology; GnRH, Gonadotropin-releasing hormone; MAPK, Mitogen-activated protein kinase; PKA, Protein kinase A.

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https://doi.org/10.1016/j.aqrep.2024.102405
Received 28 March 2024; Received in revised form 9 September 2024; Accepted 1 October 2024
Available online 15 October 2024

1. Introduction

Epigenetic changes serve as mediators of environmental adaptations, responding to both internal and external stimuli. Among epigenetic mechanisms, DNA methylation plays a fundamental role in regulating gene expression in response to environmental shifts, such as changes in salinity, temperature, or exposure to toxins (Law and Holland, 2019). This mechanism involves the conversion of cytosine to 5′-methylcytosine by DNA methyltransferases (DNMTs), while Ten-Eleven Translocation (TET) methylcytosine dioxygenases (*TET1*, *TET2* and *TET3*) promote demethylation of the methylated cytosines. These modifications predominantly occur at CpG, CHG or CHH (where H stands for A, T or C). DNA methylation is almost exclusively found in CpG dinucleotides, with the cytosines on both strands being usually methylated, influencing transcription cascades, genomic imprinting, chromatin structure, and genome stability (Firmino et al., 2017; Illingworth and Bird, 2009; Jaenisch and Bird, 2003; Robertson, 2005; Zhang et al., 2023). While DNA methylation has been extensively linked to gene expression, its precise mechanism remains elusive. Traditionally, higher methylation levels have been associated with gene repression. However, positive associations between DNA methylation and gene expression have also been observed. Decreased expression has been associated with hypermethylation of CpG sites in the promoter and gene body regions. Conversely, increased expression has been linked to hypermethylation of CpG sites downstream of the gene body (Qin et al., 2021; Salem et al., 2022). The effects of DNA methylation on gene expression extend beyond promoter regions, as evidenced by negative correlations between methylation of the first intron and gene expression (Yang et al., 2016; Anastasiadi et al., 2018; Mukiibi et al., 2022; Salem et al., 2022). Furthermore, methylation patterns exhibit significant tissue specificity, contributing to the maintenance of cell type differentiation and functional specialization within the organism (Li and Zhang, 2014; Ambrosi et al., 2017).

While epigenetics holds great promise for improving aquaculture, practical applications remain scarce. Several studies have linked epigenetic mechanisms with commercially important traits in aquaculture species including salmonids and shellfish (Gavery and Roberts, 2017). For instance, the impact of epigenetics on fish sex determination has been extensively described (Piferrer et al., 2019). Modifications in DNA methylation have been associated with temperature-induced sex differentiation (Navarro-Martín et al., 2011) in sea bass and half-smooth tongue sole and show to have transgenerational effects. The latter has also served as a model to study the role of epigenetic regulation in environmental sex differentiation (Shao et al., 2014).

The Senegalese sole (*Solea senegalensis*, Kaup 1858) is an emerging aquaculture flatfish species of high commercial importance. However, Senegalese sole aquaculture faces several challenges. High mortality during larval metamorphosis stages, the lack of effective strategies to control infectious diseases (tenacibaculosis, photobacteriosis, vibriosis), and the inability of soles born and reared in captivity (F1) to reproduce are significant barriers. These F1 males exhibit poor reproductive performance compared to wild specimens due to their absence of courtship behaviour (Riesco et al., 2019), lacking prespawning chasing or paired synchronised spawning (Carazo et al., 2011). Additionally, other reproductive issues include reduced fertilization capacity in F1 males, involving low sperm volume and quality (Forné et al., 2011; Chauvigne et al., 2017). Despite these challenges, progress has been made in addressing these issues temporarily through the development of artificial fertilization and cryopreservation protocols, as well as nutritional approaches to improve reproductive status (Agulleiro et al., 2006; Guzmán, Ramos, et al., 2009;Chauvigne et al., 2017; Riesco et al., 2017). Hormonal induction in F1 sole has yielded some success, with females responding to gonadotropin-releasing hormone agonists (GnRHa) slow-release implants and males to human chorionic gonadotropin (hCG) or homologous recombinant gonadotropins injections (Chauvigne et al., 2017; Chauvigneé et al., 2018). While hormonal therapies offer a potential solution to these reproductive dysfunctions, the results are still not optimal, indicating a need for continued research in this area.

The methylation status of sperm DNA may have a substantial impact on the sperm quality. Alterations in DNA methylation have indeed been associated with male infertility, observed also in fish (Liu et al., 2019; Woods et al., 2018). Previous studies in the striped bass sole have examined the sperm quality of both wild-born and F1 males using sperm samples, however they did not find any significant differences in methylation between the wild and F1 groups (Woods et al., 2018). Endocrine differences during the spawning period, such as those related to the dopaminergic pathway (Guzmán et al., 2009; Carazo et al., 2011; Chauvigne et al., 2017) or sperm DNA methylation status, could also impact sperm quality (Gunes et al., 2016; Woods et al., 2018). Additionally, miRNAs have been shown to play important roles in the control of reproductive functions in male fertility and in early embryo development. For instance, miRNAs like miR-17a, miR-181–5p, and miR-206–3p are known to promote myogenesis, and their expression levels are influenced by temperature (Campos et al., 2014). A previous by Riesco et al. (2019) study observed the differential expression in miR-let7-d and miR-200a-5p which are correlated with spermatogenesis and the dopaminergic pathway respectively, when comparing wild and F1 sole males (Riesco et al., 2019). Addressing these challenges is crucial for the growth of *S. senegalensis* aquaculture and the development of effective breeding programs (Benzekri et al., 2014; de la Herrán et al., 2023).

In aquaculture production, fish phenotypes are influenced by multiple external and internal factors, including temperature, nutrition, toxins, salinity, or population density. The underlying mechanism often involves distinct DNA methylation patterns (Labbé et al., 2017). Among the various techniques used to study methylation in aquaculture, Reduced Representation Bisulfite Sequencing (RRBS) stands out as a popular choice. RRBS allows for genome-scale DNA methylation analysis at the single nucleotide level, targeting relevant functional elements such as CpG islands and promoter regions (Bock et al., 2010; Squazzo, 2015; Bertucci et al., 2021). Previous studies in *S. senegalensis* have explored the relationship between temperature and the epigenetic regulation of myogenesis (Campos et al., 2013; Carballo et al., 2018). Investigations into the effects of diet on genome methylation in larvae have also been conducted (Canada et al., 2016). High temperatures applied during sex-differentiation cause masculinization in several fish species, associated to the repression of the aromatase *cyp19a1a* gene which is involved in estrogen synthesis (Baroiller and D'Cotta, 2016). One of the mechanisms of this temperature masculinisation is through the hyper-methylation of the *cyp19a1a* promoter seen also in a number of flatfish species (Navarro-Martin et al., 2011). This factor should be considered, especially given the interest in cultivating female populations due to their larger size. Therefore, in the Senegalese sole production it is also necessary to consider the temperature effects on the sex-ratio, particularly since females are the desired sex due to their enhanced growth-rate (Carballo et al., 2018). Daily temperatures of ~22°C and of ~19°C at night applied to larvae soles from 1 to 97 days post-hatching, caused ratios of 70 % female ratios with higher estradiol levels (Blanco-Vives et al., 2011). This initial approach in Senegalese sole focuses on the role of methylation, aiming to identify the potential impact of methylation on specific genes associated with maturation and reproduction while the observed methylation differences could be potentially linked to captive conditions.

Given the behavioural-environmental factors contributing to reproductive disruption observed in Senegalese sole aquaculture, it is crucial to understand the role of epigenetics, by identifying methylation differences on specific genes associated with maturation and reproduction including genes involved in spermatogenesis, as well as measuring the impact of captivity. In this study, we utilized RRBS to obtain meaningful DNA methylation profiles from Senegalese sole testes samples. These samples included both wild individuals and F1 offspring, representing both sexually mature and immature stages. We meticulously examined

methylation differences and annotated them to specific genes based on their genomic co-localization in the Senegalese sole genome. This analysis focuses on investigating DNA methylation differences as a potential regulatory mechanism influencing gene expression. By comparing F1 males with their wild counterparts, we can explore how this regulatory mechanism varies between the two groups. Such insights may have implications for reproductive performance in Senegalese sole.

2. Materials and methods

2.1. Gonadal tissue collection

Testes samples for methylation analyses were collected from 12 male *S. senegalensis* specimens. These samples came from two distinct populations: an artificially bred population (F1) at the Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), and a wild population from the southwest of Spain (Bay of Cádiz, Andalusia). Sampling of mature individuals was conducted through the spawning season, between the months of May and June during the years 2021 and 2022. Immature individuals were collected consistently throughout the entire year. The F1 males were born and raised in captive conditions, reaching sexual maturity under these stable conditions. The differences are not confined to temperature alone, but extend to other factors such as feeding patterns, population density, and oxygen levels, among others. These environmental factors within the captive settings could potentially serve as epigenetic modulators. Despite reaching sexual maturity, these F1 individuals exhibit an inability to reproduce effectively. Consequently, we considered both the origin of the individuals and their maturity level, as we delve into aspects related to sexual maturity and reproduction. As a result, we identified four distinct groups for our study: mature wild males (MW), immature wild males (IW), mature F1 males (MF), and immature F1 males (IF). The study included a total of 6 adult males (4 wild adults and 2 F1 adults) and 6 immature males (4 wild juveniles and 2 F1 juveniles). The stage of sexual maturation was assessed at the time of sampling, using the size and weight of the individuals as indicators. Mature males were identified phenotypically and collected during the spawning months. Their maturity was further verified through gonadal dissection, conducted both before and after the procedure, and by evaluating whether sperm production was occurring. The adult male average length and weight were 54.02 ± 1.84 cm (mean ± S.D.) and 1858.00 ± 444.31 g, respectively; juvenile males had an average length of 25.63 ± 1.31 cm and an average weight of 219.70 ± 28.62 g. As for the F1 specimens, adult males had an average length of 53.55 cm ± 1.06 cm and an average weight of 1990 g ± 25.24 g. The juvenile F1 males had an average length of 24.5 cm ± 0.10 cm and an average weight of 217 g ± 28.62 g. The sampled fish were transported to the laboratory in oxygenated containers. Prior to dissection, the fish were anesthetized using clove oil at a concentration of 90–100 mg/L. After dissection, gonad samples were removed and preserved in RNAlater® TissueProtect (Qiagen) at −80 C until DNA extraction. This experimental procedure adhered to the guidelines set by the European Union Council (86/609/EU) and was approved by the Ethics Committee of the University of Cadiz, Spain.

2.2. DNA extraction

The genomic DNA from the testes was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. After extraction, the DNA underwent purification and size selection using the HighPrep™ PCR Clean-up System (MAGBIO). To assess quality and concentration, we used a NanoDrop™ 2000 C spectrophotometer and a Qubit 4 fluorometer (ThermoFisher), respectively.

2.3. Reduced-representation bisulfite sequencing libraries preparation

Bisulfite-converted reduced representation genomic DNA libraries were prepared from previously obtained DNA samples using the Diagenode Premium Kit, following the manufacturer's instructions. Briefly, 100 ng of DNA from each sample was digested with the restriction enzyme *MspI* for 12 hours. This step was followed by an end preparation, adaptor ligation and size selection. Sample concentration was quantified individually in duplicate by qPCR, and samples with similar concentrations were pooled together in groups of six. These pooled samples were then subjected to bisulfite conversion. To monitor bisulfite conversion efficiency monitoring, both methylated and unmethylated control DNA were included. Subsequently, RRBS libraries were enriched by PCR and purified using the AMPure XP Bead-Based Reagent from Beckman Coulter. For quality control, an assessment of the RRBS libraries was performed by running each library pool on an Agilent 2200 Bioanalyzer (Agilent Technologies) to verify the fragment size distribution. Finally, the libraries were quantified using a Qubit fluorometer, pooled, and sequenced (150 bp paired-end sequencing) on the Novaseq Illumina platform.

2.4. Reduced Representation Bisulfite Sequencing data processing

For quality control of raw sequencing data, we utilized FastQC 0.12.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (March 2024) (Wingett and Andrews, 2018). Subsequently, the raw data underwent processing following the methodology outlined in Mukiibi et al. (2022). We removed Illumina sequencing adapters, low-quality base calls (Phred quality score < 20), and short reads (<20 bp) using Trim Galore 0.6.61. (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) (March 2024). To facilitate RRBS alignment, we performed in silico bisulphite conversion of the *S. senegalensis* reference genome (https://ftp.ensembl.org/pub/rapid-release/species/Solea_senegalensis/GCA_919967415.2/ensembl) (March 2024) (de la Herrán et al., 2023). The alignment of RRBS sequence reads to the converted reference genome, as well as methylation profiling, were carried out using Bismark 0.22.3. Additionally, the same Bismark script was employed for methylation calling of each cytosine from the alignments, resulting in CpGs coverage files for further analyses.

2.5. Differential methylation data analyses

Previous methylation coverage output files were used as input files for differential methylation analysis, performed as described by Mukiibi et al. (2022) using Bioconductor edgeR 3.42.2 R package (bioconductor.org/packages/edgeR). CpG sites always methylated/unmethylated and those with low coverage (< 8 reads per sample) were not considered. To explore the methylation patterns, we employed multi-dimensional scaling (MDS) analysis using the R prcomp function. Additionally, we ensured comparability by normalizing the CpG read counts within the sample libraries, scaling them to the average sample library size. For differential methylation analysis between studied groups, we conducted likelihood ratio tests to compare methylation rates. Significantly differentially methylated CpG sites were identified using a false discovery rate (Benjamini–Hochberg correction) threshold of 0.01. Our study involved four distinct groups: wild mature males, wild immature males, F1 mature males, and F1 immature males. Based on the number of samples, we conducted several differential methylation profile comparisons: F1 vs. Wild (F/W), Mature F1 vs. Mature Wild (MF/MW), Immature F1 vs. Immature Wild (IF/IW), Immature Wild vs. Mature Wild (IW/MW), and Immature F1 vs. Mature F1 (IF/MF). Finally, we visualized the methylation differences between studied groups using volcano plots and heatmaps created with the R package gplots v3.1.1. After identifying and profiling the CpG sites, we performed a functional annotation step using HOMER software v4.1 (http://homer.ucsd.edu/homer/) (March 2024) (Heinz et al., 2010). This annotation indicated their positions relative to gene promoters, exons, introns, or transcription start/termination sites, based on the senegalese sole annotation file (https://ftp.ensembl.org/pub/rapid-release/species/

Solea_senegalensis/GCA_919967415.2/ensembl/geneset/2022_08/) (March 2024) (de la Herrán et al., 2023).

3. Results

3.1. RRBS data and methylation analysis

Sequenced RRBS libraries from male gonadal tissue yielded on average 42.45 ± 23.92 million (mean ± S.D., n = 12) raw paired end reads. After filtering, 41.73 ± 24.27 million reads remained. In total, over 400 million read pairs were analysed using Bismark to identify methylated cytosine residues. The mapping efficiency was 62.43 % ± 0.05, with an average unique alignment rate of 30.43 ± 2.24 % (Table 1). Following quality control, each sample profiled a mean of 0.77 ± 0.39 million CpG sites, of which approximately 41.95 ± 0.24 % were methylated to some extent. Cytosines in the CHG or CHH context exhibited very low levels of methylation.

In the F1 samples, a higher percentage of methylated cytosines in CpG sites was observed compared to the wild samples. Additionally, the immature wild group exhibited a greater proportion of methylated cytosines in CpG sites when compared to its corresponding mature group. We analysed the average number of CpGs across the 21 chromosomes in the *S. senegalensis* genome (Supplementary Table 1 and Fig. 1 & 2).

3.2. Methylation profiling

A multi-dimensional scaling plot of the overall methylation levels shows differentiation between F1 and wild groups, with the mature wild group showing the clearest differences (Fig. 3). We were unable to have more than 2 replicates for the F1 groups due to their limited number, which might have brought some bias in the analysis. We intend to repeat this study with larger sample size. Considering the number of samples, we conducted five differential methylation profile comparisons: F1 vs. Wild (F/W), Mature F1 vs. Mature Wild (MF/MW), Immature F1 vs. Immature Wild (IF/IW), Immature Wild vs. Mature Wild (IW/MW), and Immature F1 vs. Mature F1 (IF/MF). We tested a total of 364,862 CpGs with sufficient coverage (>8 counts) and variation in methylation levels across samples. Only CpG sites with a false discovery rate <0.01 (corrected using the Benjamini–Hochberg method) were considered significantly differentially methylated. The differential methylation analysis identified a total of 4181 CpG sites that were significantly differentially methylated (referred to as DMCpGs) across the five comparisons: F1/W (387), MF/MW (657), IF/IW (818), IW/MW (553), and IF/MF (1766) (Supplementary Table 2 and Fig. 4 & 5).

Table 1
Average alignment quality and methylated cytosines context in *S. senegalensis* for the four study groups: Mature wild male, Immature wild male, Mature F1 male and Immature F1 male. **Notes:** The average alignment quality (Mean ± S.D. %) is shown for unique, multiple, and total alignments. The average methylated cytosines number observed (Mean ± S.D. %) is shown for CpG, CHG, and CHH context (where H = C, T, or A).

Average alignment (Mean ± S.D. %)	**Mature Wild**	**Immature Wild**	**Mature F1**	**Immature F1**	**Sample Overall**
Unique alignment	30.72 ± 3.01	30.79 ± 1.96	29.21 ± 1.87	32.79 ± 0.10	30.43 ± 2.24
Multiple alignment	4.33 ± 1.40	4.6 ± 1.61	5.76 ± 0.40	6.36 ± 0.65	5.00 ± 1.40
Total alignment	35.05 ± 3.96	35.39 ± 3.27	34.97 ± 1.47	39.15 ± 0.42	38.84 ± 3.15
Average methylated C (Mean ± S.D. %)	**Mature Wild**	**Immature Wild**	**Mature F1**	**Immature F1**	**Sample Overall**
CpG context	27.60 ± 0.21	38.45 ± 0	51.10 ± 0.11	68.50 ± 0	41.95 ± 0.24
CHG context	0.80 ± 0	0.90 ± 0	0.95 ± 0	1.10 ± 0	0.91 ± 0
CHH context	0.73 ± 0	0.78 ± 0	0.80 ± 0	0.90 ± 0	0.78 ± 0
Unknown (CN or CNH)	1.95 ± 0.01	2.50 ± 0.01	2.95 ± 0	4.10 ± 0	2.66 ± 0.01
Number CpG sites identified (Mean ± S.D. M)	**0.68 ± 0.47**	**1.01 ± 0.62**	**0.74 ± 0.01**	**1.31 ± 0.27**	**0.96 ± 0.48**
Number CpGs >8 counts coverage (Mean ± S.D. M)	**0.54 ± 0.29**	**0.89 ± 0.54**	**0.65 ± 0**	**1.13 ± 0.21**	**0.77 ± 0.39**

3.3. DMCpG genomic annotation

The DMCpG sites were annotated based on their co-localization with genes, following the annotation of the Senegalese sole genome. They were further classified based on their position within the context of the gene, including near gene promoters/transcription start site (TSS, ±1kbp), exons, introns, intergenic regions, or transcription termination sites (TTS, ±1kbp). When comparing F1 and wild groups, most DMCpGs were in intronic and promotor regions, and 34 % of the DMCpGs annotated as intronic regions were specifically located in the first intron. The average methylation difference, measured as log2fold (logFC), was 0.95 ± 0.28, with the highest value being 2.05. In the comparisons made between subgroups, where we must consider the lack of replicates, the average differences between comparisons ranged between 0.96 ± 0.03 and 2.11 ± 0.09. The highest and lowest rates are observed in IF/MF and IW/MW, respectively.

In the comparison between F1 and wild groups, the identified DMCpGs were associated with approximately 300 different genes. Additionally, when comparing immature and mature wild-type groups, the annotation results revealed more than 400 genes annotated to the identified DMCpGs. Similarly, most of the subgroup's comparisons revealed DMCpGs associated with about 400 genes. Upon annotation, certain DMCpG sites were found to be located near or within various transcription factors (*sox2, sox10, sox11a, GATA6*).

KEGG and GO enrichment analysis for differentially methylated genes (Aleksander et al., 2023; Ashburner et al., 2000) were performed in Novogene's Novomagic Platform (https://eu-magic.novogene.com/) (March 2024) (data not shown). Considering the previously mentioned issues related to this species' farming, the KEGG enrichment analysis results for the F1/W and IW/MW groups comparison were particularly interesting. This was due to the presence of enriched pathways such as the MAPK and GnRH signaling pathways.

While acknowledging the limitations due to the lack of replicates, our findings reveal CpG methylation disparities associated with genes involved in sex determination and reproduction. Notably, F1 individuals exhibit higher methylation levels when compared to the wild-type, and there are variations in methylation between mature and immature wild-type individuals, with the latter showing higher methylation. Further research is needed to understand the potential impact of methylation on these biological pathways and their proper development in immature and F1 individuals, complemented by expression data.

4. Discussion

Currently, there is a specific reproductive dysfunction unique to F1 males of *S. senegalensis* requiring studies to explore the underlying culturing factors (Imsland et al., 2004; Carrington and Secombes, 2006; Martín et al., 2019; Piferrer et al., 2019). Our study aimed to uncover potential differences in testes from wild individuals and those born and reared in captivity, by analysing testicular DNA methylation patterns. We therefore compared the methylation patterns between wild (W) and F1 (F) groups, as well as between sexually immature (I) and mature (M) individuals within the same origin. Significant methylation differences

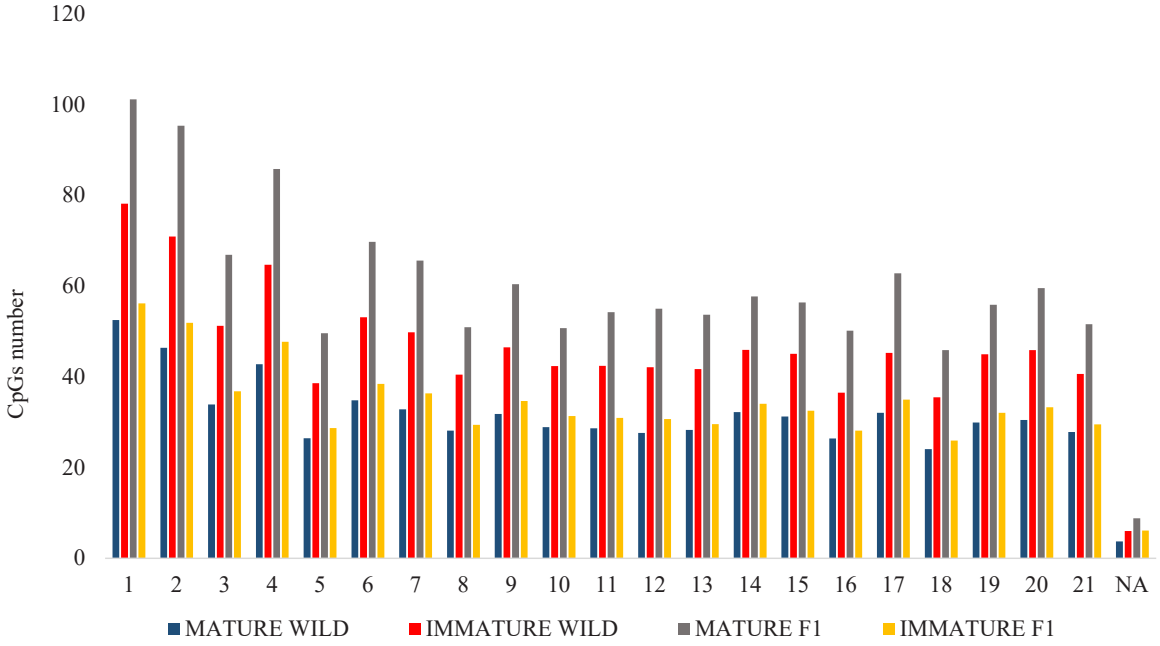

Figure 1

Fig. 1. Average number of CpGs (n · 10^3) per chromosome in the four *S. senegalensis* study groups (Mature wild male, Immature wild male, Mature F1 male and Immature F1 male). **Notes:** The average number of CpG sites is expressed in thousands. X-axis numbers represent the 21 chromosomes in *S. senegalensis*. "NA" represents CpGs located in scaffolds not associated to chromosomes.

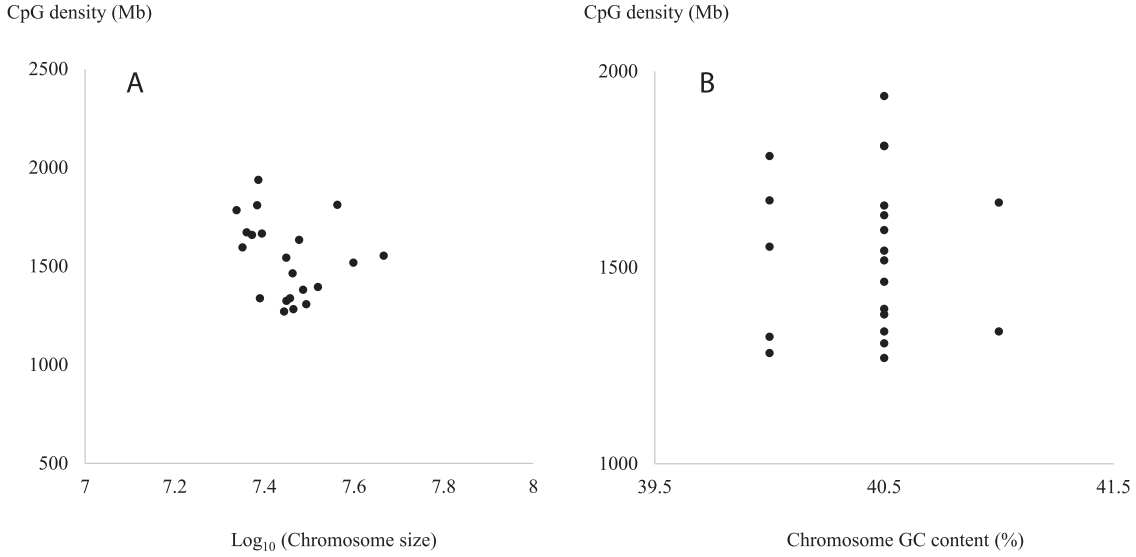

Figure 2

Fig. 2. CpG density distribution (per Mb) based on chromosome sizes (A) and GC% content in chromosomes (B). **Notes:** The dots represent the observed density across 21 chromosomes in *S. senegalensis*.

were found through DNA bisulfite conversion. The most notable findings revealed higher methylation levels in F1 individuals compared to the wild type, as well as differences in methylation when comparing wild individuals at different stages of maturity. We located the differential methylation regions to explore the possible effects on gene regulation. Although we did not perform transcriptome analysis here, our findings are promising, uncovering potential candidate genes implicated in males' development and reproduction. Nevertheless, we are conscious that our sample size for the farmed juveniles and adults was small and might have reduced certain variability. Here, we focused our approach on the general comparison between F1 and wild individuals, and the immature and mature wild groups. We believe that our study, despite its limitations, makes a valuable contribution to the field and hope that it will pave the way for future research with larger sample sizes.

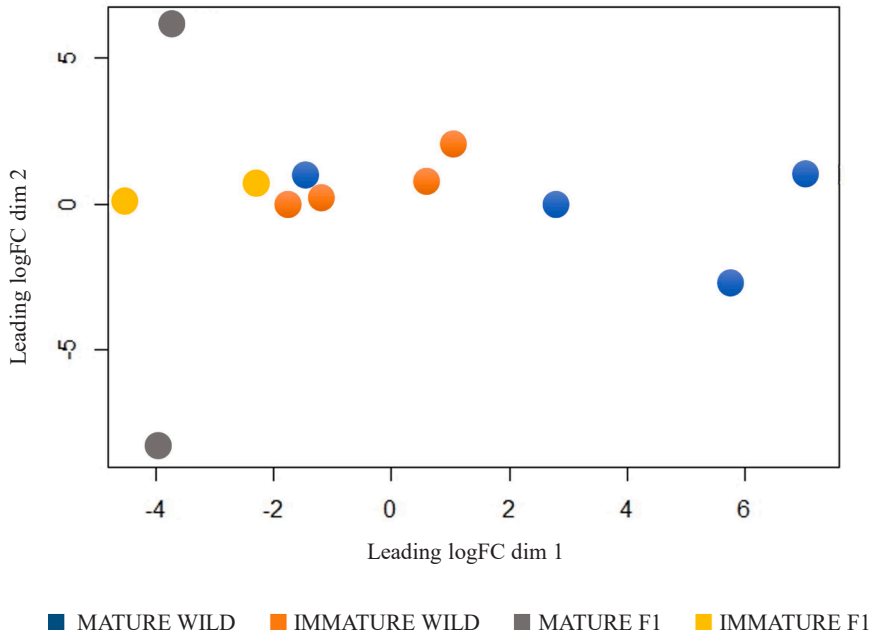

Figure 3

Fig. 3. Multidimensional scaling (MDS) plot generated using the edgeR package showing differences in methylation profiles between *S. senegalensis* samples. **Notes:** The four study groups (Mature wild male, Immature wild male, Mature F1 male and Immature F1 male) are represented in four different colours, while coloured dots represent different samples, corresponding to its categorical group. The distance between the sample (dots) indicates similarity between the *S. senegalensis* samples of each group.

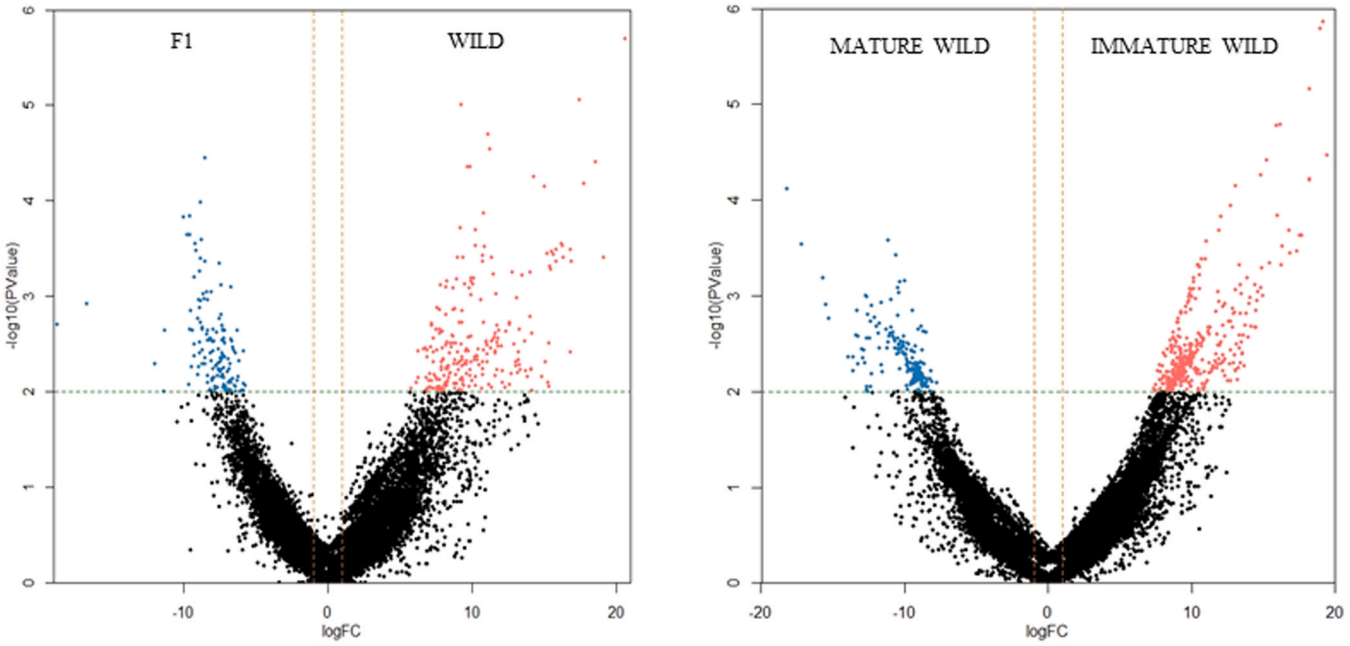

Fig. 4. Volcano plots for differential methylation between study groups. **Notes:** The volcano plots were created using the R package gplots v3.1.1. The different volcano plots represent the different performed comparisons with n>3 in *S. senegalensis*: F1/Wild and Immature Wild Vs. Mature Wild. Fold change value and p-value are represented in the x-axis and y-axis, respectively. When the established significance limits are exceeded, the samples represented by dots are displayed coloured red (positive fold change values) and blue (negative fold change values).

4.1. CpG identification

Our results show consistent methylation patterns across all chromosomes. Interestingly, no significant correlation was found between methylation levels and the number of CpG sites or CG content. The number of CpGs identified was slightly higher in the MF group followed by the IF group. Across chromosomes, the CpG count remained relatively consistent, with a higher number observed in the larger chromosomes (e.g. Chr1, Chr2 and Chr4), as expected ($r = 0.8$; $r^2 = 0.65$). CG content did not significantly impact CpG density, which remained

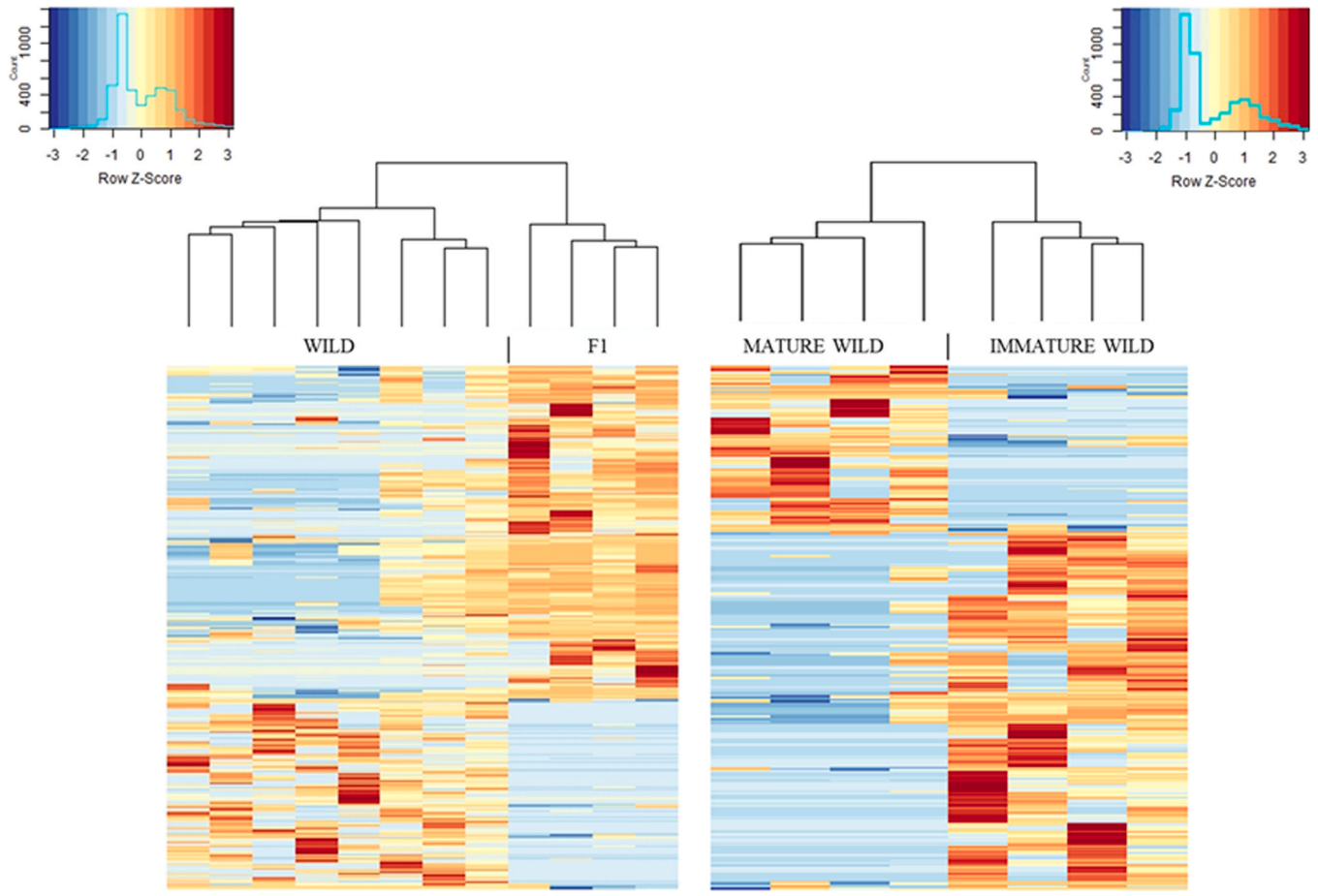

Fig. 5. Heatmaps showing clustered samples based on differentially methylated CpG sites when performing comparisons between the study groups with n>3 in *S. senegalensis*: F1/Wild and Immature Wild Vs. Mature Wild. **Notes:** The clustering of the methylation profiles was performed using the R package gplots v3.1.1. The colour scale from blue to red corresponds to the methylation values (fold change) ranging from −2–2. The histogram inside the colour scale represents the number count of CpG sites.

similar across all chromosomes. A similar scenario was described by Han and Zhao (2008) who studied CpG island density in a comparative analysis of several fish genomes (tetraodon, stickleback, medaka, zebrafish). In zebrafish, no correlation was found between identified CpG density and GC content, a feature attributed to the similar GC content across chromosomes (Han and Zhao, 2008). Similarly, in *S. senegalensis*, only minor differences in CpG density exist between chromosomes, all of which exhibit a similar GC content (~40 %).

4.2. Differential methylation analysis

The differential methylation analysis revealed significant differences across all comparisons. Notably, in the general comparison between F1 and wild individuals, the F1 group exhibited a higher number of up-methylated CpGs (246) compared with the wild group (141). In addition, F1 showed higher logFC values (0.8 ± 0.16 vs. 1.04 ± 0.29). The IW/MW comparison showed a larger number of DMCpGs (553) but similar logFC values (average logFC value = 1.04 ± 0.22). These differentially methylated sites, visually represented in heatmaps (Fig. 5), show samples cluster based on differentially methylated CpG sites. The differences between immature and mature wild males are apparent. A similar pattern was observed in the case of F1 individuals, although further study with a larger sample size is necessary. These findings suggest that the testes methylation profile undergoes changes in individuals during the transition from immaturity to sexual maturity. These differences in methylation between wild and hatchery-born F1 offspring suggest epigenetic modifications triggered by rearing in captivity as previously observed on Nile tilapia and Pacific salmon (Le Luyer et al., 2017; Podgorniak et al., 2022). Sole breeders raised in captivity do not exhibit the reproductive behaviours observed in wild breeders, especially prespawning chase or paired synchronized spawning, which has been pointed out to be the primary cause of reproductive failure (Carazo et al., 2013). While there are more reproductive issues besides the lack of courtship, certain aspects of the rearing conditions from larval development such as nutrition and endocrine aspects, and specially the higher temperature and/or lack of thermal fluctuation may affect the methylation profile of F1 individuals (Riesco et al., 2019).

4.3. DMCpGs annotation

The identified DMCpGs were annotated based on their position and co-localization with genes in the Senegalese sole genome (de la Herrán et al., 2023). The mapping efficiency was of 62.43 % which falls in the range of RRBS libraries (El Kamouh et al., 2023) and perhaps from the quality of the genome assembly. Most CpG islands are located within gene promoters (approximately 70 %) (Cedar and Bergman, 2012; Moore et al., 2013). Hypermethylation of promoter-associated CpG islands is generally associated with transcriptional silencing and genomic imprinting (Antequera, 2003; Han and Zhao, 2008; Deaton and Bird, 2011). Similarly, methylation of transcription start/end regions and first introns are also linked to decreased gene expression (Anastasiadi et al., 2018). Here, the genomic context of the DMCpGs was generally similar to that of all the CpGs analysed. A similar scenario is described in Nile tilapia by Podgorniak et al. (2022). Regarding the

methylation differences observed in DMCPGs, logFC values consistently showed higher methylation levels in intron regions, while methylation differences in exon and promoter areas were smaller (lower logFC values). Hence, it suggests that the methylation landscape in intron regions of sole might be important, as it may impact gene expression, as observed in previous studies (Anastasiadi et al., 2018).

The annotation of DMCpGs based on their gene proximity resulted in the identification of over 700 genes showing differential methylation in those comparisons with n>3: F1/W and IW/MW (Supplementary Table 2). Genes linked to reproduction, metabolism, growth, immunity, and epigenetics (Podgorniak et al., 2022) are particularly interesting and directly relevant to aquaculture. Specifically, in the Senegalese sole since females exhibit faster growth, while F1 males experience reproductive dysfunction (de la Herrán et al., 2023). Therefore, genes directly or indirectly associated with reproduction and sex determination, or sex differentiation are especially relevant. Among the annotation results, we highlight genes linked to functions of interest in *S. senegalensis* aquaculture, including development, and functionality of testes (Cross et al., 2020). The results of the KEGG enrichment analysis for the F1/W and IW/MW comparisons (Supplementary figures 1 & 2) are particularly noteworthy due to the enrichment of the MAPK and GnRH signaling pathways.

Given the higher methylation observed in F1 when compared to the wild group, and in the immature wild group when compared to its mature counterpart, it is crucial to consider the CpG methylation differences associated with genes previously linked to sex determination and reproduction (de la Herrán et al., 2023), gonadal differentiation (Zhu et al., 2019), and general development (Hagiwara, 2011; Portela-Bens et al., 2017). Similarly to our results on sole, Anastasidi et al., 2019 analyzed differences in sea bass DNA methylation between early domesticated and wild fish, identifying fifteen genes in sea bass that showed similarities to the genes affected in salmon domestication (Le Luyer et al., 2017). Several of these genes were observed in our study including protocadherins (*pcdh8* and *pcdh11*), phosphatases (*dusp1 and dusp6*) and homeobox genes (*hoxa11a, hoxb1a, hoxb2a, hoxc9a and hoxc12a*) among others (Supplementary Table 2). These results may suggest a similar phenomenon in the Senegalese sole, demonstrating that the farming environment influences methylation in the initial stages of domestication.

In addition to the commonly differentially methylated regions, it is crucial to highlight genes that have been previously studied in this species and are related to reproduction. The annotation results revealed DMCpG sites located near or within various transcription factors, including *sox* (*sox2, sox10, sox11a*) (Hu et al., 2021) and *dmrt2* gene, which play essential roles in testes development and functionality (Portela-Bens et al., 2017; Cross et al., 2020).

4.4. Key sexual differentiation and sperm maturation genes linked to differences in the methylation levels between Wild and F1 groups

In fish, a highly diverse range of chromosome SD systems and several master SD genes have been previously reported (Cioffi et al., 2017; Martínez et al., 2014). These include transcription and growth factors (*dmy, sox2, sox3, gsdf, amh)* (Herpin et al., 2021; Matsuda et al., 2002; Takehana et al., 2014), as well as genes related to the steroidogenic pathway (*bcar1, hsd17b1*) (Bao et al., 2019; Koyama et al., 2019; Pan et al., 2019). The master sex determinant in *S. senegalensis* is the Follicle-stimulating hormone receptor (*fshr*) gene (de la Herrán et al., 2023). We found no difference in methylation levels between our comparisons. Although *fshr* was not detected following the functional annotation of DMCpGs, we located the genes annotated that surround the *fshr* region (chromosome 12: 9372–11,072 kb) (Supplementary Table 2).

Specifically, when comparing F1/W groups, the aryl hydrocarbon receptor nuclear translocator *(arnt)* gene showed higher methylation in the wild group (a promoter located up-methylated CpG). *Arnt* plays a role in the regulation of transcription of downstream genes such as the cytochrome P450 family of genes (CYPs), while *cyp19a1a* is involved in estrogen production and female sex differentiation) in several flatfish species (Van Nes and Andersen, 2006; Portela-Bens et al., 2017; Shankar et al., 2020). Additionally, two DMCpGs between IW and MW groups (one up-methylated and one down-methylated) were identified in intergenic positions close to *aqp1a.2*, a gene involved in mediating egg hydration (Tingaud-Sequeira et al., 2008) and sperm motility activation (Zilli et al., 2009). These findings further support the hypothesis that methylation plays a crucial role in spermatogenesis.

Furthermore, *sox2* along *sox10* exhibited higher methylation in immature wild fish compared to the mature counterpart. In fish, *sox* genes play essential roles in embryogenesis and ontogenesis, and they are linked to the brain-pituitary-gonadal axis of reproduction (Anitha and Senthilkumaran, 2021). In our study, several DMCpGs were annotated to various *sox* family transcription factor genes (including *sox1a, sox2, sox6 sox8a, sox9a, sox9b, sox10, sox11a*). Additionally, other transcription factors (*tbx3a, tbx3b, GATA6*) also showed differential methylation. However, further validation is necessary.

Additionally, three up-methylated intergenic CpGs in the IW/MW comparison were annotated to *GATA6*, a transcription factor involved in gonadal cell proliferation, differentiation, and endoderm development (Liu et al., 2016). In *C. semilaevis*, *GATA6* plays an essential function in gonadal development and reproduction. Notably, a study by Liu et al. (2016) evaluated DNA methylation in *C. semilaevis* males, females, and pseudo males. Although no significant differences were observed in the overall methylation levels, the pattern of methylation sites differed. Interestingly, pseudo males exhibited higher expression of the *GATA6* gene compared to females. These findings suggest that the unequal methylation states among these sites may influence transcription and expression, leading to a dimorphic pattern between sex groups (Liu et al., 2016). While our results demonstrate methylation differences between immature and mature males, further analysis is necessary to study their connection to gene expression.

The *dmrt* genes (double-sex and mab-3-related transcription factor) are important due to their role in testes development and functioning (Winkler et al., 2004; Zhu et al., 2019). Similar to *sox6*, which is noted in the comparisons between subgroups, the *egr1* transcription factor also plays an important role in *dmrt1* regulation in the testes (Lei and Heckert, 2002). *Egr1* was annotated in the F1/W groups comparison to a first-intron located up-methylated CpG. While *dmrt1* is specifically expressed in the gonads and is required for testes differentiation (Lei and Heckert, 2002), *egr1* is suggested to play a critical role in *dmrt1* expression promoter function. Additionally, DMCpGs were annotated to transcription factors *dmrt2a* and *dmrt2b* in the IW/MW and IF/MF groups, respectively. *Dmrt2a* showed a down-methylated CpG located in the first intron. In the flatfish *Cynoglossus semilaevis, dmrt1, dmrt2* and *dmrt3*, are involved in male germ cell maturation and gonadal differentiation. Their expression is negatively correlated with the methylation level of the associated promoter (Zhu et al., 2019). These results point towards methylation differences in these reproduction-related genes between wild-type and dysfunctional F1 individuals, in addition to a possible difference in *dmrt2a* expression linked to differences in methylation relative to maturity in the case of wild-type individuals.

Overall, the observed methylation differences, especially those involving genes linked to gonadal maturation and development, should be considered in sole. These differences should be substantiated through expression analysis, to understand their role in testes differentiation and development, and the potential implications of gene repression. Particularly, the increased methylation in intron regions could lead to the inhibition of expression of these specific genes, as previously evidenced (Anastasiadi et al., 2018).

In our research, the mitogen-activated protein kinase (MAPK) pathway consistently appeared in the enrichment analyses when associating gene sets with statistically significant pathways. These findings revealed distinct methylation patterns across all compared groups.

Specifically, when comparing wild males to F1, the MAPK pathway showed an association, involving up-methylated CpGs. Additionally, the GnRH signaling pathway was also observed in the IW/MW groups for up-methylated genes. Gonadotropin-releasing hormone (GnRH) stimulates the mitogen-activated protein kinase (MAPK) cascade via the protein kinase A (PKA) signalling pathway in tilapia. This activation is involved in gene expression, particularly for GPα and LHβ, although not for FSHβ (Gur et al., 2001; Wei et al., 2020). The GnRH signalling pathway was observed in specific groups of up-methylated genes. These findings underscore differences in methylation patterns within the MAPK pathway during the transition from immaturity to maturity in individuals. Furthermore, these findings suggest that methylation may contribute to the reproductive determinant process and differs between F1 males and wild-type males.

5. Conclusions

We have conducted a thorough analysis of the methylation profile in the testicular tissue of *S. senegalensis*, with a specific focus on genes associated with reproductive traits. This includes those related to SD systems and spermatogenesis, encompassing key genes such as transcription and growth factors, as well as genes involved in the steroidogenic pathway. Our findings reveal consistent higher methylation levels in F1 individuals when compared to wild individuals, as well as higher methylation in immature versus mature individuals in the case of wild fish. These findings are particularly significant given the reproductive dysfunction observed in F1 male soles. The methylation differences observed between them and their wild counterparts, especially in genes linked to sexual differentiation, provide valuable insights into the reproduction of S. senegalensis. The absence of courtship in F1 individuals, coupled with these results, provides a preliminary molecular basis (novel findings to our knowledge) that could account for the behavioural differences between these two classes. This is entirely in line with the distinct cultivation conditions of both classes, with F1 experiencing a constant temperature versus the temperature fluctuations encountered by wild individuals.

The implications of these findings are profound. They not only enhance our understanding of the reproductive biology of this species, but also have the potential to inform and improve aquaculture practices and fish welfare. Future studies should consider the breeding of this species at varying temperatures given the potential impact of this factor on methylation. The possible repercussions on gene expression, as an epigenetic effect, should also be considered in future transcriptomic analysis. By shedding light on the epigenetic factors influencing sole reproduction, we can develop more effective strategies to support the health and productivity of captive-bred populations, thereby contributing to the sustainability and success of aquaculture operations.

Funding

This work was financially supported by the Junta de Andalucía [FEDER P20-00938; FEDER CCMM-00014]; and the University of Cádiz [R93REC/2019]. Diego Robledo acknowledges is funded by the Axencia Galega the Innovación (GAIN, Xunta de Galicia) as part of the Oportunius programme, and also by BBSRC Institute Strategic Grants to the Roslin Institute [BBS/E/20002172, BBS/E/D/30002275, BBS/E/D/10002070 and BBS/E/RL/230002A]. The open access fee was co-funded by the QUALIFICA Project (QUAL21-0019, Junta de Andalucía).

CRediT authorship contribution statement

Daniel Ramirez: Writing – original draft, Software, Methodology, Formal analysis. **Carolina Peñazola:** Methodology. **Robert Mukiibi:** Software, Formal analysis. **María Esther Rodríguez:** Supervision, Methodology. **Laureana Rebordinos:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Diego Robledo:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation. **Helena D'Cotta:** Formal analysis, Data curation.

Declaration of Competing Interest

Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. In addition, authors declare that the manuscript is original, and has not been published before, and is not currently being considered for publication elsewhere. They also confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

Acknowledgments

The authors acknowledge Manuel A. Merlo and Silvia Portela-Bens for their technical help in the collection of samples. They also thank Manuel Manchado from the IFAPA "El Toruño", Cádiz (Spain) and the Servicio Central de Investigación en Cultivos Marinos (UCA) for providing samples for the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102405.

Data Availability

No data was used for the research described in the article.

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