GENETICS

Genomic imprinting-like monoallelic paternal expression determines sex of channel catfish

Wenwen Wang[1]†, Yujia Yang[1]†, Suxu Tan[1]†, Tao Zhou[1,2], Yang Liu[1], Changxu Tian[1], Lisui Bao[1], De Xing[1], Baofeng Su[1], Jinhai Wang[1], Yu Zhang[1], Shikai Liu[1], Huitong Shi[1], Dongya Gao[3], Rex Dunham[1], Zhanjiang Liu[3]*

The X and Y chromosomes of channel catfish have the same gene contents. Here, we report allelic hypermethylation of the X chromosome within the sex determination region (SDR). Accordingly, the X-borne *hydin-1* gene was silenced, whereas the Y-borne *hydin-1* gene was expressed, making monoallelic expression of *hydin-1* responsible for sex determination, much like genomic imprinting. Treatment with a methylation inhibitor, 5-aza-dC, erased the epigenetic marks within the SDR and caused sex reversal of genetic females into phenotypic males. After the treatment, *hydin-1* and six other genes related to cell cycle control and proliferative growth were up-regulated, while three genes related to female sex differentiation were down-regulated in genetic females, providing additional support for epigenetic sex determination in catfish. This mechanism of sex determination provides insights into the plasticity of genetic sex determination in lower vertebrates and its connection with temperature sex determination where DNA methylation is broadly involved.

INTRODUCTION

Sex determination is such an important process for sexual organisms that one would expect its mechanisms to be highly conserved during evolution, but they are remarkably diverse, particularly among fish, amphibians, and reptiles. The high levels of diversity in the mechanisms of sex determination are partially related to the various levels of sex chromosome differentiation. Among lower vertebrates, various modes and genes for sex determination have evolved. For example, of some 270 fish species where studies of sex determination have been conducted (*1*), XY (male heterogametic) and ZW (female heterogametic) sex determination systems have been reported (*2*); monogenic and polygenic sex determination systems have been demonstrated (*3*, *4*); and various sex determination genes have been identified, such as *sdy* (sexually dimorphic on the Y chromosome) in rainbow trout (*Oncorhynchus mykiss*) (*5*), *amhy* (Y-linked anti-Müllerian hormone) in Patagonian pejerrey (*Odontesthes hatcheri*) (*6*), *amhr2* (anti-Müllerian hormone receptor type II) in tiger pufferfish (*Takifugu rubripes*) (*7*), *amh* (anti-Müllerian hormone) in silverside (*Hypoatherina tsurugae*) (*8*), and *dmrt1* (doublesex- and mab-3–related transcription factor-1) in spotted scat (*Scatophagus argus*) (*9*). Different master sex determination genes were reported in closely related medaka species, e.g., *dmy* (the DM-domain gene on the Y chromosome) in Japanese rice fish (*Oryzias latipes*) (*10*) and *gsdfY* (gonadal soma derived growth factor on the Y chromosome) in *Oryzias luzonensis* (*11*). Even within the same species of *O. latipes*, different master sex determination genes were reported (*12*–*17*), suggesting rapid evolution of sex determination mechanisms in teleosts.

Channel catfish (*Ictalurus punctatus*) is a lower teleost fish species. The genetic sex of channel catfish is determined by an XY sex determination system (*18*, *19*), but its sex chromosomes are cytologically indistinguishable (*20*). In addition to genetic factors, its sex determination is also elastic and can be affected by high temperature (*19*) and steroid hormones (*21*). With a given genetic composition, gonadal sex can be reversed by treatment with sex hormones or elevated temperature (*22*–*24*), suggesting that sex determination and/or differentiation can be exerted at the level of gene expression. However, with channel catfish, only pseudo-females (sex reversal of genetic males to phenotypic females) have been achieved, no matter what the treatments were. With many fish species, such as zebrafish (*25*), medaka (*26*), tilapia (*27*), and half-smooth tongue sole (*28*), androgen and high-temperature treatment led to sex reversal into pseudo-males, but these treatments led to sex reversal of males into pseudo-females with channel catfish (*19*, *23*), suggesting that channel catfish may have a unique mechanism for sex determination.

Through genetic linkage mapping, chromosome 4 was identified as the sex chromosome in channel catfish (*29*), and the sex determination region (SDR) was mapped to an 8.9-Mb region (*18*). Further fine mapping was not practically feasible due to the lack of recombination within the SDR, even when unrelated wild fish were used (*18*). Reference genomic sequences were generated from a double haploid XX female fish (*30*) and from a YY male fish (*18*), making it possible to compare the sequences of the X and Y chromosomes. However, from the point of protein-coding genes, identical gene content of 950 genes was found on both sex chromosomes (*18*), and identical gene content of 123 genes was found within the SDR on X or Y chromosome, suggesting that the sex of channel catfish is not determined by the presence of a Y-linked protein-coding gene(s) that is absent from X chromosome.

Although genetic sex is determined at the time of fertilization, the gonadal feminization of channel catfish starts around 19 days post-fertilization (dpf), whereas its testicular formation starts 90 to 102 dpf (*19*). This differential sex differentiation offers a good

[1]The Fish Molecular Genetics and Biotechnology Laboratory, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Auburn, AL, USA. [2]Fujian Key Laboratory of Genetics and Breeding of Marine Organisms, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China. [3]Department of Biology, College of Arts and Sciences, Syracuse University, Syracuse, NY, USA.
†These authors contributed equally to this work.
*Corresponding author. Email: johnliu@syr.edu

window of opportunity to study molecular mechanisms underlining sex determination and differentiation. In the present study, we confirmed the observation of an epigenetically marked locus within the SDR (*31*) and determined allelic methylation and allelic expression of the genes within the SDR in genetic males (XY) of channel catfish during early gonadal differentiation. Here, we report allelic hypermethylation of the SDR on the X chromosome that silenced the expression of the X chromosome–borne *hydin-1* gene (hydrocephalus-inducing protein 1 or HYDIN axonemal central pair apparatus protein-like 1), whereas the Y chromosome–borne *hydin-1* gene was undermethylated and expressed. Thus, in genetic females, *hydin-1* gene is not expressed, leading to sex differentiation into females; in genetic males, the Y-borne *hydin-1* is expressed, leading to sex differentiation into males. Furthermore, blocking of methylation using a methylation inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), led to demethylation in many CpG sites, especially within the SDR, making the originally hypermethylated SDR on the X chromosome undermethylated, which in turn allowed expression of *hydin-1*, leading to sex reversal from genetic females to phenotypic males. These results indicated that the monoallelic expression of the Y-borne *hydin-1* gene, coupled with silencing of the X chromosome-borne *hydin-1* gene through methylation, was responsible for sex determination in channel catfish. This mechanism may provide insights into genetic sex determination (GSD) and environmental sex determination (ESD), such as temperature sex determination (TSD), in a broad spectrum of lower vertebrates, as well as environmental modulation of sex differentiation in various organisms.

RESULTS

The SDR was differentially methylated

The XY sex determination system of channel catfish predicted a Y-linked factor for sex determination, but extensive comparative analysis of genomic sequences of the X and Y chromosome revealed no differences in gene contents (*18*). We hypothesized that monoallelic expression of some gene(s) on the Y chromosome may provide the basis for sex determination. Sex can be determined at the level of gene expression where one or more Y chromosome–borne gene(s) were expressed, but their counterpart(s) on the X chromosome were not expressed; this monoallelic expression may be controlled by differential methylation. To test this hypothesis, we conducted whole-genome bisulfite sequencing (WGBS) and RNA sequencing (RNA-seq) analyses. A total of 11.1 billion WGBS reads were obtained (table S1). An average of 25.6 Gb of data (~32× genome coverage) were obtained for each sample (n = 63). Across the genome, the levels of methylation were similar between genetic females and males (table S2). However, most of differentially methylated sites (DMSs) between females and males were found on the sex chromosome, suggesting involvement of DNA methylation in sex determination in channel catfish. As shown in Fig. 1, a differentially methylated region was identified within the SDR, with hypermethylation in genetic females and hypomethylation in genetic males, making the SDR epigenetically marked. The differential methylation was not only most obvious early in development and sex differentiation at 3 and 9 dpf but also visible at 12 and 16 dpf. Even after the gonadal differentiation at 135 dpf, levels of methylation in the SDR were still higher in genetic females than in genetic males (Fig. 1).

Table 1. Protein-coding genes in the EML expressed at higher levels (>2.0 fold) in genetic males compared to females of channel catfish, *I. punctatus,* at 3, 16, and 135 dpf. Gene abbreviations: *hydin-1*, hydrocephalus-inducing protein homolog 1; *spred3*, sprouty related EVH1 domain containing 3; *sphkap*, SPHK1 interactor, AKAP domain containing; *carmil2*, capping protein regulator and myosin 1 linker 2.

Gene	Differential expression			Allele-specific expression		
	Fold change (male/female)			Fold change (male Y/male X)		
	3 dpf	16 dpf	135 dpf	3 dpf	16 dpf	135 dpf
hydin-1	84.8	9.8	23.2	51.1	17.9	35.2
spred3	3.0	–	–	–	–	–
sphkap	2.1	2.3	–	1.5	–	–
carmil2	2.1	2.2	10.3	–	–	–

The differential methylation in the SDR is allele specific and inherited

Not only the SDR was differentially methylated, but also methylation was allele specific. We developed 8791 X allele– and Y allele–specific single-nucleotide polymorphisms (SNPs) (table S3), which allowed tracing of X and Y alleles in males. As shown in Fig. 2A, the X alleles were hypermethylated within the SDR, while the Y alleles were hypomethylated. This allele-specific methylation (ASM) was observed throughout the period of sex differentiation, as determined at 3, 9, 12, 16, and 135 dpf. To determine whether the methylation patterns were inherited, ASM profiles of sperm were determined and compared to those of embryos or fingerlings at 3, 9, 12, 16, and 135 dpf. In sperm, the X alleles within the SDR were hypermethylated, with most of the CpG sites being methylated at around 90%, whereas the Y alleles were hypomethylated, with most of the CpG sites being methylated at less than 10% (Fig. 2B), with the same patterns as observed in genetic males at 3, 9, 12, 16, and 135 dpf (Fig. 2A), indicating that the allelic methylation patterns were inherited from the parents.

Allelically methylated genes within the SDR are differentially expressed

Parallel to the WGBS analysis, we conducted RNA-seq analysis to determine differentially and allele-specifically expressed genes within the SDR. A total of 3.67 billion reads were generated, and the detailed information of RNA-seq samples is summarized in table S4. As shown in Table 1, of the 123 genes within the SDR, four genes, *hydin-1*, *spred3* [sprouty-related Ena/Vasodilator-stimulated phosphoprotein homology-1 (EVH1) domain containing 3], *sphkap* [sphingosine kinase 1 (SPHK1) interactor, A-kinase anchoring protein (AKAP) domain containing], and *carmil2* (capping protein regulator and myosin 1 linker 2), were differentially expressed at 3 dpf between genetic females and genetic males. *Hydin-1* gene was markedly differentially expressed between genetic females and genetic males, with 84.8, 9.8, and 23.2 times more expression in genetic males than in genetic females at 3, 9, and 135 dpf, respectively (Table 1). The other three genes were differentially expressed at one or more time points, but only two to three times more in genetic males than in genetic females (Table 1).

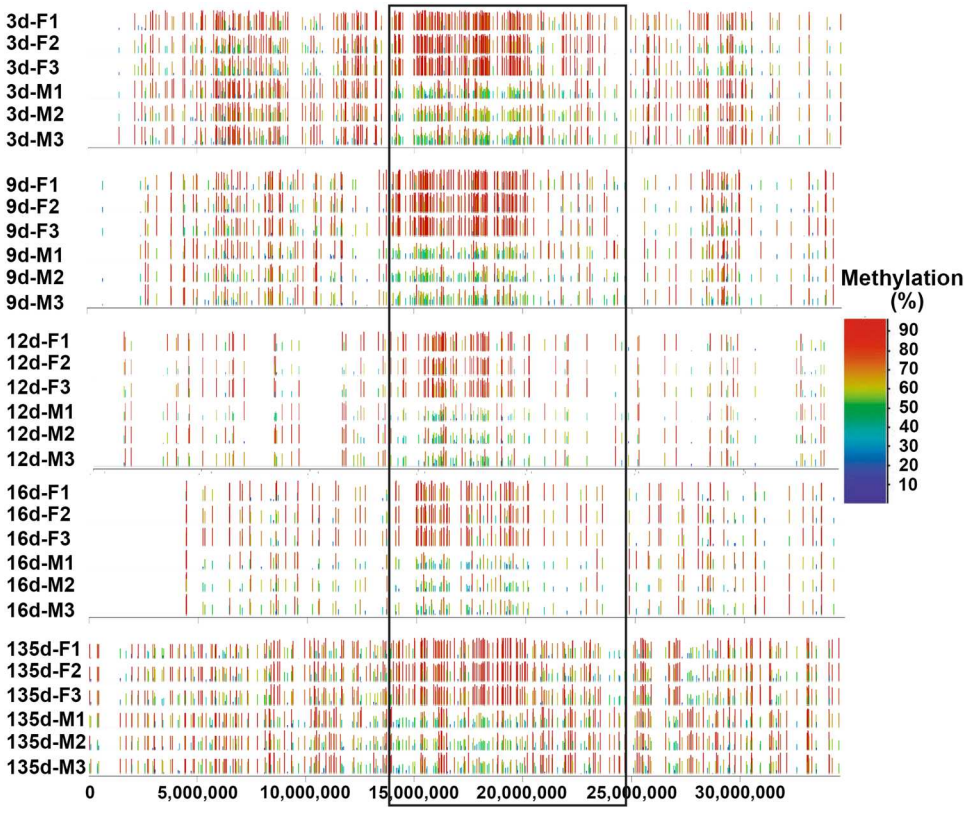

Fig. 1. Epigenetically marked locus within the SDR of channel catfish, *I. punctatus*. The distribution of differentially methylated sites (DMSs) between genetic females and males on chromosome 4 at positions as shown along *x* axis. Samples are marked on the left margin, with three genetic female samples (F1, F2, and F3) and three genetic male samples (M1, M2, and M3) at each time point of 3 days (3d), 9 days (9d), 12 days (12d), 16 days (16d), and 135 days (135d) after fertilization. SDR is highlighted with a black box. Levels of methylation at DMSs are indicated on the *y* axis with the color codes as marked on the right margin, ranging from blue (low) to red (high). Note hypermethylation in genetic females and hypomethylation in genetic males within the SDR. EML, epigenetically marked locus.

Hydin-1 was the only gene that was not only differentially methylated and differentially expressed between genetic females and males but also allele-specifically methylated and monoallelic paternally expressed at all tested times (Table 1). Eight CpG sites within the *hydin-1* gene were hypermethylated with the X allele at 3 dpf as compared with the Y allele, and this pattern, for the most part, continued at 16 dpf (Fig. 3A). Treatment with 5-aza-dC, a methylation inhibitor, led to reduce levels of methylation such that methylation was no longer differential between X and Y alleles at several CpG sites (Fig. 3B). This reduction of methylation of the X alleles was correlated with increased expression of *hydin-1* gene in the treated females. However, expression from X alleles was still low, as compared with high levels of expression of the Y chromosome–bearing *hydin-1* (Fig. 3, C and D).

Blocking of methylation caused sex reversal

A methylation inhibitor, 5-aza-dC, was used to determine the effect of genome methylation on sex differentiation of channel catfish. Genetic sex was determined using polymerase chain reaction (PCR), where homozygous XX genotype of genetic females produced one PCR band, while heterozygous XY genotypes of genetic males produced two bands (*32*). Phenotypic sex cannot be determined until after gonadal differentiation. Although female gonadal differentiation starts around 19 dpf, male gonadal differentiation starts between 90 and 102 dpf, and phenotypic sex cannot be determined until 110 dpf (*19*). We determined phenotypic sex at 135 dpf and found sex-reversed fish that had the genotype of females with just one PCR band but exhibited male phenotypic sex with testes rather than ovaries (Fig. 4A). At the highest tested concentration of aqueous exposure of 24 μM, sex reversal from genetic females to phenotypic males (pseudo-males) was 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 (Fig. 4B). Treatments at even higher concentrations were prohibited because of mortalities. Treatment with 5-aza-dC had a major effect on survival, causing substantially more mortalities, especially after 20 dpf (Fig. 4C). 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 (Fig. 4C). Because phenotypic sex could not be determined before 110 dpf, it was apparent that the high mortality rate prohibited the opportunity to detect additional sex-reversed fish.

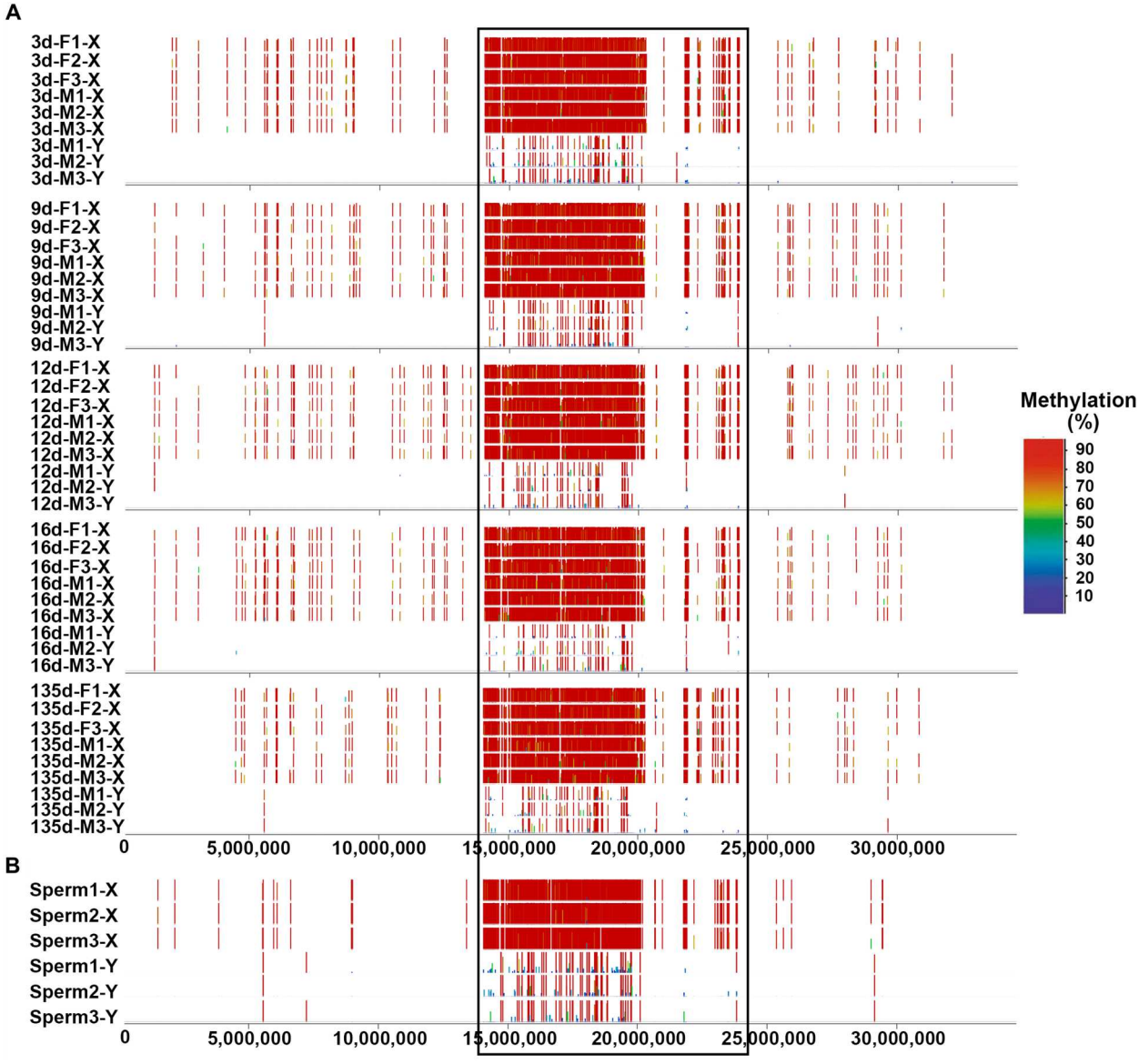

Fig. 2. ASM of EML was inherited from gametes in channel catfish, *I. punctatus*. (**A**) ASM between X and Y chromosomes in normal females and males at 3, 9, 12, and 16 dpf. (**B**) ASM between X and Y chromosomes in sperms. SDR is located within the black box. F1, F2, and F3 are genetic female samples 1, 2, and 3, respectively. M1, M2, and M3 are genetic male samples 1, 2, and 3, respectively. X, alleles from the X chromosome; Y, alleles from the Y chromosome. Positions along chromosome 4 are indicated on *x* axis, whereas the levels of methylation at DMSs are indicated on the *y* axis with color codes as shown on the right margin, ranging from low (blue) to high (red).

Methylation inhibitor caused an overall reduction of genome methylation

The effect of 5-aza-dC on methylation was observed with reduced levels of methylation at C, CpG, CHG, and CHH sites (fig. S1). The levels of methylation at CpG sites were initially increased at 3 dpf after treatment of 5-aza-dC, presumably due to exposure to the adverse treatment but were significantly reduced at 9, 12, and 16 dpf, with the largest reduction in genetic females at 12 and 16 dpf. At 135 dpf, the levels of methylation at CpG sites were just slightly lower in 5-aza-dC–treated samples than controls, regardless of genetic females or males (Fig. 4D). The distribution of methylation reduction was found on all 29 chromosomes, without a particular pattern (Fig. 4E).

Methylation inhibitor erased the epigenetic marks within the SDR

One obvious question was if the treatment of 5-aza-dC influenced the epigenetic marks within the SDR and, consequently, on the expression of genes within the SDR. When differentially methylated CpG sites between genetic females and males were mapped along the 29 chromosomes, the treatment of 5-aza-dC diminished the strong epigenetic marks of differentially methylated CpG sites between genetic females and males, especially at 12 and 16 dpf

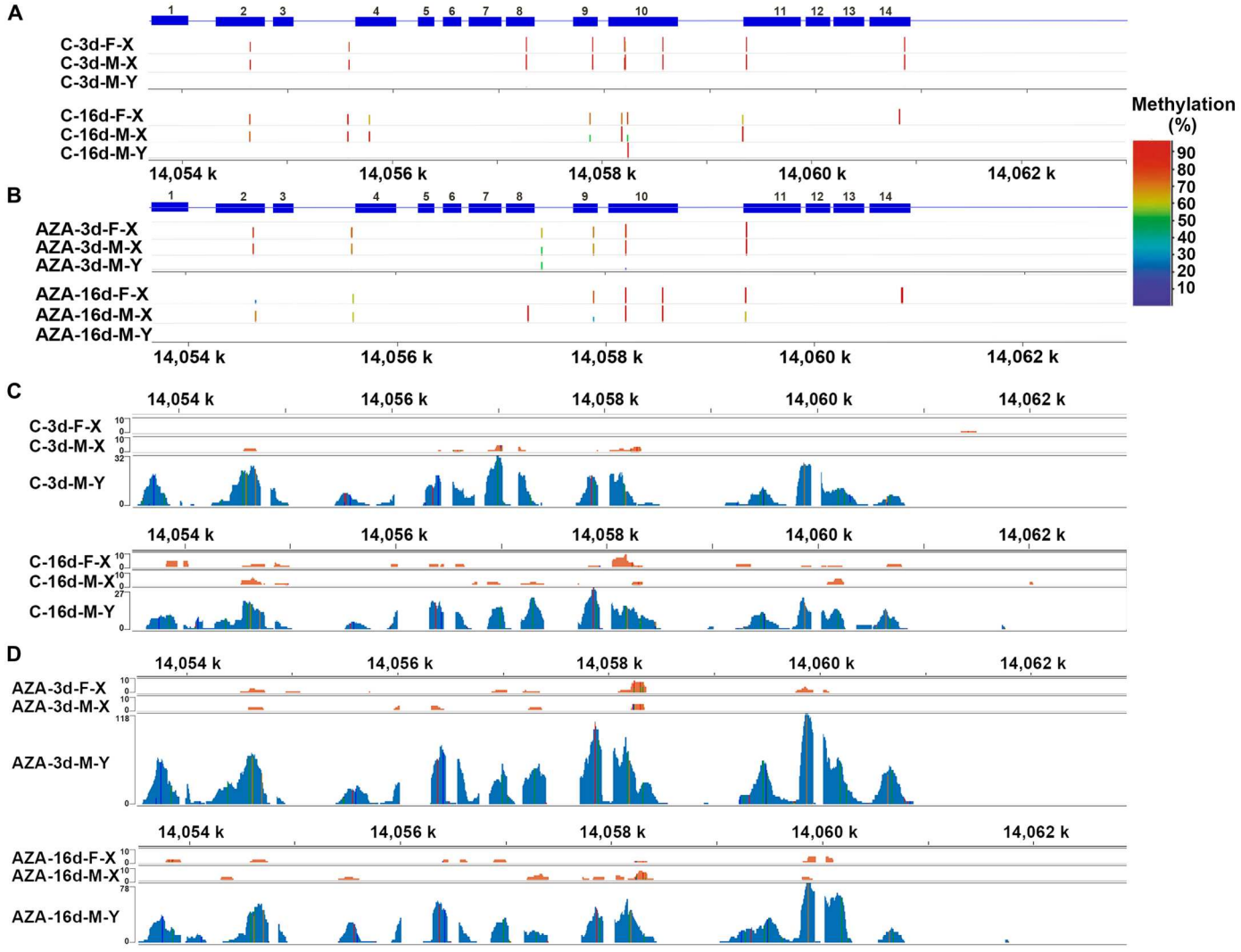

Fig. 3. ASM and expression of *hydin-1* gene during early sex differentiation of channel catfish, *I. punctatus*. (**A** and **B**) Comparison of methylation levels at DMSs between X and Y alleles of *hydin-1* gene at 3 and 16 dpf in control females and males (A) and in 5-aza-dC–treated females and males (B). Blue rectangular boxes represent exons of hydin-1 gene, whose positions on chromosome 4 are indicated on the *x* axis (in kb), and the levels of methylation at DMSs are indicated on the *y* axis with color codes as shown on the right margin. (**C** and **D**) Comparison of expression levels between X and Y alleles of *hydin-1* gene at 3 and 16 dpf in control females and males (C) and in 5-aza-dC–treated females and males (D). The positions of RNA-seq reads are indicated on the *x* axis, and read depths are indicated on the *y* axis, with reads from the X allele shown in orange and reads from the Y allele shown in blue. All samples are indicated on the left margin: C, control group; AZA, 5-aza-dC–treated group; F, female; M, male.

(Fig. 5A). Most of these were caused by the reduction of hypermethylation in genetic females. In control fish, the SDR was epigenetically marked with hypermethylation on the X alleles and hypomethylation on the Y alleles. Treatment with 5-aza-dC notably reduced the level of differential methylation between females and males, especially in the SDR (Fig. 5B). The hypermethylated sites were notable at 9 dpf, and this trend continued such that the epigenetic marks were almost completely erased at 12 and 16 dpf (Fig. 5B).

Differentially methylated genes (DMGs) in the SDR after treatment are listed in table S5. Fifty-nine genes of a total of 123 within the SDR were differentially methylated. 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, again presumably as an immediate response to the adverse treatment. Of the 59 DMGs within the SDR, only three genes, *hydin-1*, *rasgrf1* (ras-specific guanine nucleotide-releasing factor 1), and *nectin1* (nectin cell adhesion molecule 1), were hypomethylated after treatment at all time points of 9, 12, and 16 dpf and in both females and males (table S5).

Demethylation of genes in the SDR is correlated with their activated expression

A total of 10 genes within the SDR were differentially expressed after 5-aza-dC treatment, as compared to controls, of which six genes were differentially expressed at 3 dpf, three genes at 16 dpf, and one gene, *hydin-1*, at both 3 and 16 dpf (Table 2). All the 10 genes were differentially expressed after treatment in females, and

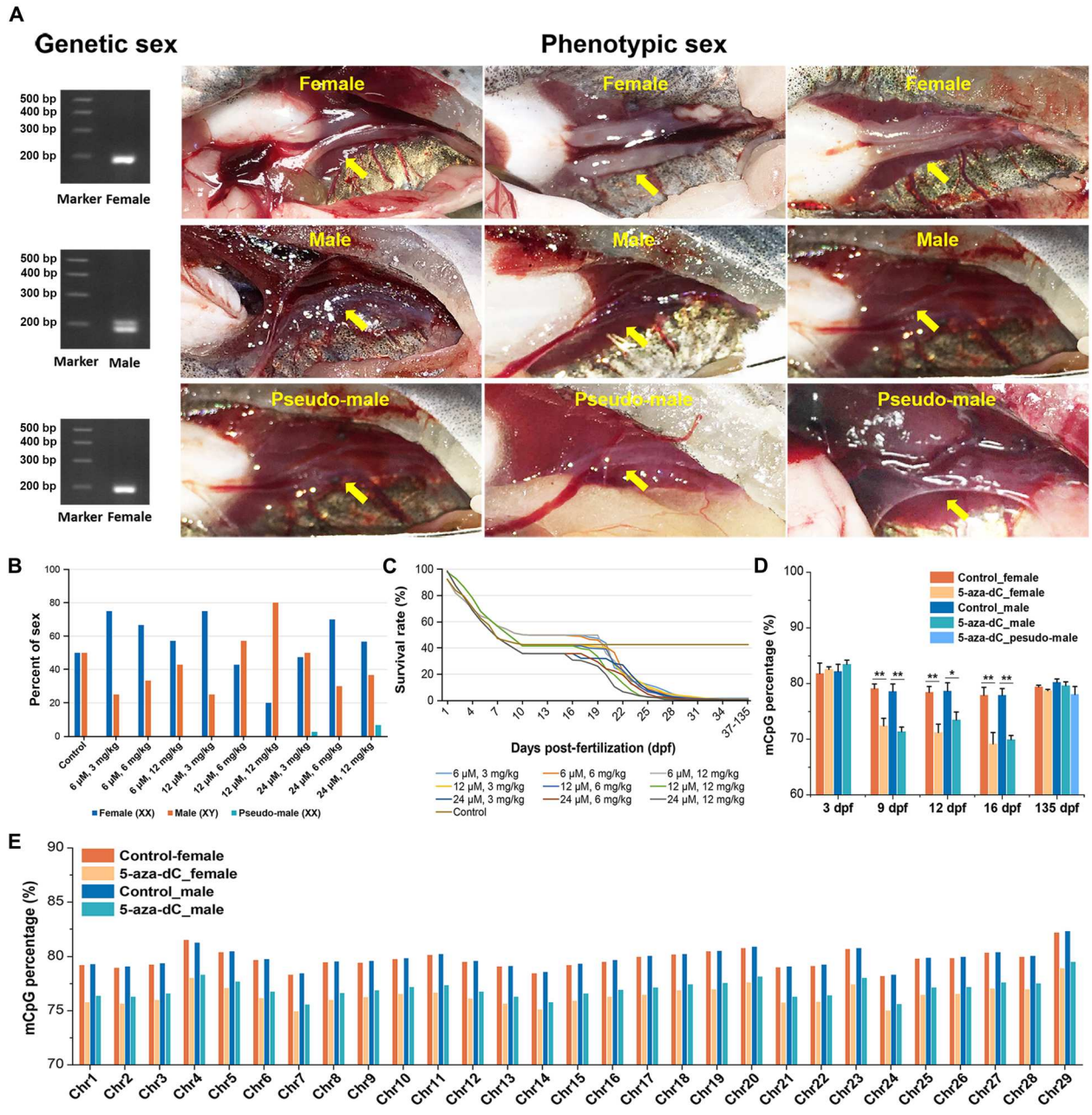

Fig. 4. Effects of 5-aza-dC on phenotypic sex, survival and genomic methylation of channel catfish, *Ictalurus punctatus*. (**A**) Identification of genetic and phenotypic sex at 135 dpf: Females had genotypes with one PCR band and well-differentiated ovaries (top panel showing three individuals); males had genotypes with two PCR bands and testes (middle panel with three individuals); sex-reversed pseudo-males had genotypes with one PCR band (genetic females) but testes (phenotypic males) (bottom panel with three individuals). bp, base pair. (**B**) Percentages of females, males, and pseudo-males at 135 dpf. (**C**) Effect of 5-aza-dC on survival rate. (**D**) Percentage of methylated CpG sites at 3, 9, 12, 16, and 135 dpf with control females, control males, and 5-aza-dC–treated females, males, and pseudo-males (24 μM aqueous before hatching, followed with 12 mg/kg in the feed). Asterisk (*) indicates statistical significance using Student's *t* test. $^{*}P < 0.05$ and $^{**}P < 0.01$. (**E**) Percentage of methylated CpG sites across the 29 chromosomes, using all methylation data.

only one gene, *tsnaxip1* (translin-associated factor X-interacting protein 1), was also differentially expressed in males. Seven of the 10 differentially expressed genes (DEGs), *hydin-1*, *spred3*, *sphkap*, *carmil2*, *slitrk3* (SLIT and NTRK-like family member 3), *hsf4* (heat shock transcription factor 4), and *tsnaxip1*, were up-regulated after 5-aza-dC treatment, and three genes, *esrrg* (estrogen-related receptor gamma-like), *pard6a* (par-6 family cell polarity regulator alpha), and *actrt3* (actin related protein T3), were down-regulated. The levels of differential expression were not notable, mostly between twofold and threefold (Table 2). Of particular interest was the differential expression of *hydin-1* after treatment with the methylation inhibitor as compared to controls. After the treatment

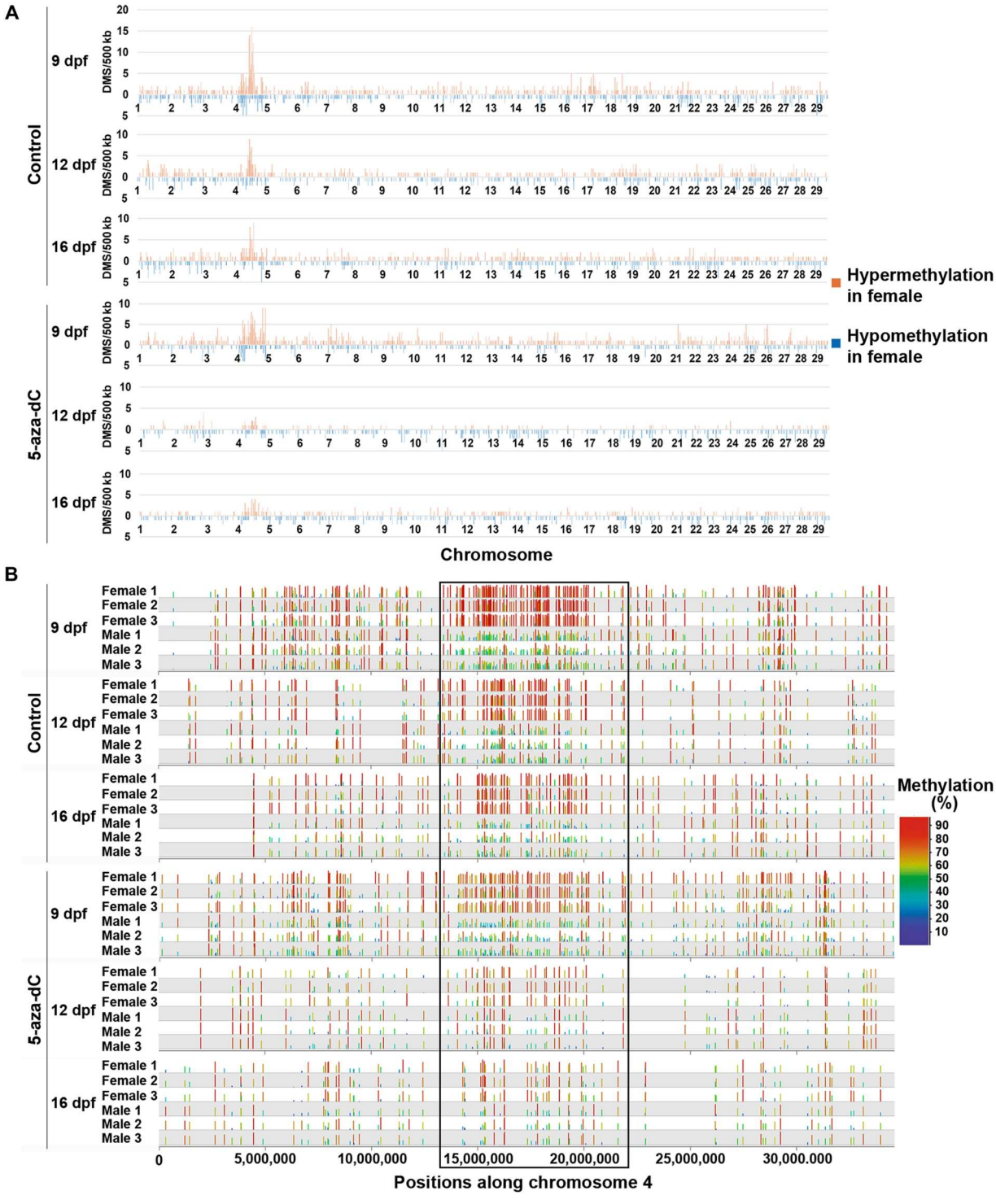

Fig. 5. Distribution of differentially methylated CpG sites after treatment of 5-aza-dC in channel catfish, *I. punctatus*. (**A**) Distribution of DMSs, expressed as DMS per 500 kb, between females and males on 29 chromosomes in control and 5-aza-dC–treated fish at 9, 12, and 16 dpf, as marked on the left margin. Positions along the 29 chromosomes are indicated on the *x* axis, and densities of DMSs, expressed as the number of DMSs per 500 kb, are indicated on the *y* axis. (**B**) Comparison of DMSs on chromosome 4 between females and males of control and 5-aza-dC–treated fish at 9, 12, and 16 dpf, with the SDR being highlighted in the black box. *X* axis indicates the positions along chromosome 4, and *y* axis indicates the levels of methylation at DMSs with color codes as shown on the right margin.

Table 2. DEGs within the SDR after treatment of 5-aza-dC in channel catfish, *I. punctatus,* at 3 and 16 dpf. Gene abbreviations: *hydin-1*, hydrocephalus-inducing protein homolog 1; *spred3*, sprouty related EVH1 domain containing 3; *sphkap*, SPHK1 interactor, AKAP domain containing; *carmil2*, capping protein regulator and myosin 1 linker 2; *hsf4*, heat shock transcription factor 4; *slitrk3*, SLIT and NTRK-like family member 3; *esrrg*, estrogen-related receptor gamma-like; *actrt3*, actin-related protein T3; *pard6a*, par-6 family cell polarity regulator alpha; *tsnaxip1*, translin-associated factor X-interacting protein 1.

Gene	Up- or down-regulated	Sex	Time point	Fold change
*hydin-1**	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
*carmil2**	Up	Female	16 dpf	2.2
hsf4	Up	Female	3 dpf	3.9
slitrk3	Up	Female	3 dpf	2.3
esrrg	Down	Female	3 dpf	2.2
actrt3	Down	Female	3 dpf	2.9
pard6a	Down	Female	3 dpf	2.3
tsnaxip1	Up/up	Female/male	16 dpf/16 dpf	3.0/3.1

*These four genes were differentially expressed in males as compared with females without treatment as well (see Table 1).

of 5-aza-dC, the expression of the X chromosome–borne *hydin-1* was up-regulated 2.2-fold at 3 dpf and was up-regulated 3.3-fold at 16 dpf (Table 2), although its expression from the X allele was still miniscule as compared to that from the Y allele (Fig. 3, C and D). It was also noteworthy with the up-regulation of three other genes, *spred3*, *sphkap*, and *carmil2*, after treatment of 5-aza-dC because these three genes, along with *hydin-1*, were differentially expressed between genetic females and males, with greater levels of expression in genetic males, without treatment.

Methylotypes in the SDR of pseudo-males are compatible with its genotypes

The treatment of methylation inhibitor, 5-aza-dC, caused sex reversal from genetic females to phenotypic males, but the sex phenotype could not be determined until gonadal differentiation at 110 dpf. We investigated the DNA methylation profiles in female (XX) ovaries, male (XY) testes, and pseudo-male (XX) testes at 135 dpf. As shown in Fig. 6A, principal components analysis (PCA) indicated that the whole-genome methylation profiles of the pseudo-males were more similar to those of normal females and males than to those of treated females and males. At 135 dpf, hypermethylation was still detected within the SDR in normal females as compared with normal males; pseudo-males exhibited hypermethylation in many sites within the SDR as compared to normal males, but not when compared with normal females (Fig. 6B), 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 (table S6).

Transcriptome of pseudo-males are compatible with its phenotypes

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 Fig. 6C, PCA revealed that time after fertilization explained the largest fraction of differences in expression. Thus, females and males, with or without treatment, were clustered close together at 3 and 16 dpf. At 135 dpf, when phenotypic sex was identified, sex had the largest influence on the profiles of gene expression. Pseudo-males had an expression profile that was more closely clustered together with males than females (Fig. 6C), which was also determined by pairwise comparison of DEGs (Fig. 6D). At 135 dpf, a total of 14,791 genes were differentially expressed between normal females and males. While 8838 DEGs were identified between pseudo-males and control females, only 25 genes were differentially expressed between pseudo-males and control males (table S7). The number of DEGs was the smallest when comparing pseudo-males with 5-aza-dC–treated males, with only 21 genes (table S7) being differentially expressed (Fig. 6D).

Of the 25 and 21 DEGs between pseudo-males and control males and between pseudo-males and 5-aza-dC–treated males, a common set of eight DEGs were found, of which three were overexpressed, and five were underexpressed in pseudo-males (table S7). The overexpressed genes in pseudo-males included *hydin-1*, *vsig1* (V-set and immunoglobulin domain-containing protein 1), and *mybpc1* (myosin binding protein C1). The underexpressed genes in pseudo-males included *cyp19a1* (cytochrome P450 family 19 subfamily A member 1), *hsd17b1* (hydroxysteroid 17-β dehydrogenase 1), *tubb5* (tubulin beta-5 chain), *tcerg1l* (transcription elongation regulator 1 like), and *znf341* (zinc finger protein 341). Apparently, the overexpressed genes in pseudo-males were enriched in masculinization, and the underexpressed genes in pseudo-males are enriched in feminization. For example, *hydin-1*, a key gene for sex differentiation into males as shown above, was expressed 60 and 120 times higher in pseudo-males than in 5-aza-dC–treated males or control males, respectively; and *visg1*, a gene that has been shown to be predominantly expressed in testicular germ cells, although not indispensable for spermatogenesis in male fertility in mice (*33*), was expressed 34 and 48 times higher in pseudo-males than in 5-aza-dC–treated males or control males, respectively. In contrast, *cyp19a1*, encoding the enzyme catalyzing estrogen biosynthesis, was expressed over 300 times lower in pseudo-males than in 5-aza-dC–treated males or control males, respectively; and *hsd17b1*, encoding the enzyme catalyzing the last step in estrogen activation, was expressed over 2000 times and 73 times lower in pseudo-males than in 5-aza-dC–treated males or control males, respectively (table S7).

The enriched terms of changes of transcriptome profiles after treatment of 5-aza-dC confirmed the similarity of expression in pseudo-males to that in males. As summarized in Fig. 6E, the enriched terms of up-regulated, DEGs in females as compared to those of normal males and pseudo-males were very similar. These enriched terms were biased toward functions of cellular growth and proliferation, such as DNA replication, DNA metabolic process, chromosome segregation, mitotic cell cycle process, organelle fission, and nuclear division. In contrast, the enriched terms of down-regulated, DEGs in females as compared to those of normal males and pseudo-males were very similar. These enriched terms

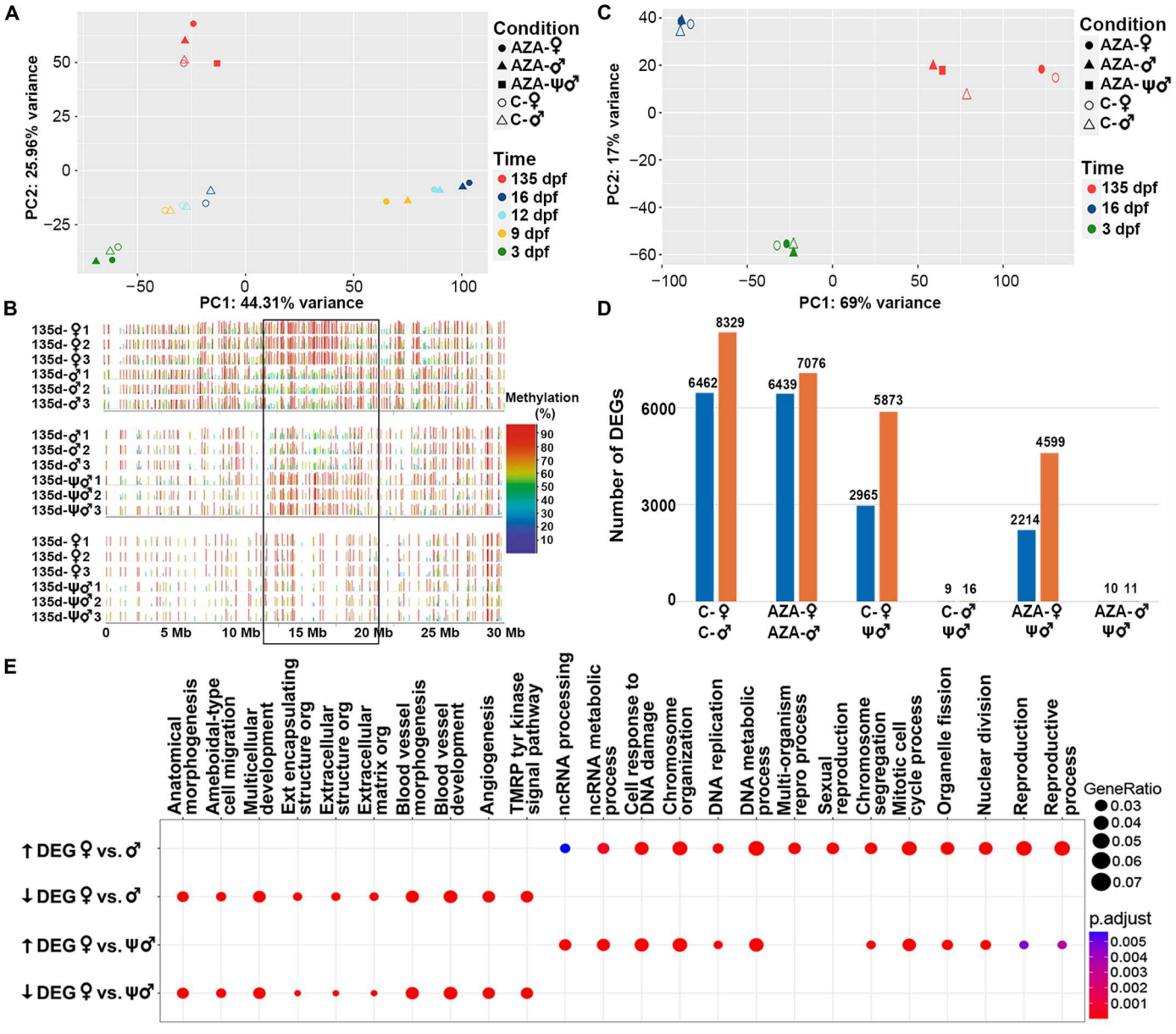

Fig. 6. DNA methylation and gene expression profiles in pseudo-males of channel catfish, *I. punctatus*. (**A**) PCA of WGBS samples using per site CpG methylation. PC1, principal component 1. (**B**) Differentially methylated CpG sites on chromosome 4 at 135 dpf, with three samples shown each for females (♀1, ♀2, and ♀3), males (♂1, ♂2, and ♂3), and pseudo-males (ψ♂1, ψ♂2, and ψ♂3). Comparisons between normal females and males, between males and pseudo-males, and between females and pseudo-males are shown on the top, middle, and lower panels, respectively, with the SDR highlighted by the black box. *X* axis indicates the location on chromosome 4; *y* axis indicates the methylation level, with color codes as shown on the right margin. (**C**) PCA analysis of RNA-seq samples using log transformation of normalized expression data in DESeq2. (**D**) Number of DEGs between sexes at 135 dpf. C-♀ versus C-♂: Control female versus control male; AZA-♀ versus AZA-♂: 5-aza-dC–treated female versus 5-aza-dC–treated male; C-♀ versus ψ♂: Control female versus pseudo-male; C-♂ versus ψ♂: Control male versus pseudo-male; AZA-♀ versus ψ♂: 5-aza-dC–treated female versus pseudo-male; AZA-♂ versus ψ♂: 5-aza-dC–treated male versus pseudo-male. For all comparisons, blue bar representedthe number of up-regulated genes, and orange bar representedthe number of down-regulated genes of the comparisons. (**E**) Enriched biological processes of up-regulated genes in females compared to males, down-regulated genes in females compared to males, up-regulated genes in females compared to pseudo-males, and down-regulated genes in females compared to pseudo-males at 135 dpf. ncRNA, noncoding RNA.

were biased toward development and sex differentiation, such as anatomical structure morphogenesis, ameboidal-type cell migration, multicellular organismal development, external encapsulating structure organization, extracellular structure organization, extracellular matrix organization, blood vessel development, angiogenesis, and transmembrane receptor protein tyrosine kinase signaling pathway. In the SDR, gene expression in pseudo-males was also similar to males. Together, it appeared that female-biased genes related to oocyte development in pseudo-males had

been suppressed, leading to expression profiles with functions similar to those of normal males.

DISCUSSION

Sex determination is one of the most fascinating areas of scientific research not only because of its fundamental importance to biology of sexual organisms but also because of the tremendous diversity of mechanisms. Here, we report an epigenetic mechanism of sex determination in channel catfish, where allele-specific hypermethylation of the SDR on the X chromosome silenced *hydin-1* gene expression in genetic females, whereas the SDR on the Y chromosome was hypomethylated, and the Y-borne *hydin-1* gene was expressed at high levels. This epigenetically regulated monoallelic, paternal origin of expression behaved much like genomic imprinting, which has never been reported from any egg-laying vertebrates such as birds or fish (*34*, *35*). This work demonstrated how important DNA methylation is for sex differentiation and thus for biology. Its importance is further elevated by the fact that environmental factors greatly affect methylation, and therefore, they can have a fundamental impact on the very existence of organisms such as fish, especially in face of climate change.

Genomic imprinting is essential to mammalian development, and it is one of the mechanisms to regulate sexual traits (*36*). However, there were no reports of monoallelic expression in teleost fish. The availability of chromosome-specific SNPs in channel catfish allowed trace of allelic expression on the sex chromosome. Although fish do not have as many sexual traits as mammals, most fish species, including channel catfish, exhibit some levels of sexual dimorphism, especially with growth and body size. Nonetheless, sex itself is surely a sexual trait, and therefore, there should be at least one difference in gene expression between genetic females and males. With channel catfish, our previous studies using genetic linkage mapping and genome-wide association studies provided solid evidence for genetic XY sex determination, and the sex determination locus was mapped to the SDR (*18*, *29*). Because of the lack of recombination within the SDR, genetic approaches are incapable to delineate to the sex determination gene. Within the SDR, however, identical gene contents were found on the X and Y chromosomes (*18*). In the present study, we provided evidence that several genes in the SDR were differentially expressed at higher levels in genetic males during early development at 3 and 16 dpf, before sex differentiation of channel catfish, where female gonadal differentiation starts around 19 dpf, and male gonadal differentiation starts between 90 and 102 dpf (*19*). These DEGs in males include *hydin-1*, *spred3*, *sphkap*, and *carmil2* (Table 1), which were of interest for their potential roles in sex determination.

The sex determination gene of channel catfish should meet the following requirements: It is located within the SDR (*18*, *29*), and it is expressed in XY males, but not expressed or expressed at low levels in XX females. *Hydin-1* met these requirements and had the essential characteristics of a sex determination gene: (i) It was expressed in genetic males but not in genetic females under normal conditions; (ii) it was monoallelically expressed exclusively from the Y chromosome; (iii) among all genes within SDR, it was the earliest differentially expressed, detected as early as 3 dpf, well ahead of sex differentiation in channel catfish; (iv) its ASM and regulated expression were inherited. In addition, *hydin-1* was markedly overexpressed in sex-reversed pseudo-males (table S7). However, *hydin* has not previously knownas a sex-determining gene in any organism, although its expression has been reported to be associated with male functions. Its mutation causes hydrocephalus in humans; it encodes a structural protein within the axoneme of sperm flagellum required for ciliary motility (*37*) and for spermiogenesis in mice (*38*–*40*). These known functions are related to male functions, but they are functions downstream of sex determination. We do not know what functions *hydin* has in early sex differentiation. Possibilities include its effect on expression of other genes in the genome that regulate sex differentiation; its roles as a promoter of proliferative growth and cancer-associated antigen (*41*) are compatible with its role as a sex determination gene. With channel catfish, female sex differentiates much earlier than male sex, and genetic males continue proliferative growth until 90 to 102 dpf when male sex starts to differentiate, while female gonadal differentiation starts 19 to 25 dpf (*19*). Enhanced proliferative growth would interfere with sex differentiation, allowing development into males. Future research is warranted to determine what functions *hydin* has and how it functions early after fertilization before sex differentiation.

Methylation inhibitor experiments supported the candidacy of *hydin-1* gene as the sex determination gene in channel catfish. The use of 5-aza-dC not only erased the epigenetic marks within the SDR but also caused sex reversal from genetic females to phenotypic males (pseudo-males). This sex reversal into pseudo-males is a "breakthrough" in channel catfish because, to date, sex reversal was only possible to produce pseudo-females no matter what the treatments have been, such as high temperature, sex hormones, and even androgens (*22*, *23*). In the present study, only a small proportion of treated fish was sex-reversed from genetic females to phenotypic males. This could be related to effective timing and effective concentrations of the treated fish as sex differentiation is irreversible. Once the differentiation occurred, sex reversal was no longer possible. Sex reversal was only observed in the treatment of the 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 before phenotypic sex can be identified, prohibiting the use of higher concentrations of the methylation inhibitor. The toxic effect of 5-aza-dC was reported in several other fish species including zebrafish (*25*, *42*) and Japanese rice fish (*43*). With channel catfish, as shown in Fig. 4C, mortality rates were very high, even at lower concentrations tested in this study, suggesting that the methylation inhibitor was generally toxic, presumably because proper methylation is a requirement of normal development and growth. This elevated sensitivity of channel catfish to 5-aza-dC could also be due to the naked body without scales, making adsorption more efficient in channel catfish as compared to fish with scales. One would expect that this sensitivity of channel catfish would increase sex reversal, but the treatment of 5-aza-dC was removed after 30 dpf because of the high mortality rate. In addition, female gonadal differentiation in channel catfish occurs much earlier, at 19 to 25 dpf, than male gonadal differentiation, at 90 to 102 dpf. This lengthy period of sex differentiation would have reduced the efficiency of sex reversal after the removal of the methylation inhibitor treatment. We realize the limitations we have with

the nonmodel species where gene knockout and knockdown systems are limited. Therefore, caution must be exercised for functional inference from the treatment of DNA methylation inhibitors because 5-aza-dC caused global demethylation that may have caused genomic deregulation of the overall development processes. Future studies are warranted to develop targeted depletion of the candidate genes through RNA interference knockdown, CRISPR knockout, or epigenomic editing.

The methylation inhibitor 5-aza-dC was used to determine epigenetic regulation of sex in zebrafish (*25*). One may argue that the effect on sex is just a general effect of 5-aza-dC, but opposite effect on sex reversal in zebrafish and channel catfish argues against that. The methylation inhibitor caused sex reversal into pseudo-females with zebrafish but into pseudo-males with channel catfish. These results supported an effect of methylation inhibitor on sex differentiation through demethylation of key genes involved in sex determination and differentiation. It makes sense that opposite direction of sex reversal was observed with zebrafish and channel catfish because zebrafish has a ZW/ZZ sex determination system (*44*), whereas channel catfish has a XX/XY sex determination system.

Epigenetic control of sex determination was described with *Drosophila melanogaster* (*45*), where the m6A pathway facilitates sex determination, and female identity in germ cells is maintained by H3K9 methyltransferase setdb1 (*46*). Epigenetic control was also reported for TSD of both plants and animals (*47*), but not for GSD. In fish and reptiles, DNA methylation–mediated control plays essential roles in TSD (*48*–*50*). In European sea bass, juvenile males have doubled DNA methylation levels in the promoter of gonadal aromatase (*cyp19a*) as compared to females; when exposed to high temperature, DNA methylation in the promoter region of gonadal aromatase *cyp19a* was increased in females (*48*). Recently, the epigenetic control of key genes for sexual development (*cyp19a1a* and *dmrt1*) in European sea bass has been reported (*51*). Considering the evolutionary position of channel catfish as a lower teleost, it is possible that such a sex determination mechanism could be operating in other lower vertebrates as well, especially those whose sex is highly vulnerable to environmental factors, such as temperature, which could function through their effects on genome methylation (*28*, *48*, *50*, *52*–*54*). Our study here has implications to connecting control mechanisms of GSD and ESD (especially TSD), in many lower vertebrates, and environmental modulation of sex differentiation in various organisms. In addition to *hydin-1*, several other genes in the SDR were differentially expressed during early sex differentiation. These included *spred3*, *sphkap*, and *carmil2* (Table 1). All these four genes were up-regulated after 5-aza-dC treatment in genetic females. In addition, *hsf4* and *slitrk3* were also up-regulated in genetic females after the treatment of the methylation inhibitor (Table 2). While these genes may be also important for sex differentiation, they were not exclusively expressed in genetic males. As a matter of fact, the levels of differential expression were modest, only two to three times during sex differentiation. *Spred3* encodes a protein with a C-terminal *Sprouty*-like cysteine-rich domain and an N-terminal EVH1 domain; it is a member of a family of proteins that negatively regulates mitogen-activated protein kinase signaling, particularly during organogenesis (*55*). Although the heat shock transcriptional factor *hsf4* could have been up-regulated in response to the adverse exposure to the methylation inhibitor, its specific up-regulation in genetic females, but not in genetic males, indicated its role in the negative regulation of female sex differentiation. *Slitrk* encodes a transmembrane protein that is involved in controlling neurite outgrowth (*56*); it is up-regulated in tumor cells in favor of cell proliferation, consistent with the sex reversal and masculinization after the treatment of the methylation inhibitor. Three genes, *esrrg*, *actrt3*, and *pard6a*, were specifically down-regulated in genetic females after 5-aza-dC treatment (Table 2). Their down-regulation paralleled to the masculinization and the slowing down of gonadal differentiation of females.

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 inherited. Hence, the methylation profiles of pseudo-males were much more similar to those of females than to those of males at 135 dpf, suggesting that "genetic memories" existed for these pseudo-males that were genetic 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 control or treated males (Fig. 6). It should be noted that the treatment of 5-aza-dC was removed after 30 dpf. While this treatment caused demethylation in the SDR and elsewhere and had its effect on masculinization and sex reversal, the treatment was not long enough to go beyond the entire period of sex differentiation and methylation reprogramming. This is consistent with the results from zebrafish where DNA methylation reprogramming was found after sex differentiation (*57*). In contrast to the situation of the methylome, the genome expression profile of pseudo-males was more similar to that of males than to that of females. These results clearly indicated the independence of genome methylation and genome expression, with regard to SDR. The "genetic memory" observed here is different from that observed in tongue sole, where methylation modification with treatment of high temperature in sex-reversed pseudo-males is globally inherited in their offspring, which can develop as pseudo-males without temperature incubation (*28*). Although we could not breed the sex-reversed pseudo-males because of mortalities, the preservation of the female profiles of methylation within the SDR of pseudo-males suggested that they would inherit the epigenetic marks as genetic females do.

In conclusion, this study demonstrated the involvement of methylation in the sex differentiation of channel catfish. The use of methylation inhibitor resulted in reduced levels of methylation, especially in 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 after treatment, *hydin*-1 was demonstrated to be allele-specifically hypomethylated and monoallelically expressed in males. The treatment of methylation inhibitor caused up-regulation of numerous 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.

MATERIALS AND METHODS

Ethical statement

The collection and treatment of fish in this study were approved by the Institutional Animal Care and Use Committee 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 United States.

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.* (*58*). Fertilized eggs were placed in suspended mesh baskets in a flow-through trough with paddle wheels until about 12 hours before hatching. During this period, embryos were taken out of the mesh basket and treated in tubs for 4 hours per day (2 hours in the morning and 2 hours in the afternoon) with 5-aza-dC (no. 11166, Cayman, Michigan, USA) at different concentrations (6, 12, and 24 μM). 5-aza-dC is a nucleoside-based DNA methyltransferase inhibitor (*59*). 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 4 hours daily. After fry started to eat feed, they were reared in flow-through troughs and fed with the 5-aza-dC diets (3, 6, and 12 mg/kg) four times a day. Treatment lasted until 30 dpf. Control groups were handled in the same way as the experimental groups except for not receiving 5-aza-dC treatment. At 135 dpf, all fish were collected, and the genetic sex was determined by PCR and gel electrophoresis. The phenotypic sex was assessed by dissecting and observing the gonads.

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, put 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, and 16 dpf, the head and tail were cut off, and the 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. In addition, sperm samples were collected from sexually mature male channel catfish. Genomic DNA from one part of each sample was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer′s protocol. RNA was extracted from the other part by the 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 (*32*).

WGBS 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), an equal amount of genomic DNA from five fish was pooled for WGBS library construction. Three replicates were conducted. Genomic DNA from each pseudo-male was used to construct libraries because only three pseudo-males were identified in treatment groups at 135 dpf. A total of 63 libraries (2 sexes × 5 time points × 2 treatments × 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 (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 (*60*). High-quality reads were mapped to the channel catfish reference genome IpCoco_1.2 (*30*) using the bisulfite alignment program Bismark v0.22.1 (*61*). 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 (*62*). 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 on the basis of the output of Bismark.

Methylation calls were analyzed using SeqMonk v1.45.2 (www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Methylation sites located on reads from the 29 chromosomes of channel catfish were imported to SeqMonk, and sites 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 was also generated by SeqMonk. To identify DMSs between two groups, the logistic regression of proportion-based statistics in SeqMonk was carried out with P value of <0.01 and a percentage difference of ≥25%. The identified genes with DMSs on their promoter [the 2 kb region upstream of transcription start site (TSS)] and gene body were selected as DMGs.

The CpG methylation clustering based on the similarity of the methylation profiles, PCA, for WGBS samples were generated using per CpG site methylation by methylKit (*63*) and plotted using ggplot2 in R (*64*). In addition, CpG methylation percentage in 3-kb region upstream and downstream of the TSS and in promoters, exons, introns, and intergenic regions was characterized on the basis of the report from Bismark.

Transcriptome sequencing and data processing

Total RNAs 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 an equal amount of RNA from three fish was 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 are also three duplicates for pseudo-male group. A total of 39 RNA libraries (2 sexes × 3 time points × 2 treatments × 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 v0.37 (*60*). Quality control of sequencing reads was carried out before and after trimming using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). All trimmed reads were mapped to the channel catfish reference genome IpCoco_1.2 (*30*) using HISAT2 v2.1.0 (*65*). HTSeq v0.11.0 was used to quantify the number of expressed transcripts (*66*). DESeq2 (*67*) was used to identify DEGs with $|\log_2(\text{fold change})| > 1$ and adjusted P value of <0.05. Gene Ontology and

Kyoto Encyclopedia of Genes and Genomes enrichment analyses of the DEGs were performed by clusterProfiler (*68*).

SNP calling and allele assignment

After aligning WGBS data to the channel catfish reference genome, Bis-SNP was used to call SNPs. Bis-SNP is based on the GATK framework to construct a bisulfite SNP calling model. Identified SNPs were filtered using the "VariantFiltration" function in GATK and "VCFpostprocess" function in Bis-SNP. Using Bismark (*61*) and HISAT2 v2.1.0 (*65*), WGBS and RNA-seq reads were respectively aligned to a customized catfish genome, with X allele– and Y allele–specific SNPs masked by the ambiguity base "N." Mapped reads were separated on the basis of X allele– and Y allele–specific SNPs using SNPsplit (*69*). The allele-specific reads were extracted from SNPsplit output using Picard/SamToFastq for further analysis of ASM and expression.

Identification of allele-specific DNA methylation and expression

The allele-specific reads from WGBS and RNA-seq datasets were aligned against the catfish reference genome IpCoco_1.2 using Bismark (*61*) and HISAT2 (*65*), respectively. ASM occurredwhen different methylation patterns were exhibited between parental alleles with a cutoff value of 80% methylation difference and adjusted P value of <0.01 (*70*). CpG sites with ASM were identified and visualized in SeqMonk (Babraham Bioinformatics, UK). The allele-specific expression was quantified using cufflinks (*71*) with aligned allele-specific RNA-seq reads.

Supplementary Materials

This PDF file includes:
Supplementary Materials
Fig. S1
Tables S1, S2, S4, and S7

Other Supplementary Material for this manuscript includes the following:
Tables S3, S5 and S6

View/request a protocol for this paper from *Bio-protocol*

REFERENCES AND NOTES

1. V. Ravi, B. Venkatesh, The divergent genomes of teleosts. *Annu. Rev. Anim. Biosci.* **6**, 47–68 (2018).
2. T. Ezaz, R. Stiglec, F. Veyrunes, J. A. Marshall Graves, Relationships between vertebrate ZW and XY sex chromosome systems. *Curr. Biol.* **16**, R736–R743 (2006).
3. J. E. Mank, J. C. Avise, Evolutionary diversity and turn-over of sex determination in teleost fishes. *Sex. Dev.* **3**, 60–67 (2009).
4. K. Kikuchi, S. Hamaguchi, Novel sex-determining genes in fish and sex chromosome evolution. *Dev. Dyn.* **242**, 339–353 (2013).
5. A. Yano, R. Guyomard, B. Nicol, E. Jouanno, E. Quillet, C. Klopp, C. Cabau, O. Bouchez, A. Fostier, Y. Guiguen, An immune-related gene evolved into the master sex-determining gene in rainbow trout, Oncorhynchus mykiss. *Curr. Biol.* **22**, 1423–1428 (2012).
6. R. S. Hattori, Y. Murai, M. Oura, S. Masuda, S. K. Majhi, T. Sakamoto, J. I. Fernandino, G. M. Somoza, M. Yokota, C. A. Strüssmann, A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 2955–2959 (2012).
7. T. Kamiya, W. Kai, S. Tasumi, A. Oka, T. Matsunaga, N. Mizuno, M. Fujita, H. Suetake, S. Suzuki, S. Hosoya, S. Tohari, S. Brenner, T. Miyadai, B. Venkatesh, Y. Suzuki, K. Kikuchi, A trans-species missense SNP in *Amhr2* is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (Fugu). *PLOS Genet.* **8**, e1002798 (2012).
8. D. K. Bej, K. Miyoshi, R. S. Hattori, C. A. Strüssmann, Y. Yamamoto, A duplicated, Truncated amh gene is involved in male sex determination in an old world silverside. *G3 (Bethesda)* **7**, 2489–2495 (2017).
9. U. F. Mustapha, D. N. Jiang, Z. H. Liang, H. T. Gu, W. Yang, H. P. Chen, S. P. Deng, T. L. Wu, C. X. Tian, C. H. Zhu, G. L. Li, Male-specific *Dmrt1* is a candidate sex determination gene in spotted scat (*Scatophagus argus*). *Aquaculture* **495**, 351–358 (2018).
10. M. Matsuda, Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, C. E. Morrey, N. Shibata, S. Asakawa, N. Shimizu, H. Hori, S. Hamaguchi, M. Sakaizumi, DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **417**, 559–563 (2002).
11. T. Myosho, H. Otake, H. Masuyama, M. Matsuda, Y. Kuroki, A. Fujiyama, K. Naruse, S. Hamaguchi, M. Sakaizumi, Tracing the emergence of a novel sex-determining gene in Medaka, Oryzias luzonensis. *Genetics* **191**, 163–170 (2012).
12. Y. Takehana, D. Demiyah, K. Naruse, S. Hamaguchi, M. Sakaizumi, Evolution of different Y chromosomes in two medaka species, Oryzias dancenaand O. latipes. *Genetics* **175**, 1335–1340 (2007).
13. T. Myosho, Y. Takehana, S. Hamaguchi, M. Sakaizumi, Turnover of sex chromosomes in *Celebensis* group medaka fishes. *G3 Genes Genomes Genet.* **5**, 2685–2691 (2015).
14. K. Tanaka, Y. Takehana, K. Naruse, S. Hamaguchi, M. Sakaizumi, Evidence for different origins of sex chromosomes in closely related oryzias fishes: Substitution of the master sex-determining gene. *Genetics* **177**, 2075–2081 (2007).
15. Y. Takehana, S. Hamaguchi, M. Sakaizumi, Different origins of ZZ/ZW sex chromosomes in closely related medaka fishes, *Oryzias javanicus* and *O. hubbsi*. *Chromosome Res.* **16**, 801–811 (2008).
16. Y. Takehana, K. Naruse, S. Hamaguchi, M. Sakaizumi, Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, Oryzias hubbsi and O. dancena. *Chromosoma* **116**, 463–470 (2007).
17. T. Nagai, Y. Takehana, S. Hamaguchi, M. Sakaizumi, Identification of the sex-determining locus in the Thai medaka, *Oryzias minutillus*. *Cytogenet. Genome Res.* **121**, 137–142 (2008).
18. L. Bao, C. Tian, S. Liu, Y. Zhang, A. Elaswad, Z. Yuan, K. Khalil, F. Sun, Y. Yang, T. Zhou, N. Li, S. Tan, Q. Zeng, Y. Liu, Y. Li, Y. Li, D. Gao, R. Dunham, K. Davis, G. Waldbieser, Z. Liu, The Y chromosome sequence of the channel catfish suggests novel sex determination mechanisms in teleost fish. *BMC Biol.* **17**, 1–16 (2019).
19. R. Patiño, K. B. Davis, J. E. Schoore, C. Uguz, C. A. Strüssmann, N. C. Parker, B. A. Simco, C. A. Goudie, Sex differentiation of channel catfish gonads: Normal development and effects of temperature. *J. Exp. Zool.* **276**, 209–218 (1996).
20. W. H. LeGrande, R. A. Dunham, R. O. Smitherman, Karyology of three species of catfishes (Ictaluridae: *Ictalurus*) and four hybrid combinations. *Copeia* **1984**, 873–878 (1984).
21. W. Wang, S. Tan, Y. Yang, T. Zhou, D. Xing, B. Su, J. Wang, S. Li, M. Shang, D. Gao, R. Dunham, Z. Liu, Feminization of channel catfish with 17β-oestradiol involves methylation and expression of a specific set of genes independent of the sex determination region. *Epigenetics* **17**, 1820–1837 (2022).
22. C. Goudie, B. Redner, B. A. Simco, K. B. Davis, Feminization of channel catfish by oral administration of steroid sex hormones. *Trans. Am. Fish. Soc.* **112**, 670–672 (1983).
23. K. B. Davis, B. A. Simco, C. A. Goudie, N. C. Parker, W. Cauldwell, R. Snellgrove, Hormonal sex manipulation and evidence for female homogamety in channel catfish. *Gen. Comp. Endocrinol.* **78**, 218–223 (1990).
24. N. Ospina-Álvarez, F. Piferrer, Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLOS ONE* **3**, e2837 (2008).
25. L. Ribas, K. Vanezis, M. A. Imués, F. Piferrer, Treatment with a dna methyltransferase inhibitor feminizes zebrafish and induces long-term expression changes in the gonads. *Epigenet. Chromatin* **10**, 1–16 (2017).
26. I. Nanda, M. Kondo, U. Hornung, S. Asakawa, C. Winkler, A. Shimizu, Z. Shan, T. Haaf, N. Shimizu, A. Shima, A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka,Oryzias latipes. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11778–11783 (2002).
27. L.-X. Sun, Y.-Y. Wang, Y. Zhao, H. Wang, N. Li, X. S. Ji, Global DNA methylation changes in Nile tilapia gonads during high temperature-induced masculinization. *PLOS ONE* **11**, e0158483 (2016).
28. C. Shao, Q. Li, S. Chen, P. Zhang, J. Lian, Q. Hu, B. Sun, L. Jin, S. Liu, Z. Wang, H. Zhao, Z. Jin, Z. Liang, Y. Li, Q. Zheng, Y. Zhang, J. Wang, G. Zhang, Epigenetic modification and inheritance in sexual reversal of fish. *Genome Res.* **24**, 604–615 (2014).
29. Y. Li, S. Liu, Z. Qin, G. Waldbieser, R. Wang, L. Sun, L. Bao, R. G. Danzmann, R. Dunham, Z. Liu, Construction of a high-density, high-resolution genetic map and its integration with BAC-based physical map in channel catfish. *DNA Res.* **22**, 39–52 (2015).
30. Z. Liu, S. Liu, J. Yao, L. Bao, J. Zhang, Y. Li, C. Jiang, L. Sun, R. Wang, Y. Zhang, T. Zhou, Q. Zeng, Q. Fu, S. Gao, N. Li, S. Koren, Y. Jiang, A. Zimin, P. Xu, A. M. Phillippy, X. Geng, L. Song, F. Sun, C. Li, X. Wang, A. Chen, Y. Jin, Z. Yuan, Y. Yang, S. Tan, E. Peatman, J. Lu,

Z. Qin, R. Dunham, Z. Li, T. Sonstegard, J. Feng, R. G. Danzmann, S. Schroeder, B. Scheffler, M. V. Duke, L. Ballard, H. Kucuktas, L. Kaltenboeck, H. Liu, J. Armbruster, Y. Xie, M. L. Kirby, Y. Tian, M. E. Flanagan, W. Mu, G. C. Waldbieser, The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nat. Commun.* **7**, 11757 (2016).

31. Y. Yang, T. Zhou, Y. Liu, C. Tian, L. Bao, W. Wang, Y. Zhang, S. Liu, H. Shi, S. Tan, D. Gao, R. A. Dunham, Z. Liu, Identification of an epigenetically marked locus within the sex determination region of channel catfish. *Int. J. Mol. Sci.* **23**, 5471 (2022).
32. P. Ninwichian, E. Peatman, D. Perera, S. Liu, H. Kucuktas, R. Dunham, Z. Liu, Identification of a sex-linked marker for channel catfish. *Anim. Genet.* **43**, 476–477 (2012).
33. Y. Jung, H. Bang, Y. H. Kim, N. E. Park, Y. H. Park, C. Park, S. R. Lee, J. W. Lee, B. S. Song, J. S. Kim, B. W. Sim, D. W. Seol, G. Wee, S. Kim, S. U. Kim, E. Kim, V-set and immunoglobulin domain-containing 1 (*Vsig1*), predominantly expressed in testicular germ cells, is dispensable for spermatogenesis and male fertility in mice. *Animals* **11**, 1037 (2021).
34. D. P. Barlow, M. S. Bartolomei, Genomic imprinting in mammals. *Cold Spring Harb. Perspect. Biol.* **6**, a018382 (2014).
35. V. Tucci, A. R. Isles, G. Kelsey, A. C. Ferguson-Smith; Erice Imprinting Group, Genomic imprinting and physiological processes in mammals. *Cell* **176**, 952–965 (2019).
36. Z. D. Smith, A. Meissner, DNA methylation: Roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220 (2013).
37. H. R. Dawe, M. K. Shaw, H. Farr, K. Gull, The hydrocephalus inducing gene product, Hydin, positions axonemal central pair microtubules. *BMC Biol.* **5**, 1–10 (2007).
38. K. F. Lechtreck, P. Delmotte, M. L. Robinson, M. J. Sanderson, G. B. Witman, Mutations in *Hydin* impair ciliary motility in mice. *J. Cell Biol.* **180**, 633–643 (2008).
39. B. E. Davy, M. L. Robinson, Congenital hydrocephalus in hy3 mice is caused by a frameshift mutation in *Hydin*, a large novel gene. *Hum. Mol. Genet.* **12**, 1163–1170 (2003).
40. R. Pereira, J. Oliveira, L. Ferraz, A. Barros, R. Santos, M. Sousa, Mutation analysis in patients with total sperm immotility. *J. Assist. Reprod. Genet.* **32**, 893–902 (2015).
41. K. Laske, Y. V. Shebzukhov, L. Grosse-Hovest, D. V. Kuprash, S. V. Khlgatian, E. P. Koroleva, A. Y. Sazykin, D. N. Penkov, P. V. Belousov, S. Stevanovic, V. Vass, S. Walter, D. Eisel, B. D. Schmid-Horch, S. A. Nedospasov, H. G. Rammensee, C. Gouttefangeas, Alternative variants of human HYDIN are novel cancer-associated antigens recognized by adaptive immunity. *Cancer Immunol. Res.* **1**, 190–200 (2013).
42. C. C. Martin, L. Laforest, M. A. Akimenko, M. Ekker, A role for DNA methylation in gastrulation and somite patterning. *Dev. Biol.* **206**, 189–205 (1999).
43. A. K. Dasmahapatra, I. A. Khan, DNA methyltransferase expressions in Japanese rice fish (*Oryzias latipes*) embryogenesis is developmentally regulated and modulated by ethanol and 5-azacytidine. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **176–177**, 1–9 (2015).
44. C. A. Wilson, S. K. High, B. M. McCluskey, A. Amores, Y. L. Yan, T. A. Titus, J. L. Anderson, P. Batzel, M. J. Carvan, M. Schartl, J. H. Postlethwait, Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains. *Genetics* **198**, 1291–1308 (2014).
45. L. Kan, A. V. Grozhik, J. Vedanayagam, D. P. Patil, N. Pang, K. S. Lim, Y. C. Huang, B. Joseph, C. J. Lin, V. Despic, J. Guo, D. Yan, S. Kondo, W. M. Deng, P. C. Dedon, S. R. Jaffrey, E. C. Lai, The m^6A pathway facilitates sex determination in *Drosophila*. *Nat. Commun.* **8**, 15737 (2017).
46. A. E. Smolko, L. Shapiro-Kulnane, H. K. Salz, The H3K9 methyltransferase *SETDB1* maintains female identity in *Drosophila* germ cells. *Nat. Commun.* **9**, 4155 (2018).
47. J. Zhang, A. Boualem, A. Bendahmane, R. Ming, Genomics of sex determination. *Curr. Opin. Plant Biol.* **18**, 110–116 (2014).
48. L. Navarro-Martín, J. Viñas, L. Ribas, N. Díaz, A. Gutiérrez, L. Di Croce, F. Piferrer, DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLOS Genet.* **7**, e1002447 (2011).
49. Y. Matsumoto, A. Buemio, R. Chu, M. Vafaee, D. Crews, Epigenetic control of gonadal aromatase (*cyp19a1*) in temperature-dependent sex determination of red-eared slider turtles. *PLOS ONE* **8**, e63599 (2013).
50. B. B. Parrott, S. Kohno, J. A. Cloy-McCoy, L. J. Guillette Jr., Differential incubation temperatures result in dimorphic DNA methylation patterning of the *SOX9* and aromatase promoters in gonads of alligator (*Alligator mississippiensis*) embryos. *Biol. Reprod.* **90**, 1–11 (2014).
51. M. V. Krick, E. Desmarais, A. Samaras, E. Gueret, A. Dimitroglou, M. Pavlidis, C. S. Tsigenopoulos, B. Guinand, Family-effects in the epigenomic response of red blood cells to a challenge test in the European sea bass (*Dicentrarchus labrax*, L.). *BMC Genomics* **22**, 111 (2021).
52. J. Han, Y. Hu, Y. Qi, C. Yuan, S. Naeem, D. Huang, High temperature induced masculinization of zebrafish by down-regulation of *sox9b* and *esr1* via DNA methylation. *J. Environ. Sci.* **107**, 160–170 (2021).
53. S. Radhakrishnan, R. Literman, J. L. Neuwald, N. Valenzuela, Thermal response of epigenetic genes informs turtle sex determination with and without sex chromosomes. *Sex. Dev.* **12**, 308–319 (2018).
54. D. Venegas, A. Marmolejo-Valencia, C. Valdes-Quezada, T. Govenzensky, F. Recillas-Targa, H. Merchant-Larios, Dimorphic DNA methylation during temperature-dependent sex determination in the sea turtle *Lepidochelys olivacea*. *Gen. Comp. Endocrinol.* **236**, 35–41 (2016).
55. R. Kato, A. Nonami, T. Taketomi, T. Wakioka, A. Kuroiwa, Y. Matsuda, A. Yoshimura, Molecular cloning of mammalian Spred-3 which suppresses tyrosine kinase-mediated Erk activation. *Biochem. Biophys. Res. Commun.* **302**, 767–772 (2003).
56. J. Aruga, K. Mikoshiba, Identification and characterization of Slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. *Mol. Cell. Neurosci.* **24**, 117–129 (2003).
57. X. Wang, X. Ma, G. Wei, W. Ma, Z. Zhang, X. Chen, L. Gao, Z. Liu, Y. Yuan, L. Yi, J. Wang, T. Tokumoto, J. Huang, D. Chen, J. Zhang, J. Liu, The Role of DNA methylation reprogramming during sex determination and transition in zebrafish. *Genomics Proteomics Bioinforma.* **19**, 48–63 (2021).
58. M. E. Gima, A. Gima, A. Hutson, A. Chaimongkol, R. Beam, D. A. Perera, R. A. Dunham, Realized heritability and response to selection for fecundity, hatching rate and fry/Kg for channel catfish females (*Ictalurus punctatus*) induced to ovulate and fertilized with blue catfish (*Ictalurus furcatus*) males for the production of hybrid catfish embryos. *Aquaculture* **420–421**, S36–S41 (2014).
59. J. K. Christman, 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: Mechanistic studies and their implications for cancer therapy. *Oncogene* **21**, 5483–5495 (2002).
60. A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
61. F. Krueger, S. R. Andrews, Bismark: A flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27**, 1571–1572 (2011).
62. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).
63. A. Akalin, M. Kormaksson, S. Li, F. E. Garrett-Bakelman, M. E. Figueroa, A. Melnick, C. E. Mason, MethylKit: A comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biol.* **13**, 1–9 (2012).
64. H. Wickham, ggplot2. *Wiley Interdiscip. Rev. Comput. Stat.* **3**, 180–185 (2011).
65. D. Kim, B. Langmead, S. L. Salzberg, HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360 (2015).
66. S. Anders, P. T. Pyl, W. Huber, HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
67. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).
68. G. Yu, L. G. Wang, Y. Han, Q. Y. He, clusterProfiler: An R package for comparing biological themes among gene clusters. *Omi. a J. Integr. Biol.* **16**, 284–287 (2012).
69. F. Krueger, S. R. Andrews, SNPsplit: Allele-specific splitting of alignments between genomes with known SNP genotypes. *F1000Research* **5**, 1479 (2016).
70. R. Shoemaker, J. Deng, W. Wang, K. Zhang, Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome. *Genome Res.* **20**, 883–889 (2010).
71. C. Trapnell, A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, L. Pachter, Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578 (2012).

Acknowledgments: We thank R. Beam and K. Veverica for support in production and maintenance of experimental fish. **Funding:** This project was supported by Agriculture and Food Research Initiative (AFRI) Competitive Grants 2019-67015-29409 from the USDA National Institute of Food and Agriculture (NIFA) Animal Reproduction Program to Z.L. and 2017-67015-26295 from the Animal Breeding, Genetics and Genomics Program to Z.L. **Author contributions:** Conceptualization: Z.L. Formal analysis: W.W., Y.Y., S.T., and Z.L. Investigation: D.G., R.D., and Z.L. Data curation: T.Z., Y.L., C.T., L.B., D.X., Y.Z., S.L., H.S., D.G., B.S., and J.W. Supervision: Z.L. Writing (original draft): Z.L., W.W., Y.Y., S.T. Writing (review and editing): D.G., R.D., and Z.L. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** Sequence data have been deposited in the NCBI BioProject database under accession nos. PRJNA553256, PRJNA826119, and PRJNA837725. All other data needed to evaluate the conclusions in the manuscript are present in the paper and/or the Supplementary Materials.

Submitted 6 May 2022
Accepted 18 November 2022
Published 21 December 2022
10.1126/sciadv.adc8786