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Sex differences in DNA methylation and expression in zebrafish brain: a test of an extended 'male sex drive' hypothesis

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ABSTRACT

The sex drive hypothesis predicts that stronger selection on male traits has resulted in masculinization of the genome. Here we test whether such masculinizing effects can be detected at the level of the transcriptome and methylome in the adult zebrafish brain.

Although methylation is globally similar, we identified 914 specific differentially methylated CpGs (DMCs) between males and females (435 were hypermethylated and 479 were hypomethylated in males compared to females). These DMCs were prevalent in gene body, intergenic regions and CpG island shores. We also discovered 15 distinct CpG clusters with striking sex-specific DNA methylation differences. In contrast, at transcriptome level, more female-biased genes than male-biased genes were expressed, giving little support for the male sex drive hypothesis.

Our study provides genome-wide methylome and transcriptome assessment and sheds light on sex-specific epigenetic patterns and in zebrafish for the first time.

Keywords: zebrafish; sexual dimorphism, male sex drive, masculinization, brain, RRBS, CpG site, CpG island, DNA methylation, RNA-Seq

Background

Phenotypic differences between the two sexes of a species are referred to as sexual dimorphism. Striking morphological differences between large unsightly females and minute parasitic males in anglerfish are one of many spectacular examples of such sexual dimorphism [1]. Sexual dimorphism manifests not only in morphological traits, but also in physiological and behavioural traits. In the organisms that do not have sex chromosomes, males and females are derived from an identical or nearly identical genome. Sex-specific gene expression (i.e., expression exclusively in one sex) or, more commonly, sex-biased gene expression (i.e., expression predominantly in one sex), is one of the main proximate causes of phenotypic differences between the sexes in these organisms. [2, 3].

The most obvious phenotypic difference between the sexes is the development of the female or male gonads. Not surprisingly, female and male gonads usually differ remarkably in the sets of highly expressed genes [4-7]. Sex-biased gene expression, although less pronounced than in the gonads, has been also found in many somatic tissues, such as liver, spleen, muscles and brain [4, 8, 9]. Brain is the second most sexually dimorphic organ after gonads. The sexually dimorphic expression in the brain is of particular interest, because it is likely to underpin behavioural differences between the sexes [10].

Males of many species exhibit a broad spectrum of sex-specific behaviours (e.g. courtship, male contest) and other phenotypic traits (e.g. ornaments, weaponry) contributing to their reproductive success. These traits can affect outcomes of male-male competition and mate choice, and thus are usually under strong sexual selection [11]. It has been hypothesized that strong sexual selection acting on males results in

genomes that progressively accumulate genetic innovations that affect male traits at a rate faster than for female-specific traits or sex-neutral traits. This effect was termed "male sex drive" [12]. Male sex drive can lead to genome masculinization, which can be manifested in two main ways: 1) the existence of larger number of genes in the genome that have male-specific effects than genes that have female-specific effects, and 2) faster rates of evolution of male-biased genes than female-biased and sex-unbiased genes, leading to larger divergence of male-biased genes at the levels of DNA, RNA and protein [3, 13]. A masuculinized transcriptome can be, therefore, characterized by a higher proportion of male-biased genes than that of female-biased genes.

Since tissue-specific transcription of genes is regulated, at least in part, by DNA methylation [14, 15], one might also expect sexually dimorphic DNA methylation to be observable in body tissues. Indeed, sex-biased DNA methylation was reported for saliva, blood and brain samples of humans [16-19]. Sex-specific methylation patterns have been also observed in the brains of mice [20] and chicken [21]. A recent study on mice revealed that DNA methylation plays a key role in suppressing masculinization in the developing brain and allows the brain to preserve its original feminized form in female animals [22]. These studies suggest that the methylation process contributes to the development of sexual dimorphism. However, there is still limited knowledge of the interplay between sex-biased DNA methylation and sex-biased gene expression [23]. In general, at least in vertebrates, high levels of DNA methylation in the promoter regions, are linked with lower levels of gene transcription [24]. Accordingly, the male sex drive hypothesis can be extended to include the methylome, generating two predictions: 1) male-biased gene expression is associated with hypomethylation of the male genome, and 2) particular genes that are highly

expressed in the males will have lower methylation levels in males than in females (i.e., are hypomethylated in males).

In this study, we use zebrafish brains to address the male sex drive hypothesis at both methylome and transcriptome levels. First, to determine global DNA methylation patterns of male and female brains we performed reduced representation bisulfite sequencing (RRBS). The characteristics of genome-wide methylation of the zebrafish brains, without differentiating between male and female samples, are presented in [25, 26]. In summary, the zebrafish RRBS genome contains higher levels of CpG methylation than mammalian RRBS genome and that high level of global CpG methylation is not exclusive to the zebrafish brain but is also found in other tissues (such as liver). Furthermore, consistent with recent base-resolution studies in zebrafish we found low levels (< 3.0%) of non-CpG methylation in zebrafish brain [25]. Further, the pooled samples (Male1 vs. Male2 and Female1 vs. Female2) showed high positive correlation on common CpG sites (covered by 10 or more sequenced reads) suggesting negligible variation between the pooled samples. Globally, Male1, Male2, Female1 and Female2 showed CpG methylation of 75.0%, 71.6%, 69.4% and 70.0% respectively (as indicated by Bismark alignment [27]). Further, the male and female methylomes also showed high positive correlation (Pearson's correlation coefficient r = 0.98) with each other [25]. Taken together, these results indicate that, overall, the DNA methylation patterns between female and male adult wild type zebrafish brains are very similar. However, some site-specific differences are still present between males and females.

Next, we generated whole genome-expression profile and integrated this information with the whole-genome scale methylome data to investigate the relationship between differentially methylated sites and corresponding gene expression levels. Further, we integrate data from previous studies to place our findings in broad context. We tested whether male-enriched expression and male hypomethylation supports the male sex drive hypothesis in zebrafish brains.

Results and discussion

Male and female brains show site-specific differential methylation

Although the global DNA methylation patterns of male and females were similar, the hierarchical clustering of the male and female methylomes (on common CpGs with high coverage) indicated the existence of some site-specific differences in methylation (Figure 1). Therefore, we aimed to identify these differences. Differential methylation analyses between male and female samples were performed on the 232581 common CpG sites (covered by 10 or more reads in all four RRBS libraries). We identified 914 CpG sites that were significantly differentially methylated between the male and female brains (with a cut-off q-value of < 0.01 after multiple test correction and with stringent cut-off percent methylation difference of $\geq 25\%$ for a CpG site). These sites were termed differentially methylated CpG sites (DMCs) (Additional file 2). The DMCs did not show significant sex bias in terms of the distribution of hypo and hypermethylated bases between sexes (Figure 2a): out of the 914 DMCs, 435 were hypermethylated and 479 were hypomethylated in males compared to females. The similar numbers of hypo and hypermethylated sites in female and male brains do not seem to support our hypothesis that, if methylation

pattern was shaped by male sex drive, males should have many more hypomethylated sites than females. However, there is a tendency in the expected direction; i.e., more hypomethylated sites in males than females. The extent of the observed bias may be influenced by the magnitude of sexual selection operating on males in this species. Zebrafish males do not differ dramatically in their appearance from females and it has been suggested that the opportunity for sexual selection could be weak in this species [28, 29].

This is the first study addressing the differences in methylation patterns between the sexes in zebrafish. Zebrafish do not have sex chromosomes (at least, in the laboratory populations), and the allelic combinations of several loci dispersed throughout the genome determine individual's sex [30, 31]. Sex-biased DMCs have been reported in other vertebrate species, such as mice and humans. However, in these species over 90% of differentially methylated sites reside on X chromosomes and are likely associated with X chromosome dosage compensation mechanisms in females [32]. Even in birds, which have ZW sex determination system, sex-biased methylation of gene promoter regions was also found on sex chromosomes, with male hypermethylation prevalent on Z chromosome (birds do not have complete dosage compensation, but have a male hypermethylated region on Z chromosome) [21]. Given this pattern, it may be more appropriate to compare sex-biased methylation on zebrafish chromosomes to that observed on autosomal chromosomes in species with dimorphic sex chromosomes.

The results of our study are concordant with those obtained for human saliva and blood samples, where small differences in the same direction were reported. Namely, in saliva samples, 307 autosomal sites were hypomethylated in males and 273 sites

were hypermethylated in males, whereas in female saliva samples these numbers were 21 and 15 respectively [16]. However, other results in human methylation studies are not consistent with these findings. For example, using blood samples, El-Maarri et al. found higher average levels of methylation in males than in females [17], although the difference was slight. Eckhardt et al., using 2,524 autosomal loci, could not detect any statistical differences between male and female samples from 12 different tissues [33]. Among the studies investigating human brain tissue, two showed more hypermethylated autosomal sites in females than in males [18, 34], and two studies reported equal proportion of the male hypo and hypermethylated autosomal DMCs between sexes [35, 36].

The distribution of the DMCs in our study varied across chromosomes; for example, chromosomes 18, 19 and 20 contained more hypermethylated DMCs (in males), whereas chromosomes 2 and 4 had higher proportion of hypomethylated DMCs in males (Supplementary Figures S1-S2 in Additional file 1). Heterogeneous distribution on the chromosomes was also noted in a study on mice brain [22]. Higher prevalence of sex-biased methylation sites on different chromosomes could be potentially linked to sex-specific functions of the genes on these chromosomes. Alternatively, these differences might be also due to the distribution of chromosome lengths, number of genes per chromosome, or CpG content of chromosomes.

In our study, only 0.39% of CpG sites were identified as differentially methylated (DMCs). Although this proportion is small, it is comparable to the results of some other vertebrate studies, when only autosomal loci are taken into account. For example, in a study on the fetal human brain methylome 1.3% of autosomal sites were differentially methylated between the sexes [35]. Similarly, a study on human adult

cortex reported 0.15% of sites on autosomes to be differentially methylated [19]. In contrast, Numata et al. found 5% of autosomal loci had significantly sex-biased methylation levels in the human prefrontal cortex [18].

Majority of the DMCs reside in gene body, intergenic regions and CpG island shores

We examined the relationship of the DMCs with CpG features to assess if they are particularly enriched for a given feature. Only a small proportion (7%) of the DMCs overlapped a core CpG island. Interestingly, 51% of the DMCs resided within CpG island shore (defined as 2 Kb from either side of a CpG island core) and 40% were outside any CpG feature (Figure 2a). Next, the relationship of DMCs with gene elements was investigated. 51% of the DMCs were located in the intergenic regions (> 5 Kb from start of a protein coding gene) and 43% were located in the gene bodies (Figure 2c). Consistent with their low overlap with core CpG islands, only 6% of the DMCs were in the promoter (defined as up to 5 Kb upstream from the start of the gene) of protein-coding genes. The proportions of DMCs in the promoter and intergenic regions is similar to the CpG distribution in zebrafish RRBS genome [25], suggesting sex-specific DMCs are not preferentially enriched in these regions. However, amongst the gene body DMCs, 69% mapped to intronic regions, but only 59% of CpGs in zebrafish are located in introns [25], indicating enrichment of sex-specific DMCs in introns.

Our findings are generally concordant with the recently reported sex-specific DNA methylation differences in mammalian brain [22]. Specifically, the majority of DMCs were found in intergenic regions and gene introns and very few were located in CpG islands. Our finding that half of the DMCs were located within CpG island shore is

intriguing. Profiling CpG island methylation differences has been a major focus of epigenetic studies for many years. CpG island shores were shown to be an important element in regulating gene function. For example, in human colon cancer patients, methylation at CpG island shores was highly variable, and more importantly, differential methylation of CpG island shores was reported to be mainly tissue-specific [37-39]. Therefore, it is plausible that CpG island shore methylation could contribute to tissue-specific methylation patterns in zebrafish males and females.

Although DNA methylation is generally considered to suppress transcription [40], this effect can vary depending on the genomic context, such as position in relation to genes. High DNA methylation in the promoter region is known to generally block transcription initiation or mark already silenced genes [41]. However, high level of gene body methylation is thought to allow efficient transcriptional elongation and repressive nonspecific intragenic transcription [42]. Methylation within the gene body may influence multiple processes, such as silencing of transposable elements embedded in gene body, transcript elongation, use of alternative intragenic promoters, and alternative splicing. A study on multiple human neural tissues showed that sex-biased splicing is more common than sex-biased expression on the autosomes [43]. Therefore, qualitative difference in gene products, rather than sex-biased expression levels, might be the key to sexual dimorphism in adult brains. Additionally, differential methylation of intergenic regions could potentially play a role in the control of gene expression, e.g. via cis-regulatory regions and enhancer regions [14, 24]. Taken together, the results of our study indicate that sex-biased methylation could potentially affect brain function, and subsequently behaviour, by influencing gene expression in a more subtle ways than gene silencing by promoter methylation.

There are many sex-biased differentially methylated genes

Next, we aimed to identify the key genes that were differentially methylated between male and females. The 914 DMCs were found to be associated with 708 protein coding genes (in Additional file 2); 346 genes were associated with hypermethylated DMCs in male brains, whereas 400 genes were associated with hypomethylated DMCs in male brains. Furthermore, 37 genes were associated with both hypo- and hypermethylated DMCs (Supplementary Figure S3 in Additional file 1). We generated separate lists of the DMCs that were far upstream from the gene (> 5 Kb from transcription start site) and of the DMCs that were harboured within a gene promoter (within 0 to 5Kb from the transcription start site) or within a gene body. We found 467 DMCs (associated with 348 genes) that were upstream from the start of a gene (78% of them showed > 20 Kb distance from the gene start). On the other hand, we found 371 genes that contained DMCs in the promoter or gene body (in Additional file 2). We identified 3 and 11 genes that contained multiple DMCs (≥ 3) in the promoter and gene body, respectively and 20 genes that showed association with multiple far upstream DMCs (≥ 3). Interestingly, the overlap of the promoter, body and upstream DMCs-associated genes was negligible (Supplementary Figures S4 – S5 in Additional file 1), suggesting that methylation change in male and female zebrafish brains occurs at different elements for different classes of genes. Functional gene enrichment analysis suggested that the male hypermethylated genes were involved in neuron morphogenesis. On the other hand, the male hypomethylated genes are associated with appendage morphogenesis and functions in extracellular matrix (Supplementary Figure S7 in Additional file 1).

Distinct CpG clusters contain consistent sex-specific DNA methylation differences in zebrafish brain

We identified 15 small clusters of DMCs (spanning 8-370 bp, median length= 44 bp) that showed sex-specific methylation differences (Table 1). These clusters contained at least three independently identified DMCs and exhibited methylation change in the same direction (i.e., consistently high or low methylation in males compared to females). Although some of the CpGs within these DMCs clusters were not identified as DMCs due to the stringent criteria used for differential methylation analysis, the majority of these non-significant CpGs showed differences in DNA methylation consistent with adjacent DMCs. Eight of these clusters reside far upstream from the start of the gene, whereas six of them were either in an exon or intron of a protein-coding gene (Table 1). Junb and mtdhb genes harboured DMCs clusters in their first exon and were within 1 Kb from the start of the gene and fam150ba contained a DMC cluster in its promoter. These results suggest that the consequences of these methylation clusters could be genomic context dependent. When we examined the CpG methylation patterns outside these clusters, we did not find significant differences between male and female samples, demonstrating the discrete nature of the clusters. Methylation pattern of the males and females in five clusters (that contained 5 or more DMCs) are shown in Figure 3.

As described, the global distribution of DMCs did not show notable preference for either male or female being hypo- or hypermethylated. However, in 10 out of the 15 identified DMC clusters where consistent methylation changes were observed, male brain samples showed hypermethylation. This finding raises the possibility that DMCs clusters are likely to be more methylated in males, despite the lack of

prevalence of particular DMCs in male and female brains (Table 1 and Figure 3). In the DMC cluster we see multiple adjacent CpG sites exhibits large methylation changes in the same direction. This result suggests that these site-specific differences are biologically determined rather than just stochastic variation.

Female brain shows higher expression in differentially expressed genes compared to male brain

We performed whole genome transcriptome analysis (using RNA Sequencing) of the adult male and female brains to compare their levels of gene expression. We obtained 190 million sequenced reads for both male and female brain transcriptome libraries (each library contained pool of three fish). A consistent observation (at various expression fold-change thresholds) was that the number of genes that were more highly expressed in females was significantly greater than for males. With a cut-off for fold-change of expression of $\log_2$ 1.2, we found 492 genes that were significantly up-regulated in female compared to male zebrafish brain and 186 genes that were up-regulated in male ($q \leq 0.05$) (Supplementary Table S1 in in Additional file 1). This result indicates female bias in gene expression and is opposite to the prediction based on male sex drive hypothesis. This result is consistent with some of the studies on vertebrates. For example, Nätt et al. [21] reports more genes with female-biased expression than with male-biased expression in brain samples from red jungle fowl and domesticated chickens (25 vs. 7 and 14 vs. 5, respectively, autosomal chromosomes only). However, a study on brains of two passerine species found opposite pattern [44]. Nugent et al. [22] found an almost equal number of genes expressed at higher levels in males or females in mice brains.

In our study we also observed higher fold change values in female-biased genes (Figure 4 and Additional file 3), which is inconsistent with our predictions based on the male sex drive hypothesis. According to male driver hypothesis, as a result of higher selection pressure on male traits, male-biased genes should have more pronounced changes in expression levels relative to female-biased genes. Again, the pattern identified in our analysis might be due to comparable levels of sexual selection acting on male and female traits in zebrafish.

Functional gene enrichment analysis suggested that the genes with male-biased expression were mainly involved in sensory perception, functions of non-motile cilium and DNA binding and transcription. On the other hand, the genes with female-biased expression were more likely to be involved in regulation of different enzymatic and biochemical activities of the cell, lipid transport and wound healing (Supplementary Figure S8 in Additional file 1).

Comparison of results with other zebrafish studies shows no consistent pattern of sex-biased transcription

Finally, we compared results of four other studies reporting gene expression from female and male zebrafish brains (Table 2). Two of these studies found overall female-biased expression and two found male-biased expression. We compared the lists of sex-biased genes between our study and the other four studies, where available, and found less than 1% overlap in gene identity (Supplementary Figure S6 in Additional file 1). The inconsistent findings of different studies may stem from the differences in the technological platforms used (e.g. RNA-seq vs. microarray), different sampling and pooling strategies, including strain and age of the fish used, and finally, data processing and stringency of the criteria used to determine

significantly sex-biased genes. In addition, the zebrafish genome is variable between strains at the base level [45], which could also account for the discrepancies. Nevertheless, these contradictory published results, together with the findings of our study, suggest minor or non-existent effects of male sex drive on gene expression pattern in the zebrafish brain.

Only a small subset of differentially methylated genes correlates with expression changes

When we compared the lists of differentially expressed genes from our study with the genes containing DMCs, only 8 of 346 male hypermethylated genes and 5 of 400 hypomethylated genes showed concomitant expression changes with differential methylation (Figure 5). Interestingly, in these methylation-expression associated genes, the DMCs were located either in gene body (5 of these 13 genes) or intergenic regions (8 of 13 genes) but not in promoter (Supplementary Table S2 in Additional file 1). Three genes containing DMC clusters demonstrated gene expression differences between sexes (Supplementary Table S3 in Additional file 1). kcnj13, associated with a hypermethylated DMC cluster in an intron, showed lower expression in male brains compared to female brains (corrected p-value = 1.88E-05, $\log_2$ fold change = 2.41). In contrast, gp1bb, associated with a hypomethylated DMCs cluster in an exon, showed lower expression in male brains compared to female brains (corrected p-value = 5.23E-09, $\log_2$ fold change = 1.39). Finally, Junb, associated with a hyper methylated DMCs cluster in an exon showed higher expression in male brains compared to female brains (corrected p-value = 2.17E-07, $\log_2$ fold change = 1.22).

Overall, the comparison of methylation status and expression levels shows no clear pattern of the relationship between methylation status and expression levels, for the differentially expressed genes between the sexes. However, three main limitations of our study might have resulted in the limited ability to link methylation and expression in zebrafish brains. First, each of the samples sequenced was pooled from several individuals and thus inter-individual variation might have masked the relationships. And third, we used whole-brain homogenates and the patterns could have been different for different brain tissues if analysed separately. However, for zebrafish, isolating single cell types is not feasible. Second, we had to use different individuals for methylome and transcriptome analysis and variation in these individuals might result in poor correlation in methylation with gene expression. However, DNA methylation is a stable and mitotically heritable epigenetic mark. Therefore if sex-specific methylation change and corresponding gene expression change in a phenomenon in zebrafish, then it is unlikely to alter in different cohort of fish. Finally, methylation is only one of several factors that regulate differential expression and thus the relationship between methylation and expression can be complex and hard to disentangle [23]. In line with this last point, we found more differentially expressed genes than differentially methylated between the sexes.

Conclusions

The main aim of this study was to test an extended version of the ‘male sex drive’ hypothesis, using zebrafish brains from the two sexes. That is, we examined differential DNA methylation and expression between the sexes. Overall, our result does not provide support in favour this hypothesis. The male and female brain tissues showed similar levels of global methylation with relatively higher prevalence of

hypomethylated DMCs in male. In addition, several discrete DMCs clusters were identified where males were hyper methylated.

One potential limitation of the study is that the sample size analysed here are small. However, in each of our library we have pool of 6 fish (i.e., 24 fish in total). This strategy lowers the possibility of inter-individual variation in methylation to a large extent. We found very high positive correlation between the male (Pearson's correlation coefficient between Male1 vs. Male2 = 0.98) and female (Pearson's correlation coefficient between Female1 vs. Female2 = 0.97) replicate samples [25] demonstrating minimal technical variation between the pooled samples. Further, These data was derived using high coverage methylation analysis using RRBS. Methylation calls from RRBS technology has been shown to be very reproducible by several groups across the world [46-53]. Further, in the identified DMC cluster we see multiple adjacent CpG sites exhibits large methylation changes in the same direction. This result suggests that these site-specific differences are biologically determined rather than just stochastic or spurious variation. Nevertheless, validation of sex specific DMRs in additional cohorts and functional study will be valuable to determine the role of these DMRs in zebrafish sex determination in future research.

At transcription level, contrary to our expectation, the gene expression seemed to be more female-biased. Notably, we found only 13 genes that showed a concordant methylation and expression pattern. We also reviewed and compared results from four other studies reporting sex differences in gene expression in zebrafish brain. We found very little consistency between results of different studies, including ours. This

inconsistency may stem from the different experimental and analytical methods used but it also suggests that the effect, if it exists, is small.

Understanding of epigenetic regulation in zebrafish genome is still very limited; therefore, further work is needed to examine epigenetic events in other tissues in this important model organism. In relation to the current study it will be intriguing to explore if the differential methylation we observed is embedded at a very early stage of development in zebrafish, before the actual sex determination occurs. This will reveal how early sex-specific epigenetic changes occur and whether other tissues carry these epigenetic marks and will also allow to investigate role of non-genetic events in regulating gene expression pattern [54]. Such study also has potential to be used for understanding the mechanisms of the development of sexual dimorphism in brain function. Also, it will be important for future studies to look at the role of DNA methylation in regulating the use of alternative promoters and alternative splicing of transcripts in zebrafish [55]. Further, it will be important to determine whether the unique DMC clusters play role in in determining sex-specific phenotypes.

Overall, we know little about developmental pathways involved in gonad differentiation, and even less about pathways involved in brain differentiation, in zebrafish. Differential brain development between males and females could be either the cause or consequence of sex-biased DNA methylation and the links between sex-specific methylation pattern and sex-specific behaviour are yet to be revealed.

Methods

Ethics statement

All zebrafish work was approved by the University of Otago Animal Ethics Committee. Animal handling and manipulations were conducted in accordance with Otago Animal Ethics Committee (protocol 48–11).

Sample collection

Adult zebrafish wild-type AB strains were used for this study. The fish were maintained at the Otago Zebrafish Facility, Department of Pathology, University of Otago. Preparation of DNA for RRBS libraries was performed as previously described [25, 26]. Briefly, brains were dissected from 12 male and 12 female adult sexually mature zebrafish and were halved through the sagittal plane. Two male and two female RRBS libraries were prepared, with each library containing a pool of six halved zebrafish brains. For RNA-Seq, brain tissues were collected from an independent cohort of adult male and female fish, and snap frozen in liquid nitrogen. The frozen samples were stored at -80 °C. One male and one female sample pool (each containing brain tissues from three adult fish) was created and RNA was extracted for library preparation.

RRBS library preparation

Genomic DNA from each pooled brain sample was extracted with PureLink Genomic DNA Mini Kit (Invitrogen) following the manufacturer's protocol. Reduced representation bisulfite sequencing libraries were prepared based on our published protocol [56, 57]. In brief, the genomic DNA was digested with MspI (New England Biolabs, Ipswich, MA) followed by end repair, addition of 3' A overhangs and addition of methylated adaptors (Illumina, San Diego, CA) to the digested fragments. Following adaptor ligation, DNA fragments ranging from 40-220 bp (pre-ligation size) were cut from a 3% (w/v) NuSieve GTG agarose gel (Lonza, Basel,

Switzerland) and subsequently bisulfite converted using the EZ DNA methylation kit (Zymo Research, Irvine, CA) with an extended incubation time of 18-20 hours. Bisulfite converted libraries were amplified by PCR reactions and sequenced on an Illumina HiSeq2000 sequencer with a single-ended 49 bp run. A total of 98 million sequenced reads were obtained from four zebrafish brain RRBS libraries.

Quality check and alignment of methylation data

Quality check of the sequenced reads was performed using FastQC software package (distributed by Babraham Institute, Cambridge, UK). Our in-house cleanadaptors program was used to assess contamination of adaptor sequences and to remove them from the sequences. Single-ended bisulfite reads were aligned against zebrafish genome assembly (Zv9) using Bismark software [58].

Analysis of differential DNA methylation

Following alignment by Bismark, the SAM files containing uniquely aligned reads were numerically sorted and then processed in R studio (version 0.97.312) using the R package methylKit [59] to produce single CpG site files. The CpG sites that were covered by at least 10 sequenced reads were retained for further analysis. The forward and reverse strand CpG site coverage essentially represent the same CpG sites; we have combined forward and reverse coverage by setting the DESTRAND = True parameter in methylKit package (default = False). Differentially methylated CpG sites were identified using methylKit algorithm [59] that used logistic regression to calculate p-values, adjusted the p-values for multiple hypothesis testing and generated q values using SLIM approach [60]. The criteria used for identification of differentially methylated CpG sites was q-value of < 0.01 and a percent methylation difference $\geq 25\%$ for each individual CpG site.

Gene and feature location of differentially methylated CpG sites

To investigate the distribution and genomic positions of the differentially methylated CpG sites (in relation to the gene and CpG features) we used identgenloc program from the DMAP package [61]. We developed the DMAP package for comprehensive analysis of RRBS and WGBS data. The identgenloc program used SeqMonk feature table information for Zv9 assembly. SeqMonk (freely distributed from Babraham Institute) provided .DAT files containing information on CpG islands and genes in zebrafish. These files were parsed by identgeneloc, returning information on proximal genes, CpG islands and exon, intron locations of differentially methylated CpG sites. Seqmonk annotations are based on Ensembl database. For the current analysis only protein coding genes were considered. UNIX awk (an interpreted programming language) commands were used for further processing of information returned by identgenloc program [62].

RNA-Seq library preparation

Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA). RNA concentrations were determined using NanoDrop 2000 (Thermo Scientific). The integrity of RNA samples was determined using an Agilent RNA 6000 Nano chip on an Agilent 2100 Bioanalyzer [63, 64]. Samples with RNA Integrity Number (RIN) value of 8-9 was used for RNA-Seq library preparation. Messenger RNA sequencing library was prepared using TruSeq total RNA sample preparation kit (Illumina; Total RNA sample preparation guide), as per the manufacturers instructions, with 3 µg input RNA per library. Quality of RNA-Seq library was checked following the manufacturer's recommended protocols. RNA sequencing of the pooled male and

female samples were performed on the Illumina HiSeq2000 (Illumina, USA) machine with single-ended 51-bp reads. One sample was sequenced per flow cell lane.

Analysis of RNA-Seq data

The sequenced reads from RNA-Seq experiments were assessed for quality and subjected to normalization (duplicate filtering) and then mapped to the zebrafish genome assembly (Zv9) with Tophat alignment tool [65]. The mapped files were then loaded into Genespring for downstream analysis. Raw read count information were generated and normalized for each gene. The normalization of raw read counts and analysis of differentially expressed genes was performed using the DESeq Bioconductor R package. DESeq estimates variance to mean relationship and uses negative binomial distribution model to determine differential expression [66]. The final list of differentially expressed genes consisted of the genes that had a False Discovery Rate (FDR) q-value less than 0.05 and showed at least 1.2 fold change in expression between male and female brains. Fold change was calculated using the formula: fold change = $\log_2$ (Normalized count for male / Normalized count for female).

Additionally, we performed microarray analysis using the Affymetrix GeneChip Zebrafish Genome Array. Six halved zebrafish brains (the other half was used for DNA methylation analysis as described above) were pooled and used for RNA extraction in duplicates for each sex respectively. Hybridizaton of probes was performed according to the manufacturer's protocol (see: http://www.affymetrix.com/catalog/131530/AFFY/Zebrafish+Genome+Array#1_1). Normalization of the raw probe intensities was performed using a Robust Multi-Array Average (RMA) approach (Irizarry, et al., 2003; Irizarry, et al., 2003). Processing of

the data was performed as we previously described [63]. Differentially expressed genes between male and female brains were identified using a p-value < 0.05 and abs(log_2FC) ≥ 1.2. However, the number of detected transcripts was very low for microarray experiments due to low detection rate and subsequently we found few very differentially expressed transcripts between male and female brain and these data are not shown. The processed microarray data for male and females are available on request.

Gene Functional Enrichment analysis

Functional annotation clustering was used to cluster similar GO terms together and results were ranked according to the Group Enrichment Score (the geometric mean (on -log scale) of member's p-values in a corresponding annotation cluster) [67]. Functional annotation clusters were given an overall term which summarised the general theme of each cluster and only clusters with enrichment scores greater than 1.5 were considered. List of protein-coding genes of zebrafish genome was used as the background for these analyses.

Data availability:

The datasets supporting this article are available in the NCBI Gene Expression Omnibus (GEO) archive. Accession number for the Brain DNA methylation data: GSE59916. The accession number for RNA-Seq data is GSE67092.

List of abbreviations used

DMCs: differentially methylated CpG sites; RRBS: reduced representation bisulfite sequencing; FDR: False Discovery Rate

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AC, SN conceptually designed the project. PAS performed the alignment of RRBS data and developed the methylation analysis pipeline. ML, EJR, ED helped in data analysis, generating relevant figures and contributed to manuscript preparation. JAH helped in zebrafish experimental work. LZ, JJ, SM, performed RNA-Seq experiments and data analysis of the transcriptome libraries. YO helped with zebrafish sample collection and RRBS. AC wrote the first draft of the manuscript with significant help from all the authors. All authors read and approved the final manuscript.

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Figures and legends

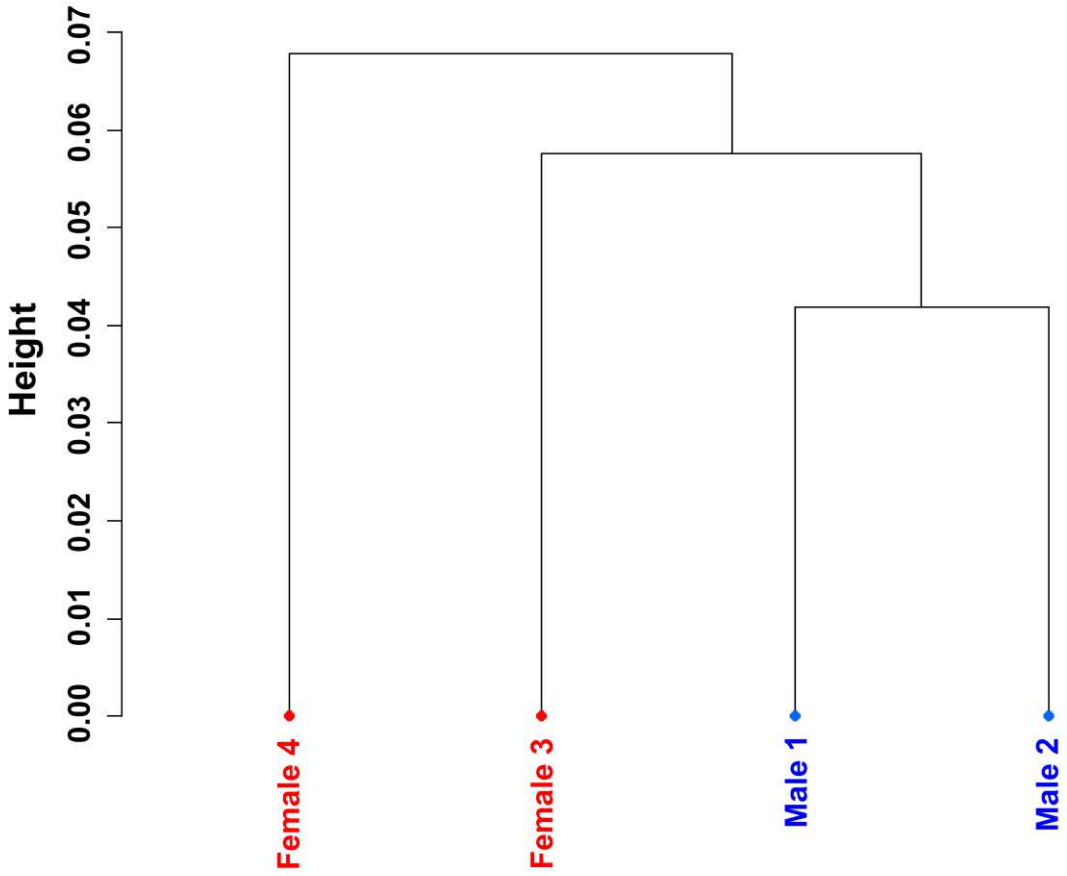

Figure 1. Unsupervised hierarchical clustering of CpG site methylation in four RRBS libraries. Only the CpG sites that were covered by 10 or more sequenced reads were included in this analysis.

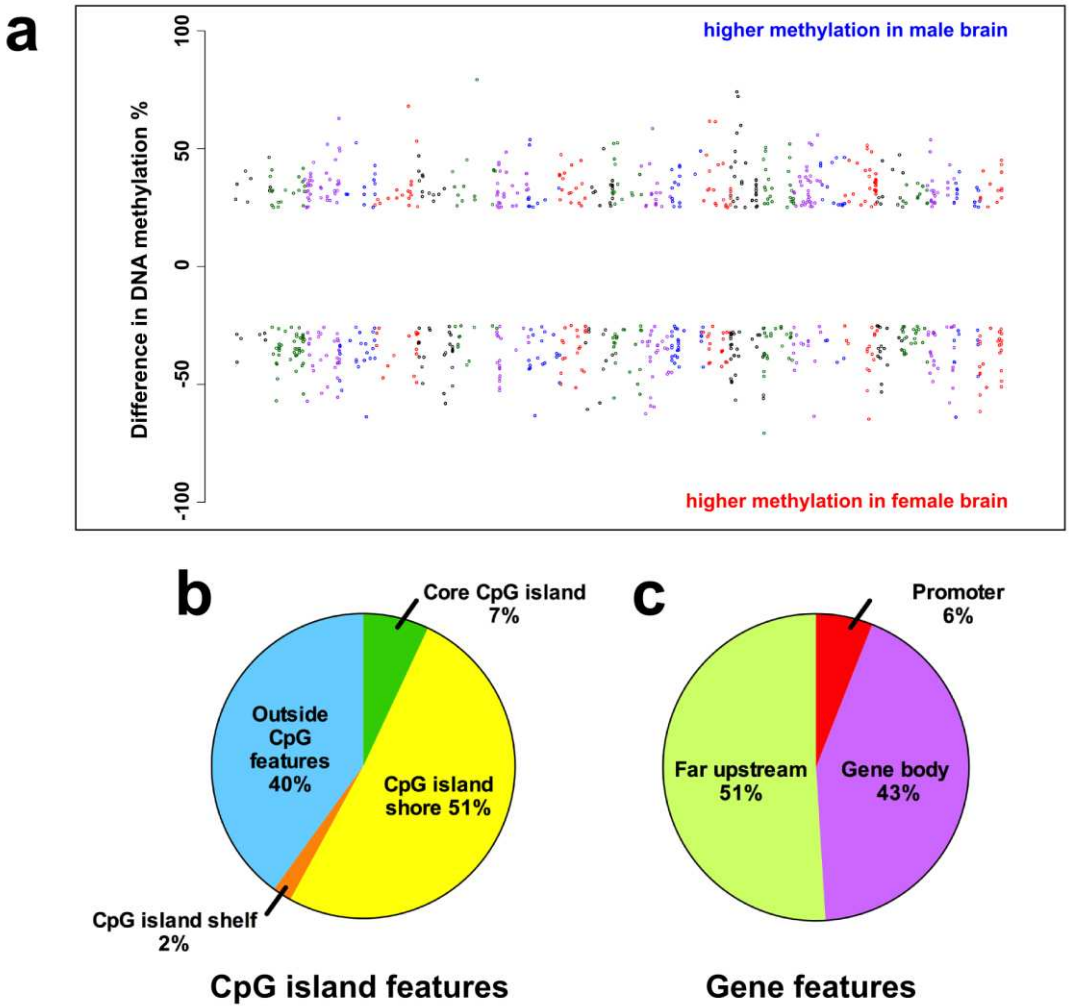

Figure 2. Chromosomal, genomic and CpG feature distribution of differentially methylated CpG sites in the brains of female and male zebrafish. 2a: Manhattan plot showing the chromosomal distribution of the DMCs. Chromosomes are shown along the x-axis (chromosomes 1 to 25). Each differentially methylated site is represented by a single data point. The y-axis depicts the % difference in DNA methylation seen at each individual site between males and females, with a positive value corresponding to higher methylation in male brains, and negative value to higher methylation in female brain tissue. Figure 2b-c: The distribution of DMCs within CpG features (b) and within different genomic elements (c).

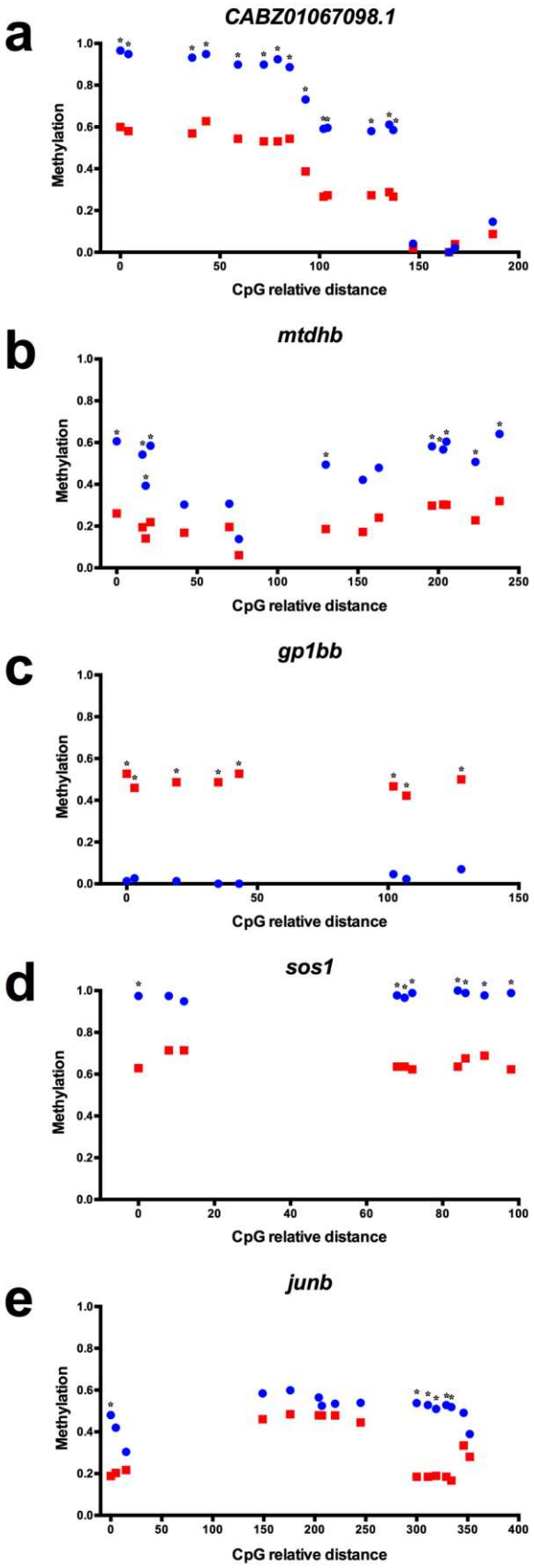

Figure 3. DMCs clusters with sexually dimorphic methylation patterns. (a-e)**.** DMC clusters with five or more DMCs are shown in the figure**.** Male brains are represented in blue colour while female brains are represented as red. Y-axis: DNA methylation level (scale of 0-1), x axis: relative distance of the DMCs in bp. Apart from the significantly differentially methylated CpG sites (DMCs) listed in the Table 1 (marked with * in the figure), the adjacent CpG sites methylation are also shown in the figure, to provide comprehensive overview of the methylation pattern in these regions.

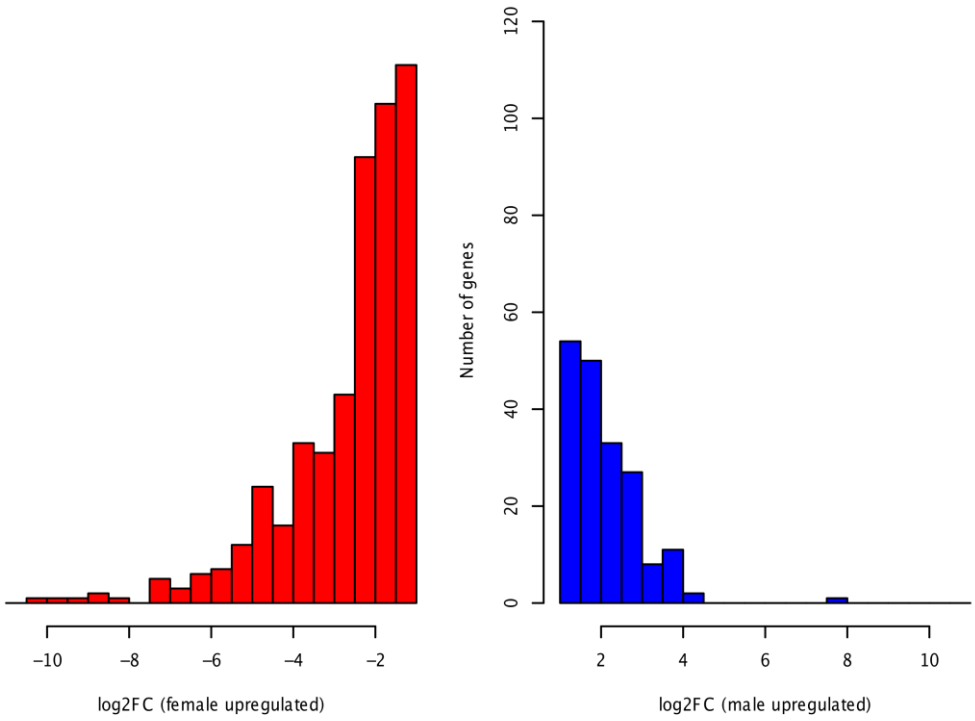

Figure 4. Expression bias for female upregulated (male downregulated) and male upregulated genes. Histograms of the distributions of fold change values for genes that have higher expression in females than in males (red, 492 genes, $\log_2$ fold change > 1.2, $q \leq 0.05$) and genes that have higher expression in males than females (blue, 186 genes). Female-biased genes were not only more numerous, but also more often exhibit higher fold change values, relative to male-biased genes.

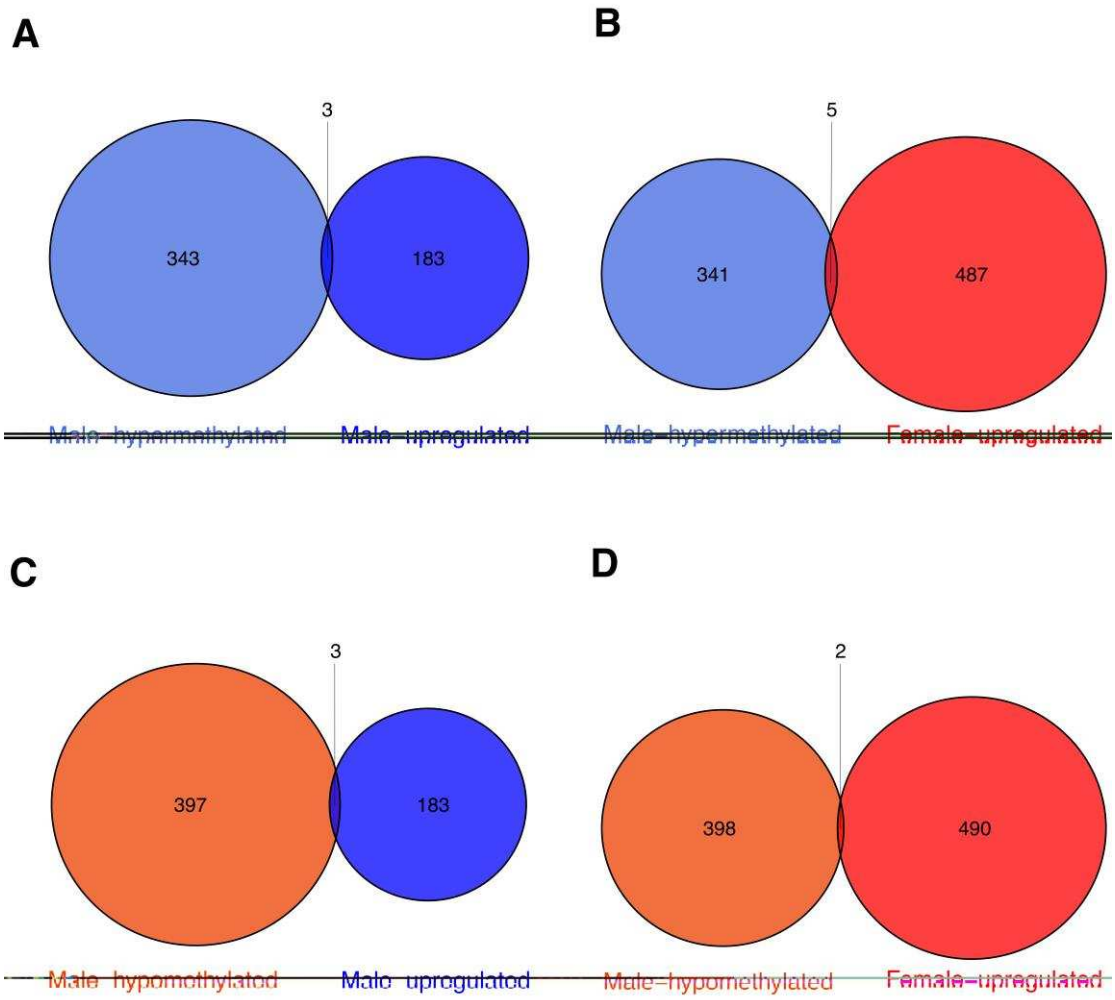

Figure 5. Overlap between differentially methylated and differentially expressed genes between zebrafish male and female brain. (A-D) different comparisons and individual overlaps between hypo and hypermethylated genes in males vs. male up- and downregulated genes (female upregulated). Shades of blue indicate male hypermethylated and male upregulated genes and shades of red indicate male hypomethylated (female hypermethylated) and female upregulated genes.

Tables and captions

Table1.

Genes related to distinct clusters of differentially methylated CpG (DMCs clusters) between male and female zebrafish brains.

Gene	Function	Number of DMCs in cluster	Chromosome	Contig length (bp)	Genomic co-ordinate	Gene relation	Hyper methylated sex
CABZ01067098.1(LOC100334776)	Phosphatase and actin regulator 1-like	14	20	137	53097848 -53097985	Intergenic (28255 bp)	Male
mtdhb	Metadherin b	10	16	238	44486902	Exon	Male

					-44487140		
gp1bb	Glycoprotein Ib (platelet), beta polypeptide	8	8	128	4778157 -4778285	Exon	Female
SOS1	Son of sevenless homolog 1	8	11	98	46305439 -46305537	Intergenic (21613 bp)	Male
Junb	Jun B proto-oncogene b	6	3	334	8435633 -8435967	Exon	Male
hecw1[1]	C2 and WW domain containing ubiquitin protein ligase	4[1]	2	33	50488990 -50489023	Intron	Female
pls3[2]	Plastin 3	4[2]	14	370	13572388	Intergenic (290864 bp)	Male

					-13572758		
kcnj13	potassium inwardly-rectifying channel	3	15	22	38879054 -38879076	Intron	Male
gk5	Glycerol kinase 5	3	2	44	16503339 -16503383	Intron	Female
fam150ba	family with sequence similarity 150, member Ba	3	23	209	35600782 -35600991	Promoter (1725 upstream)	Male
mrps35	Mitochondrial Ribosomal Protein S35	3	26	24	2248265-2248289	Intergenic (127528 bp)	Male
trim35-31	Tripartite motif containing 35-31	3	3	8	6085712-6085720	Intergenic (12577 bp)	Male

grhl1	Grainyhead-like 1	3	17	19	32646679-32646698	Intergenic (14530 bp)	Female
rhcgl1	Rhesus blood group, C glycoprotein, like 1	3	6	40	36135672-36135712	Intergenic (24639 bp)	Male
si:ch211-245h14.1	Ensembl:ENSDARG00000073913 (predicted protein coding)	3	20	13	38564459-36135672	Intergenic (14284 bp)	Female

[1] Only 4 out of 5 DMCs form the cluster. One DMC (chr2: 50513951) was in an exon and was far apart.

[2] Only 4 out of 5 DMCs form the cluster. One DMC (chr14: 13420480) was far apart.

Table2.

Comparison of the results of studies reporting gene expression in the male and female zebrafish brains.

Reference	Cut-off P-value	Cut-off log_2FC	Number of male-biased genes	Number of female-biased genes	Overall result
Santos et al., 2008	≤ 0.05*	≥ 1.2	18	24	Female-biased
Sreenivasan et al., 2008	≤ 0.05*	≥ 1.5	NA	NA	Female-biased^
Wong et al., 2014	≤ 0.05*	Not used	48	13	Male-biased
Arslan-Ergul and Adams, 2014	≤ 0.05	Not used	655	254	Male-biased

Our study	≤ 0.05*	≥ 1.2	186	492	Female-biased

* indicates use of FDR-corrected P-values. NA stands for “not available”. ^ - based on authors’ conclusion

Availability of supporting data

Additional Files:

Additional file 1 (docx): Figures S1-S8 and Tables S1-S3.

Additional file 2 (.xls): Complete list of differentially methylated CpG sites (DMCs) and lists of significant differentially methylated genes in different genomic elements with their associated DMC frequency.

Additional file 3 (.xls.): List of differentially expressed genes (including P-values, gene name and annotation and normalized RPKM values) between male and female zebrafish brains.

List of abbreviations used

DMCs: differentially methylated CpG sites; RRBS: reduced representation bisulfite sequencing; FDR: False Discovery Rate

Highlights

1. The global DNA methylation profile of male and female zebrafish brain is similar

2. Site-specific methylation differences exist between adult male and female brain

3. Distinct CpG clusters are differentially methylated between male and female brain

4. Differentially methylated CpGs are enriched in gene body, intergenic regions

5. More female-biased genes are expressed than male-biased genes in zebrafish brain