Epigenomic and transcriptomic alterations of channel catfish exposed to DNA methyltransferase inhibitor and estradiol

by

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Abstract

Teleost fish exhibit a great level of diversity of sex determination. With channel catfish (*Ictalurus punctatus*), a lower teleost, sex determination genes have not been identified. However, the sex determination region (SDR) was found to be differentially methylated between the X- and Y-alleles, with the Y-alleles being hypomethylated, suggesting that methylation may be involved in sex determination in channel catfish. We tested the effect of a methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), on whole genome methylation and sex differentiation. 5-aza-dC caused genome-wide reduction of DNA methylation, with the largest reduction in females, and led to sex reversal from genetic females to phenotypic males. Whole genome bisulfate sequencing (WGBS) and RNA-Seq analysis revealed that methylation levels in the SDR were significantly reduced, especially in females, and a set of genes were sex-specific differentially expressed in females, including seven genes, *hydin*, *spred3*, *slitrk3*, *tsnaxip1*, *carmil2*, *sphkap*, and *hsf4*, that were up-regulated, and three genes, *esrrg*, *pard6a*, and *actrt3*, that were down-regulated in females. Taken together, this work provided a direct evidence for the involvement of methylation in sex determination in channel catfish. It was possible that demethylation in the sex determination region resulted in expression of key gene(s), such as *hydin*, for differentiation into males, which in turn activating expression of a set of genes that "slow-down" sex

differentiation into females, leading to sex reversal from genetic females to phenotypic males.

Exogenous estrogen 17β-estradiol (E2) has been shown to effectively induce feminization in teleosts. However, the molecular mechanisms underlying the process remain unclear. Here, we determined global DNA methylation and gene expression profiles of channel catfish during early sex differentiation after E2 treatment. Overall, the levels of global DNA methylation after E2 treatment were not significantly different from those of controls. However, a specific set of genes were differentially methylated, which involved in many sex differentiation-related pathways, such as MARK signaling, adrenergic signaling, Wnt signaling, GnRH signaling, ErbB signaling, and ECM-receptor interactions. Many genes involved in these pathways were also differentially expressed after E2 treatment. Specifically, E2 treatments resulted in upregulation of female-related genes and downregulation of male-related genes in genetic males during sex reversal. However, E2-induced sex reversal did not cause sex-specific changes in methylation profiles or gene expression within the sex determination region (SDR), although expression of over a dozen of genes were mostly up-regulated after E2 treatment, especially at 16 days post fertilization (dpf), suggesting that E2-induced sex reversal was a downstream process independent of the sex determination process that was dependent on sex-specific methylation within the SDR.

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Chapter 1 Introduction

1.1 Sex determination and differentiation in fish

Sex determination is the process that directs the development of germ cells and the formation of either a testis or an ovary in sexual reproducing organisms. Sex differentiation refers to the sex-specific response of tissues to hormones produced by the gonads after they have differentiated in a female or male pattern (MacLaughlin & Donahoe, 2004). Bipotential primordia exists in the early embryo, which means that each embryo has the potential to differentiate as either sex by activating one developmental pathway and shutting down the other (Capel, 2017).

What initiates the differentiation of the gonad as a testis or ovary? Teleost fish exhibits diversity and complexity of sex determination mechanisms, such as self-fertilizing hermaphrodites (Weibel et al., 1999), sequential hermaphrodites (Avise & Mank, 2009), genetic sex determination and environmental sex determination (Baroiller et al., 2009). *Rivulus marmoratus* is a synchronous hermaphroditic fish, the only known vertebrate that reproduces by internal self-fertilization (Harrington, 1961). Other strategies include sequential hermaphrodites, which are protogynous species in which an individual begins reproductive life as a female and later may switch to male, protandrous species in which a fish starts as a male and later may switch to female, and serial bi-directional sex changers (Avise & Mank, 2009), such as groupers, seabasses, wrasses, and parrotfishes (Erisman et al., 2013). Environmental factors can determine

or influence sex differentiation of many fish species. Temperature is the most common environmental factor affecting sex (Baroiller & D'Cotta, 2001). Density, pH, salinity, and hypoxia have also been shown to influence the sex ratio of fish species (Lejeune, 1987; Abucay et al., 1999; Shang et al., 2006; Reddon & Hurd, 2013; Kuwamura et al., 2014).

Among fish species that exhibit genetic sex-determination, chromosomal sex determination (CSD) and polygenic (multigenic or multifactorial) sex determination (PGSD) have been found (Mank & Avise, 2009). In CSD, sex is determined by a primary switch located on one or both members of a differentiated sex chromosomal pair (Liew et al., 2012). Some fish, including channel catfish (*Ictalurus punctatus*) (Patiño et al., 1996), rainbow trout (*Oncorhynchus mykiss*) (Yano et al., 2012) and medaka (*Oryzias latipes*), employ male heterogametic system (XX-XY). Others, such as turbot (*Scophthalmus maximus*) (Martínez et al., 2009), Chinese tongue sole (*Cynoglossus semilaevis*) (Shao et al., 2014) and blue tilapia (*Oreochromis aureus*), use a female heterogametic system (ZW-ZZ). In PGSD, the genes with strong influence on sex determination and/or gonad differentiation are distributed throughout the genome and the combination of their alleles determines the sex (Liew et al., 2012). European seabass (Vandeputte et al., 2007) and a handful of cichlid species from Lake Malawi (Ser et al., 2010) exhibited this system.

1.2 Sex determining genes in fish

The master sex determination gene is quite diverse among teleosts, with over a dozen sex determination genes being identified from various fish species, in contrast to the evolutionary conservation of SRY/Sry gene among mammals. The first sex-determining master gene *DMY* in fish species was identified in medaka (*Oryzias latipes*) (Matsuda et al., 2002). The recombinant breakpoint analysis and deletion analysis of the Y chromosome of a congenic XY female medaka were employed to restrict the sex determining region to a 250 Kb. Only 27 genes were predicted in this region by shotgun sequencing, in which, only *DMY* gene was Y specific. During normal development, *DMY* was expressed only in somatic cells in the testis. *DMY* was necessary for normal male development and falls within the sex-determining region of the Y chromosome (Matsuda et al., 2002). Additionally, the phylogenetic analysis indicated that *DMY* was probably derived from *DMRT1*.

By using a genetic mapping approach, Myosho et al. (2012) identified that *GsdfY* (gonadal soma derived growth factor on the Y chromosome) had replaced *DM*Y as the master sex determining gene in *Oryzias luzonensis*, a closely related species of medaka. It was found that this gene is a somatic factor controlling the proliferation of primordial germ cells and spermatogonia in rainbow trout (Sawatari et al., 2007), and its expression in gonads was also observed in medaka and zebrafish (Shibata et al., 2010; Gautier et al., 2011). *GsdfY* showed high expression specifically in males during sex

differentiation in *O. luzonensis*. Furthermore, the presence of a genomic fragment that included *GsdfY* could convert XX individuals into fertile XX males.

A male-specific gene, *sdY*, was identified in rainbow trout, and was predominantly expressed during testicular differentiation (Yano et al., 2012). The *sdY* gene encodes a truncated, divergent protein presenting significant similarities with the carboxy-terminal domain of Irf9 proteins that are members of the interferon regulatory factor family. The gene expression level of *sdY* reached the peak at approximately 40 to 60 days post fertilization (dpf), but only in male differentiating gonads, and this high expression was sustained until 90 dpf. Additionally, *sdY* is tightly linked with the sex locus on the Y chromosome. Overexpression of *sdY* in rainbow trout genetic females showed that 12% genetic females that were transgenic had a clear testis structure. Additionally, the targeted inactivation of *sdY* in males using zinc-finger nuclease induced ovarian differentiation. Together, these results demonstrated that *sdY* is a novel vertebrate master sex determining gene.

Anti-Müllerian hormone (*amh*) was observed to be master sex determining gene in the teleost fish Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012). Two different *amh* transcripts originated from two different loci are present in *O. hatcheri*, and one of them was present only in the male genome and responsible for the early transcription of *amh* in XY gonads; thus, named *amhy* (Y chromosome-specific *amh*). *Amhy* was expressed much earlier than the autosomal *amh* (6 d after fertilization vs. 12 weeks after fertilization) and was localized to presumptive Sertoli cells of XY males

during testicular differentiation. Moreover, *amhy* knockdown in XY embryos resulted in the up-regulation of *foxl2* and *cyp19a1a* mRNAs and the development of ovaries. These results suggested that *amhy* was the male master sex determining gene in *O. hatcheri*. *Amh* was also identified as the male master sex determining gene in the Northern pike, *Esox lucius* (Pan et al., 2019). PCR amplification on genomic DNA of phenotypic females and males showed that one *amh* copy was present in all phenotypic females and males, while the genomic sequence of the testis-specific *amh* was only present in phenotypic males, which indicates that the genomic sequence of this testis-specific copy of *amh* in the Y chromosome, *amhby*.

The male genome was assembled using Nanopore long reads and identified a ~300 kb male specific region. *Amhby* was the only non-transposable element, protein-coding gene in this locus. *Amhby* was high expressed in male gonads before the first signs of histological differentiation between female and male gonads. All *amhby* knockout mutants failed to develop a normal testis. *Amhby* was functionally necessary and sufficient to trigger testicular development, and was expressed in the male gonadal primordium, fulfilling key requirements for a classic master sex determining gene.

1.3 Epigenetics of sex change in fish

The epigenetic regulatory mechanisms involved in sex determination and gonadogenesis have been widely described (Bromfield et al., 2008; Khalil & Wahlestedt, 2008; Menger et al., 2010; Hales et al., 2011; Piferrer, 2013). By changing

the activity of genes, epigenetic mechanisms enable organisms to respond to internal or external environments and integrate genomic and environmental information, so that organisms can produce a specific phenotype (Turner, 2009). Modern epigenetic studies focus on heritable modifications of DNA, histones, and chromatin structure (Henikoff & Greally, 2016). Those modifications can regulate gene expression through preventing or favoring the binding and access of transcription factors or regulating chromatin remodeling proteins.

DNA methylation, a key epigenetic modification, is prevalent in eukaryotes (Colot and Rossignol 1999; Suzuki and Bird 2008), and involves the addition of a methyl (CH3) group to the fifth carbon of cytosine (5mC) by DNA methyltransferases (DNMTs) (Yong et al., 2016). DNA methylation occurs frequently in CpG dinucleotides (CpGs) but is also observed in CHG and CHH nucleotides (H = A, T or C). Methylation is involved in gene expression regulation (Jaenisch & Bird, 2003). Promoter methylation is usually negatively correlated with gene expression, which may be because DNA methylation changes chromatin structure and exerts suppression on transcriptional activity (Jones 2012). Gene body methylation has long been ignored, but might be involved in differential promoter usage. DNA methylation exhibits dramatic changes at intron-exon boundaries, suggesting an association with alternative splicing (Laurent et al. 2010; Regulski et al. 2013), which is an important mechanism of post-transcriptional regulation for increasing proteomic diversity and mediating gene expression (Nilsen & Graveley, 2010; Kalsotra & Cooper, 2011).

Chinese tongue sole (*Cynoglossus semilaevis*) is a marine fish that has both ZW chromosomal GSD and temperature-dependent ESD. Under normal temperature (22°C), about 14% of ZW genetic females are reversed to phenotype males (pseudo-males), while at the higher temperature (28°C), the sex-reversal rate was 73% (Chen et al., 2014). The offspring of pseudo-males exhibit an extremely high sex reversal rate (~94%), even when cultured in 22°C (Chen et al., 2014). The methylation pattern of pseudo-male testes and their offspring male testes were similar to those of normal male testes, and the overall methylation levels in testes were ~10% higher than ovaries (Shao et al., 2014). The genome-wide methylation patterns of genetic females have been accurately shaped to the patterns of normal males after phenotypic sex reversal, implying that the DNA methylation changes after sex reversal were associated with gonad differentiation in Chinese tongue sole (Shao et al., 2014). Many sex-determination-related genes displayed significant differential methylation patterns between testes and ovaries (e.g., *dmrt1*, *gsdf*, *amh*, *amhr2*, *wt1a*, and *wt1b*). These results indicated that epigenetic regulation plays crucial roles in sexual reversal of Chinese tongue sole (Shao et al., 2014).

In the European sea bass (*Dicentrarchus labrax*), juvenile males have hypermethylation in the *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a) promoter compared with females (Navarro-Martín et al., 2011). Exposure to high temperature increased the *cyp19a1a* promoter methylation and down-regulated the *cyp19a1a* expression, leading to male development (Navarro-Martín et

al., 2011). In Japanese flounder (*Paralichthys olivaceus*) and Nile tilapia (*Oreochromis niloticus*), the promoter region of *cyp19a* in testis was more highly methylated than in ovary and the methylation level was negatively correlated with gene expression (Wen et al., 2014).

Modifications in DNA methylation patterns have also been studied in hermaphroditic fish. The rice field eel (*Monopterus albus*) is a protogynous hermaphrodite fish that changes sex naturally from a functional female to a functional male. The continuous decrease expression of gonadal *cyp19a1a* occurred during the natural sex change from functional female to functional male (Zhang et al., 2013). Zhang et al. (2013) found that the *cyp19a1a* promoter was hypermethylated in testis compared with ovary, blocking its activation by gonadotropins through a cAMP-dependent pathway. During sex change from female to male, the DNA methylation levels of the promoter of *cyp19a1a* increased and expression level decreased in the gonads. Treatment with DNA methylation inhibitor 5-aza-2′-deoxycytidine in gonadal differentiation reversed natural sex change in the rice field eel. DNA methylation level of *cyp19a1a* promoter was decreased and the expression of *cyp19a1a* was up-regulated. This study revealed that DNA methylation was responsible for abrogating the stimulatory effects of gonadotropin on testicular *cyp19a1a* gene expression in rice field eel. The epigenetic modifications of *cyp19a1a* may represent a crucial mechanism for establishing the sexually dimorphic expression of gonadal *cyp19a*1a in teleosts and possibly other vertebrates.

Bluehead wrasse (*Thalassoma bifasciatum*), a small coral reef fish, could change the sex rapidly in response to a social cue (Warner & Swearer, 1991). Most bluehead wrasse begin their reproductive life as female, however rapidly and completely reverse sex in the absence of a socially dominant male (Godwin, 2009). Todd et al. (2019) found that DNA methylation progressively increased as ovaries became testes and DNA methylation, and gene silencing were coupled in a similar fashion throughout sex change. The methylation patterns of key sex determining genes provides evidence for the role of DNA methylation reprogramming in gonadal transformation. The transcriptional start site of gene *cyp19a1a* was hypermethylated as gene silencing progressed during sex change. Additionally, the promoter was progressively demethylated during sex reversal as the male determining gene *dmrt1* was up-regulated.

Barramundi (*Lates calcarifer*), also known as Asian seabass, is a large hermaphrodite fish in Southeast Asia and Australia (Banh et al., 2017). Testicular tissues of barramundi develop during the second to sixth month of age. Males are fully mature at about 3–4 years and sex reversal to female occur at 4–8 years of age (Banh et al., 2017). In a breeding environment, the sex reversal can occur at early as 2-3 years old (Guiguen et al., 1994). Domingos et al. (2018) found the promoters and first exons of *dmrt1*, *cyp19a1*, *amh* and *nr5a2* showed significant differences in DNA methylation level between ovaries and testes. Nonfunctional sex-specific isoforms for *dmrt1* in females and *cyp19a1* in males were identified. Additionally, the promoter of *dmrt1* was hypermethylated and the promoter of *cyp19a1* were demethylated during barramundi

sex reversal, which provides evidence for epigenetics and alternative splicing affecting genes in sex differentiation pathways (Domingos et al., 2018).

Taken together, these observations reveal that DNA methylation dynamics are critical for both maintaining sexual identity and reprogramming sexual fate in fish. Epigenetic modifications emerge as a critical link between environmental stimuli, sex change, and subsequent maintenance of sexual phenotype.

1.4 Sex determination mechanism in channel catfish

Ictalurid catfish have been an important commercial and aquaculture fish for several decades in the United States. Channel catfish (*Ictalurus punctatus*) are native east of the Rocky Mountains and have been introduced to almost all parts of North America, as well as South America, Europe, and Asia (Dunham & Elaswad, 2018). Some catfish species exhibit strong sexual dimorphism with the males growing faster by approximately 37-60% than the females (Brooks et al., 1982; Simco et al., 1989; Kelly, 2004). Understanding the sex determining gene and the sex determination mechanisms will facilitate the application of mono-sex culture technologies and their application in catfish aquaculture.

With catfish, success of sex manipulations is limited at the level of research. Sex reversal with catfish has been mostly successful to achieve high percentage of females (not desired), but not for high percentage of males (Goudie et al., 1983; Davis et al., 1992). Androgen treatment of other fishes leads to sex reversal into males, while

estrogen or androgen treatment led to sex reversal into females in catfish, suggesting its unique mechanism of sex differentiation (Goudie et al., 1983). In catfish, androgens were converted immediately into estrogens upon administration such that they were sex reversed into females as if they were treated with estrogens with the help of cytochrome P450 aromatase (Kazeto & Trant, 2005). Channel catfish possess male heterogametic sex determination system (XX/XY) (Davis et al., 1990; Tiersch et al., 1990). Production of YY males is possible, but not in mass quantities to serve as the brood fish for mating with regular females (XX) to produce all-male (XY) populations. Therefore, understanding of sex determination is crucially important in order to provide the technological foundation for the application of sex manipulation techniques to enhance catfish aquaculture production.

The X and Y chromosome of channel catfish has been sequenced and assembled using a XX female and a YY male, respectively (Liu et al., 2016; Bao et al., 2019), which provided crucial resources for research on sex determination mechanism and sex determination genes. Through genetic linkage mapping and genome-wide association studies (GWAS), the sex determination locus was located in a narrow region of 8.9 Mb on chromosome 4 (Li et al., 2015; Bao et al., 2019). Unlike the obviously morphological distinct sex chromosomes in other species, channel catfish X and Y chromosome sequences were quite similar without sex-specific genes, suggesting that channel catfish sex may be determined by the variation in epigenetics and regulation of gene expression within the sex determination region (Bao et al., 2019).

Transcriptome analyses were conducted to determine differentially expressed genes between channel catfish females and males (Sun et al., 2013; Zeng et al., 2016; Bao et al., 2019). By comparing the transcriptome expression profiles in the testis and other tissues, more than 5,000 genes had > 5-fold higher expression in the testis than in other tissues. Of these genes, a large number of genes involved in gonad genesis, spermatogenesis, testicular development and differentiation and sex determination were identified and many spermatogenesis genes were distributed on the Y chromosome (Sun et al., 2013). To identify potential genes involved in initial gonadal differentiation phase, gene expression of male and female gonads at 90, 100, and 110 dpf were compared. Transcriptomic profiles of male gonad showed drastic changes during 100–110 dpf and a group of genes involved in germ cells development were up-regulated. Male-preferential genes, such as *gsdf*, *cxcl12*, as well as other cytokines mediated the development of the gonad into a testis were identified between testes and ovaries (Zeng et al., 2016). In the sex determination region (SDR, a narrow region of 8.9 Mb on chromosome 4), one of the alternatively spliced transcripts of the breast cancer anti-estrogen resistance protein 1 (*BCAR1*) was expressed exclusively in genetic males (XY genotype), but not in genetic females (XX genotype), suggesting the candidacy of *BCAR1* as the sex determination gene in catfish and the potential involvement of alternative splicing in sex determination of catfish (Bao et al., 2019). The candidate genes identified in above studies set the foundation for further studies on sex determination and differentiation in teleost.

Epigenetic regulation of sex determination was also conducted in channel catfish (Yang et al., manuscript under revision). The chromosome 4 was differentially methylated in genetic females and males with female hypermethylated and male hypomethylated, especially in the SDR. Furthermore, it was found that most of the differentially methylated CpG sites had X and Y allele specificity and corresponding genes were expressed allele-specifically, reflecting that the sex of channel catfish was determined through sex-biased transcription and allele-biased gene expression.

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Chapter 2 Methylation inhibitor 5-aza-2′-deoxycytidine causes masculinization during early sex differentiation of channel catfish

2.1 Abstract

Teleost fish exhibit a great level of diversity for sex determination. Sex determination genes have not been identified for channel catfish (*Ictalurus punctatus*), a lower teleost. The sex determination region (SDR) was found to be differentially methylated between the X- and Y-alleles, with the Y-alleles being hypomethylated, suggesting that methylation may be involved in sex determination. In this study, we tested the effect of a methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), on whole genome methylation and sex differentiation. 5-aza-dC caused genome-wide reduction of DNA methylation, with the largest reduction in females, which led to sex reversal from genetic females to phenotypic males. Whole genome bisulfate sequencing (WGBS) and RNA-Seq analysis revealed that methylation levels in the SDR were significantly reduced, especially in females, and a set of genes were sex-specific differentially expressed in females, including seven genes, *hydin*, *spred3*, *slitrk3*, *tsnaxip1*, *carmil2*, *sphkap*, and *hsf4*, that were up-regulated, and three genes, *esrrg*, *pard6a*, and *actrt3*, that were down-regulated in females. This work provided direct evidence for the involvement of methylation in sex determination in channel catfish. It is possible that demethylation in the sex determination region resulted in expression of key gene(s), such as *hydin*, for differentiation into males, which in turn activating

expression of a set of genes that “slow-down” sex differentiation into females, leading to sex reversal from genetic females to phenotypic males.

2.2 Introduction

Mechanisms of sex determination vary greatly among various animals in the evolutionary spectrum of species. In lower classifications of animals such as insects and worms, gene dosage is important for the determination of sex. For example, sex is determined by the ratio of X chromosomes to autosomes in Drosophila melanogaster flies and Caenorhabditis elegans worms where XX animals become hermaphrodites (in worms) or females (in flies), whereas XY and XO animals become males (Cline and Meyer, 1996). In higher vertebrates such as mammals, sex is determined by a dominant Y-linked sex determination gene SRY/Sry, where XY animals become males whereas XX animals become females (Sinclair et al., 1990). In most birds, heterogametic ZW animals become females whereas homogametic ZZ animals become males, and DMRT1 dosage is essential for testis development (Ioannidis et al., 2021).

In teleosts, mechanisms of sex determination are tremendously diverse, including hermaphroditism, genetic sex-determination (both XY and ZW systems), and environmental sex determination (Devlin, et al., 2002; Volff and Schartl, 2002; Charlesworth, 2004). Among genetically sex-determined species, monofactorial and polygenic systems have been found (Mank and Avise 2009; Kikuchi and Hamaguchi 2013). Not only the mechanism sex determination at chromosomal level of is diverse

among teleosts, so are the sex determination genes with over a dozen sex determination genes already identified from various fish species (Matsuda et al., 2002; Hattori et al., 2012; Myosho et al., 2012; Yano et al., 2012; Pan et al., 2019), in contrast to the evolutionary conservation of SRY/Sry gene among mammals. Different master sex determination genes have been reported in very closely related species as shown in ricefish, such as *Oryzias dancena*, *O. latipes* and *O. hubbsi* (Takehana et al., 2007b, 2007a, 2008; Tanaka et al., 2007; Nagai et al., 2008; Myosho et al., 2015), and even in different populations of the same species, as in the case of the Southern platyfish (*Xiphophorus maculatus*) (Volff and Schartl, 2002), suggesting very rapid evolution of sex determination mechanisms in teleost.

To date, most studies have been conducted with higher teleost fish species such as fugu, medaka, platyfish, rainbow trout, northern pike, and tilapia. We have been working on sex determination of channel catfish (*Ictalurus punctatus*), a lower teleost species in the order of Siloriformes. Genetic linkage mapping demonstrated that sex was controlled by a mono genetic factor located on chromosome 4 that was inherited through Mendelian segregation, suggesting a single genetic locus was involved in sex control in channel catfish (Li et al., 2015). Genomic sequencing of a XX female fish (Liu et al., 2016) and a YY male fish (Bao et al., 2019), followed by comparative sequence and transcriptome analysis provided no gene candidates for sex determination. The sex chromosomes, both X and Y, each harbored 950 genes and all these genes were transcribed. A sex-specific transcript variant of *BCAR1* gene was identified only in

males and its knockout led to females (Bao et al., 2019). However, additional research revealed that sex-specific alternative splicing was not specific to *BCAR1* gene, but rather common among many genes, suggesting that *BCAR1* may be involved in sex determination pathway, but it was not the sex determination gene.

In our following research, Yang et al. (manuscript under revision) found an epigenetically marked locus that overlapped with the sex determination region (SDR). Within the SDR, the X-alleles were found to be hypermethylated whereas the Y-alleles hypomethylated. Many genes within the SDR were allele-specific and differentially expressed between genetic females and males, suggesting a unique sex determination mechanism in channel catfish involving differential methylation within the SDR and thereby differential expression of genes.

Epigenetic modification has been found to be involved in sex determination and gonadogenesis (Bromfield et al., 2008; Khalil and Wahlestedt, 2008; Menger et al., 2010; Hales et al., 2011; Piferrer, 2013). Specifically, DNA methylation has been found to be associated with sex determination in some teleosts (Piferrer, 2013). For example, in European sea bass (*Dicentrarchus labrax*), juvenile males had hypermethylation in the *cyp19a* promoter compared with females (Navarro-Martín et al., 2011). Exposure to high temperature during the early life of European sea bass increased the *cyp19a* promoter methylation of females and down-regulated the gene expression, leading to generation of a high percentage of males (Navarro-Martín et al., 2011). However, such

effect of methylation on sex determination was considered a downstream event for sex determination.

Methylation inhibitors, such as 5-aza-2'-deoxycytidine (5-aza-dC), can be used to study the effect of methylation in sex determination (Jüttermann et al., 1994; Stresemann and Lyko, 2008). Zhang et al. (2013) found that 5-aza-dC treatment reversed the natural sex change of rice field eel (*Monopterus albus*). Ribas et al. (2017) reported an increase in the number of females in zebrafish after 5-aza-dC treatment with different doses at different developmental stages. In an experiment where adult female zebrafish were fed with 5-aza-dC for 32 days, the growth and global DNA methylation level of the liver were lower than the control fish (Olsvik et al., 2014). Furthermore, adult stickleback (*Gasterosteus aculeatus*) fed with 5-aza-dC exhibited global DNA changes in both testes and ovaries (Aniagu et al., 2008).

The objective of this study was to directly test the effect of a methylation inhibitor, 5-aza-dC, on whole genome methylation and sex differentiation. Here we report that 5-aza-dC methylation inhibitor caused sex reversal from genetic females to phenotypic males; the methylation levels in the SDR were significantly reduced, especially in females; and a set of genes were sex-specific differentially expressed in females, providing direct evidence for the involvement of methylation in sex determination in channel catfish.

2.3 Materials and methods

2.3.1 Ethical statement

The collection and treatment of fish in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. All animal procedures were carried out according to the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the USA.

2.3.2 Treatment with 5-aza-dC

The channel catfish females and males used as parents were reared at the Fish Genetics Research Unit at the EW Shell Fisheries Research Center, Auburn University. Artificial spawning and fertilization were conducted as described in Gima et al. (2014). Fertilized eggs were placed in suspended mesh baskets in a flow-through trough with paddle wheels until about 12 h before hatching. During this period, embryos were taken out of the mesh basket and treated in tubs for four hours per day (two hours in the morning and two hours in the afternoon) with DNA methylation inhibitor 5-aza-dC (No. 11166, Cayman, Michigan, USA) at different concentrations (6, 12 and 24 μM). Near hatching, eggs and the fry were reared in tubs with clean pond water and treated with different treatment concentrations (6, 12 and 24 μM) for four hours daily. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at different doses (3, 6 and 12 mg/kg) three times per day until 30 days post fertilization (dpf). Control

groups were handled in the same way as the experimental groups except for not receiving 5-aza-dC treatment. At 135 dpf, we collected all fish and determined their genetic sex by PCR (Ninwichian et al., 2012) and gel electrophoresis, and their phenotypic sex by observing the gonads.

2.3.3 Sample collection and DNA/RNA extraction

The samples from the high concentration treatment group (24 μM, 12 mg/kg) were collected at 3, 9, 12 and 16 dpf. At 135 dpf, gonads in all treatment groups were collected. Fish were euthanized with tricaine mesylate (MS-222) before sample collection. Each sample was placed in a 1.5 ml tube, placed into liquid nitrogen immediately and stored at −80°C for subsequent DNA and RNA extractions. The control samples at each time point were also collected.

At 3 dpf, each sampled egg was split into two equal parts. At 9, 12, 16 dpf, head and tail were cut off and abdomen was then divided into two equal parts from the spine for each fry. At 135 dpf, the gonad of each fish was also divided into two parts. Genomic DNA from one part of each sample was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. RNA was extracted from the other part by RNeasy Plus Universal Tissue Mini Kit (Qiagen). Genomic DNA and RNA were quantified using Nanodrop 2000 and qualified by 1% agarose gel electrophoresis. Small amount of DNA was used to determine genetic sex of each sample via PCR validation using the sex-linked microsatellite marker AUEST0678 (Ninwichian et al., 2012).

2.3.4 Whole-genome bisulfite sequencing and data analysis

For each time point (3, 9, 12, 16 and 135 dpf), each sex type (female, male and pseudo-male) and each group (control and treatment), equal amount of genomic DNA from five fish were pooled for whole-genome bisulfite sequencing (WGBS) library construction. Three replicates were conducted. Genomic DNA from one pseudo-male was used to construct library, because only three pseudo-males were identified in treatment groups at 135 dpf. A total of 63 libraries (2 sexes × 5 time points × 2 treatment × 3 replicates + 3 pseudo-males) were prepared and sequenced by Illumina Novaseq platform with paired-end (PE) 150 (CD Genomics, Shirley, NY, USA).

Quality assessment was performed on raw sequencing reads using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were then trimmed to remove adaptor sequences, ambiguous nucleotides, short length (< 36 bp), and low-quality reads (quality score < 20) using Trimmomatic v0.37 (Bolger et al., 2014). High-quality reads were mapped to the channel catfish reference genome (Liu et al., 2016) using the bisulfite alignment program Bismark v0.22.1 (Krueger and Andrews, 2011). The two strands of channel catfish reference assembly have been modified in silico to convert all C's to T's, using 'bismark_genome_preparation' tool with indexing format following Bowtie2 requirements (Langmead & Salzberg, 2012). Methylation level on each site was determined using 'bismark_methylation_extractor'

tool. Overall levels of methylation in CpG, CHG and CHH context and their percentages were calculated based on the output of Bismark.

Methylation calls were analyzed using SeqMonk v1.45.2 (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Methylation loci located on reads from the 29 chromosomes of channel catfish were imported to SeqMonk, and loci covered by at least 10 reads were retained for further analysis. Methylation percentages were calculated using the 'bisulfite methylation over feature' pipeline in SeqMonk with default parameters. The distribution of hypomethylated/hypermethylated CpGs per chromosome were also generated by SeqMonk. To identify differentially methylated sites (DMSs) between two groups, the logistic regression of proportion based statistics in SeqMonk was carried out with *p* value < 0.01 and a percentage difference ⩾ 25%. The identified genes with DMSs on their promoter (the 2Kb region upstream of TSS) and gene body were selected as differentially methylated genes (DMGs).

The CpG methylation clustering based on the similarity of the methylation profiles, principal component analysis (PCA) for WGBS samples were generated using per CpG site methylation by methylKit (Akalin et al., 2012) and plotted using ggplot2 in R (Wickham, 2011). Additionally, CpG methylation percentage in 3Kb region upstream and downstream of the transcription start site (TSS) and in promoters, exons, introns and intergenic regions were characterized.

2.3.5 Transcriptome sequencing and data processing

Total RNA of nine females and nine males from the control and treatment group (24 μM, 12 mg/kg) at 3, 16 and 135 dpf were extracted, and equal amount of RNA from three fish were pooled per replicate (three replicates) for Ribo-Zero (ribosomal RNA depletion) and strand-specific RNA-seq library construction. The RNA from one pseudo-male was used to build library as one replicate at 135 dpf. There were also three duplicates for pseudo-male group. A total of 39 RNA libraries (2 sexes × 3 time points × 2 treatment × 3 replicates + 3 pseudo-males) were prepared and sequenced by Illumina Novaseq platform with PE150 (CD Genomics, Shirley, NY, USA).

Adapters and low-quality reads (quality score < 20) were trimmed by Trimmomatic v 0.37 (Bolger et al., 2014). Quality control of sequencing reads were carried out before and after trimming using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All trimmed reads were mapped to the channel catfish reference genome (Liu et al., 2016) using HISAT2 v2.1.0 (Kim et al., 2015). HTSeq v0.11.0 was used to quantify the number of expressed transcripts (Anders et al., 2015). DESeq2 (Love et al., 2014) was utilized to identify differentially expressed genes (DEGs) with $|\log_2(\text{fold change})| > 1$ and adjusted p value < 0.05. GO and KEGG enrichment analyses of the DEGs were performed by clusterProfiler (Yu et al. 2012).

2.3.6 Correlation between methylation and gene expression

To examine the relationship between methylation and gene expression, we calculated the average methylation level of promoter and gene body of each gene and integrated it with gene expression level (FPKM) obtained from the same samples. Spearman correlation was used to assess the relationship between methylation and expression, with a significance level of 5%.

2.4 Results

2.4.1 Methylation inhibitor causes sex reversal to males

A methylation inhibitor, 5-aza-dC, was used to determine the effect of genome methylation on sex differentiation of channel catfish. The treatment was applied by exposing fertilized embryos of channel catfish to various concentrations (6, 12 and 24 μM) of 5-aza-dC for the first 10 days after fertilization, followed by feeding with feed containing various concentrations of 5-aza-dC (3, 6 and 12 mg/kg) for three more weeks, and then the fish were cultured under normal water and feeding conditions until termination of experiments (135 dpf). At the highest tested concentration of aqueous exposure of 24 μM, pseudo-male fish (male phenotypically but female genetically) were observed, although at low proportions: one fish was identified as a sex-reversed pseudo-male at 24 μM aqueous exposure followed by feeding with pellets containing 5-aza-dC at 3 mg/kg, and two fish were identified as sex-reversed pseudo-males at 24

μM aqueous exposure followed by treatment with feed containing 5-aza-dC at 12 mg/kg, but no sex reversed pseudo-males were identified in any other combinations of treatments tested (Figure 1A).

Treatment with 5-aza-dC had a major effect on survival, causing significantly more mortalities, especially after 20 dpf (Figure 1B). This effect was correlated with the concentrations of 5-aza-dC, as greater percentages of fish died and died earlier when the aqueous exposure of 5-aza-dC was at 24 μM, than at 12 and 6 μM (Figure 1B). The average survival rate of treatment groups had further dropped to approximately 7% at 25 dpf, which was significantly lower than the control which had an average survival rate of 42% (Student's t-test, $p < 0.05$). Experiments were attempted with 5-aza-dC concentrations at 48 μM, but no fish survived long enough to be included in the experiments (data not shown).

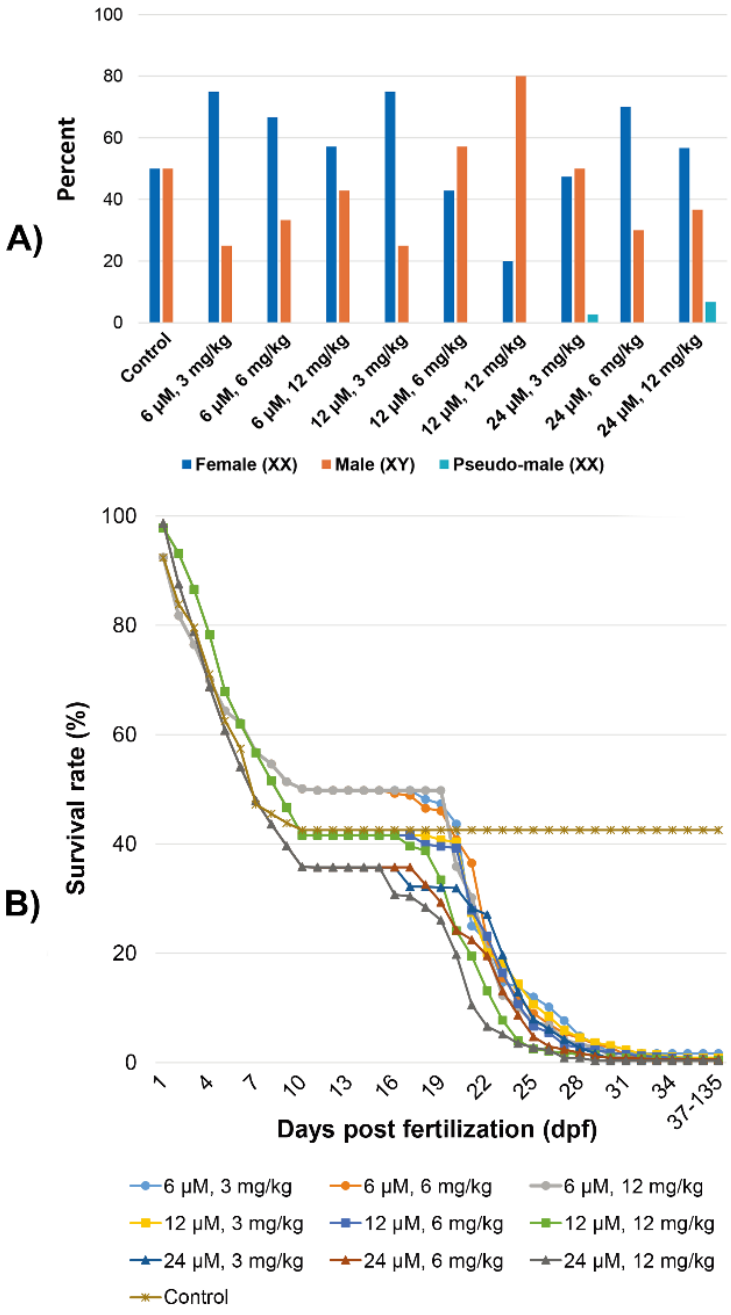

Figure 1. Effect of DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), exposure on and phenotypic sex and survival rate of channel catfish, *Ictalurus punctatus*. A) Phenotypic sex ratio of fish treated with different concentrations of 5-aza-dC at 135 days post fertilization (dpf). **B)** Survival rate of fish treated with different concentrations of 5-aza-dC from 1-135 dpf.

2.4.2 Effect of 5-aza-dC on whole-genome methylation

To determine the effect of 5-aza-dC on methylation, whole genome bisulfate sequencing (WGBS) was conducted with DNA extracted from genetic females and males at 3, 9, 12, 16 and 135 dpf, with and without the treatment of 5-aza-dC. At 135 dpf, WGBS was also conducted for pseudo-males. Out of the 63 libraries, a total of 10.8 billion sequence reads (1,613 Gb data) were obtained (Table 1). An average of 25.6 Gb data with 32× genome depth was obtained for each sample. After quality control, 10.3 billion clean reads were mapped to channel catfish reference genome (Liu et al., 2016) with an average mapping rate of 72% (Table 1).

Table 1. Whole-genome bisulfite sequencing statistics of each sample in control group and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treatment of channel catfish, *Ictalurus punctatus*, at 3, 9, 12 and 16 days post fertilization (dpf). 5-aza-dC treated samples were collected from the group of 24 μM immersion concentration and 12 mg/kg feeding concentration.

Treatment	Time point (dpf)	Sex	Raw reads number	Raw read length (bp)	Clean reads number	Average length after trim (bp)	Percentage kept after trimming (%)	Percentage of genome mapping (%)
5-aza-dC	3	Female 1	216,108,260	150	204,084,106	135.7	94.4	68.3
		Female 2	175,877,132	150	170,370,992	136.2	96.9	75.1
		Female 3	174,794,222	150	169,519,742	136.3	97.0	74.1
		Male 1	198,881,088	150	187,333,996	135.3	94.2	70.0
		Male 2	141,377,352	150	135,482,044	134.9	95.8	71.7
		Male 3	161,568,942	150	155,594,464	135.5	96.3	73.1
	9	Female 1	209,368,778	150	199,511,328	135.5	95.3	64.5
		Female 2	162,588,130	150	157,880,590	136.4	97.1	75.8
		Female 3	171,639,806	150	165,973,862	136.0	96.7	73.7

		Male 1	212,130,538	150	200,021,018	135.6	94.3	65.6
		Male 2	157,542,334	150	152,576,410	136.1	96.8	74.2
		Male 3	135,948,820	150	132,035,312	136.4	97.1	75.7
	12	Female 1	206,441,156	150	194,555,040	135.3	94.2	64.7
		Female 2	137,526,460	150	131,054,706	135.7	95.3	74.2
		Female 3	181,902,278	150	174,838,654	135.9	96.1	74.2
		Male 1	194,388,734	150	184,012,232	134.2	94.7	64.2
		Male 2	151,272,034	150	145,061,082	135.7	95.9	72.6
		Male 3	166,530,770	150	159,176,676	135.8	95.6	73.2
	16	Female 1	215,131,324	150	203,345,962	134.8	94.5	65.5
		Female 2	146,725,926	150	140,761,558	135.8	95.9	73.9
		Female 3	158,823,106	150	152,263,190	135.9	95.9	74.2
		Male 1	209,428,742	150	197,353,358	135.3	94.2	67.5
		Male 2	159,859,722	150	153,814,610	135.8	96.2	74.4
		Male 3	184,225,826	150	175,109,808	135.6	95.1	73.5
	135	Female 1	168,993,540	150	160,561,174	135.7	95.0	74.5
		Female 2	107,156,864	150	102,852,178	136.3	96.0	71.8
		Female 3	172,545,356	150	161,624,842	135.7	93.7	73.7
		Male 1	172,707,626	150	167,080,940	136.6	96.7	75.1
		Male 2	120,477,374	150	115,646,698	136.5	96.0	75.4
		Male 3	154,281,720	150	148,849,082	135.9	96.5	74.7
		Pseudo-male 1	134,546,102	150	129,783,698	135.9	96.5	76.1
		Pseudo-male 2	167,721,340	150	160,476,794	135.8	95.7	74.5
		Pseudo-male 3	181,581,100	150	175,502,174	136.6	96.7	75.2
Control	3	Female 1	345,685,824	150	327,677,646	135.9	94.8	58.3
		Female 2	140,864,064	150	136,486,040	136.3	96.9	74.7
		Female 3	120,541,148	150	116,739,280	136.3	96.8	74.3
		Male 1	213,929,120	150	201,048,358	134.8	94.0	68.6
		Male 2	194,422,884	150	188,664,696	136.5	97.0	70.4
		Male 3	172,887,794	150	167,387,418	136.0	96.8	73.1
	9	Female 1	228,954,292	150	214,604,062	134.9	93.7	70.5
		Female 2	133,074,412	150	129,061,392	136.0	97.0	74.5
		Female 3	157,151,454	150	152,146,984	136.1	96.8	74.0
		Male 1	203,264,380	150	190,625,574	135.2	93.8	70.9
		Male 2	161,979,334	150	156,973,716	136.2	96.9	75.4
		Male 3	151,741,286	150	147,083,840	136.3	96.9	75.4
	12	Female 1	199,783,000	150	188,724,374	135.3	94.5	68.5
		Female 2	145,486,116	150	141,122,516	136.2	97.0	74.5
		Female 3	146,248,642	150	141,764,376	136.3	96.9	74.0
		Male 1	207,651,494	150	194,856,592	135.4	93.8	65.9
		Male 2	115,401,630	150	112,230,394	136.5	97.3	74.6
		Male 3	170,840,078	150	163,482,030	135.9	95.7	74.0
	16	Female 1	207,651,280	150	195,213,666	135.0	94.0	64.9
		Female 2	166,213,152	150	158,924,996	135.7	95.6	71.6
		Female 3	148,201,866	150	142,077,826	135.9	95.9	74.9
		Male 1	223,459,364	150	210,800,260	135.5	94.3	58.5

	Male 2	146,688,252	150	141,173,790	135.9	96.2	71.8
	Male 3	123,657,446	150	118,925,408	136.0	96.2	73.4
135	Female 1	164,732,928	150	157,440,060	136.0	95.6	75.4
	Female 2	169,126,828	150	161,977,062	136.0	95.8	75.0
	Female 3	154,476,974	150	148,455,022	136.0	96.1	75.2
	Male 1	162,671,832	150	153,086,316	135.7	94.1	73.8
	Male 2	156,461,188	150	146,221,376	135.7	93.5	74.0
	Male 3	111,079,854	150	106,432,352	136.4	95.8	74.3
Total		10,754,420,418	150	10,275,515,742	135.819	95.7	72.1

On average, 8.2% of the total number of Cs were methylated for all samples (Table 2). The mC percentage of both females and males were significantly decreased after 5-aza-dC treatment (Student's t-test, $p < 0.05$) (Figure 2A). The CpG methylation was the predominant form of DNA methylation. Among the methylated Cs, the vast majority (~93%) were in the CpG context, while only 1.7% were methylation at the CHG sites and 5.3% for CHH sites (Table 2). Of the CpG sites, 79.4% were methylated in control samples, and treatment of 5-aza-dC reduced the mCpG percentage to 75.4% on average (Figure 2A; Table 2). At the whole genome level, the mCpG percentage of females and males in treatment were indistinguishable from control at 3 dpf (Figure 2B). Methylation levels were significantly reduced by the treatment at 9, 12 and 16 dpf in both females and males (Student's t-test, $p < 0.05$) (Figure 2B). No significant difference between treated groups and controls was detected at 135 dpf, presumably due to withdraw of 5-aza-dC treatment after 30 dpf.

Table 2. Methylated cytosines detected of each sample in control groups and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treatments of channel catfish, *Ictalurus punctatus*, at 3-, 9-, 12- and 16 days post fertilization

(dpf). 5-aza-dC treated samples were collected from the group of 24 µM immersion concentration and 12 mg/kg feeding concentration.

Treatment	dpf (days post fertilization)	Sex	Context	# Total C's	# Methylated C's	Ratio (%)
		Female 1	C	2,354,192,310	193,297,775	8.21
			CpG	223,688,975	183,976,890	82.25
			CHG	517,042,918	2,432,236	0.47
			CHH	1,613,460,417	6,888,649	0.43
		Female 2	C	2,152,410,323	179,725,798	8.35
			CpG	200,540,609	165,300,000	82.43
			CHG	461,871,855	3,528,298	0.76
			CHH	1,489,997,859	10,897,500	0.73
		Female 3	C	2,114,867,324	177,984,978	8.42
			CpG	196,695,983	163,441,426	83.09
			CHG	451,230,948	3,533,438	0.78
			CHH	1,466,940,393	11,010,114	0.75
	3	Male 1	C	2,185,906,079	181,097,686	8.28
			CpG	208,656,761	172,418,835	82.63
			CHG	480,212,868	2,275,871	0.47
			CHH	1,497,036,450	6,402,980	0.43
		Male 2	C	1,592,638,097	137,309,376	8.62
			CpG	151,210,968	126,554,273	83.69
			CHG	338,964,595	2,618,152	0.77
			CHH	1,102,462,534	8,136,951	0.74
		Male 3	C	1,859,841,389	160,721,158	8.64
			CpG	176,143,365	148,027,382	84.04
			CHG	394,807,890	3,080,813	0.78
			CHH	1,288,890,134	9,612,963	0.75
5-aza-dC		Female 1	C	2,137,040,895	161,133,048	7.54
			CpG	207,435,209	152,743,955	73.63
			CHG	476,455,457	2,174,675	0.46
			CHH	1,453,150,229	6,214,418	0.43
		Female 2	C	2,073,012,175	153,668,367	7.41
			CpG	192,929,756	140,106,733	72.62
			CHG	444,773,004	3,237,981	0.73
			CHH	1,435,309,415	10,323,653	0.72
		Female 3	C	1,980,761,299	144,913,111	7.32
			CpG	186,244,075	132,251,771	71.01
			CHG	422,871,237	3,006,250	0.71
			CHH	1,371,645,987	9,655,090	0.70
	9	Male 1	C	2,198,266,649	155,333,929	7.07
			CpG	207,962,210	147,104,379	70.74
			CHG	487,146,643	2,116,620	0.43
			CHH	1,503,157,796	6,112,930	0.41
		Male 2	C	1,939,504,103	143,557,511	7.40
			CpG	181,309,057	130,950,095	72.22
			CHG	414,132,035	2,996,231	0.72
			CHH	1,344,063,011	9,611,185	0.72
		Male 3	C	1,757,174,761	128,193,923	7.30
			CpG	164,025,701	116,793,279	71.20
			CHG	376,355,295	2,719,751	0.72
			CHH	1,216,793,765	8,680,893	0.71
			C	1,956,158,082	142,863,125	7.30

	Female 1	CpG	195,130,956	135,598,066	69.49
		CHG	442,790,557	1,898,484	0.43
		CHH	1,318,236,569	5,366,575	0.41
	Female 2	C	1,630,900,874	124,086,951	7.61
		CpG	157,708,512	114,323,447	72.49
		CHG	350,679,374	2,341,919	0.67
		CHH	1,122,512,988	7,421,585	0.66
	Female 3	C	2,099,011,848	156,604,577	7.46
		CpG	201,703,096	144,359,403	71.57
		CHG	449,247,346	2,926,382	0.65
		CHH	1,448,061,406	9,318,792	0.64
12	Male 1	C	1,944,942,246	144,042,989	7.41
		CpG	190,217,160	136,678,557	71.85
		CHG	437,223,877	1,905,607	0.44
		CHH	1,317,501,209	5,458,825	0.41
	Male 2	C	1,717,497,837	135,776,030	7.91
		CpG	168,365,147	125,891,297	74.77
		CHG	367,930,247	2,368,004	0.64
		CHH	1,181,202,443	7,516,729	0.64
	Male 3	C	1,910,145,526	147,546,644	7.72
		CpG	185,434,013	136,645,781	73.69
		CHG	409,425,757	2,610,278	0.64
		CHH	1,315,285,756	8,290,585	0.63
	Female 1	C	2,234,872,234	152,100,023	6.81
		CpG	213,168,891	143,633,129	67.38
		CHG	497,447,334	2,147,055	0.43
		CHH	1,524,256,009	6,319,839	0.41
	Female 2	C	1,643,891,330	125,131,423	7.61
		CpG	160,901,919	114,870,011	71.39
		CHG	354,050,938	2,456,573	0.69
		CHH	1,128,938,473	7,804,839	0.69
	Female 3	C	1,873,902,621	136,801,107	7.30
		CpG	182,300,817	125,443,403	68.81
		CHG	405,228,457	2,727,237	0.67
		CHH	1,286,373,347	8,630,467	0.67
16	Male 1	C	2,246,577,759	159,897,222	7.12
		CpG	218,844,479	151,391,729	69.18
		CHG	505,806,224	2,190,475	0.43
		CHH	1,521,927,056	6,315,018	0.41
	Male 2	C	1,887,036,488	140,350,007	7.44
		CpG	184,724,238	129,000,404	69.83
		CHG	408,025,253	2,728,400	0.67
		CHH	1,294,286,997	8,621,203	0.67
	Male 3	C	2,094,019,343	158,393,763	7.56
		CpG	205,600,905	145,299,847	70.67
		CHG	452,205,563	3,144,831	0.70
		CHH	1,436,212,875	9,949,085	0.69
	Female 1	C	2,080,726,042	172,648,199	8.30
		CpG	203,333,751	159,555,982	78.47
		CHG	452,597,823	3,152,677	0.70
		CHH	1,424,794,468	9,939,540	0.70
135	Female 2	C	1,412,524,702	117,527,184	8.32
		CpG	138,710,931	109,002,517	78.58
		CHG	308,546,001	2,055,133	0.67
		CHH	965,267,770	6,469,534	0.67
	Female 3	C	1,963,111,582	163,369,284	8.32
		CpG	191,340,518	151,166,963	79.00

			CHG	427,341,292	2,940,561	0.69
			CHH	1,344,429,772	9,261,760	0.69
		Male 1	C	1,998,982,981	164,786,892	8.24
			CpG	193,998,299	153,053,041	78.89
			CHG	432,439,778	2,797,014	0.65
			CHH	1,372,544,904	8,936,837	0.65
		Male 2	C	1,508,516,363	127,469,082	8.45
			CpG	148,431,235	118,645,418	79.93
			CHG	329,610,253	2,125,606	0.64
			CHH	1,030,474,875	6,698,058	0.65
		Male 3	C	1,879,965,160	158,665,423	8.44
			CpG	184,535,158	147,905,087	80.15
			CHG	407,194,991	2,578,881	0.63
			CHH	1,288,235,011	8,181,455	0.64
		Pseudo-male 1	C	1,707,467,283	139,315,820	8.16
			CpG	164,433,781	129,943,592	79.02
			CHG	370,931,805	2,240,088	0.60
			CHH	1,172,101,697	7,132,140	0.61
		Pseudo-male 2	C	1,953,324,916	157,782,491	8.08
			CpG	191,470,475	146,367,271	76.44
			CHG	421,098,095	2,708,167	0.64
			CHH	1,340,756,346	8,707,053	0.65
		Pseudo-male 3	C	2,133,891,433	175,888,478	8.24
			CpG	206,522,283	162,600,515	78.73
			CHG	460,922,380	3,167,284	0.69
			CHH	1,466,446,770	10,120,679	0.69
Control	3	Female 1	C	3,081,530,240	241,066,481	7.82
			CpG	288,202,887	229,412,251	79.60
			CHG	681,469,498	3,089,023	0.45
			CHH	2,111,857,855	8,565,207	0.41
		Female 2	C	1,725,776,287	147,279,068	8.53
			CpG	163,394,388	135,466,422	82.91
			CHG	370,048,011	2,899,964	0.78
			CHH	1,192,333,888	8,912,682	0.75
		Female 3	C	1,447,893,814	122,441,845	8.46
			CpG	136,369,334	113,046,246	82.90
			CHG	309,980,802	2,302,796	0.74
			CHH	1,001,543,678	7,092,803	0.71
		Male 1	C	2,297,014,760	190,687,305	8.30
			CpG	225,357,541	181,888,380	80.71
			CHG	516,914,949	2,400,108	0.46
			CHH	1,554,742,270	6,398,817	0.41
		Male 2	C	2,267,305,743	191,361,225	8.44
			CpG	213,317,294	176,065,086	82.54
			CHG	487,669,710	3,770,433	0.77
			CHH	1,566,318,739	11,525,706	0.74
		Male 3	C	1,981,654,607	170,582,508	8.61
			CpG	186,173,546	155,036,716	83.28
			CHG	422,070,032	3,756,253	0.89
			CHH	1,373,411,029	11,789,539	0.86
	9	Female 1	C	2,544,983,936	197,708,354	7.77
			CpG	239,755,267	187,733,186	78.30
			CHG	560,165,759	2,584,888	0.46
			CHH	1,745,062,910	7,390,280	0.42
		Female 2	C	1,656,309,101	132,889,219	8.02
			CpG	154,273,649	121,980,992	79.07
			CHG	355,795,212	2,634,255	0.74

			CHH	1,146,240,240	8,273,972	0.72
		Female 3	C	1,924,833,750	158,119,289	8.21
			CpG	181,628,339	145,224,479	79.96
			CHG	411,711,900	3,104,502	0.75
			CHH	1,331,493,511	9,790,308	0.74
		Male 1	C	2,303,271,224	177,351,726	7.70
			CpG	219,102,500	168,609,962	76.95
			CHG	510,771,355	2,283,756	0.45
			CHH	1,573,397,369	6,458,008	0.41
		Male 2	C	2,034,504,313	168,408,590	8.28
			CpG	195,013,780	154,954,004	79.46
			CHG	441,362,779	3,298,283	0.75
			CHH	1,398,127,754	10,156,303	0.73
		Male 3	C	1,907,510,492	153,790,239	8.06
			CpG	178,097,058	141,066,983	79.21
			CHG	409,894,315	3,070,968	0.75
			CHH	1,319,519,119	9,652,288	0.73
	12	Female 1	C	2,189,621,524	173,218,746	7.91
			CpG	213,022,368	164,612,081	77.27
			CHG	492,123,129	2,274,992	0.46
			CHH	1,484,476,027	6,331,673	0.43
		Female 2	C	1,866,816,499	152,255,696	8.16
			CpG	177,856,962	140,455,808	78.97
			CHG	401,930,671	2,862,847	0.71
			CHH	1,287,028,866	8,937,041	0.69
		Female 3	C	1,856,293,448	147,595,683	7.95
			CpG	172,923,829	136,743,805	79.08
			CHG	396,670,017	2,607,156	0.66
			CHH	1,286,699,602	8,244,722	0.64
		Male 1	C	2,166,130,479	167,495,114	7.73
			CpG	206,169,914	158,937,056	77.09
			CHG	481,739,010	2,228,965	0.46
			CHH	1,478,221,555	6,329,093	0.43
		Male 2	C	1,531,907,768	123,168,708	8.04
			CpG	144,284,557	114,134,259	79.10
			CHG	327,267,071	2,173,957	0.66
			CHH	1,060,356,140	6,860,492	0.65
		Male 3	C	1,980,266,461	164,577,950	8.31
			CpG	191,041,781	152,559,102	79.86
			CHG	426,176,165	2,929,812	0.69
			CHH	1,363,048,515	9,089,036	0.67
	16	Female 1	C	2,202,366,106	165,953,711	7.54
			CpG	206,305,152	157,448,120	76.32
			CHG	487,902,302	2,172,315	0.45
			CHH	1,508,158,652	6,333,276	0.42
		Female 2	C	1,810,959,041	152,402,525	8.42
			CpG	177,984,394	140,284,562	78.82
			CHG	389,110,289	2,924,120	0.75
			CHH	1,243,864,358	9,193,843	0.74
		Female 3	C	1,833,209,017	157,303,861	8.58
			CpG	184,969,130	145,418,616	78.62
			CHG	403,019,041	2,942,269	0.73
			CHH	1,245,220,846	8,942,976	0.72
		Male 1	C	2,107,201,749	161,579,857	7.67
			CpG	200,451,506	153,295,563	76.48
			CHG	470,593,762	2,127,192	0.45
			CHH	1,436,156,481	6,157,102	0.43

		Male 2	C	1,712,115,614	143,975,421	8.41
			CpG	169,157,863	132,598,613	78.39
			CHG	371,924,992	2,774,491	0.75
			CHH	1,171,032,759	8,602,317	0.73
		Male 3	C	1,444,917,292	120,390,610	8.33
			CpG	141,550,955	111,408,605	78.71
			CHG	311,971,436	2,173,588	0.70
			CHH	991,394,901	6,808,417	0.69
	135	Female 1	C	1,980,317,579	160,396,984	8.10
			CpG	186,554,145	147,846,747	79.25
			CHG	426,006,059	2,989,167	0.70
			CHH	1,367,757,375	9,561,070	0.70
		Female 2	C	2,064,844,333	168,793,245	8.17
			CpG	197,511,885	156,487,867	79.23
			CHG	445,597,156	2,953,988	0.66
			CHH	1,421,735,292	9,351,390	0.66
		Female 3	C	1,895,249,402	155,703,924	8.22
			CpG	181,344,982	144,560,468	79.72
			CHG	408,722,242	2,664,530	0.65
			CHH	1,305,182,178	8,478,926	0.65
		Male 1	C	1,857,097,896	155,438,994	8.37
			CpG	180,906,251	144,137,740	79.68
			CHG	399,238,760	2,684,034	0.67
			CHH	1,276,952,885	8,617,220	0.67
		Male 2	C	1,812,089,674	153,347,640	8.46
			CpG	177,367,497	142,309,640	80.23
			CHG	391,654,427	2,639,650	0.67
			CHH	1,243,067,750	8,398,350	0.68
		Male 3	C	1,448,388,281	121,986,797	8.42
			CpG	139,805,633	112,965,604	80.80
			CHG	313,198,328	2,141,317	0.68
			CHH	995,384,320	6,879,876	0.69

To determine whether the effects of 5-aza-dC pervade the entire genome, the mCpG sites were mapped to the reference genome sequence. As shown in Figure 2C, 5-aza-dC reduced the level of methylation of all chromosomes in the genome, although the methylation levels were different across the genome. No significant differences between females and males were identified.

To characterize methylation in different genomic regions, we examined the correlation between methylation level and the distance from the TSS as well as mCpG percentage of various genomic elements. The average mCpG density was as low as 28% near the TSS and increased sharply in the upstream and downstream regions (Figure

2D). 5-aza-dC treatment exhibited lower mCpG density than control at each site near the TSS, with no difference between females and males (Figure 2D). Introns showed the highest mCpG density (80%), followed by intergenic regions (78%) and exons (73%), whereas promoters only had 55% mCpG (Figure 2E). The methylation levels of these genomic elements were significantly reduced in females and males after 5-aza-dC treatment (Figure 2E).

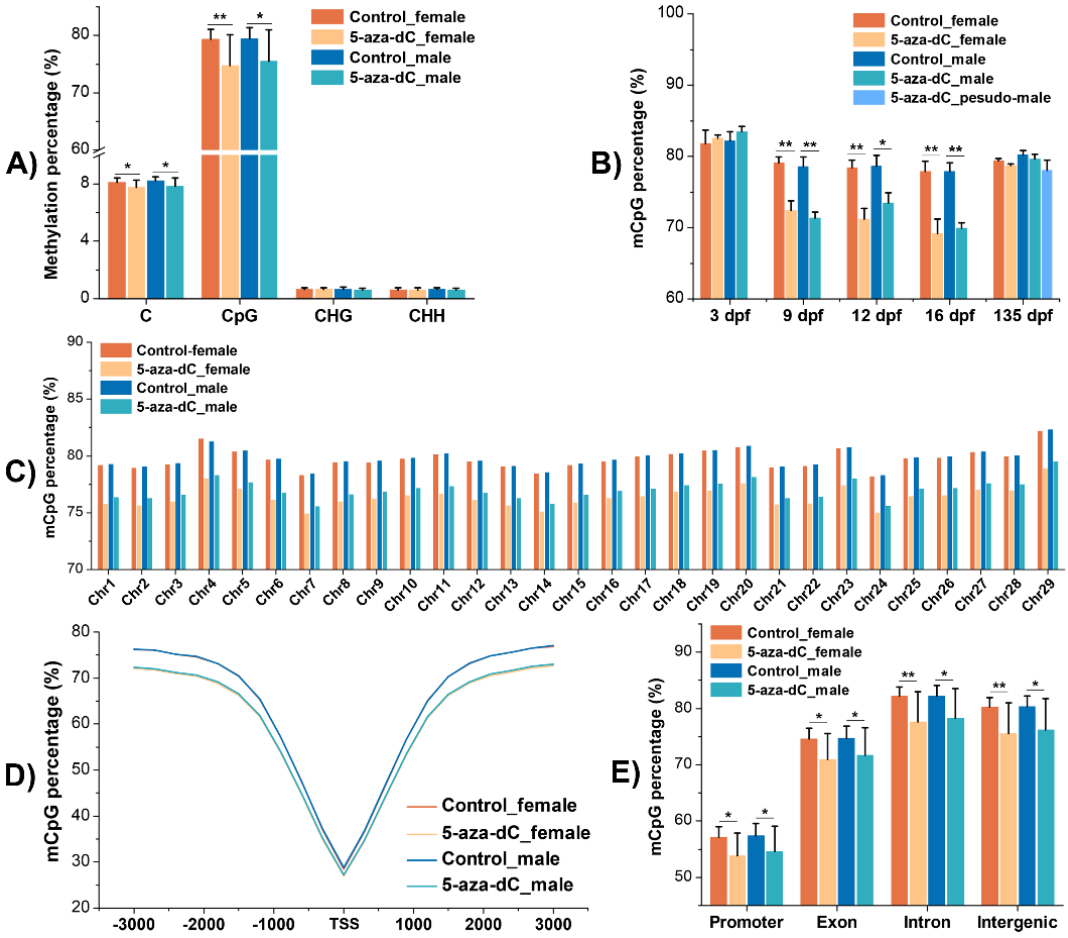

Figure 2. Genome methylation features of female and male in control and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treated channel catfish, *Ictalurus punctatus*. 5-aza-dC treated samples were collected from the group

of 24 μM immersion concentration and 12 mg/kg feeding concentration. **A)** Methylation percentage within each of C, CpG, CHG, and CHH contexts. **B)** Relative CpG methylation density (the ratio of mCpGs to CpGs) at 3-, 9-, 12-, 16- and 135 days post fertilization (dpf). **C)** Relative CpG methylation density of each chromosome. **D)** Relative CpG methylation density in 3Kb region upstream and downstream of the transcription start site (TSS). **E)** The percentage of mCpGs in different genomic elements. Student's t test, $*p < 0.05$, $**p < 0.01$.

2.4.3 Differentially methylated CpG sites and genes after 5-aza-dC treatment

We employed SeqMonk to perform pairwise comparisons of 5-aza-dC treatment and control in females and males at 3, 9, 12, 16 and 135 dpf by sites with ⩾ 10× coverage to detect differentially methylated CpG sites (DMSs). At 3 dpf, the numbers of overall hypermethylated sites were actually greater for the 5-aza-dC treatment than that with controls, in both females and males. At 9 dpf, the number of DMSs with hypomethylation was much greater than that of hypermethylated DMSs after treatment. Similar patterns held true for 12 and 16 dpf, and the extent of the numbers was even greater for hypomethylation in treated fish as compared with controls. At 135 dpf, however, the numbers of DMSs were much smaller, and the overall numbers of hyper- and hypomethylated sites were also similar between the treated and control samples (Figure 3A).

Genes that harbored DMSs in their promoters, exons, or introns were identified as differentially methylated genes (DMGs). The number of DMGs at each time point was summarized in Figure 3B, and they mirrored those patterns of DMSs (Figure 3A). Compared with control, many CpG sites and genes were differentially hypomethylated with 5-aza-dC treatment, especially at 9, 12 and 16 dpf, with more and more hypo-DMSs and DMGs identified over time (Figure 3A and 3B). In either the DMSs or DMGs, the numbers of hypomethylated sites or genes were more dramatic in females than in males. However, similar to the numbers of DMSs, at 135 dpf, the numbers of DMGs were similar, with or without treatment, and the numbers of hypomethylated genes were just slightly more in treated samples than in controls. The numbers of DMGs were more evident in females than in males (Figure 3B).

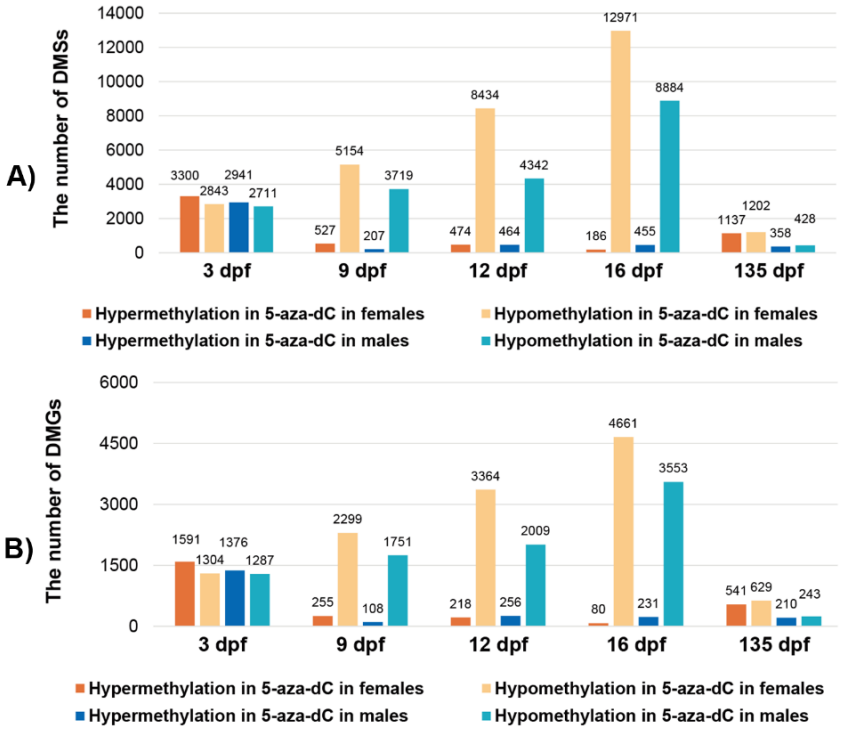

Figure 3. Statistics of differential methylation after DNA methylation inhibitor 5-aza-dC treatment in channel catfish, *Ictalurus punctatus*. For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 days post fertilization (dpf). Numbers of differentially methylated CpG sites (DMSs) (**A**) and differentially methylated genes (DMGs) (**B**) between control and 5-aza-dC treated samples at 3, 9, 12, 16 and 135 dpf.

2.4.4 Differential methylation within the SDR with 5-aza-dC treatment

Although the effect of 5-aza-dC on methylation was genome wide, one obvious question was what the methylation inhibitor 5-aza-dC did to the epigenetic marks within the SDR and consequently, to the expression of genes within the SDR. DMSs were mapped to chromosomes and those on chromosome 4, and especially those within SDR, were determined. Methylation of a large number of sites within the SDR were inhibited with the treatment at 9, 12 and 16 dpf, leading to hypomethylation in treated samples. This was more evident in females than in males (compare Figures 4A and 4B). Additionally, greater similarities of methylation levels within the SDR between treated females and males than between normal females and males were observed at 9, 12 and 16 dpf (Figure 5).

Fifty-nine genes of a total of 123 within the SDR, were differentially methylated, suggesting that the genes within the SDR were more differentially methylated than genes across the genome. Most of the DMGs within the SDR were hypomethylated after treatment, and those that were hypermethylated were mostly limited to one time point, 3 dpf. Of the 59 differentially methylated genes within the SDR, only three genes, *hydin*, *rasgrf1*, and *nectin1*, were hypomethylated at all time points 9, 12 and 16 dpf, after the treatment of 5-aza-dC, and in both females and males. Three genes, *bncr*, *sphkap*, and *mef2a*, exhibited gender-specific hypomethylation for at least two of the three time points of 9, 12 and 16 dpf in females, after the treatment with 5-aza-dC, but not in the males. At 135 dpf, four genes in the SDR, *rasgrf1*, *golgb1*, *edc4*, and *gnao1a*, were hypomethylated after treatment in treated females, but no genes in the males were differentially methylated.

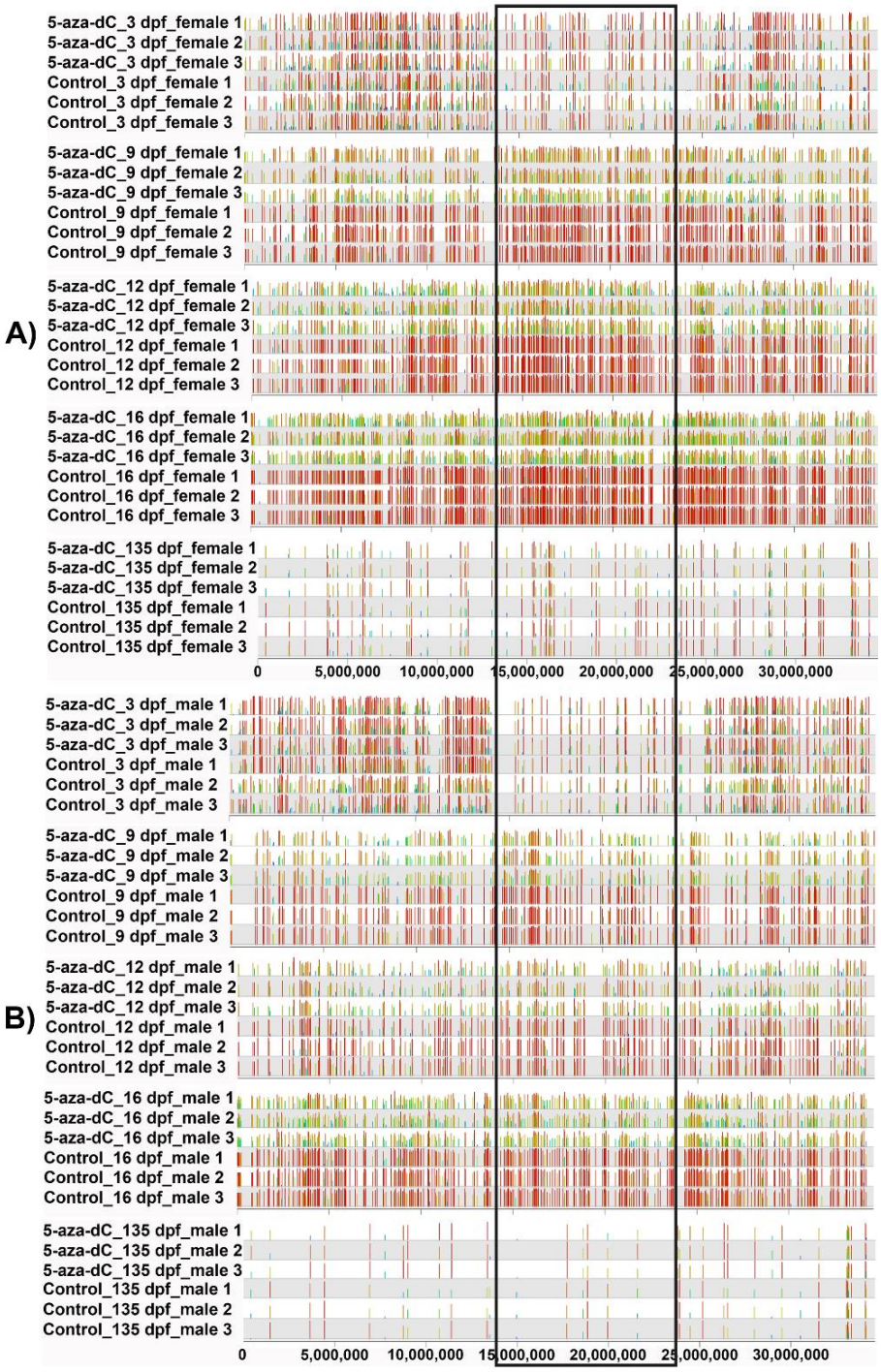

Figure 4. The distribution of differentially methylated CpG sites (DMSs) on chromosome 4 after DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treatment at 3-, 9-, 12-, 16- and 135 days post fertilization (dpf) in channel

catfish, *Ictalurus punctatus*. For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 µM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf. **A**) The DMSs between 5-aza-dC treated and control females. **B**) The DMSs between 5-aza-dC treated and control males. The sex determination region (SDR) is located within the black box.

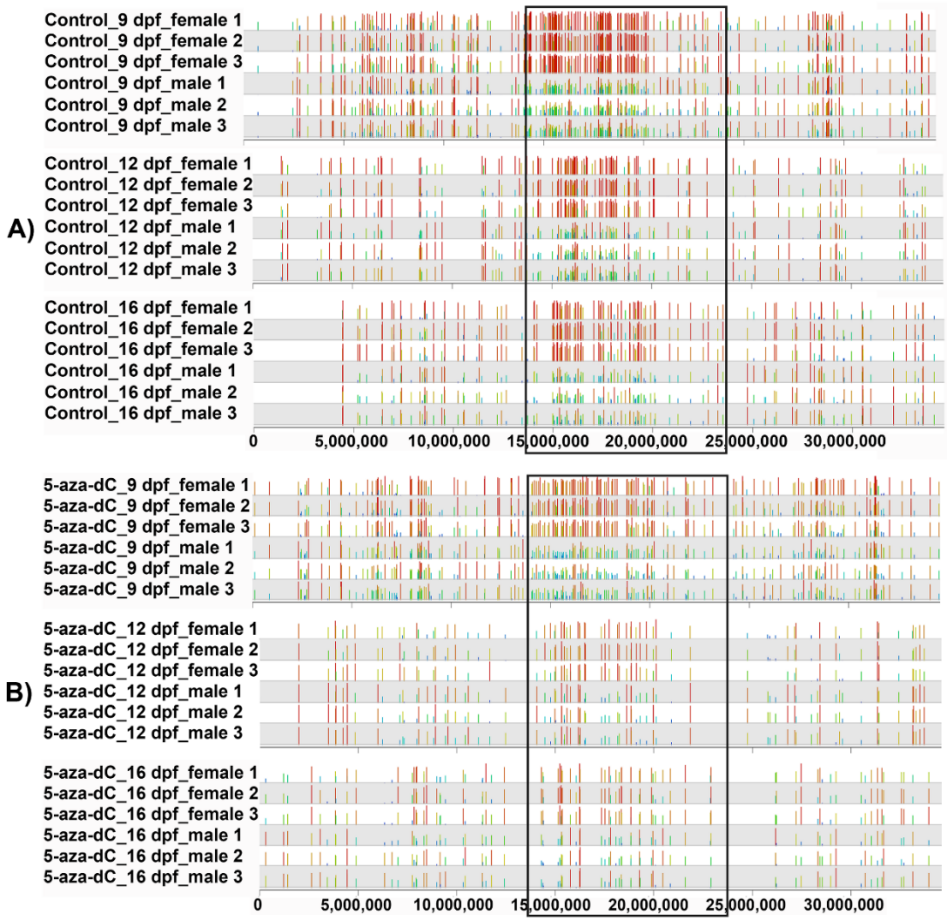

Figure 5. The distribution of differentially methylated CpG sites between females and males on chromosome 4 in control (A) and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treated fish (B) at 9-, 12- and 16 days post

fertilization (dpf). For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf. All differentially methylated CpG sites are shown on chromosome 4, with the sex determination region (SDR) within the black box. Note the drastic methylation differences in controls between females and males within SDR. 5-aza-dC caused drastic reduction in methylation, which led to less dramatic differences between female and male fish after treatment.

2.4.5 Differentially expressed genes with the methylation inhibitor

To determine the effects of 5-aza-dC on genomic expression, RNA-Seq was conducted from samples with and without the treatment with each sex (female, male, and pseudo-male) at each time point (3, 16 and 135 dpf). The detailed information of RNA-seq including the number of sequencing reads, clean reads, and mapped reads in each group is summarized in Table 3.

Treatment with 5-aza-dC caused differential expression of a large number of genes (Table 4). At 3 dpf, 342 and 198 genes were up-regulated in females and males, respectively, and the numbers of down-regulated genes were much larger in females (763) than in males (only 36). At 16 dpf, the numbers of up- and down-regulated genes were similar in females and males. However, at 135 dpf, the numbers of up- and down-regulated genes in females and males were again quite different, with 753 genes up-

regulated in females, and 342 in males, while the numbers of down-regulated genes were drastically different, with only 28 genes being down-regulated in females but 461 in males (Table 4). GO enrichment analysis was conducted using DEGs. Up-DEGs were overrepresented in the biological process of cell adhesion, immune response, embryonic organ development. In contrast, down-DEGs were enriched in DNA replication and cell cycle process, suggesting that methylation inhibitor had the effect of blocking DNA synthesis and inhibiting cell proliferation.

To gain insight into roles of the up-regulated DEGs after 5-aza-dC treatment, these genes were compared to genes that were differentially expressed between untreated females and males at the same time. At 3 dpf, a significant number of genes (137) that were up-regulated in females after 5-aza-dC treatment overlapped with differentially expressed genes with higher expression in control males; Similarly, a significant number of genes (285) that were down-regulated in females after 5-aza-dC treatment overlapped with DEGs with higher expression in control females compared to males at 3 dpf. The number of such overlapped genes was largest between up-regulated genes in females after 5-aza-dC treatment and those that were differentially expressed genes with higher expression in control males compared to females at 135 dpf (Figure 6). These patterns of overlapped genes suggested that the treatment of 5-aza-dC induced a set of genes in females that were normally differentially more expressed in males, and suppressed a set of genes in females that were normally differentially expressed more in females.

Table 3. Summary of RNA sequencing of control and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treated channel catfish (*Ictalurus punctatus*) at different time points. For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 days post fertilization (dpf).

Treatment	Time point	sex	Raw reads number	Clean reads number	Percentage of genome mapping (%)
5-aza-dC	3 dpf	Female 1	103,317,466	95,220,354	95.57
		Female 2	130,516,192	120,623,058	96.20
		Female 3	156,501,466	144,537,658	95.20
		Male 1	180,257,392	165,939,492	95.54
		Male 2	214,031,088	197,656,302	95.93
		Male 3	180,311,304	166,584,418	95.96
	16 dpf	Female 1	148,739,948	137,659,652	92.39
		Female 2	87,220,870	80,013,494	93.93
		Female 3	101,763,058	92,475,108	92.43
		Male 1	118,283,986	108,891,616	94.28
		Male 2	101,168,972	93,388,184	92.97
		Male 3	166,646,584	153,267,326	92.88
	135 dpf	Female (XX) 1	48,554.902	44,355,196	93.91
		Female (XX) 2	49,081,500	44,975,796	93.66
		Female (XX) 3	47,495,144	43,837,088	93.94
		Pseudo-male (XX) 1	49,179,980	45,234,602	95.69
		Pseudo-male (XX) 2	48,064,422	44,488,928	95.69
		Pseudo-male (XX) 3	49,949,760	46,382,924	95.20
		Male (XY) 1	40,582,456	36,922,006	94.57
		Male (XY) 2	43,681,816	39,687,460	94.11
Control	3 dpf	Female 1	72,643,092	70,148,600	95.03
		Female 2	74,322,010	72,267,692	95.44
		Female 3	68,131,342	62,647,874	93.94
		Male 1	80,741,474	74,253,364	94.52
		Male 2	74,595,348	68,513,364	94.68
		Male 3	127,090,676	117,264,528	95.24
	16 dpf	Female 1	138,510,360	128,100,168	94.97
		Female 2	110,243,572	102,298,614	95.43
		Female 3	217,606,286	200,844,896	93.70
		Male 1	146,420,038	135,184,216	78.16
		Male 2	106,126,404	98,076,294	89.23
		Male 3	119,085,546	110,045,476	92.39
	135 dpf	Female 1	45,780,938	42,621,368	95.10
		Female 2	62,928,846	59,045,314	95.06
		Female 3	56,622,110	53,036,034	94.86
		Male 1	52,534,630	49,120,326	95.71
		Male 2	48,992,736	45,481,860	95.47
		Male 3	48,481,516	44,262,316	91.86
Total			3,667,698,883	3,435,352,966	94.0

Table 4. The numbers of differentially expressed genes (DEGs) after treatment with DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), at 3, 16 and 135 days post-fertilization (dpf) in channel catfish (*Ictalurus punctatus*). For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 µM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf.

Time point (dpf)	Up-DEGs in females	Down-DEGs in females	Up-DEGs in males	Down-DEGs in males
3	342	763	198	36
16	373	246	327	350
135	753	28	342	461

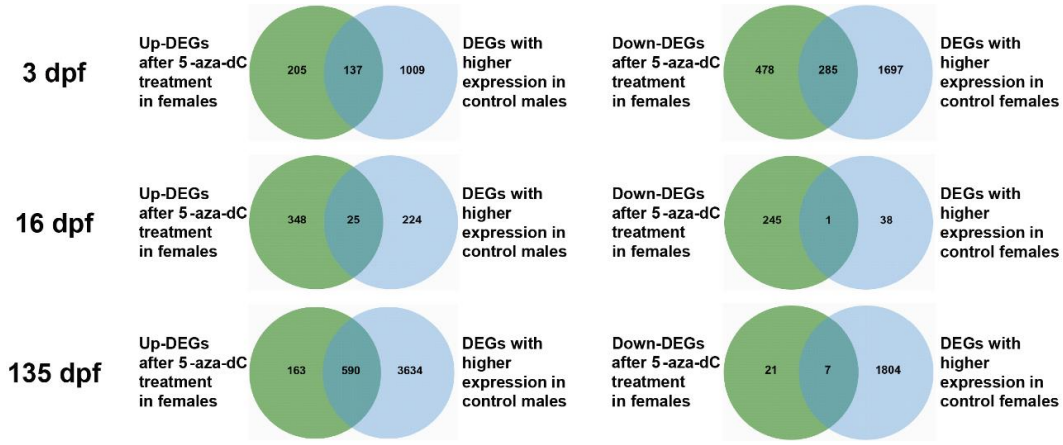

Figure 6. The shared genes between the up-regulated differentially expressed genes (up-DEGs) after DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), treatment in females and DEGs with higher expression in control males compared to females and between down-DEGs after treatment in females

and DEGs with higher expression in control females compared to control males at 3-, 16- and 135 days post fertilization (dpf) in channel catfish, *Ictalurus punctatus*. For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf.

2.4.6 Differentially expressed genes within the SDR

A total of ten genes within the SDR on chromosome 4 were differentially expressed after 5-aza-dC treatment, with six genes differentially expressed at 3dpf, and three genes at 16 dpf, and one gene, *hydin*, at both 3 and 16 dpf (Table 5). All the 10 genes were differentially expressed after the treatment in females, and only one, *tsnaxip1*, was also differentially expressed in males. Seven genes, *hydin*, *spred3*, *slitrk3*, *hsf4*, *tsnaxip1*, *carmil2*, and *sphkap*, were up-regulated in females after 5-aza-dC treatment, and three genes, *esrrg*, *pard6a*, and *actrt3*, were down-regulated in females after 5-aza-dC treatment. The levels of differential expression were not dramatic, mostly between two to three folds (Table 5).

Table 5. Differentially expressed genes (DEGs) within the sex determination region (SDR) after the treatment of DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC), in channel catfish (*Ictalurus punctatus*) at 3- and 16 days post fertilization (dpf). For 5-aza-dC treatment, starting from the fertilized eggs, fish

were with 5-aza-dC four hours per day at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf. Gene abbreviations: *hydin*, hydrocephalus-inducing protein homolog; *spred3*, sprouty related EVH1 domain containing 3; *sphkap*, SPHK1 interactor, AKAP domain containing; *esrrg*, estrogen-related receptor gamma-like; *actrt3*, actin related protein T3; *tsnaxip1*, translin-associated factor X interacting protein 1; *carmil2*, capping protein regulator and myosin 1 linker 2; *pard6a*, par-6 family cell polarity regulator alpha; *hsf4*, heat shock transcription factor 4; *slitrk3*, SLIT and NTRK like family member 3.

Gene	Up or down regulated	Sex	Time point	Fold change
hydin	Up/up	Female/Female	3 dpf/16 dpf	2.2/3.3
spred3	Up	Female	3 dpf	2.9
sphkap	Up	Female	16 dpf	2.2
esrrg	Down	Female	3 dpf	2.2
actrt3	Down	Female	3 dpf	2.9
tsnaxip1	Up/up	Female/male	16 dpf/16 dpf	3.0/3.1
carmil2	Up	Female	16 dpf	2.2
pard6a	Down	Female	3 dpf	2.3
hsf4	Up	Female	3 dpf	3.9
slitrk3	Up	Female	3 dpf	2.3

2.4.7 DNA methylation in pseudo-males

The treatment of methylation inhibitor caused sex reversal from genetic females to phenotypic males, but the sex phenotype could not be determined until 135 dpf. We investigated the DNA methylation profiles in normal female (XX) ovaries, normal male (XY) testes and pseudo-male (XX) testes at 135 dpf. As shown in figure 7A, PCA

indicated that the whole genome methylation profiles of the pseudo-males were more similar to those of normal females than to normal males. At 135 dpf, hypermethylation was still observed within the SDR in normal females as compared with normal males (Figure 7B). Pseudo-males exhibited hypermethylation in many sites within the SDR as compared to normal males (Figure 7C), but not when compared with normal females (Figure 7D), suggesting that the methylation profiles within the SDR in pseudo-males were more similar to those of normal females than to those of normal males. However, the number of DMSs between pseudo-males and normal males was much less than that between normal females and males, as well as DMGs.

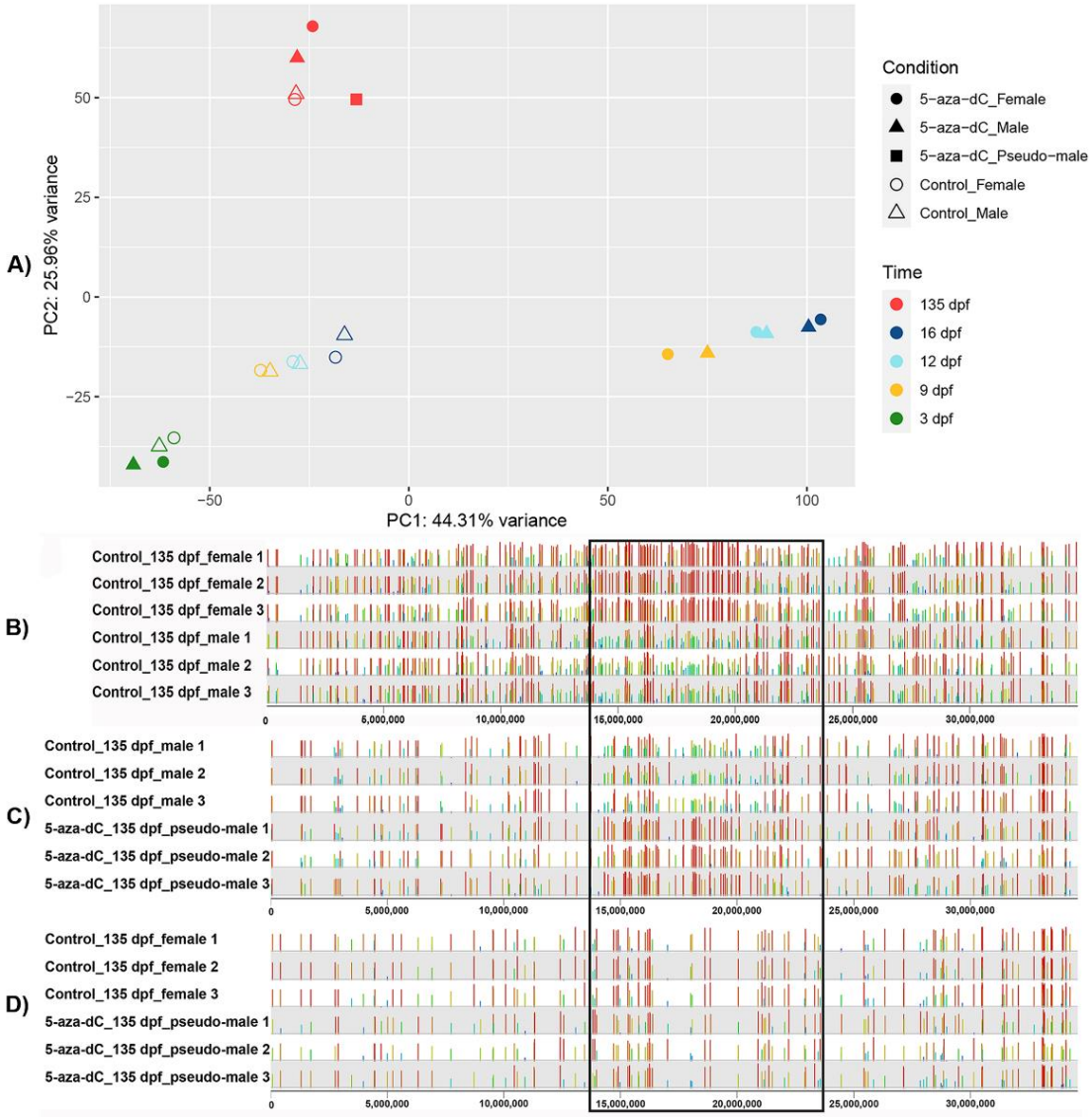

Figure 7. DNA methylation of channel catfish (*Ictalurus punctatus*) pseudo-males.

A) Principal component analysis (PCA) of WGBS samples using per site CpG methylation. The distribution of differentially methylated CpG sites (DMSs) on chromosome 4 between females and males (**B**), between males and pseudo-males (**C**), and between females and pseudo-males (**D**) at 135 days post fertilization (dpf).

2.4.8 Gene expression in pseudo-males

Genome-wide transcriptome analysis revealed that expression profiles of pseudo-males were much more similar to those of normal males than to normal females. As shown in Figure 8A, PCA revealed that time after fertilization was the major effect explaining differences in expression. Thus, males and females, with or without treatment, were clustered close together at 3 and 16 dpf. At 135 dpf, when pseudo-males could be identified, sex had the largest influence on the profiles of gene expression. Thus, female and male samples were clustered together within the same sex. Pseudo-males had an expression profile that was even more closely clustered together with treated males than control males (Figure 8A).

Genome expression of the pseudo-males was most similar to that of treated males, as also determined by pairwise comparison of differentially expressed genes (Figure 8B). At 135 dpf, a total of 14,791 genes were differentially expressed between normal females and males. While 8,838 DEGs were identified between pseudo-males and control females, only 25 genes were differentially expressed between pseudo-males and control males. Similarly, 6,813 genes were differentially expressed between pseudo-males and treated females, only 21 genes were differentially expressed between pseudo-males and treated males.

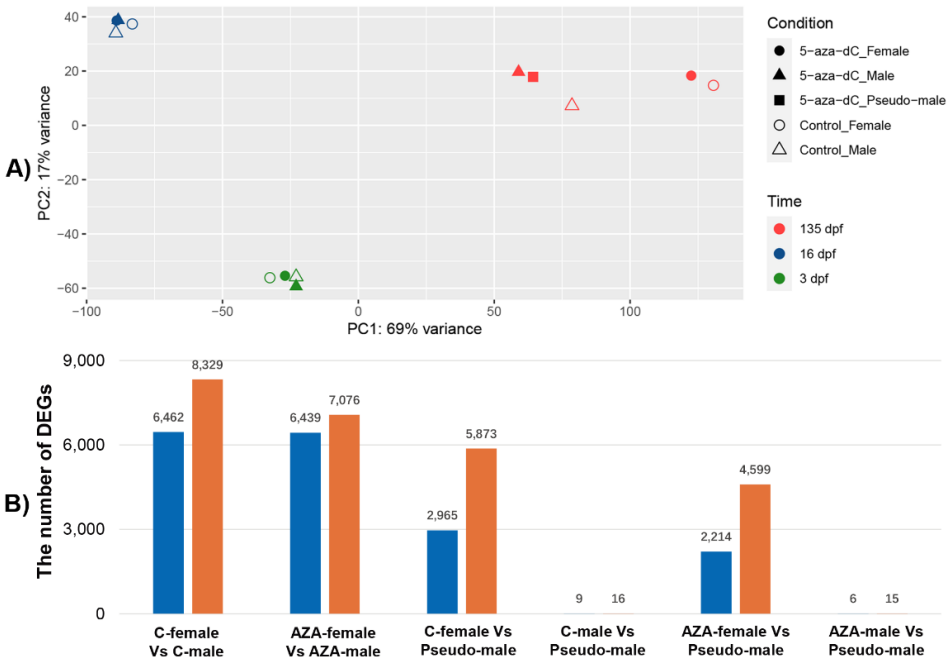

Figure 8. Gene expression of channel catfish (*Ictalurus punctatus*) pseudo-males. **A)** Principal component analysis (PCA) of RNA-seq samples using regularized-logarithm transformation of normalized expression data in DESeq2. **B)** The number of differentially expressed genes (DEGs) between sexes at 135 days post fertilization (dpf). C: Control group; AZA: DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC).

GO enrichment showed that DEGs that were up-regulated in normal females relative to pseudo-males were overrepresented in biological processes of reproductive process, regulation of cell cycle, and somite development (Figure 9A). In contrast, DEGs that are up-regulated in pseudo-male testes were enriched in tube morphogenesis, embryonic organ development, and cell adhesion (Figure 9A). Furthermore, enrichment analysis of DEGs between females and males in the control was also conduced (Figure

9B). Up-regulated DEGs in females were mainly related to reproduction, female gamete generation, cell cycle, and oogenesis, and the enrichment GO of up-regulated DEGs were blood vessel development, cell adhesion, and G protein-coupled receptor signaling pathway. In the SDR, the gene expression of pseudo-males was also similar to normal males. Taken together, it appears that female-bias gene related to oocyte development in pseudo-males had been suppressed, leading to the function closer to normal males.

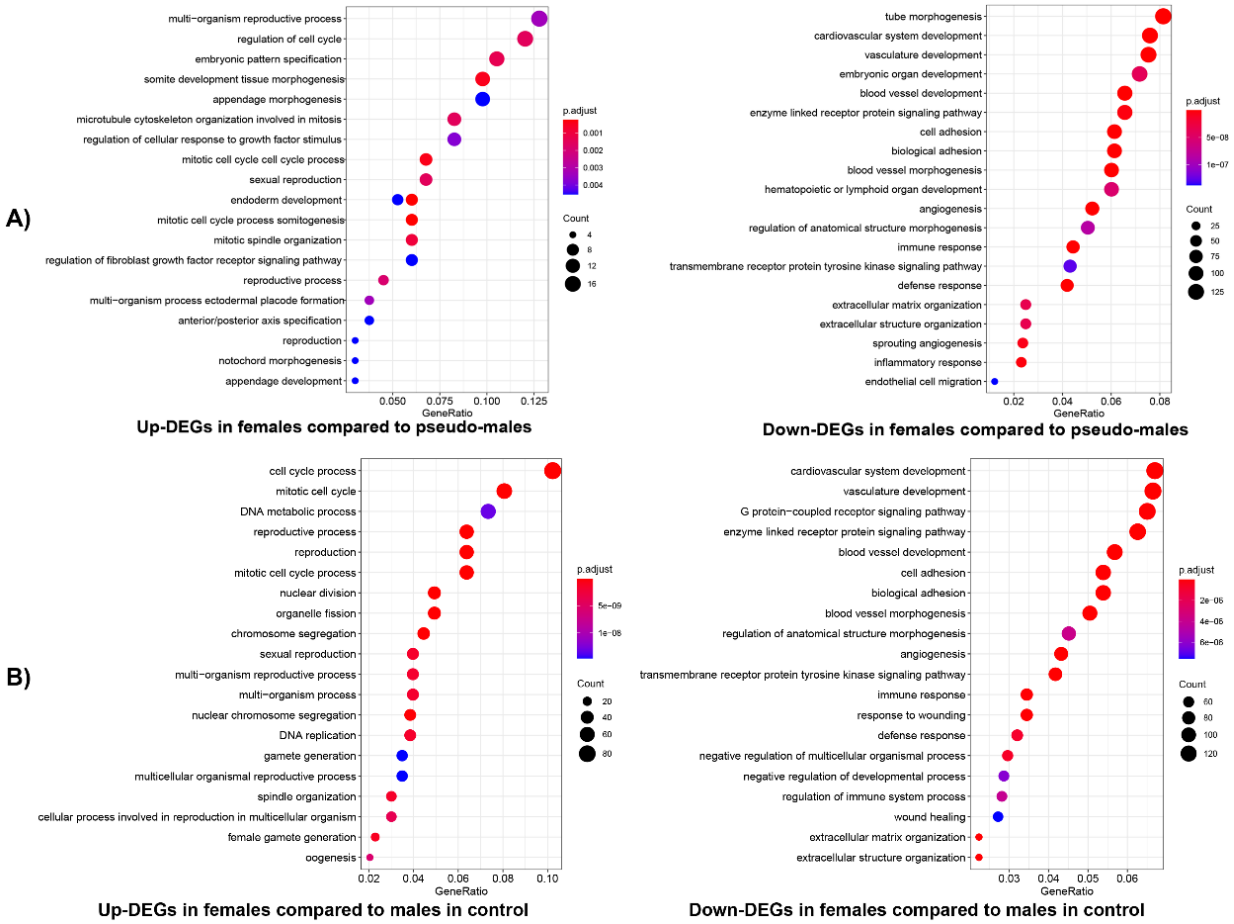

Figure 9. Top enriched biological processes of differentially expressed genes (DEGs) between different gonad samples of channel catfish, *Ictalurus punctatus*,

at 135 days post fertilization (dpf). **A**) Top enriched biological processes of up-regulated DEGs and down-regulated DEGs in control females compared with pseudo-males. **B**) Top enriched biological processes of up-regulated DEGs and down-regulated DEGs in control females compared with control males.

2.4.9 Correlation between methylation and gene expression

To explore the potential regulatory role of DNA methylation during critical period of sex differentiation in channel catfish, we integrated the methylation and gene expression datasets obtained from the same samples. In all samples, a negative correlation was observed between promoter methylation and gene expression as well as between gene body methylation and gene expression, however, the levels of correlation were not strong (Figure 10A).

In addition, we investigated the shared genes among DMGs and DEGs in each comparison group after treatment (Figure 10B). For the most part, DMGs and DEGs after treatment with 5-aza-dC were independent of each other. Only a small number of DMGs were also differentially expressed. For instance, of the 1,304 hypomethylated genes after 5-aza-dC treatment in females at 3 dpf, only 51 genes were differentially expressed, of which 23 genes were up-regulated while 28 genes were down regulated. Similar relationships were observed in both males and females, and at 3, 16 and 135 dpf. Of the shared genes located within the SDR, *hydin* and *sphkap* were

hypomethylated and up-regulated after 5-aza-dC treatment in females at 16 dpf, while *pard6a* was hypermethylated and down-regulated at 3 dpf (Figure 10B).

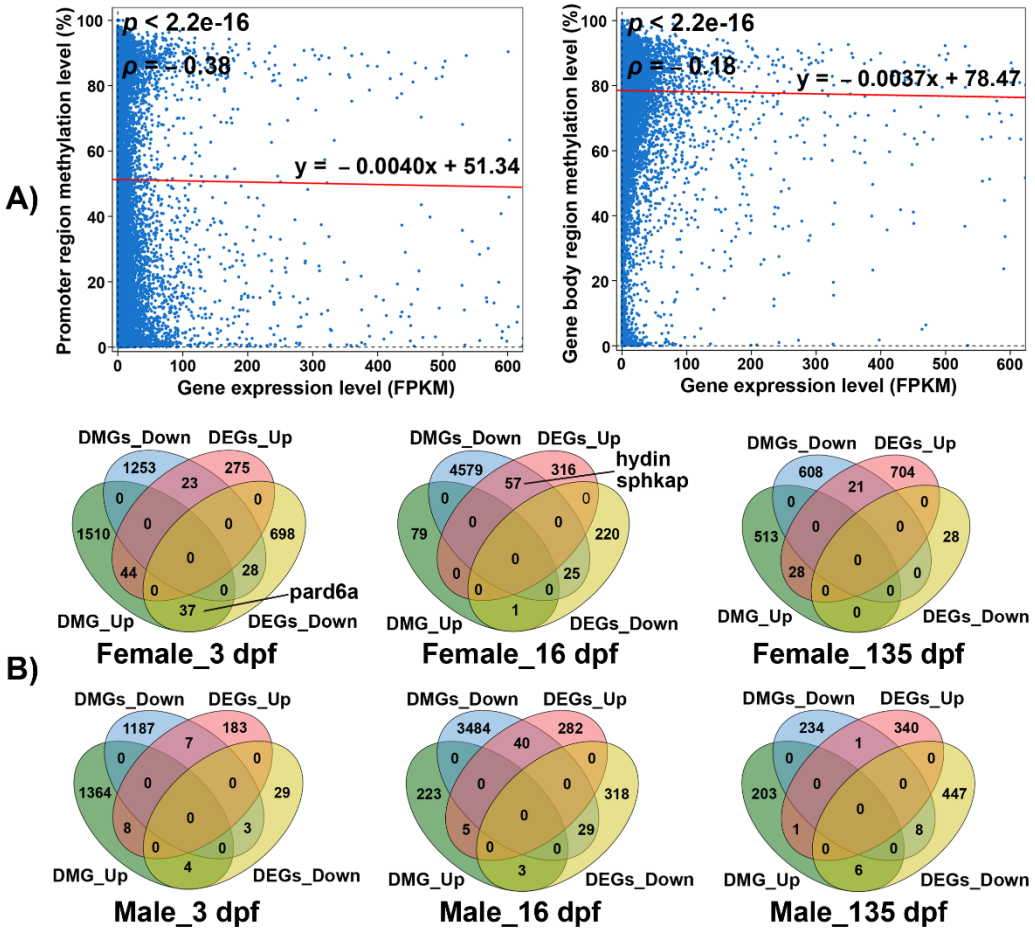

Figure 10. Correlation between methylation and gene expression in channel catfish (*Ictalurus punctatus*). A Correlation between gene expression and methylation in promoter and gene body of female samples at 3 days post fertilization (dpf) in control. The trend line indicates the linear correlation between methylation and gene expression. **B** The differentially expressed genes (DEGs) overlapped with differentially methylated genes (DMGs) after DNA methylation inhibitor 5-aza-dC treatment. For 5-aza-dC treatment, starting from the fertilized eggs, fish were with 5-aza-dC four hours per day

at the concentration of 24 μM. After the yolk-sac was absorbed, the fish were fed diets containing 5-aza-dC at a dose of 12 mg/kg three times per day until 30 dpf. The shared genes within the sex determination region (SDR) were *pard6a*, par-6 family cell polarity regulator alpha, *hydin*, hydrocephalus-inducing protein homolog, and *sphkap*, SPHK1 interactor, AKAP domain containing.

2.5 Discussion

Teleosts display tremendous diversity of sex determination mechanisms. Our previous research examined channel catfish using genetic linkage mapping provided solid evidence for genetic sex determination (Li et al., 2015; Zeng et al., 2016). Fine genetic mapping and association studies confirmed a single locus on chromosome 4 that determines sex in channel catfish. This sex determination region was narrowed down to approximately 8.9 Mb region that were defined as the sex determination region (SDR) (Bao et al., 2019). Further delineation of the SDR region was not possible through genetic approaches because there was no recombination within the SDR. Bao et al. conducted comparative genome analysis across the entire chromosome 4, but found no difference in gene contents between the X and the Y chromosome, suggesting that the sex was not controlled by a dominant gene on Y chromosome that was absent from the X chromosome (Bao et al., 2019). Through comparative analysis of whole genome methylation profiles, we identified an epigenetically marked locus that physically overlapped with the sex determination region (SDR). In the SDR, the X-

alleles were hypermethylated while the Y-alleles were hypomethylated (Yang et al., manuscript under revision). Based on the observed allele-specific differential methylation and differential expression of a set of genes within the SDR, we proposed that sex determination in channel catfish, a lower teleost, was regulated by methylation. However, there was no direct evidence to support the involvement of methylation in sex determination, other than the observed allele-specific methylation and expression of a set of genes, and differences of methylation profiles and expression in genetic females and males. In the present study, we directly tested the effect of methylation on sex differentiation by using a methylation inhibitor. We found that the methylation inhibitor 5-aza-dC caused sex reversal from genetic female to phenotypic male (pseudo-male), providing a direct evidence for the involvement of methylation in sex differentiation. Although methylation has been reported as being involved in the sex determination processes, this study demonstrated methylation as a primary factor for sex determination in channel catfish, serving as a novel sex determination mechanism. Considering the evolutionary position of channel catfish as a lower vertebrate and lower teleost, it is possible that such a sex determination mechanism may be operating in other lower vertebrates and invertebrates as well, especially those whose sex are highly vulnerable to environmental factors, such as temperature, which could function through their effects on genome methylation (Navarro-Martín et al., 2011; Parrott et al., 2014; Venegas et al., 2016; Navara, 2018; Radhakrishnan et al., 2018; Han et al., 2021).

Only a small proportion of treated fish were sex reversed from genetic females to phenotypic males. Perhaps the ideal the treatment regime was not identified. Sex reversal was only observed in treatment of highest aqueous concentration (24 μM) of the methylation inhibitor, but not at lower concentrations, suggesting that critical concentration of the inhibitor when applied at the earliest timing may be required for sex reversal. We attempted to test concentrations higher than 24 μM, such as 48 μM, but all fish died after treatment, preventing the use of higher concentrations of the methylation inhibitor. The aqueous application of the inhibitor, followed by feeding, was technically easy to perform, but the actual effective concentration and effective timing may be variable among individuals of treated fish. It was possible that only those individuals that received the high concentrations of the inhibitor at the earliest times were sex reversed.

5-aza-dC is toxic and can cause growth arrest, developmental perturbations, and various types of malformations, which has been observed in zebrafish (Martin et al. 1999; Riobas et al. 2017) and Japanese rice fish (*Oryzias latipes*) (Dasmahapatra and Khan, 2015). With channel catfish, mortality rates of treatments were very high, even at lower concentrations tested in this study, suggesting that the methylation inhibitor is generally toxic, presumably because proper methylation is a requirement of normal development and growth.

The methylation inhibitor caused genome-wide reduction of methylation levels. This is consistent with reports from other species including mammals (Ferguson et al.,

1997; Mossman et al., 2010), and teleosts, such as zebrafish (Martin et al. 1999; Ribas et al. 2017), stickleback (*Gasterosteus aculeatus*) (Aniagu et al., 2008) and fathead minnow (*Pimephales promelas*) (Futami et al., 2019). In spite of the genome-wide effect, 5-aza-dC apparently had greater effects with genomic regions that were normally hypermethylated, such as the SDR in females. As such, 5-aza-dC caused great numbers of CpG sites and greater numbers of genes to become hypomethylated in females than in males after treatment. In terms of the levels of methylation in the SDR, the application of the methylation inhibitor caused more dramatic reduction of methylation levels in females than in males, leading to greater similarities of methylation levels between treated females and males than between normal females and males.

Sex differentiation in channel catfish exhibits drastic sexual dimorphism with the females being differentiated much earlier than the males. Female gonad differentiation is visible at 19 dpf while male gonad differentiation is undetectable until 90 dpf (Patiño et al., 1996). From the perspective of time sequence of the sex differentiation processes, an individual either starts to differentiate into a female, upon expression of a set of genes required of female gonadal sex differentiation, or not until later to differentiate into a male, upon genetic composition of XY chromosomes in which female gonadal sex differentiation-related genes are not expressed. The treatment of the embryos early during development with the methylation inhibitor changed the landscape of methylation, which in turn, changed expression profiles including those genes within the SDR that have important sex determination roles. This hypothesis predicted that the

treatment of genetic females (XX) with the methylation inhibitor would increase expression of the "maleness" genes, and decrease the expression of the "femaleness" genes. Analysis of DEGs after the treatment of 5-aza-dC provided evidence for this hypothesis. A large number of genes that were up-regulated with the treatment of 5-aza-dC actually were differentially expressed with greater levels of expression in control females than in control males. This implied that inhibition of methylation led to increased expression of "maleness" genes, and decreased expression of "femaleness" genes.

It is noteworthy that several genes in the SDR showed sex-specific methylation and expression, as well as differential methylation and differential expression after 5-aza-dC treatment. In our previous study (Yang et al., manuscript under revision), *hydin* (hydrocephalus-inducing protein homolog) was a likely candidate for sex determination, because it located in the SDR, exhibited allelic hypermethylation in females, almost exclusively expressed in males in an allele-specific manner. The results of this study support the potential candidacy of *hydin* as the sex determination gene in channel catfish. After 5-aza-dC treatment, the methylation level of *hydin* was reduced in females and its expression was up-regulated in females at both 3 and 16 dpf. *Hydin* was found in the flagellar proteomes of *Chlamydomonas reinhardtii* (Pazour et al., 2005), and its knock down in *C. reinhardtii* caused an unusual form of flagellar paralysis (Lechtreck and Witman, 2007). Loss of *hydin* resulted in slow growth and a severe motility defect in *Trypanosoma brucei* (Broadhead et al., 2006; Dawe et al., 2007), suggesting that

hydin plays a role in formation, function or maintenance of cilia. *Hydin* mutations cause hydrocephalus in mice and human, which is caused by defects in the ependymal cilia of the brain (Davy and Robinson, 2003; Doggett et al., 2006; Lechtreck et al., 2008). In mice, *hydin* was mainly detected in testis, including the developing spermatocytes, spermatocytes within the seminiferous tubules of the testis and the lining of the oviduct, extending out to the fimbriae (Davy and Robinson, 2003). This, together with the results of from *C. reinhardtii* and *T. brucei*, suggested that *hydin* may be involved in the formation of sperm flagella and motility. Such functional aspects of *hydin* were not in conflict with its candidacy as a sex determination gene. However, these are speculative, as the functions of *hydin* in teleost has not been studied.

Other than *hydin*, the only other gene in the SDR, which was hypermethylated in the normal female, hypomethylated and up-regulated after the treatment of 5-aza-dC, was *Sphkap* (SPHK1-interactor and AKAP domain-containing protein). *Sphkap* is an anchoring protein, which binds to the c-AMP-dependent protein kinase (Lacana et al., 2002). Its expression was found to be higher in male than in female of channel catfish (Bao et al., 2019; Yang et al., manuscript under revision). However, considering the delayed up-regulation, it was unlikely that *sphkap* was the very upstream sex determination gene.

It was generally believed that methylation of gene promoters was correlated with transcription repression (Li and Zhang, 2014), while gene body DNA methylation was found to be positively correlated with gene expression (Kulis et al., 2013). Recent

studies, however, challenged the traditional views and demonstrated that some transcription factors prefer to bind to methylated CpG sites (Hu et al., 2013; Yin et al., 2017), suggesting that methylation-mediated gene expression regulation is more complicated. In this study, we investigated the relationship between differential methylation and differential gene expression in both promoters and gene bodies. The methylation levels at promoters were found to be negatively correlated with gene expression, consistent with the inhibitory role of DNA methylation. From a genome-wide perspective, the methylation of gene bodies also showed a negative correlation with gene expression, suggesting that DNA methylation may have complicated regulatory mechanism on gene expression. In either case, however, the correlation coefficient was very low. It was possible that only a fraction of genes whose expression was controlled by methylation. This was supported by the fact that expression of the vast majority of down-methylated genes were not up-regulated, and vice versa (Figure 9). Therefore, the relationship between methylation and expression is far more complicated.

The patterns of methylation and expression in pseudo-males also suggested that methylation and expression may be quite independent. While genome expression appeared to be largely dictated by functional requirement, the memories of methylation patterns may be deeply genetic. As such, the methylation profiles of pseudo-males were much more similar to those of normal females than to those of normal males at 135 dpf, suggesting that “genetic memories” existed for these pseudo-males that were

genetically females. This was more evident in the SDR where hypermethylation persisted throughout the 135 dpf in females and pseudo-males, but hypomethylation was found in normal or treated males. In contrast, genome expression profiles of pseudo-males were most similar to those of 5-aza-dC treated males and normal males than to those of treated or normal females. These results clearly indicated the independence of genome methylation and genome expression.

In conclusion, this study demonstrated involvement of methylation in sex differentiation of channel catfish. The use of methylation inhibitor resulted in reduced levels of methylation, especially in otherwise hypermethylated female genomes. The reduced methylation in the SDR was observed in females, and accordingly, a number of genes in the SDR were up-regulated. Among the demethylated and up-regulated genes, *hydin* gene previously demonstrated to be allele-specifically demethylated and expressed in males. The treatment of methylation inhibitor caused up-regulation of a large number of genes that were preferentially expressed in normal males, and down-regulation of a large number of genes that were preferentially expressed in normal females, revealing genome expression signatures leading to sex reversal from genetic females to phenotypic males.

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Chapter 3 Feminization of channel catfish with 17β-estradiol involves methylation and expression of a specific set of genes independent of the sex determination region

3.1 Abstract

Exogenous estrogen, 17β-estradiol (E2), has been shown to effectively induce feminization in teleosts. However, the molecular mechanisms underlying the process remain unclear. Here, we determined global DNA methylation and gene expression profiles of channel catfish (*Ictalurus punctatus*) during early sex differentiation after E2 treatment. Overall, the levels of global DNA methylation after E2 treatment were not significantly different from those of controls. However, a specific set of genes were differentially methylated, which included many sex differentiation related pathways, such as MARK signaling, adrenergic signaling, Wnt signaling pathway, GnRH signaling, ErbB signaling, and ECM-receptor interaction. Many genes involved in these pathways were also differentially expressed after E2 treatment. Specifically, E2 treatments resulted in upregulation of female-related genes and downregulation of male-related genes in genetic males during sex reversal. However, E2-induced sex reversal did not cause sex-specific changes of methylation profiles or gene expression within the sex determination region (SDR), although expression of over a dozen genes were mostly up-regulated after E2 treatment, especially at 16 dpf, suggesting that E2-

induced sex reversal was a downstream process independent of the sex determination process that was dependent of sex-specific methylation within the SDR.

3.2 Introduction

Fish species account for more than half of all vertebrate species, and they exhibit very diverse reproductive strategies and sexual patterns (Devlin and Nagahama, 2002; Kobayashi et al., 2013). Among them, gonochoristic species have only ovarian or testicular tissues after sex determination and differentiation, while sequential hermaphrodites can change from male to female or from female to male in their life cycle. For instance, zebrafish (*Danio rerio*) is a gonochoristic species without sex switch as adults (Kossack and Draper, 2019), whereas bluehead wrasse (*Thalassoma bifasciatum*) exhibited dramatic and complete female-to-male sex reversal following social stresses (Todd et al., 2019). This diversity and plasticity in fishes provides excellent models to study sex determination and differentiation in vertebrates.

Sex determination leads to the binary fate of ovary or testis. In species where sex is determined by genetic factors, the potential of the individual to become a specific sex with either ovary (female) or testis (male) is determined by their chromosomal composition. However, in many species, especially with lower teleost fish, sex differentiation may be delayed and altered by environment or sex-specific responses of tissues to hormones produced by the gonads (MacLaughlin and Donahoe, 2004). Sex steroids are involved in the natural process of sex differentiation and maintenance

(Nakamura et al., 2003; Li et al., 2019), and the administration of exogenous sex steroids can affect the process (Pandian and Sheela, 1995). Exogenous sex steroids, estrogens and androgens, can cause sex reversal at the phenotypic level even if the genetic sex is fixed. In many species of fish, estrogens cause feminization whereas androgens cause masculinization (Bhandari et al., 2005; Pandian & Kirankumar, 2003; Pandian & Sheela, 1995).

Paradoxically, in some species, such as channel catfish (*Ictalurus punctatus*), exogenous application of sex steroids, either estrogens or androgens, causes feminization (Goudie et al., 1983; Davis et al., 1990), presumably because of conversion of androgens into estrogens in intestine and liver after application (Lou et al., 2002). Estrogens are produced from androgens by a key enzyme, cytochrome P450 aromatase, mainly encoded by *cyp19a1* in fish (Simpson et al., 1994, Zhang et al., 2014).

Exogenous estrogen, 17β-estradiol (E2), has been reported to effectively induce feminization in more than 50 fish species, including, but not limited to the taxonomic families of Cyprinidae, Salmonidae, Cichlidae and Ictaluridae (Pandian and Sheela, 1995; Piferrer, 2001), but the underlying mechanisms of the steroid-induced sex reversal remain elusive in fish species.

In model organisms, estrogen is involved in the regulation of methylation and transcription (Zhao et al., 2017; Zheng et al., 2019; Kovács et al., 2020). After E2 exposure in zebrafish, enriched genes included those involved in calcium signaling pathway, ECM-receptor interaction, gap junction, and cell adhesion molecules, and

genes related to ovaries and development were significantly up-regulated (Zheng et al., 2019). E2 exposure caused global genomic hypermethylation in male gonads of stickleback (*Gasterosteus aculeatus*) (Aniagu et al., 2008).

Channel catfish and its hybrid with male blue catfish, *I. furcatus*, account for more than 50% of aquaculture in the United States. After E2 exposure, channel catfish undergo sex reversal from males to females. However, the changes at the molecular level are unknown. The objective of this study was to determine global methylation and expression profiles of channel catfish after treatment with E2. Here we report the dynamic changes of genome expression of a large set of genes involved in female gonadal sex differentiation, whereas whole-genome level of methylation was not significantly modified after E2 treatment.

3.3 Materials and methods

3.3.1 Ethical statement

This study was performed according to the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the USA. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University.

3.3.2 Experimental design and sample collection

Fertilized eggs of channel catfish from Fish Genetics Laboratory, Auburn University, were hatched in suspended mesh baskets placed in the flow-through paddle wheel troughs until an estimated period of 12 h before hatching. Starting within the first 2 h post-fertilization, embryos were taken out of the trough and treated in the tubs with 17β-estradiol (E2) (E8875, Sigma-Aldrich, Saint Louis, USA) for four hours per day (two hours in the morning and two hours in the afternoon) at the concentration of 400 μg/L. After the yolk-sac was absorbed, the hatched fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post-fertilization (dpf). Control fish were reared in the same way as the treatment, except that E2 was not added during the whole process.

The samples from treatment and control groups were collected at 3, 9, 12, 16 and 110 dpf. Embryos or fry were euthanized with MS222, then placed in a 1.5 ml tube and plunged into liquid nitrogen immediately. For fish at 110 dpf, gonads could be observed with naked eyes, and the phenotypic sex was determined by gonad morphology. Gonads of all fish alive at 110 dpf were collected. Samples were stored at −80°C freezer for subsequent DNA and RNA extractions.

3.3.3 DNA and RNA extraction

All samples collected were split into two parts to extract DNA and RNA separately. Each fertilized egg was equally divided into two halves at 3 dpf. Head and

tail were cut off and the abdomen was divided longitudinally into two equal parts from the spine for each fry at 9, 12, 16 dpf. Each gonad at 110 dpf was also divided into two parts.

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. Total RNA was extracted by RNeasy Plus Universal Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA and RNA were quantified using Nanodrop 2000 by 1% agarose gel electrophoresis. Additionally, small amount of DNA was utilized to determine genetic sex of each sample via PCR analysis using a sex-linked microsatellite marker AUEST0678 (Ninwichian et al., 2012).

3.3.4 Whole-genome bisulfite sequencing (WGBS) and sequence data analysis

For time points at 3, 9, 12, 16 and 110 dpf, equal amount of genomic DNA from five fish samples of genetic females or males in the treatment or control groups were pooled as one replicate for WGBS libraries construction. At 110 dpf, five DNA samples from pseudo-female gonads were also pooled for library preparation. Three replicates were used for each time point, each sex and each treatment. A total of 63 libraries (2 sexes × 5 time points × 2 treatment × 3 replicates + 3 pseudo-females) were prepared and sequenced by Illumina Novaseq platform with paired-end (PE) 150 (CD Genomics, Shirley, NY, USA).

Quality control of raw WGBS reads was performed by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic v0.37 (Bolger et al., 2014) was used to remove adaptor sequences, ambiguous nucleotides, short length (< 36 bp), and low-quality reads (quality score < 20). Before mapping, trimmed reads were transformed into fully bisulfite-converted forward (C to T) and reverse read (G to A conversion of the forward strand) versions by the bisulfite alignment program Bismark v0.22.1 (Krueger and Andrews, 2011). Channel catfish reference genome (Liu et al., 2016) was also converted (C to T and G to A converted) by 'bismark_genome_preparation' tool in the Bismark v0.22.1. Then, sequence reads were aligned to converted versions of genome using Bowtie 2 (Langmead & Salzberg, 2012). Methylation level on each site was extracted using 'bismark_methylation_extractor' tool. Upon completion, a run report including percentage methylation of cytosines in CpG, CHG or CHH context, number of methylated and unmethylated cytosines, and the percent methylation value (context) was produced.

Methylation levels were analyzed using SeqMonk v1.45.2 (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). The methylation sites located on the 29 chromosomes of channel catfish were imported to SeqMonk, and minimum read count per position was set to 10 when defining the probe. Methylation percentages were calculated using the 'bisulfite methylation over feature' pipeline in SeqMonk with default parameters. A statistical test between two groups was

performed by the logistic regression of proportion-based statistics in SeqMonk with *p*-value < 0.01 with a minimum observation of 10. The determination of differentially methylated sites (DMSs) between two groups also required a percentage difference great than 25%.

Differentially methylated genes (DMGs) were defined as DMSs located in their promoter [the 2Kb region upstream of transcription start site (TSS)] or gene body. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the DMGs were performed using the R package clusterProfiler (Yu et al., 2012).

Principal component analysis (PCA) was conducted using methylKit (Akalin et al., 2012), and plotted by ggplot2 in R (Wickham, 2011). CpG methylation levels of features in the channel catfish genome and average CpG methylation percentage near TSS of different groups were calculated with the results from Bismark.

3.3.5 RNA-seq samples and data analysis

For the RNA-seq libraries, RNA from three samples of the same sex (female and male) and same group (treatment and control) were pooled to produce a single replicate for each category at 3, 16 and 110 dpf. Three replicates were prepared for each group. Additionally, RNA from sex-revered fish at 110 dpf were also extracted. A total of 39 libraries were generated and sequenced by Illumina Novaseq platform with PE150 (CD Genomics, Shirley, NY, USA).

Raw reads were trimmed by Trimmomatic v 0.37 with length > 36 bp and quality score > 20 (Bolger et al., 2014). Quality of each sample was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The analysis proceeds by mapping reads to channel catfish reference genome (Liu et al., 2016) with HISAT2 v2.1.0 (Kim et al., 2015). The alignments were saved as SAM format, which were converted and sorted to bam files with SAMtools (H. Li et al., 2009). HTSeq v0.11.0 was used to quantify the number of expressed transcripts (Anders et al., 2015). Differentially expressed genes (DEGs) were identified with DESeq2 with $|\log_2(\text{fold change})| > 1$ and adjusted p value < 0.05 (Love et al., 2014). Functional enrichment analysis of DEGs was conducted in R using clusterProfiler (Yu et al. 2012).

3.4 Results

3.4.1 Feminization by E2 exposure

The genetic and phenotypic sex of experimental fish were determined at 110 dpf. Of a total of 233 fish that were treated with E2, 101 were genetic females and 132 were genetic males. All genetic females were phenotypic females. Among the 132 genetic males, 116 were sex reversed to phenotypic females (pseudo-females), with a sex reversal rate of 87.9%. In total, 217 phenotypic females (91.8%) were identified among the E2 treated fish (Figure 11). The sex ratio of control was near 1:1, including 81 females and 78 males, with no fish being sex reversed (Figure 11).

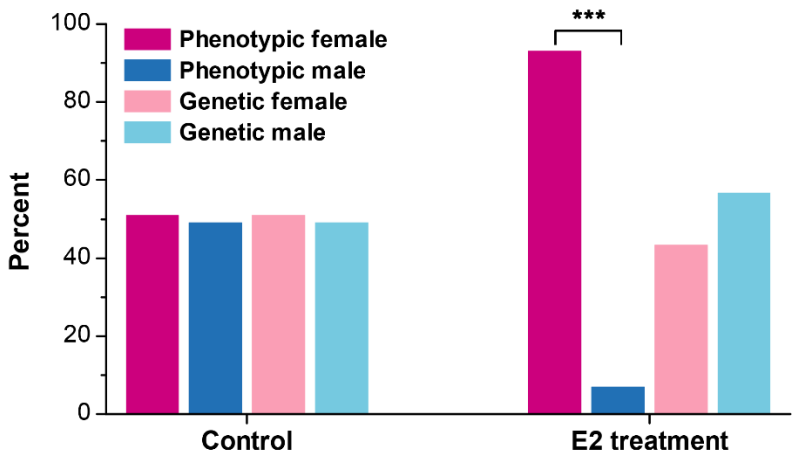

Figure 11. The percent of genetic and phenotypic female and male channel catfish (*Ictalurus punctatus*) in the control and 17β-estradiol (E2) treatment at 110 days post fertilization (dpf). For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 µg/L. After the yolk-sac was absorbed, the fish were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 dpf. ***, $p < 0.001$ by the Chi-square goodness-of-fit test.

3.4.2 Genome-wide DNA methylation profiles after E2 exposure

To determine the effect of E2 on genome methylation, whole genome bisulfate sequencing (WGBS) was conducted with samples of females and males at 3, 9, 12, 16 and 110 dpf, with and without E2 treatment. Samples from pseudo-females at 110 dpf after E2 exposure were also analyzed. A total of 1,794 Gb methylome data with 12 billion sequence reads were produced, which yielded an average depth of 35× per sample. Of these datasets, an average of 163 million methylated cytosines (mCs) per

sample were identified, accounting for 8% of total sequenced cytosines. Among them, 95% of mCs were in CpG context, and nearly 80% of total CpG were methylated (Figure 12A). No significant difference in genome methylation levels was observed between E2 treatment and control in both females and males (Figure 12A). Likewise, the percentage of mCpG between E2 treatment and control at each time point (Figure 12B) were also similar. The overall mCpG percentage was declining with gonadal differentiation over time from 3 to 110 dpf. Additionally, slight increases of mCpG percentage were observed after E2 treatment at 3, 9 and 16 dpf in both females and males, but a slight decrease was observed at 110 dpf in females; all these differences were not significant ($p > 0.05$) (Figure 12B). The methylation level of pseudo-females was most similar to that of E2-treated females at 110 dpf (Figure 12B).

Principal component analysis (PCA) revealed that the methylation patterns were initially clustered by development, but then by treatment (Figure 12C). Samples of E2 treatment and control were effectively separated at 9, 12 and 16 dpf. At 110 dpf, pseudo-females clustered together with both control females and E2-treated females, while control males and E2-treated males were more different, suggesting that the pseudo-females were functioning as females with regard to whole-genome methylation patterns.

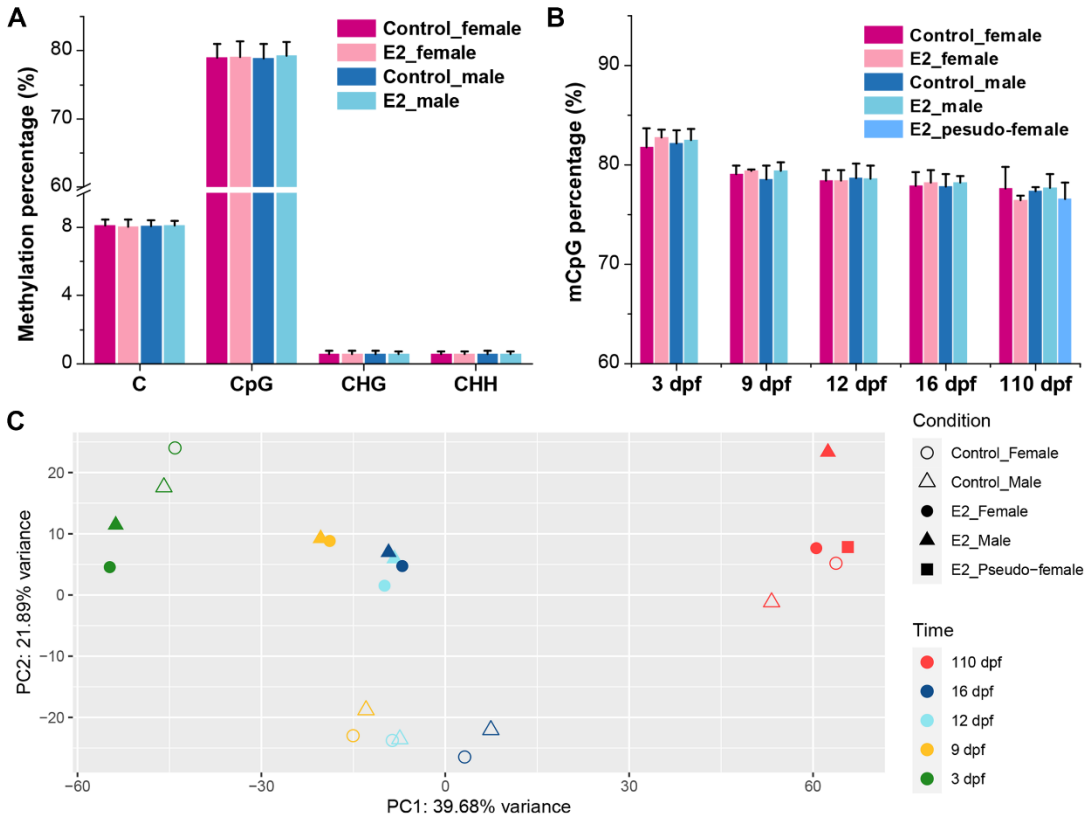

Figure 12. Genome-wide methylation levels of the control and 17β-estradiol (E2) treated channel catfish, *Ictalurus punctatus*. For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 µg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization. **A** Methylation percentage in the contexts of C, CpG, CHG, and CHH. **B** Relative CpG methylation density (the ratio of mCpGs to CpGs) at different time points. dpf: days post fertilization. **C** Principal component analysis (PCA) of all whole-genome bisulfite sequencing (WGBS) samples based on CpG methylation levels.

3.4.3 Differentially methylated genes and involved gene pathways after E2 exposure

Although the overall levels of methylation after E2 treatment were not significantly ($p > 0.05$) different from those of controls, a large number of genes were differentially methylated after E2 treatment (Figure 3A). The numbers of differentially methylated genes (DMGs) in males were greater than that in females at both 3 and 9 dpf. At 12 dpf, the numbers of DMGs in females and males became smaller (Figure 13A) than those at 3 and 9 dpf, and they were similar between females and males. At 16 dpf, the numbers of DMGs in females and males remained low, and they were even smaller in males than in females. At 110 dpf, the numbers of DMGs became very large, with females harboring more DMG than males (Figure 13A).

To gain initial insight into the nature of DMGs after E2 treatment in early developmental stages (3, 9, 12 and 16 dpf), the involved pathways were determined through KEGG pathway enrichment analysis. As shown in Figure 13B, hypermethylated genes in females were enriched in 10 pathways, including calcium signaling, MARK signaling, adrenergic signaling, Wnt signaling, vascular smooth muscle contraction, apelin signaling, melanogenesis, gap junction, GnRH signaling, and adipocytokine signaling. Hypomethylated genes in females were involved in ErbB signaling (Figure 13C).

There were remarkable similarities of pathways involving genes that were hypermethylated in females to those that were hypomethylated in males. The top three pathways involving the largest numbers of genes were the same pathways hypermethylated in females and hypomethylated in males (compare Figure 13B with Figure 13E). Genes involved in ErbB signaling pathway were hypomethylated in both females and males (Figure 13C and 13E). Interestingly, genes involved in certain pathways were both hypermethylated and hypomethylated in males, including cell adhesion molecules and ECM-receptor interaction (Figure 13D and 13E).

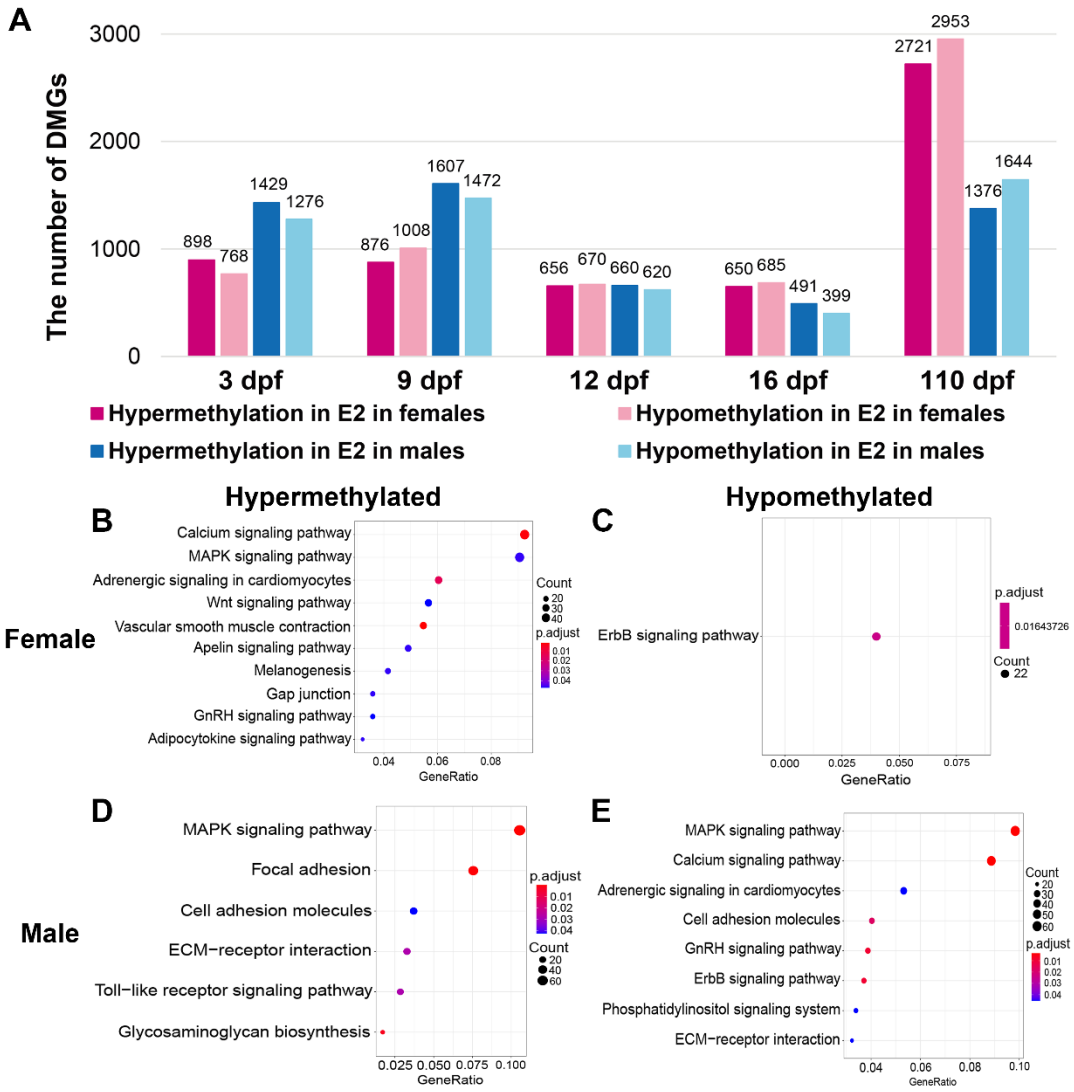

Figure 13. Differential methylation after 17β-estradiol (E2) treatment in channel catfish, *Ictalurus punctatus*. For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 µg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization (dpf). **A** Comparison of differentially methylated genes (DMGs) after E2 treatment in

females and males at 3, 9, 12, 16 and 110 dpf. **B-E** KEGG (kyoto encyclopedia of genes and genomes) pathway enrichment analysis of hypermethylated differentially methylated genes (DMGs) in females (**B**), hypomethylated DMGs in females (**C**), hypermethylated DMGs in males (**D**), and hypomethylated DMGs in males (**E**) at 3 and 16 dpf after E2 treatment.

3.4.4 Differential expression after E2 exposure

RNA-Seq was conducted to determine genomic expression after E2 treatment. A total of 3,950 million raw reads were produced, and 3,689 clean reads were generated. The average mapping rate was 93.4%. Expression levels were compared between treatments and controls in both females and males. At 3 dpf, 208 and 283 genes were up-regulated in females and males, respectively; 610 and 99 genes were down-regulated in females and males, respectively (Table 6). At 16 dpf, many more genes were differentially expressed in both females (3,636 up and 1,875 down) and males (2,575 up and 1,492 down), but at 110 dpf, the number of differentially expressed genes in females were relatively small, with 876 up-regulated genes and 213 down-regulated genes. However, the number of differentially expressed genes in males were extremely large, with 5,665 up-regulated genes and 4,609 down-regulated genes (Table 6), presumably because of the transition from a genetic male to a phenotypic female.

Table 6. The numbers of differentially expressed genes (DEGs) after 17β-estradiol (E2) treatment at 3-, 16- and 110 days post fertilization (dpf) in channel catfish,

***Ictalurus punctatus*.** For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 μg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 dpf. Up-DEGs: up-regulated differentially expressed genes; down-DEGs: down-regulated differentially expressed genes.

Time point (dpf)	Up-DEGs in females	Down-DEGs in females	Up-DEGs in males	Down-DEGs in males
3	208	610	283	99
16	3,636	1,875	2,507	1,492
110	876	213	5,665	4,609

At 110 dpf, gonads were differentiated such that phenotypic sex could be determined. KEGG enrichment analysis was conducted using RNA-Seq datasets of 3 and 16 dpf, to reveal gene pathways involved in E2-induced sex reversal. As expected, enriched gene pathways among up-regulated genes in females and males were very similar, with each enriched for gene pathways of neuroactive ligand-receptor interaction, calcium signaling, MARK signaling, adrenergic signaling in cardiomyocytes, cell adhesion molecules, and cardiac muscle contraction. The enrichment of these pathways apparently reflected significant advances toward female sex differentiation with both genetic females and genetic males, although the numbers of genes involved in these enriched pathways in genetic females were larger (compare Figure 14A with 14C). In contrast, a much larger numbers of pathways were enriched among down-regulated genes in both females and males. Most of the down-regulated

gene enriched pathways were shared between females and males, including DNA replication, focal adhesion, cardiac muscle contraction, cell cycle, ECM-receptor interaction, fatty acid metabolism, glycolysis-pyruvate metabolism, amino acid metabolism, and terpenoid backbone biosynthesis (Figures 14B and 14D). Most of these enriched pathways were involved in growth rather than differentiation, turning the course of prolonged cellular growth as naturally occurring in males, to gonadal sex differentiation as naturally occurring in females.

PCA was conducted using genomic expression datasets of RNA-Seq at 3, 16 and 110 dpf. As shown in Figure 14E, the developmental time accounted for the largest proportion of variance of gene expression. As such, samples of 3, 16 and 110 dpf were mostly clustered within their own group at the same time point after fertilization, especially at 3 dpf. Over time, the treatment effect accounted for increasing amount of variance of expression. At 16 dpf, the control females and males clustered together, while the treated females and males clustered together. This pattern, however, did not hold true for samples at 110 dpf when control females, treated males, treated females, and pseudo-females clustered together, whereas control males were very distant (Figure 14E), suggesting all treated fish were most similar to females with regard to genome expression patterns at 110 dpf.

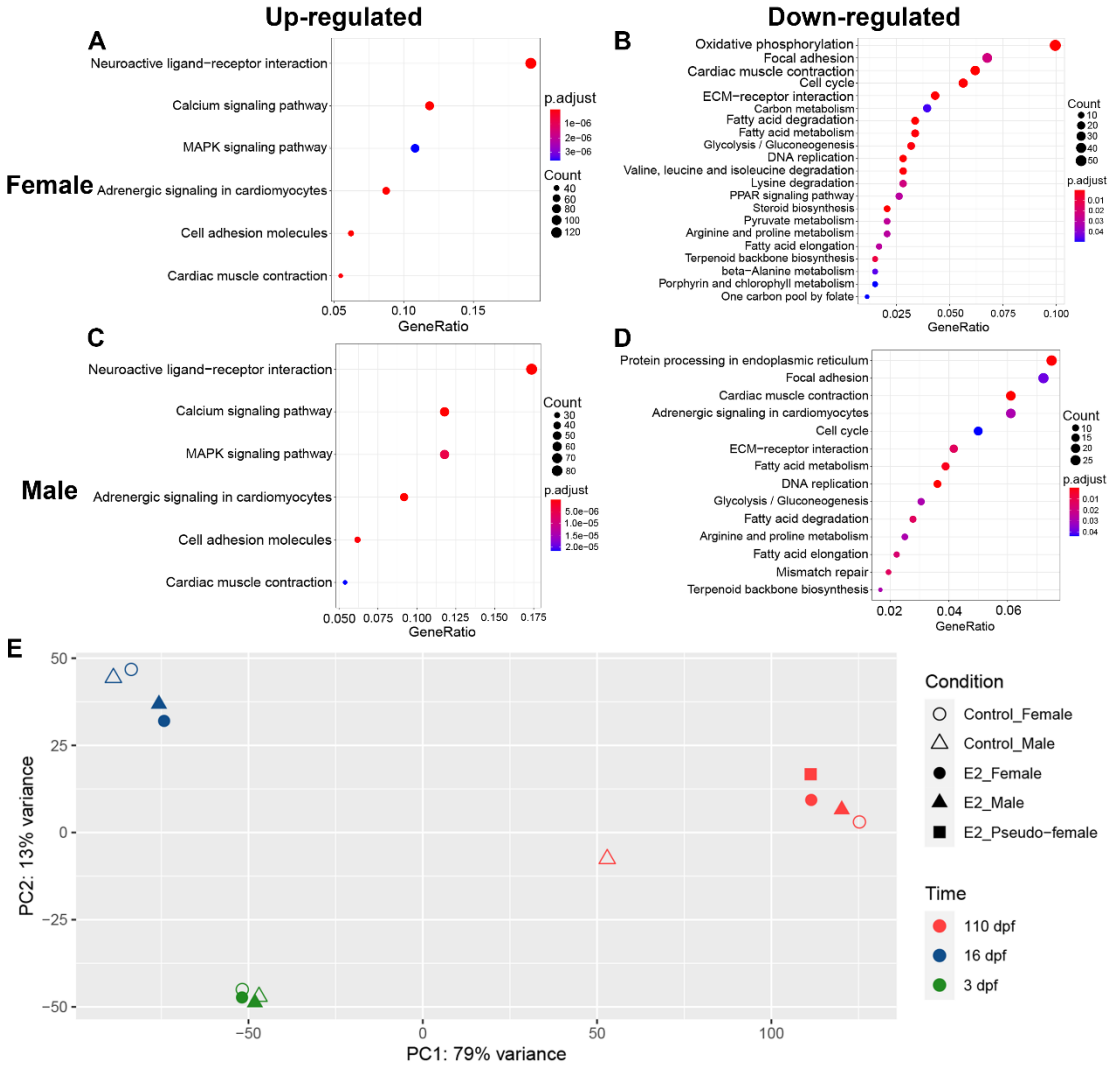

Figure 14. Differential gene expression after 17β-estradiol (E2) treatment in channel catfish, *Ictalurus punctatus*. For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 µg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization (dpf). **A-D** KEGG (kyoto encyclopedia of genes and genomes) pathway enrichment analysis of up-regulated differentially expressed genes (DEGs) in females (**A**), down-

regulated DEGs in females (**B**), up-regulated DEGs in males (**C**), and down-regulated DEGs in males (**D**) at 3 and 16 dpf after E2 treatment. **E** PCA of RNA-seq samples using regularized-logarithm transformation of normalized expression data in DESeq2.

3.4.5 Methylation and gene expression in the sex determination region (SDR) after E2 treatment

As with the whole genome, treatment of E2 did not have a specific effect on methylation within the SDR with regard to differences between females and males. As shown in Figure 15, hypermethylation was observed with females in both control and E2-treated fish at 3, 9, 12 and 16 dpf. The overall DMSs between females and males were strikingly similar, with or without treatment, suggesting that E2-induced sex reversal was independent of methylation within the SDR.

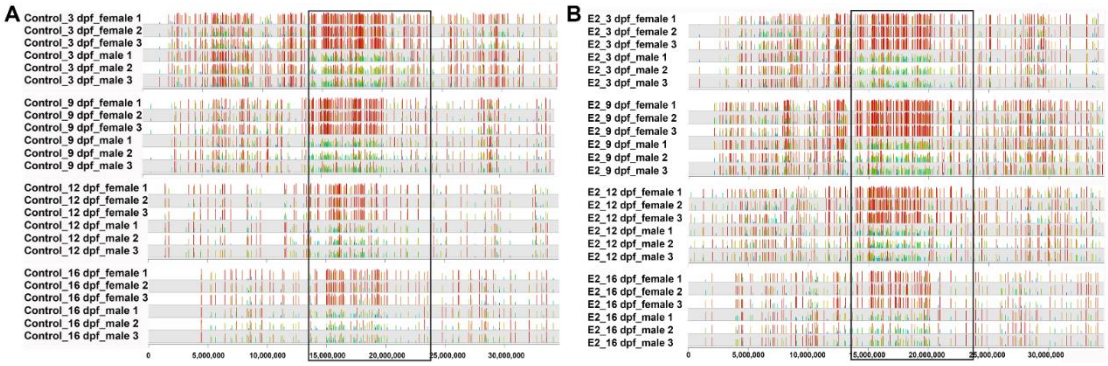

Figure 15. The distribution of differentially methylated CpG sites between females and males of channel catfish (*Ictalurus punctatus*) on chromosome 4 in control (A) and 17β-estradiol (E2) treatment (B). For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the

concentration of 400 μg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization. The sex determination region (SDR) is located within the black box.

The DMGs and DEGs within the SDR after E2 treatment were summarized in Table 7. A total of 13 genes within the SDR were found to be differentially methylated at least at one time point at 3 or 16 dpf. The patterns of methylation, however, were more dynamic than trending. For example, *hydin* gene was hypermethylated in females at 3 dpf but not in 16 dpf; while in males it was hypomethylated at 3 dpf, but hypermethylated at 16 dpf. There was no clear relationship between methylation and expression of these 13 genes; only six genes, *hydin*, *spred3*, *rasgrf1*, *reln*, *esrrg*, and *sphkap*, of the 13 genes were differentially expressed in at least one sex or at one time point (Table 8). An additional 21 genes were differentially expressed, but were not differentially methylated, again suggesting that methylation and expression regulation within the SDR region was independent after E2 treatment.

Table 7. Differentially methylated genes (DMGs) and differentially expressed genes (DEGs) within the sex determination region (SDR) after 17β-estradiol (E2) treatment in channel catfish, *Ictalurus punctatus*. For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated in the tubs with E2 for four hours per day at the concentration of 400 μg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post

fertilization (dpf). Hyper and hypo indicated hyper and hypomethylated genes, respectively, and up and down indicated up-regulated and down-regulated genes, respectively, as compared to the control. The numbers of in the parenthesis indicated percentage of difference in methylation, or fold change in expression. "-" indicates no significant difference after E2 treatment.

Gene	Female				Male			
	3 dpf		16 dpf		3 dpf		16 dpf	
	Methylation	Expression	Methylation	Expression	Methylation	Expression	Methylation	Expression
hydin	Hyper (31)	-	-	Up (15.2)	Hypo (-25)	-	Hyper (26)	Up (3.7)
aplp2	Hypo (-32)	-	-	-	Hyper (28)	-	-	-
rasgrf1	-	-	-	Up (8.0)	Hyper (31)	-	-	Up (4.9)
alpk3a	Hyper (-31)	-	-	Down (2.2)	-	-	-	-
nectin1	Hypo (-27)	-	-	-	-	-	-	-
reln	-	-	-	Up (8.9)	-	-	Hyper (26)	Up (7.3)
ltbp4	-	-	-	-	Hypo (-25)	-	-	-
golgb1	-	-	-	-	Hypo (-33)	-	-	-
ppfia1	Hypo (-38)	-	-	-	-	-	-	-
zbbx	-	-	Hypo (-27)	-	-	-	-	Up (2.6)
mbtps1	-	-	Hypo (-29)	-	Hyper (32)	-	Hyper (39)	-
bcar1	Hypo (-37)		Hyper (25)	-	-	-	-	-
pard6a	Hyper (29)	Down (2.0)	-	-	Hypo (-37)	-	-	-
kcnj1	-	-	-	Up (2.9)	-	-	-	-
ehd2b	-	Down (2.1)	-	-	-	-	-	-
idh2	-	-	-	Down (4.1)	-	-	-	Down (2.8)
ttc36	-	-	-	Up (2.3)	-	-	-	-
slc28a1	-	-	-	-	-	-	-	Down (2.1)
treh	-	-	-	-	-	-	-	Down (2.4)
spred3	-	-	-	Up (11.0)	-	-	-	Up (4.0)
dner	-	-	-	Up (15.2)	-	-	-	Up (6.0)
sphkap	-	-	-	Up (9.4)	-	-	-	Up (5.5)
sptbn4	-	-	-	Up (2.9)	-	-	-	Up (2.7)
esrrd	-	-	-	Up (6.6)	-	-	-	Up (4.0)
shank2	-	-	-	Up (7.6)	-	-	-	Up (7.9)
serpini1	-	-	-	Up (2.9)	-	-	-	Up (2.1)
wdr49	-	-	-	-	-	-	-	Up (2.2)

necab2	-	-	-	Up (5.0)	-	-	-	Up (3.0)
slc38a8	-	-	-	Up (7.9)	-	-	-	-
nutf2	-	-	-	Down (2.2)	-	-	-	-
tsnaxip1	-	-	-	Up (4.7)	-	-	-	Up (4.9)
carmil2	-	-	-	Up (6.2)	-	-	-	Up (3.2)
gnao1a	-	-	-	Up (3.6)	-	-	-	Up (2.5)
nr2f2	-	-	-	Up (2.7)	-	-	-	Up (3.3)

3.4.6 DNA methylation and gene expression in pseudo-females

As described above, E2 treatment caused sex reversal of 87.9% of genetic males to phenotypic females. To elucidate methylation and gene expression in pseudo-females, patterns of DNA methylation, both within the SDR and the whole genome, and profiles of gene expression were compared between females, males and pseudo-females. As shown in Figure 16, methylation profile within the SDR of pseudo-females at 110 dpf was more similar to those of males than to those of females. Thus, for most of the DMSs, hypermethylation was observed within females in the control (Figure 16A). Similarly, many of the hypermethylated sites within females as compared with males remained hypermethylated when compared with pseudo-females (Figure 16B). In contrast, there were few DMSs between control males and pseudo-females at 110 dpf (Figure 16C), suggesting a high level of similarity of methylation between pseudo-females and males.

The methylation profile in the whole genome was different from those of the SDR. As shown in Figure 16D, when the whole genome differentially methylated genes were compared, the largest difference were found to be between control females and control males. Thus 437 DMGs were hypermethylated in control females and 860

DMGs were hypomethylated in control females when compared with control males. The differences between control females and pseudo-females were very small, with only 28 DMGs being hypermethylated in control females and 20 DMGs being hypomethylated in control females. When pseudo-females were compared with control males, the difference was larger than the difference between control females and pseudo-females, but much smaller than the difference between control females and males (Figure 16D). Taken together, the whole genome methylation profiles of pseudo-females were most like those of control females.

Whole genome expression of pseudo-females at 110 dpf was also much similar to that of females. As shown in Figure 16E, a total of 13,762 genes (6,624 higher in females, and 7,138 higher in males) were differentially expressed between control females and control males at 110 dpf; and a total of 10,630 genes (4,820 higher in females, and 5,810 higher in pseudo-females) were differentially expressed between control males and pseudo-females at 110 dpf, but the number of differentially expressed genes between control females and pseudo-females were much smaller, only 2,064 (254 higher in control females and 1,810 higher in pseudo females).

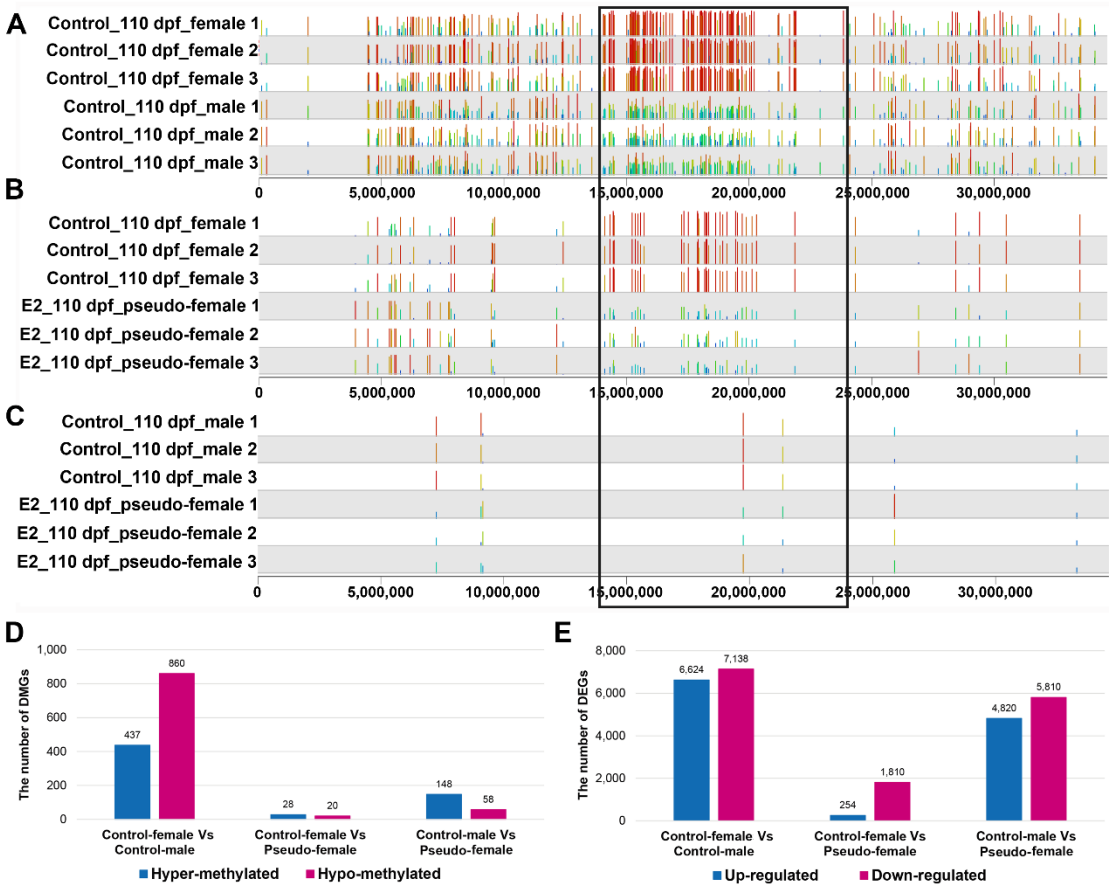

Figure 16. Methylation and gene expression in pseudo-females of channel catfish, *Ictalurus punctatus*. Pseudo-females were induced by 17β-estradiol (E2). Starting within the first 2 h post-fertilization, embryos were treated with E2 for four hours per day at the concentration of 400 μg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization (dpf). **A-C** The distribution of differentially methylated CpG sites (DMSs) on chromosome 4 between control females and control males (**A**), between control females and pseudo-females (**B**), and between control males and pseudo-females (**C**) at 110 dpf. The black box indicates the sex determination region (SDR). **D** The number of

differentially methylated genes (DMGs) in the genome between different gonads at 110 dpf. **E** The number of differentially expressed genes (DEGs) between different gonads at 110 dpf.

3.4.7 Methylation and expression of some known sex-related genes

Methylation and expression of a set of 12 female sex-related genes and 15 male sex-related genes were examined in E2-treated genetic males. The detailed description and function of these genes were provided in Table 8. Methylation and expression profiles of these sex-related genes between E2-treated genetic males and control males were compared. As shown in Figure 7, in most cases, female sex-related genes were up-regulated following E2 treatment, especially at 16 dpf, while the expression of most male sex-related genes was down-regulated, especially at 110 dpf.

The effect of E2 treatment on methylation profiles with these genes did not show a specific pattern. Diverse relationships between methylation and gene expression were observed (Figure 17). For example, female-related genes, *cyp19a1*, *figla*, and *hsd17b1* exhibited an inverse correlation between methylation and gene expression, while no obvious correlation was found in some male-related genes (*dmrt1*, *pld1a*, *amh*, *amhr2*, *cyp1a1*, *pla2g4ab*, and *rspo1*), and in some female-related genes (*foxl2a*, *cyp11a*, and *hsd17b2*). Additionally, the methylation of some genes was positively correlated with gene expression, including *fshr*, *gnrhr1*, *cyp17*, *tcf21*, *fstl1b*, *pdgfrb*, and *wt1b*.

Table 8. Function and description of 27 known sex-related genes and their position in channel catfish, *Ictalurus punctatus*.

Gene	Gene description	Function	Chromosome	Position
cga	glycoprotein hormones alpha chain	positive regulation of steroid biosynthetic process	9	18984588-18985570
fshr	follicle stimulating hormone receptor	female gonad development	13	9048018-9059888
foxl2a	forkhead box L2a	ovarian follicle development	20	7377238-7378555
cyp19a1	P450 aromatase	female gonad development; response to estradiol	14	6214147-6221356
lhb	luteinizing hormone subunit beta	female gonad development; oocyte maturation	3	27435496-27436326
hsd17b1	17beta-estradiol 17-dehydrogenase	estrogen biosynthetic process	2	5829548-5834170
figla	folliculogenesis specific bHLH transcription factor	oocyte development	22	1523093-1528335
lhcgr	luteinizing hormone/choriogonadotropin receptor	estrogen biosynthetic process	3	29782796-29822870
gnrhr1	gonadotropin-releasing hormone II receptor	gonadotropin-releasing hormone receptor activity	24	8425678-8436242
cyp11a	cytochrome P450 cholesterol side chain cleavage	response to steroid hormone; C21-steroid hormone biosynthetic process	14	13031854-13036707
hsd17b2	17β-estradiol 17-dehydrogenase / 3alpha(17beta)-hydroxysteroid	estrogen biosynthetic process	14	13514586-13522293
cyp17	steroidogenic cytochrome P450 17-hydroxylase/lyase	female sex determination; sex differentiation	3	16299948-16303015
ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	spermatogenesis; male meiotic nuclear division	16	8126488-8142399
dazl	deleted in azoospermia-like	spermatogenesis; germ cell development	12	13239403-13254199
amhr2	anti-Mullerian hormone receptor type 2	male gonad development; sex differentiation	9	1574413-1583086
pld1a	phospholipase D1a	response to peptide hormone; regulation of microvillus assembly	17	507297-531071
dhh	desert hedgehog signaling molecule	spermatid development; male sex determination	15	20042069-20047894
pdgfrb	platelet-derived growth factor receptor, beta polypeptide	male gonad development	14	20070334-20118667
wt1a	WT1 transcription factor a	sex determination; negative regulation of female gonad development; germ cell development	8	19199196-19220219
cyp1a1	cytochrome P450 family 1 subfamily A1	flavonoid metabolic process	4	30337881-30342050
tcf21	transcription factor 21	sertoli cell differentiation; negative regulation of androgen receptor signaling pathway; reproductive structure development	15	21326786-21329283
dmrt1	doublesex and mab-3 related transcription factor 1	spermatogenesis; sex differentiation; male sex determination	22	8179274-8202749
wt1b	WT1 transcription factor b	sex determination	4	7228800-7249006

amh	anti-Mullerian hormone	sperm capacitation; development of primary male sexual characteristics	10	26083644-26087738
rspo1	R-spondin 1	Wnt signaling pathway	1	21785602-21811658
fstl1b	follistatin-like 1b	cell differentiation; regulation of BMP signaling pathway	6	16043272-16110119
pla2g4ab	cytosolic phospholipase A2	regulation of cell cycle	25	19185998-19201116

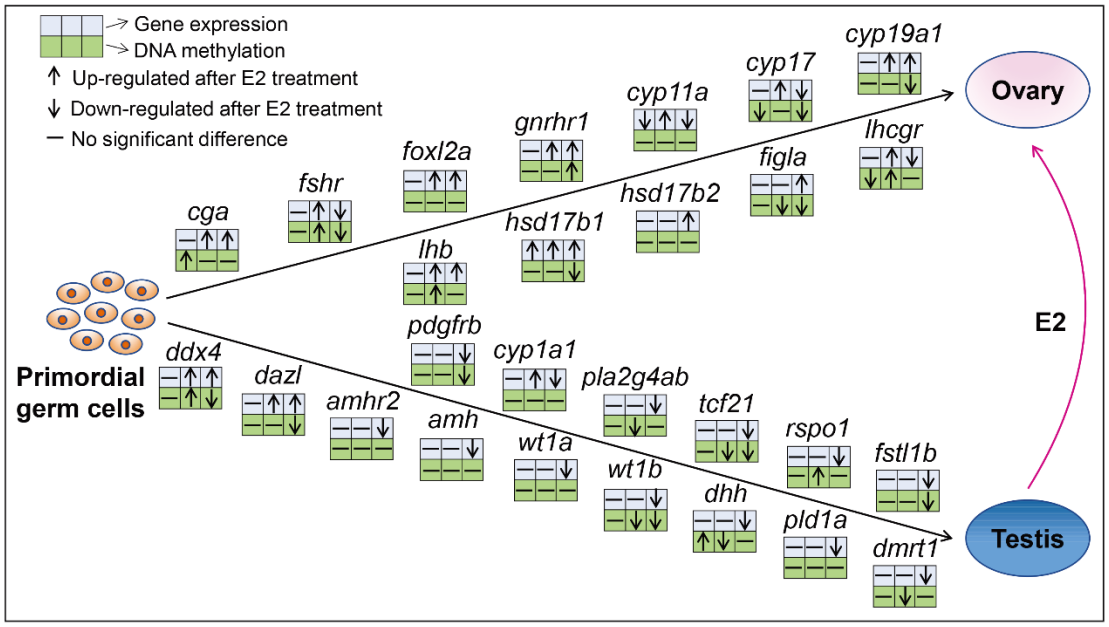

Figure 17. Gene expression and methylation changes of sex-related genes in the process of sex reversal from genetic males to phenotypic females after 17β-estradiol (E2) exposure in channel catfish, *Ictalurus punctatus*. For E2 treatment, starting within the first 2 h post-fertilization, embryos were treated with E2 for four hours per day at the concentration of 400 µg/L. After the yolk-sac was absorbed, the fry were fed diets containing E2 at a dose of 200 mg/kg three times per day until 110 days post fertilization (dpf). For each gene, the three upper blue squares and the three lower green squares represent gene expression and methylation, from the comparison between E2 treated males and control males at 3 dpf (first square), between E2 treated males and

control males at 16 dpf (second square), and between E2-induced pseudo-females and control males at 110 dpf (third square). The details of these genes are listed in the Table 8.

3.5 Discussion

It is well known that the sex of most fish can be reversed at the undifferentiated stage of gonad by administration of high doses of exogenous 17β-estradiol (E2) (Imai et al., 2005; Seki et al., 2006; Lin et al., 2012; Karsli et al., 2016; Li et al., 2019). However, the molecular mechanisms underlying sex reversal is still not fully understood. In this study, whole genome DNA methylation and gene expression profiles were analyzed in females and males of channel catfish during the time of gonadal sex differentiation after exposure to E2. The treatment of E2 caused sex reversal of over 87% of genetic males into phenotypic females (pseudo-females), but the global methylation profiles were not significantly modified in either females or males. However, a specific set of genes, especially those that were normally highly expressed in females, were both differentially methylated and expressed after treatment, suggesting that epigenic regulation plays crucial roles in sex reversal.

DNA methylation is highly plastic, playing important roles in response to environmental cues (Manolakou et al. 2006; Sun et al. 2016). Increasing numbers of reports indicated that external stimuli may cause epigenetic changes in various organisms (Strömqvist et al., 2010; Sun et al., 2016; Banh et al., 2021). For instance,

female-to-male sex reversal occurred under high temperature in Nile tilapia (*Oreochromis niloticus*), and both females and males showed increased methylation levels (Sun et al., 2016). The hatchery reared (freshwater) and seawater (migratory) brown trout (*Salmo trutta*) showed significant differences in genome-wide methylation patterns, and salt-rich diets affected genome-wide methylation in hatchery reared brown trout (Morán et al., 2013). E2 exposure caused global genomic hypermethylation in male gonads of stickleback (Aniagu et al., 2008).

In the current study, whole-genome methylation level was not significantly different after E2 treatment in either females or males. However, a large number of DMSs and DMGs were identified in both females and males. DMGs after E2 exposure were significantly enriched in pathways involved in gonadal differentiation, such as calcium signaling pathway, MAPK signaling pathway, adrenergic signaling pathway, Wnt signaling pathway, GnRH signaling pathway, ErbB signaling pathway, and ECM-receptor interactions. GnRH signaling pathway is a key regulator of the reproductive system (Kraus et al., 2001). The binding of GnRH to the receptor triggers a series of signal transduction events and leads to the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Bliss et al. 2010). The ErbB receptors signal through Akt, MAPK and many other pathways to regulate cell proliferation, migration, differentiation, apoptosis, and cell motility (Marmor et al., 2004). Studies revealed that erB1 and erbB4 receptors signaling in glial cells controlled female sexual development and the onset of female puberty in mammals (Prevot et al.,

2003, 2005). ECM-receptor interaction is involved in a variety of cellular activities such as adhesion, migration, differentiation, proliferation, and apoptosis (Zhang et al., 2016), and can regulate the synthesis of steroid hormone (Wang et al., 2000). ECM-receptor interaction was associated with the primordial follicle activation and follicular development in mice (Xiong et al., 2019), which was also implicated in ovarian function in pigs (Wang et al., 2000), goats (Wang et al., 2016), cattle (Lan et al., 2016), and tilapia (Wang et al., 2021). MAPK signaling pathway integrated with GnRH signaling pathway is necessary for normal fertility (Bliss et al., 2010). MAPK signaling pathway mediates the balance between ovary- and testis-promoting genes and is related to the gonadal sex reversal in mice and humans (Bogani et al., 2009; Pearlman et al., 2010; Gierl et al., 2012; Warr et al., 2012; Loke et al., 2014)

It was interesting to note that most of the enriched pathways with genes hypermethylated in E2-treated females overlapped with those with genes hypomethylated in E2-treated males, suggesting sex-specific methylation and regulated expression of the same set of genes were important for female gonadal sex differentiation, leading to sex reversal from genetic males to phenotypic females.

Up-regulated DEGs after E2 exposure were enriched in ligand-receptor interaction, calcium signaling, MARK signaling and cell adhesion molecules pathways, while down-regulated DEGs were involved in growth rather than differentiation. Given that female gonads differentiate well ahead of male gonadal sex differentiation (female differentiation at around 19 dpf, male at over 90 dpf) (Patiño et al., 1996), these

results were logical because increased expression of genes leading to female gonadal sex differentiation should have promoted sex differentiation into females, rather than the normal situation in genetic males that continued with prolonged cell growth and proliferation.

Despite regulated methylation and expression of a specific set of genes, E2-induced sex reversal appeared to be independent of methylation and regulated expression in the SDR. The methylation profiles within the SDR remained similar before and after treatment with E2, in both females and males. Although over a dozen genes within the SDR were differentially expressed after the treatment of E2, their expression also appeared to be independent of the methylation within the SDR. These results suggested that the E2-treated males, even the pseudo-females at 110 dpf, exhibited a methylation profile within the SDR similar to that of normal males. However, in terms of whole genome methylation profiles, the pseudo-females exhibited a pattern extremely similar to that of normal females at 110 dpf. Similarly, for gene expression, the E2-treated fish were increasingly more like normal females. The numbers of DEGs between control females and pseudo-females were the smallest of the three pairs of comparisons.

The estrogen related genes are likely downstream of the sex determination process (Capel, 2017; Purcell et al., 2018). In channel catfish, sex is determined genetically by inheritance, with XY being males. Through genetic linkage mapping and genome-wide association studies, the sex determination locus of channel catfish was

mapped to chromosome 4 within the SDR (Li et al., 2015; Bao et al., 2019). This region was shown to have sex-specific DNA methylation, and the sex-specific methylation in turn dictated sex- and allele-specific expression of a set of genes within the SDR (Yang et al., manuscript under revision). In this study, DMGs and DEGs within the SDR exhibited dynamic changes at different time points without specific correlations with sex. Additionally, the DMSs within the SDR between females and males were strikingly similar in E2 treatment and control, suggesting that E2-induced sex reversal was independent of the SDR, and was the downstream of the sex determination pathway.

Sex development is regulated by a network of genes which generate gene cascade leading to establishment of sex phenotypes (Angelopoulou et al., 2012; Munger and Capel, 2012). The functions of these sex-related genes are highly conserved across species. Our results revealed that majority of genes documented to be associated with sex differentiation and ovarian steroidogenesis in other vertebrates had a similar overall expression patterns in channel catfish. Most of those genes known to be highly expressed during female sex differentiation in other species were up-regulated in E2-treated males, whereas most of those genes known to be highly expressed during male sex differentiation in other species were down-regulated in E2-treated males compared to control males.

E2 treatment induced expression of a specific set of genes important for female sex differentiation, including *cyp19a1*, *cyp17*, *cyp11a*, *gnrhr1*, *foxl2a*, *cga*, *lhb*, *hsd17b1*, *hsd17b2*, and *figla*. These findings are consistent with those reported in Asian

seabass (*Lates calcarifer*) implanted with E2 (Banh et al., 2021). *Cyp19a1* is a key gene in the conversion of endogenous androgens to estrogen and ovarian differentiation in various teleosts. It is regulated by sex-biased methylation and involved in DNA methylation mediated sex reversal following heat induction in the European sea bass (Navarro-Martín et al., 2011). In this study, DNA methylation in promoter of *cyp19a1* was reduced and gene expression was increased in pseudo-females compared with control males. *Foxl2* is a conserved gene with function of ovarian differentiation in vertebrates (Ottolenghi et al., 2005; Yao, 2005). Studies have demonstrated that *foxl2* was a direct transcriptional activator of the *cyp19a1* gene, which was observed in goat (Pannetier et al., 2006) and chicken (Govoroun et al., 2004). The expression pattern of *foxl2* was also found to be synchronized with *cyp19a1* in teleosts, such as air-breathing catfish (*Clarias gariepinus*) (Sridevi and Senthilkumaran, 2011; Sridevi et al., 2012), zebrafish (Caulier et al., 2015), Nile tilapia (Wang et al., 2007), medaka (*Oryzias latipe*s) (Nakamoto et al., 2006), rainbow trout (Baron et al., 2004), spotted scat (*Scatophagus argus*) (Liu et al., 2015) and black porgy (*Acanthopagrus schlegeli*) (Wu et al., 2008). In the present study, *foxl2a* was up-regulated after E2 exposure since 16 dpf, without significant changes in DNA methylation, which is similar to the study in Asian seabass (Banh et al., 2021). The results implied that exogenous estrogen induced upregulation of *foxl2* in channel catfish genetic males, which resulted in increased expression of *cyp19a1*, leading to the conversion of endogenous androgens to estrogens. The accumulation of endogenous estrogen and the reduction of androgens were considered

to be the determinant of sex differentiation in teleost fish (Piferrer, 2001, 2013), explaining the conversion of genetic males to phenotypic females after E2 exposure. Furthermore, *figla* is another female-specific gene which plays important roles in folliculogenesis, fertilization and early development (Joshi et al., 2007; Qin et al., 2018). Disruption of *figla* gene led to an all-male phenotype in zebrafish (Qin et al., 2018). In this study, the promoter methylation of *figla* was decreased and its expression was significantly increased after E2 treatment.

Phenotypic sex is governed by an antagonistic interaction between feminizing and masculinizing gene networks (Capel, 2017). E2 treatment also suppressed a specific set of genes important for male sex differentiation, leading to sex reversal from genetic males to phenotypic females. These genes included *amhr2*, *amh*, *wt1a*, *wt1b*, *dhh*, *pld1a*, *dmrt1*, *pdgfrb*, *cyp1a1*, *pla2g4ab*, *tcf21*, *rspo1*, and *fstl1b*. *Dmrt1* is essential to testis determination in birds, reptiles, amphibians and teleost fish (Ferguson-Smith, 2006; Smith et al., 2009; Matson and Zarkower, 2012; Capel, 2017; Cui et al., 2017). Exogenous estrogen exposure has been observed to reduce *dmrt1* expression in red-eared slider turtle (*Trachemys scripta*), pejerrey (*Odontesthes bonariensis*) and rainbow trout (Marchand et al., 2000; Murdock and Wibbels, 2006; Fernandino et al., 2009). In addition, *amh* is also involved in testicular development and function (Matuszczak et al., 2013). Exposure to 17α-ethinylestradiol suppressed the expression of *amh* in zebrafish (Schulz et al., 2007). Taken together, increased expression of female sex-

related genes and reduced expression of male sex-related genes worked together for sex reversal of genetic males to phenotypic females.

DNA methylation was generally thought to be negatively correlated with gene expression, while recent studies revealed a more dynamic and complex association between DNA methylation and expression than previously known (Siegfried and Simon, 2010; Wagner et al., 2014; Moarii et al., 2015). In this study, we observed negative relationship between methylation and gene expression in female-related genes (*cyp19a1*, *figla*, and *hsd17b1*), but most male-related genes did not show obvious correlation between methylation and gene expression, suggesting that the regulation of genes involved in sex reversal may be more complex, with some being epigenetically regulated, while many others may be regulated by other factors including being a secondary response after the primary response to the estrogen treatment.

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