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ORIGINAL ARTICLE

Comparative Transcriptome Analysis Reveals Differentially Expressed Genes and Signaling Pathways Between Male and Female Red-Tail Catfish (*Mystus wyckioides*)

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Abstract

Sexual dimorphism is widespread in fish species. The red-tail catfish (*Mystus wyckioides*) is a commercially important catfish in the lower reaches of the Lancang River and the Mekong basin, and it shows a growth advantage in males. Here, RNA-seq was for the first time used to explore the gene expression difference between the sexes in the hypothalamus and pituitary of red-tail catfish, respectively. In the hypothalamus, 5732 and 271 unigenes have significantly higher and lower expressions, respectively, in males compared with females. KEGG analysis showed that 212 DEGs were annotated to 216 signaling pathways, and enrichment analysis suggested different levels of cAMP and glutamatergic synapse signaling between male and female hypothalami and some of the DEGs appear involved in gonad development and growth. In the pituitary, we found only 19 differentially expressed unigenes, which were annotated to 32 signaling pathways, most of which play important roles in gonad development.

Keywords Transcriptome · Hypothalamus · Pituitary · Growth · Gonad development

Introduction

Sexual dimorphism commonly exists in gonochoristic animals and involves many aspects of biology (Mei and Gui 2015), including body size (Ma et al. 2016; Rideout et al. 2015; Shine 1986; Wu et al. 2015), brain morphology (Gorski et al. 1978; Moriarty 1975), buccal morphology (Okuda et al. 2002), organ morphology (Adamson and King 1984; Yasuda et al. 2005), pituitary and gonadal hormones (Jalouli et al. 2003; Zimmerberg and Farley 1993), or even life expectancies (Shi et al. 2017). Fish are the most species-rich vertebrate lineage including almost half of the extant vertebrate species (Betancur-R et al. 2017). Liu first reported sex reversal phenomenon in ricefield eel (*Monopterus albus*), which initiated a new field on revealing the sex differentiation of fish (Bullough 1947). During the last decades, large numbers of basic studies have been reported on fish sexual dimorphism and sex determination and differentiation (Devlin and Nagahama 2002; Kobayashi et al. 2013; Scott et al. 1989; Wu et al. 2015; Li and Gui 2018a, b, Li et al. 2018).

Hypothalamus and pituitary are key endocrine glands for controlling instinctive behaviors and hormone secretion, such as metabolism, feeding, stress response, and reproduction (Dhillo et al. 2005; Harris et al. 1978; Rivier and Rivest 1991; Suchecki et al. 1993). Although the reproductive behaviors and strategies are diverse in vertebrates, the endocrine network of reproduction is conserved (hypothalamic-pituitary-gonadal axis) (Sower et al. 2009). When gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus, the gonadotropins (GTHs) are secreted in pituitary as a

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response. In the following, the GTHs stimulate gonads, where sex hormones are released and gonad maturation is initiated (Nozaki 2013). In addition, other adenohypophysial hormones such as growth hormone (GH), adrenocorticotropin, and prolactin are also responsive to the hypothalamic-pituitary system (Blackwell and Guillemin 1973; Heinz et al. 1995; Saga et al. 1993; Sayers et al. 1980; Salemi et al. 2007). In conclusion, hypothalamic-pituitary system is a vital element leading to physiological divergence, including sex-dependent dimorphic growth pattern (Wang et al. 2009).

Red-tail catfish (*Mystus wyckioides*) belongs to the catfish family Bagridae and is named after the extending filament-like ornament in its red tail. It is a native species of the Lancang River (Tippayadara et al. 2016). Interestingly, just like the other two fish in Bagridae, *Pelteobagrus fulvidraco* (Wang et al. 2009; Dan et al. 2013) and *Pseudobagrus ussuriensis* (Pan et al. 2015; Wang et al. 2009), the significant sex-dependent growth difference has been observed in red-tail catfish. However, previous studies of red-tail catfish have only focused on artificial breeding, karyotype analysis, and muscle nutrition analysis (Tippayadara et al. 2016). In the present study, we performed Illumina RNA-seq to identify differentially expressed genes and related pathways in the hypothalamic-pituitary system to reveal the molecular mechanism of sexual difference between male and female red-tail catfish. This study is the first comparative transcriptome analysis in red-tail catfish and reveals significant expression differences between males and females in hypothalamus and pituitary.

Results

Size Dimorphism Between Males and Females

Aquaculture practices and field measurement have identified the sexual dimorphism in growth. In this study, 55 4-year-old individuals (27 males and 28 females) were randomly selected, and it showed that the males had a significant growth advantage over females based on the values of body weight and body length. The average weight of females and males was 9.06 ± 2.74 kg and 11.44 ± 3.95 kg, respectively, which indicated that the weight of males was 26.27% heavier than that of females ($p < 0.01$) (Fig. 1a). As for the length, the average length of females and males was 93.56 ± 15.82 cm and 98.55 ± 15.83 cm, respectively, and it showed that the males were 5.33% longer than the females ($p < 0.05$) (Fig. 1b).

Illumina Sequencing, De Novo Assembly, and Functional Annotation

To identify differentially expressed mRNAs in hypothalamic-pituitary system between male and female red-tail catfish, two different tissues (hypothalamus and pituitary) of mature male and female adults were collected and the total RNAs from each tissue was extracted for cDNA library construction. After removing low-quality reads, a total of 47.1–48.5 (GC content = 45%, Q30 = 92.44%) and 46.6–48.3 million (GC content = 47%, Q30 = 92.51%) clean reads were obtained in the hypothalamus and pituitary, respectively (Supplementary Table 1). After trimming, these high-quality reads were de novo assembled by Trinity version20131110. Finally, a total of 89,487 unigenes with an average length of 1027.52 bp and N50 of 1792 bp were obtained in the hypothalamus, and 85,143 unigenes with average length of 1019.74 bp and N50 of 1775 bp were received in the pituitary (Supplementary Table 2). The length distributions of de novo assembled unigenes were shown in Supplementary Fig. 1.

The unigenes of two tissues were blasted against the NR, SWISSPROT, and KOG databases by using BLASTX with a threshold value of $1e^{-5}$. In NR annotation, more than half of the annotated unigenes (54.83% unigenes in the hypothalamus and 54.13% unigenes in the pituitary) were overlapped with known genes of *Ictalurus punctatus* (a closely related species of red-tail catfish) (Supplementary Fig. 2A and B). The numbers of annotated unigenes against databases are shown in a Wayne chart (Supplementary Fig. 2C and D). Besides that, about 60% unigenes did not match with any database entries, which may represent some non-coding RNAs or unknown protein coding genes in red-tail catfish.

Screening and Identification of Sexual Differentially Expressed Unigenes

To identify differentially expressed unigenes (DEGs) between males and females, the expression levels of assembled unigenes were determined using the reads per kilobase per million (RPKM) method. In this study, we focused on DEGs in the hypothalamus and the pituitary, respectively. Genes were considered DEGs if they showed a fold change ≥ 2 and $p < 0.05$ (FDR test). In the hypothalamic comparison, a total of 6003 DEGs were screened; among them, 5732 DEGs were highly expressed in males and 271 DEGs were highly expressed in females (Fig. 1c). In the pituitary, 1083 DEGs were found between genders, of which 480 were expressed significantly high in males and 603 were expressed significantly high in females (Fig. 1d). Among them, only 4 common DEGs (*TAAR*, *Pcdh16*, *Gcnt3*, *Amhr2*) were identified in both of the hypothalamus and pituitary (Supplementary Table 3).

Some important genes (*GnRH*, *GnRHR*, *GHRH*, etc.) involved steroid secreting in the hypothalamic-pituitary-gonadal (HPG) system, genes related to growth (*IGF1/2*) and aromatase secreting (*CYP19A*) were analyzed by performing RPKM and qRT-PCR. According to normalized value of RPKM and qRT-PCR in our studies, the greater expression of *PACAP*, *NPY*, *KISS1R*, *IGF-1* was identified in the hypothalamus of a male, and *CYP19A* is highly expressed in the hypothalamus of a

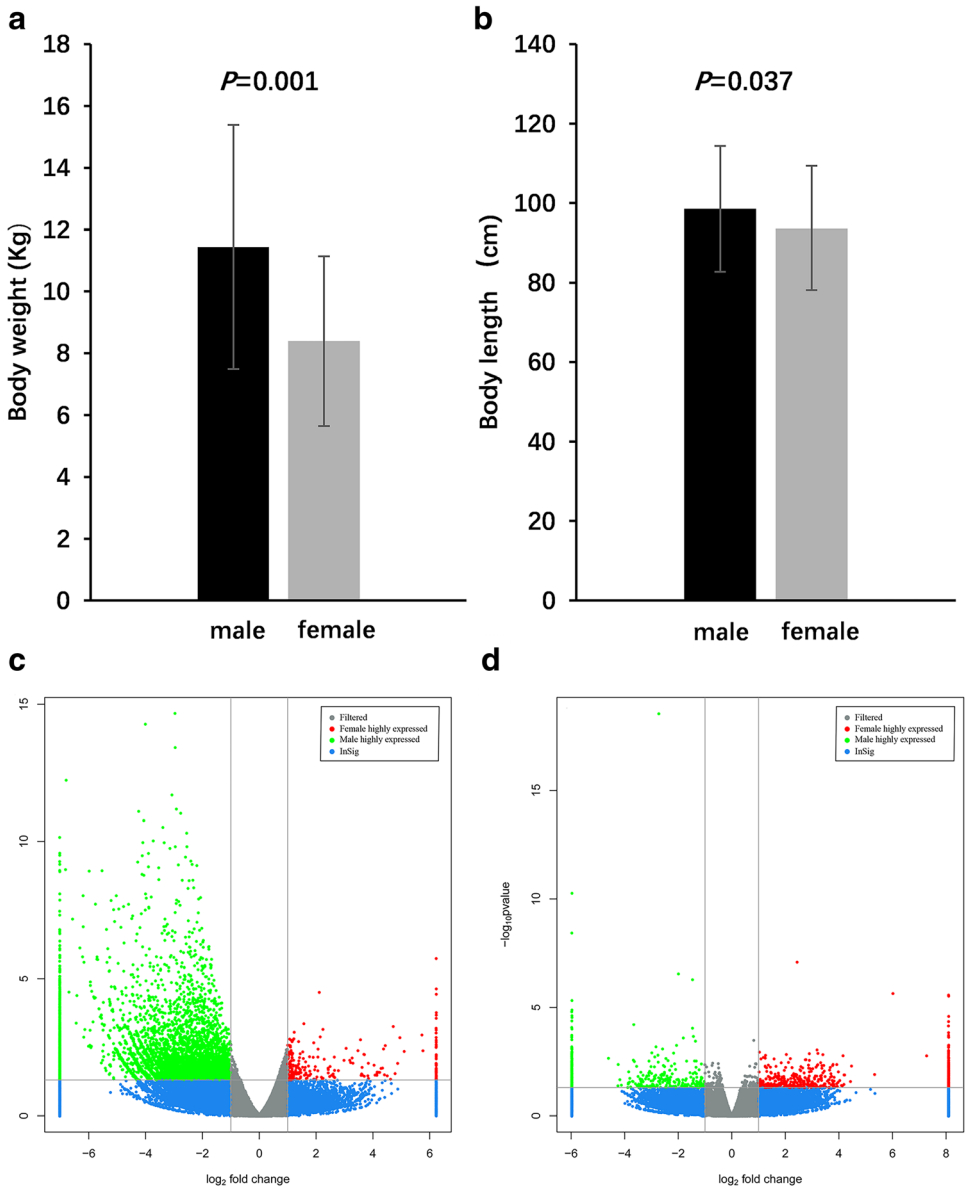

Fig. 1 **a** Statistics of body weight of 55 4-year-old red-tail catfish between sexes (27 males and 28 females, $p = 0.001$). **b** Statistics of body length of 55 4-year-old red-tail catfish between sexes (27 males and 28 females, $p = 0.037$). Difference of unigene expression between the hypothalamic-pituitary axis in male and female red-tail catfish. The *x*-axis presents level of differential expression, and the *y*-axis presents value of significant difference. **c** DEGs in the hypothalamus of male and female. **d** DEGs in the pituitary of male and female

female (Fig. 2a). In the pituitary, *FSHβ* and *LH* are mainly expressed in female and a higher expression of *PACAP* was identified in male (Fig. 2b). Linear regression analysis revealed that the positive correlation between RPKM and qRT-PCR-dependent unigene expression persisted in both of the hypothalamus and pituitary with $R^2 = 0.86$ and 0.89, respectively (Supplementary Fig. 3A and B).

GO Enrichment Analysis of DEGs

For both the hypothalamus and pituitary, DEGs were searched against the Gene Ontology database (www.geneontology.org) to infer their function according to GO annotation. In the hypothalamus, 405 DEGs were annotated to 1585 GO terms accompanied by 345 and 60 DEGs with relatively higher expression in male and female hypothalami, respectively. Ninety-eight male-highly expressed unigenes (MEGs) were annotated to nucleus (GO:0005634), 95 MEGs were annotated to cytoplasm (GO:0005737), 71 MEGs were annotated to plasma membrane (GO:0005886), 70 MEGs were annotated to DNA binding (GO:0003677), 70 MEGs were annotated to metal ion binding (GO:0046872), etc. Twenty-six female-highly expressed unigenes (FEGs) were annotated to nucleus (GO:0005634), 18 FEGs were annotated to cytoplasm (GO:0005737), 17 FEGs were annotated to ATP binding (GO:0005524), 11 FEGs were annotated to DNA binding (GO:0003677), 9 FEGs were annotated to transcription DNA-templated (GO:0006351), etc. (Fig. 3a).

In pituitary, a total of 47 DEGs were annotated into 348 GO terms. Among them, 16 male-highly expressed unigenes were related to 164 GO terms, 4 MEGs were annotated to metal ion binding (GO:0046872), 4 MEGs were annotated to plasma

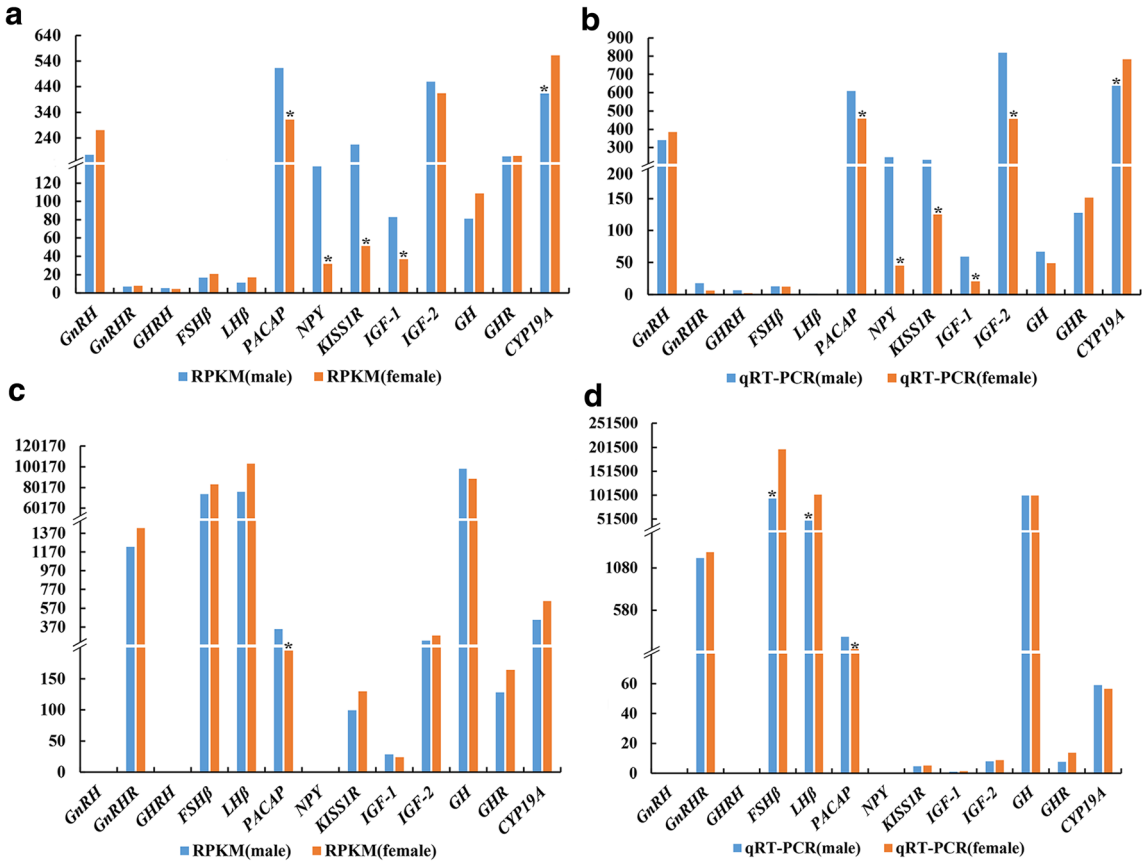

Fig. 2 RNA-seq expression data and qRT-PCR data of selected genes involved steroid secreting in the hypothalamic-pituitary-gonadal (HPG) system. Asterisks represent significant difference ($p < 0.05$) between sexes. **a** Normalized RPKM data of selected genes in the hypothalamus. **b** Normalized qRT-PCR data of selected genes in the hypothalamus. **c** Normalized RPKM data of selected genes in the pituitary. **d** Normalized qRT-PCR data of selected genes in the pituitary

membrane (GO:0005886), 4 MEGs were annotated to cytoplasm (GO:0005737), 3 MEGs were annotated to extracellular space (GO:0005615), 3 MEGs were annotated to calcium ion binding (GO:0005509), etc. Thirty-one female-highly expressed unigenes were annotated to 219 GO terms. Among them, 8 FEGs were annotated to integral component of membrane (GO:0016021), 5 FEGs were annotated to extracellular exosome (GO:0070062), 5 FEGs were annotated to nucleus (GO:0005634), 4 FEGs were annotated to cytoplasm (GO:0005737), 3 FEGs were annotated to protein homodimerization activity (GO:0042803), etc.(Fig. 3b).

KEGG Enrichment Analysis of DEGs Between Males and Females

KEGG enrichment annotation can help us clarify the function of enriched DEGs in the metabolic pathways. In the hypothalamus, 212 DEGs were annotated to 216 KEGG pathways accompanied by 171 and 41 DEGs with relatively higher expression in males and females, respectively. In detail, most of the male-highly expressed unigenes (MEGs) were assigned to cAMP signaling pathway (30 MEGs, ko04024), glutamatergic synapse (30 MEGs, ko04724), circadian entrainment (29 MEGs, ko04713), calcium signaling pathway (29 MEGs, ko04020), retrograde endocannabinoid signaling (26 MEGs, ko04723), dopaminergic synapse (26 MEGs, ko04728), amphetamine addiction (24 MEGs, koko05031), insulin secretion (23 MEGs, ko04911), oxytocin signaling pathway (23 MEGs, ko04921), cGMP-PKG signaling pathway (22 MEGs, ko04022), etc. (Fig. 4a). Most of the female-highly expressed unigenes (FEGs) were assigned to cell adhesion molecules (CAMs) (4 FEGs, ko04514), aminoacyl-tRNA biosynthesis (3 FEGs, ko00970), hepatitis C (3 FEGs, ko05160), leukocyte transendothelial migration (3 FEGs, ko04670), cell cycle (3 FEGs, ko04110), viral carcinogenesis (3 FEGs, ko05203), tight junction (3 FEGs, ko04530), and axon guidance (3 FEGs, ko04360) (Fig. 4b).

The cAMP signaling pathway is involved in some fundamental functions including body growth and spermatogenesis (Skålhegg et al. 2002). Gamma-aminobutyric acid (GABA) B receptor 1 (*GABBR1*), adenylate cyclase 5 (*ADCY5*), rap guanine nucleotide exchange factor 3 (*RAPGEF3*), calcium/calmodulin-dependent protein kinase 2 (*CAMK2*), peroxisome proliferator-activated receptor alpha (*PPARα*), and T

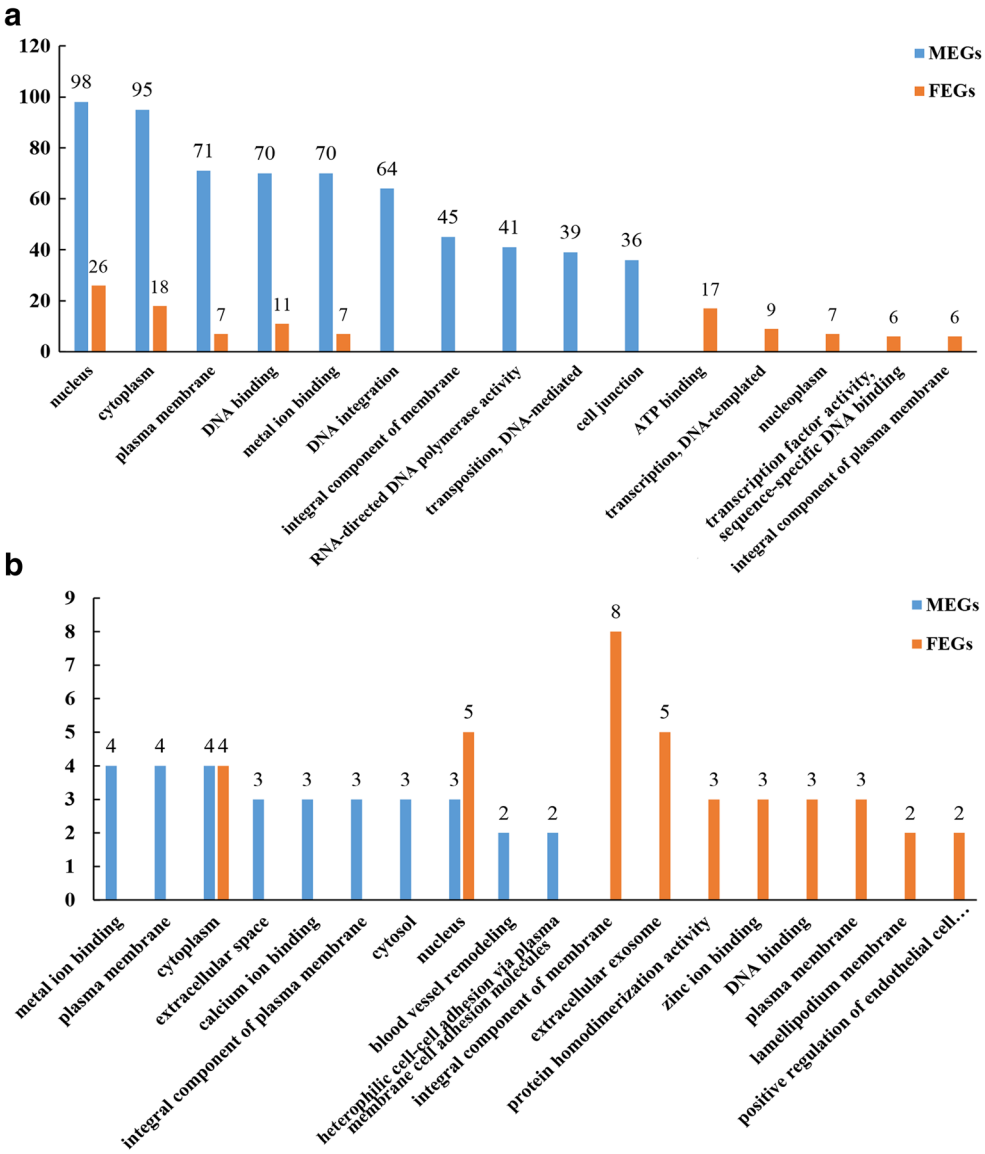

Fig. 3 Functional classification of male-highly expressed unigenes (MEGs) and female-highly expressed unigenes (FEGs) based on Gene Ontology (GO) terms. The *x*-axis is level 2 name of GO term, and the *y*-axis is the number of differently expressed unigenes. **a** The GO classification of male-highly expressed unigenes (MEGs) in the hypothalamus. **b** The GO classification of female-highly expressed unigenes (FEGs) in the pituitary

lymphoma invasion and metastasis-inducing protein 1 (*TIAM1*) were also identified as highly expressed in males (Fig. 5). Previous studies confirmed that the cAMP pathway is regulated by some glutamate receptors, which are essential in male development (Mao and Wang 2002; Winder and Conn 1993). In the glutamatergic synapse signal pathway, the glutamate receptors *GRIA1*, *GRIA2*, *GRIA3*, *GRIA4*, *GRIN1*, and *GRIN 2B* were expressed about 3.13-, 3.59-, 2.94-, 3.62-, 3.93-, and 3.16-fold higher in males compared with females (Fig. 6). Interestingly, the different expressions of *CACNA1D* between sexes were found in 17 KEGG pathways, *ADCY5* were concentrated in 13 pathways, and *PPP3C* were concentrated in 9 pathways, which were enriched in most of significant KEGG pathways that may play vital role in growth sexual dimorphism or sex differentiation of red-tail catfish.

In the pituitary, only 18 DEGs were annotated to 30 pathways, 9 DEGs were highly expressed in males, and 9 DEGs were highly expressed in females. For example, the anti-Mullerian hormone type 2 receptor (*AMHR2*) was only identified in males and was enriched in the cytokine-cytokine receptor interaction and TGF-beta signaling pathway. While cyclin B2 (*CCNB2*), progesterone receptor (*PGR*), peroxisomal biogenesis factor 11 alpha (*PAX11A*), and cytochrome P450 family 26 subfamily A (*CYP26A*) were enriched in oocyte meiosis, p53, peroxisome, and retinol metabolism signaling pathway with higher expression in females. The desert hedgehog (*DHH*) was enriched in hedgehog signaling pathway and only identified in males (Table 1).

qRT-PCR Confirmation for DEGs Between Sexes

To verify accuracy of the RNA-seq data, DEGs related to mainly enriched signal pathway were randomly chosen and validated by qRT-PCR. In the hypothalamus, we analyzed

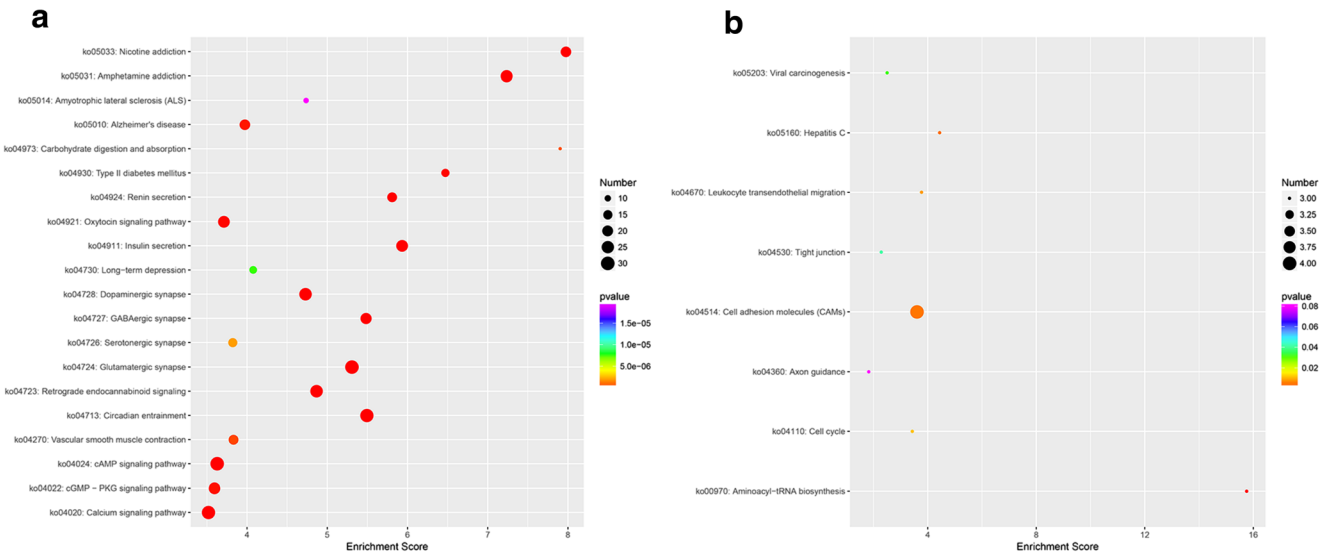

Fig. 4 TOP-enriched KEGG signal pathways in the hypothalamus. The *x*-axis represents KEGG enrichment score, the size of the dots represents numbers of DEGs, and the color of dots represent *p* value. **a** TOP-enriched KEGG signal pathways with male-highly expressed unigenes (MEGs). **b** TOP-enriched KEGG signal pathways with female-highly expressed unigenes (FEGs)

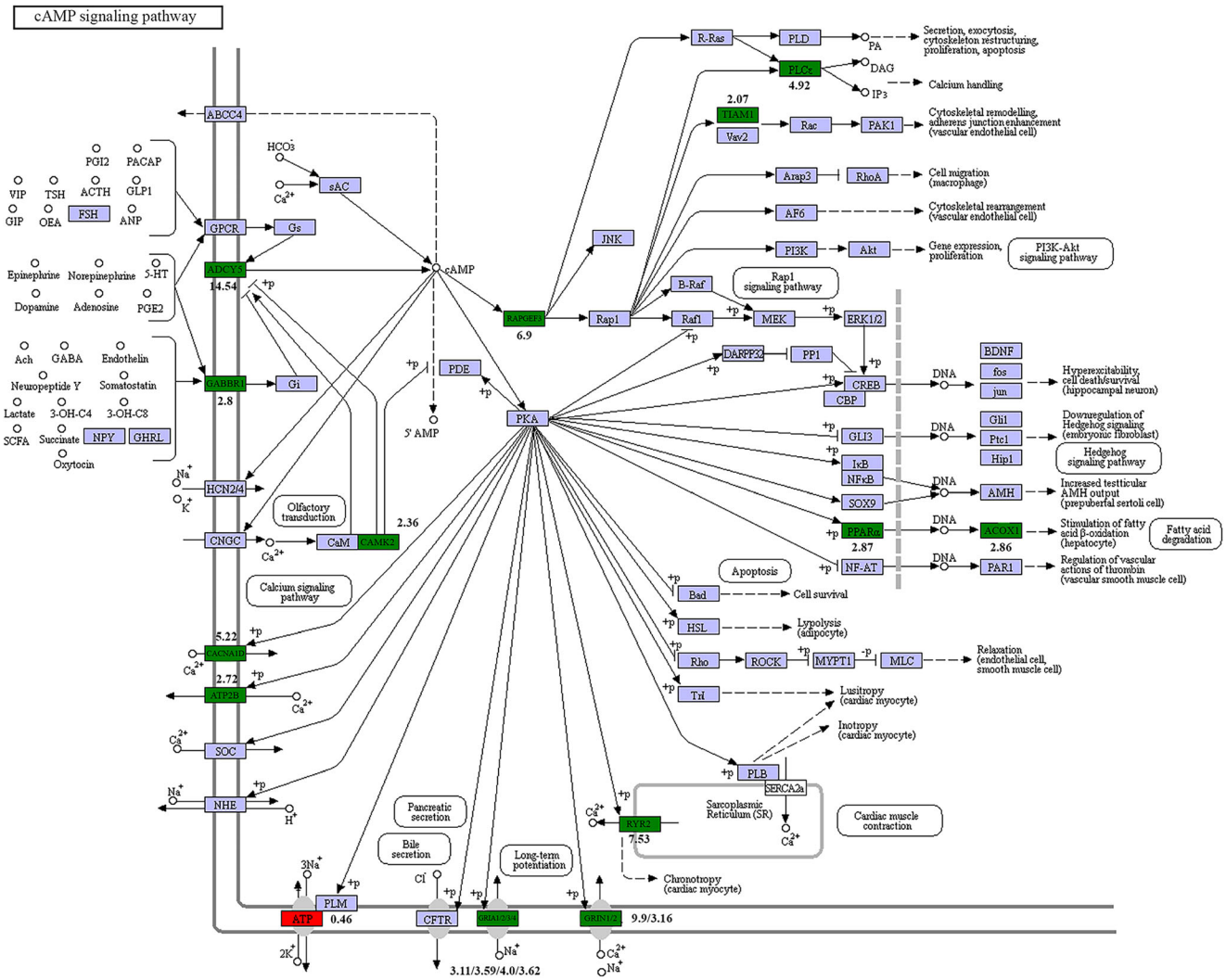

Fig. 5 DEGs involved in the cAMP signaling pathway. Male-highly expressed unigenes are represented in green and female-highly expressed unigenes are represented in red. The number is the value of fold change

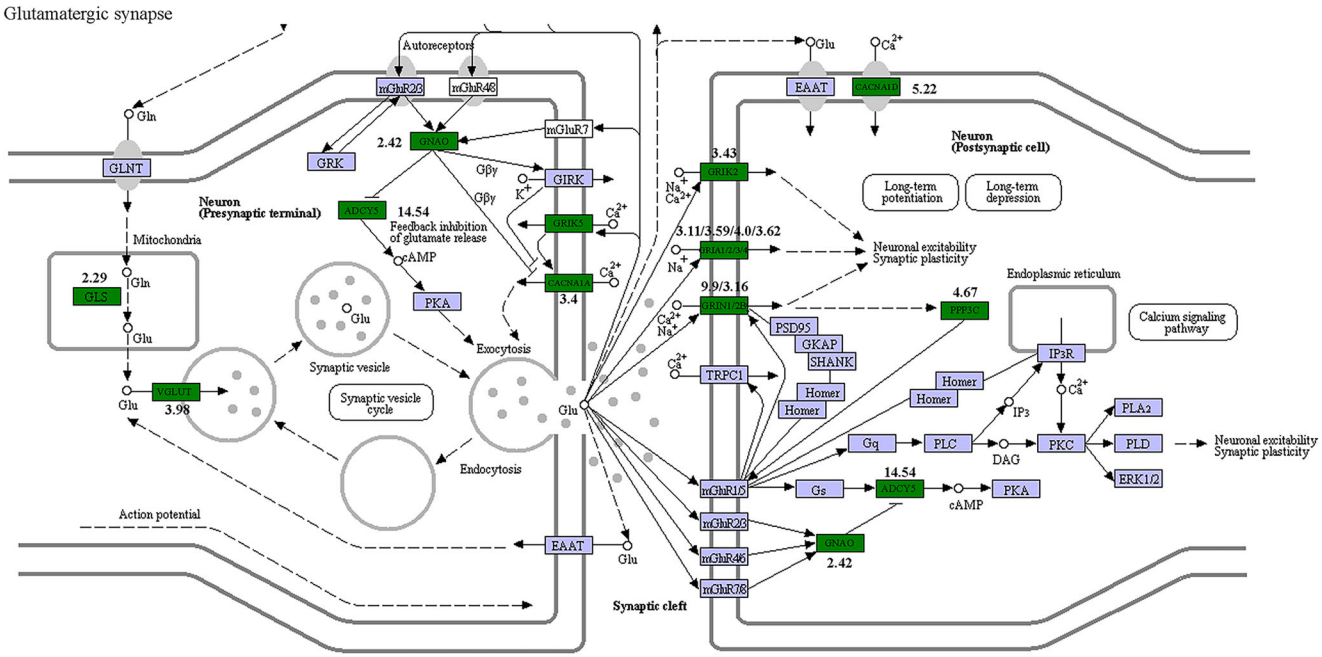

Fig. 6 DEGs involved in the glutamatergic synapse signal pathway. Male-highly expressed unigenes are represented in green and female-highly expressed unigenes are represented in red. The number is the value of fold change

fourteen DEGs, such as *CACNA1D*, *RAPGEF3*, *TIAM1*, *PPARα*, *CAMK2*, *PPP3C*, *GABBR1*, *ADCY5*, *GIRIA1*, *GIRIA2*, *GIRIA3*, *GIRIA4*, *GIRIN1*, and *GIRIN2B*, which were highly expressed in males (Fig. 7a), which was corresponded with expression data generated by qRT-PCR except *CAMK2* (Fig. 7b). In the pituitary, RNA-seq showed that four genes (*CYP26A*, *PEX11A*, *PGR*, *CCNB2*) were highly expressed in the female, while *DHH* and *Amhr2* were highly expressed in the male (Fig. 7c). Except for *CCNB2*, the qRT-PCR analysis confirmed the findings of the RNA-seq data (Fig. 7d). Linear regression analysis confirmed that the positive correlation between RPKM and qRT-PCR dependent unigenes expression persisted in both of the hypothalamus and pituitary with $R^2 = 0.87$ and 0.89, respectively (Supplementary Fig. 3C and D).

Discussion

The hypothalamic-pituitary system is the most vital system for regulating reproduction and associated behaviors, and it is conserved from jawless fishes to mammals (Sower et al. 2009). The HiSeq platform has been used to compare DEGs between sexes and provide precious data for studying the sexual differentiation and its mechanism (Lin et al. 2017; Wu et al. 2015). The red-tail catfish is a native and distinctive fish in the Lancang River and also shows sexual dimorphism, such that males have a faster growth rate than females. Therefore, we performed transcriptome sequencing to identify differentially expressed genes in the hypothalamic-pituitary system between males and females in red-tail catfish.

Reproduction of animals is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. In detail, if the hypothalamus is stimulated, gonadotropin-releasing hormone (GnRH) will be secreted to stimulate releasing of pituitary gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) (Macmanes et al. 2017; Sower et al. 2009). In this study, genes of *GnRH*, insulin-like growth factor I (*IGF1*), pituitary adenylate cyclase–activating polypeptide (*PACAP*), norepinephrine, neuropeptide Y (*NPY*), and kisspeptin (*KISS1/KISS1R*) were for the first time identified in the hypothalamus and pituitary of red-tail catfish. We found several key endocrine genes of GH/IGF axis highly expressed in males, coinciding with a higher level of *IGF1* in male yellow catfish (Ma et al. 2016), and whose deficiency leads to dwarfism (Liu and Leroith 1999; Meyer et al. 2004; Petkovic et al. 2007). In the present work, a higher expression of *KISS1R* was also identified in the hypothalamus of a male, and previous studies showed that *KISS1R* was highly expressed in testis (Lapatto et al. 2007; Tariq et al. 2013) and brain, which was crucial for the onset of puberty in both sexes and essential for testicular function (Filby et al. 2008; Mohamed et al. 2007; Navarro et al. 2004). In our study, a higher expression of *PACAP* and *GHRH* was also identified in males, which was identical with previous findings in the half-smooth tongue sole (Ji et al. 2011). We found that *LHβ* and *FSHβ* are mainly expressed in the pituitary of females, which are the most important gonadotropins and are associated with oocyte development (Hassin et al. 1999).

Table 1 Illumina expression for genes found to be enriched in KEGG term of the pituitary

Gene	RPKM		*p*	KEGG term
	Male	Female		
AMHR2	17.29	0	0.001	Cytokine-cytokine receptor interaction
				TGF-beta signaling pathway
CCNB2	5.73	46.07	0.041	Oocyte meiosis
				p53 signaling pathway
				Cell cycle
				FoxO signaling pathway
				Cellular senescence
PGR	0	9.54	0.032	Oocyte meiosis
PdxK	14.95	4.46	0.026	Vitamin B_6 metabolism
Gcnt3	0	29.64	0.036	Mucin type O-glycan biosynthesis
Odc1	0.63	41.68	0.0000023	Glutathione metabolism
				Arginine and proline metabolism
Ccl20	17.18	49.52	0.037	TNF signaling pathway
				Cytokine-cytokine receptor interaction
Myh	0	9.53	0.028	Tight junction
Taar	0	8.42	0.043	Neuroactive ligand-receptor interaction
Dchs1	11.97	0.63	0.042	Hippo signaling pathway—fly
				Hippo signaling pathway—multiple species
Mpdz	28.88	5.47	0.022	Hippo signaling pathway—fly
				Tight junction
Meth	15.18	1.55	0.045	Selenocompound metabolism
				One carbon pool by folate
				Cysteine and methionine metabolism
Afmid	118.78	45.42	0.003	Glyoxylate and dicarboxylate metabolism
				Tryptophan metabolism
Itga4	9.52	0.25	0.023	ECM-receptor interaction
				Cell adhesion molecules (CAMs)
				Regulation of actin cytoskeleton
				Focal adhesion
				PI3K-Akt signaling pathway
Ncf2	8.42	0	0.043	Phagosome
PAX11A	26.92	65.57	0.036	Peroxisome
CYP26A	50.11	103.83	0.026	Retinol metabolism
DHH	8.81	0	0.035	Hedgehog signaling pathway

It is very interesting to identify the DEGs, which are parts of specific signaling pathways that regulate sex differentiation and sexual size dimorphism. To this end, KEGG enrichment analysis was performed. In the hypothalamus, the top 20 signaling pathways were identified. The cAMP signaling pathway plays essential roles in postnatal body growth and spermatogenesis, as loss of cAMP-dependent protein kinases reduced *IGF1* mRNA in the liver and would lead to defective sperm motility (Skålhegg et al. 2002; Liu and Leroith 1999). *GRIAs* and *GRIN1* are important receptors of the glutamatergic synapse pathways. Defect of GRIAs leads to much smaller body size and reduced weight (Jia et al. 1996; Zamanillo et al. 1999). Moreover, *GRIA2/4* was found to be functional in motility of spermatozoa by regulating miR-141-3p (Wu et al. 2015). Interestingly, the voltage-dependent calcium channel L type alpha-1D (*CACNA1D*) was confirmed to be enriched in most of KEGGs (17 of 20 KEGGs). Functional analysis found that CACNA1D-knockout mice were smaller than their littermates, as *CACNA1D* defect leads to significant reduction of β-cell proliferation in islets (Namkung et al. 2001). *ATP2B*$^{-/-}$

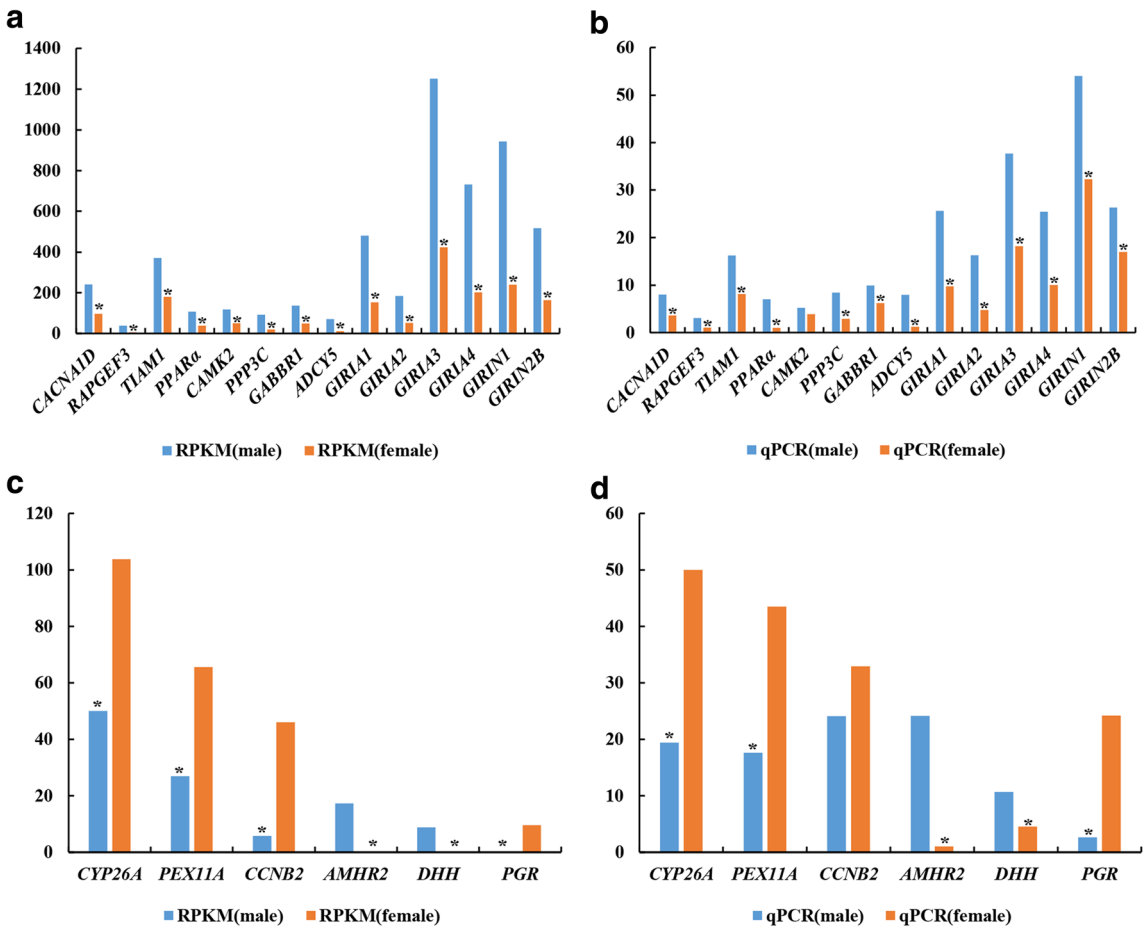

Fig. 7 Concordance of RNA-seq data (RPKM) with qRT-PCR relative expression. Asterisks represent significant difference ($p < 0.05$) in gene expression between sexes. **a** Normalized RPKM data of selected genes in the hypothalamus. **b** Normalized qRT-PCR data of selected genes in the hypothalamus. **c** Normalized RPKM data of selected genes in the pituitary. **d** Normalized qRT-PCR data of selected genes in the pituitary

mouse also showed a lower body weight (Ver et al. 2001). *ATP2B* and *CACNA1D* are members of the histocompatibility complex I (Seuánez et al. 1997), and we suspect that *CACNA1D* may interact with *ATP2B* and regulate growth.

In the hypophysis, the cytokine-cytokine receptor interaction and oocyte meiosis pathways are indispensable during ovary development and oogenesis, because loss of function of *REC8*, *AMHR2*, and *PGR* leads to female infertility (Bannister et al. 2004; Hernandez Gifford et al. 2009; Lydon et al. 1995; Zhu et al. 2015). In our enriched KEGGs, *PEX11A* and *DHH* were also identified. Previous studies demonstrated that a significantly lower PEX11 may lead to higher body weight (Weng et al. 2013), and DHH appears in pre-Sertoli cells, displays male-specific transcription, and is essential for spermatogenesis (Bitgood et al. 1996).

Taken together, our study provided a new window for revealing the sexually/differently expressed genes in the hypothalamic-pituitary system. Further work will be undertaken to explore some important genes in this work to reveal sex differentiation and growth dimorphism in red-tail catfish.

Materials and Method

Sample Collection and Growth Comparison Between Males and Females

All experimental procedures of this study were permitted by the Institutional Animal Care and Institute of Hydrobiology, Chinese Academy of Sciences. The red-tail catfish involved in this study was collected in Xishuangbanna, Yunnan Province, China. Fifty-five 4-year-old individuals including 27 males and 28 females from the same parent were randomly selected. Body weight and length were compared by *t* test between males and females. All fish was dissected, and sex was confirmed anatomically.

Libraries Construction and Sequencing

Tissues of the hypothalamus and pituitary were sampled from 3 adult male and female individuals, respectively, and the sex was confirmed by mature testis and ovary anatomically. Total RNA was extracted by Trizol method with manufacturer's protocol (Ambion), and Agilent 2100 Bioanalyzer (Agilent Technologies) was used to perform RNA integrity assessment. In each biological replicate, 50-μg total RNA of each tissue was used for RNA library construction. The procedures of RNA purification and synthesis of cDNA were performed by using TruSeq Stranded mRNA LTSample Prep Kit (Illumina). In detail, the mRNA was purified and concentrated by magnetic beads and then fragmented into short fragments to serve as templates for synthesizing the first strand cDNA. The double strand cDNA was synthesized and purified by agarose gel purification; after that, the cDNA fragments were coupled by sequencing adaptors at the 5′/3′ ends. The libraries of the hypothalamus and pituitary were sequenced with HiSeq 2000 platform. After wiping out the adaptor and low-quality bases, the clean paired-end reads were assembled in *Trinity.pl* script with the following parameters (-seqType fq -min_contig_length 200 -JM 400G -left $R1 -right $R2 -SS_lib_type RF -CPU 80) for each of tissue assemblies (Grabherr et al. 2011). The *Trinity_stats.pl* script was performed to obtain assembly metrics. Finally, unigenes were obtained by performing TGICL version 2.1 with default parameters (Pertea et al. 2003).

Analysis of Differentially Expressed Unigenes

To detect the differentially expressed unigenes (DEGs) between sexes in the hypothalamus and pituitary, DESeq package with the negative binomial distribution was performed to quantify the expression of two expression profiles. Fold change was performed to quantificate the differential expression and only DEGs with fold change ≥ 2, adjusted p value ≤ 0.05 (Wang et al. 2010). The false discovery rate test (adjusted p value) was applied to correct significant levels by eliminating random errors and fluctuations (Ott et al. 2012).

GO/KEGG Enrichment Analysis

The differentially expressed unigenes (DEGs) were blasted against database of NR, SWISSPROT, KOG, GO, and KEGG ($p < 0.05$). The statistical significance of the GO/KEGG enrichment was evaluated by the hypergeometric distribution testing, $p = 1-\sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$, in which N represents the number of unigenes with GO/KEGG annotation, n represents number of DEGs with GO/KEGG annotation, M represents number of unigenes with one specific GO/KEGG annotation, and m represents the number of DEGs with one specific functional annotation (Hassin et al. 1999). A smaller p value presented a more abundant enrichment.

Quantitative Real-Time PCR

For verifying the results of analysis of DEGs, total RNA of the hypothalamus and pituitary from both sexes was reverse-transcribed by using the PrimeScriptRT reagent Kit (Takara) following the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) reaction was performed using a Roche LightCycler 480 instrument with SYBR Green PCR master mix (Roche). Five biological replicates were performed in each reaction. The expression of β-actin was used as reference to normalize the Ct values to conduct the $2^{-\Delta\Delta Ct}$ method (Nolan et al. 2006). The differential expression analysis was confirmed by ANOVA analysis (Anderson 2010). The sequences of primers involved in this study were Supplementary Table 4.

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Compliance with Ethical Standards

All experiments and animal treatments were approved by the Institute of Hydrobiology Institutional Animal Care and Use Committee (approval ID, keshuizhuan 0829).

Conflict of Interest The authors declare that they have no competing interests.

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