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Transcriptomics-based analysis of neurotoxic and reproductive effects in turbot (*Scophthalmus maximus*) after exposure to tris (2-chloroethyl) phosphate (TCEP)

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Abstract

Background Tris (2-chloroethyl) phosphate (TCEP), a widely used flame retardant, is widespread in the environment and potentially harmful to organisms. However, the specific mechanisms of TCEP-induced neurological and reproductive toxicity in fish are largely unknown. Turbot (*Scophthalmus maximus*) is cultivated on a large scale, and the emergence of pollutants with endocrine disrupting effects seriously affects its economic benefits. This study aimed to investigate the toxic effects of TCEP on turbot by integrating physio-biochemical and transcriptomic analyses.

Results TCEP exposure induced severe neuroendocrine disrupting effects in turbot. Firstly, the hormone levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), and 11-ketotestosterone (11-KT) were significantly decreased under prolonged TCEP stress, which may have a negative impact on normal reproductive function. We identified and summarized representative differentially expressed genes (DEGs) and their functions, such as endocrine system and oxidative stress. Pathway enrichment showed that the toxicological characteristics of TCEP on turbot were neuroendocrine regulation disorders, including oxidative phosphorylation, apoptosis, steroid biosynthesis, GnRH signaling pathway and so on. Weighted gene co-expression network analysis (WGCNA) also revealed key genes involved in these pathways. Among these genes, those encoding the components of the electron transport chain presented an initial increase in expression followed by a decrease, indicating that TCEP stress might affect mitochondrial function and lead to cell damage. This finding was also supported by the upregulation of apoptosis-related gene expression. Moreover, acute exposure to TCEP regulated MAPK-mediated transduction and regulation of GnRH signaling, thereby altering the expression of hypothalamic-pituitary-gonadal (HPG) axis-related genes.

Conclusions These findings revealed the endocrine disrupting effects of TCEP on turbot and identified biomarkers related to reproductive toxicity, providing early warning for the monitoring of healthy aquaculture.

Keywords Turbot, Tris (2-chloroethyl) phosphate (TCEP), Transcriptomics, Reproductive toxicity

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Introduction

Due to their adverse health effects, polybrominated diphenyl ethers (PBDEs) have been gradually banned in many countries, and organophosphorus flame retardants (OPFRs) have become the main substitutes [1]. In recent years, the global production of OPFRs has increased rapidly, reaching an annual output value of 3×10^6 tons in 2018, 10 times more than in 2011 [2]. As one of the most representative OPFRs, TCEP has been widely used in various industries, such as furniture, electronics, and building materials [3]. TCEP is added to the material through physical mixing and has a high water solubility (25 °C, 7820 mg/L) and a low octanol-water partition coefficient (log Kow = 1.47), so high concentrations of TCEP have been frequently detected in various environmental media [4]. During an investigation in 2023, a variety of organophosphate esters (OPEs) were detected in China's Bohai Sea, with TCEP concentrations ranging from 13.75 to 1721.29 ng/L [5]. The highest concentration of TCEP (87.4 mg/L) was recorded in raw water from a Japanese sea-based wastewater disposal site [6]. TCEP tends to occur with bioaccumulation and cascade amplification effects in the food chain [7]. TCEP concentrations were detected in shrimp and crabs collected from aquafarms of the Beibu Gulf, China, with values ranging from 21.3 to 138 ng/g dw (mean 55 ng/g) [8]. These data indicated that TCEP has become a pervasive emerging pollutant in the environment that may impact the health of aquatic organisms.

In recent years, several studies have shown that TCEP exposure may result in neurotoxicity, developmental toxicity, reproductive toxicity, and even carcinogenesis in organisms [9, 10]. For example, TCEP exposure significantly downregulated key genes and protein markers related to neurodevelopment in zebrafish [11]. After 30 days of TCEP exposure, the survival rate, body weight, and specific growth rate of juvenile yellow catfish were found to be diminished [12]. TCEP has also been shown to be an endocrine disrupting chemical [13]. TCEP exposure decreased testosterone (T) levels in mice, and the levels of genes involved in T synthesis were similarly downregulated [14]. Notably, the specific mechanisms of the neurotoxicity and reproductive toxicity of TCEP to fish and other aquatic organisms are still poorly understood.

Turbot (*Scophthalmus maximus*) is a commercially significant aquaculture species in China. It is a highly nutritious fish that has been successfully farmed and bred in China since its introduction in 1992. By the end of 2019, the total cultivation area of turbot in China's system demonstration zones was 6.0652 million m^3. In Europe, Spain is the main region for farmed turbot, with 7,450 tons produced in 2018 [15]. Owing to the accumulation of pollutants in the seawater, the turbot aquaculture industry is seriously threatened. Precocious sexual maturation in fish can increase disease susceptibility and health problems, in addition, gonadal development and maturation consume a considerable amount of energy, thereby reducing the overall growth rate and final body weight of fish and, ultimately leading to decreased production [16]. For instance, Atlantic halibut males grow relatively slowly due to precocious sexual maturation [17]. Precocious European sea bass grew up to 18% less in weight than their counterparts during their second annual cycle of life [18]. However, the impact of new pollutants, including TCEP, on turbot culture has not been fully recognized in recent years.

In teleost fish, the reproductive endocrine system is regulated by the hypothalamic-pituitary-gonadal (HPG) axis [19]. The hypothalamus governs the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by secreting gonadotropin-releasing hormone (GnRH), thus controlling gonadal development and the secretion of sex hormones, estradiol (E2) and 11-ketotestosterone (11-KT) [20]. LH and FSH are key regulators of the reproductive axis. They stimulate the gonads to produce a variety of sex steroid hormones at different stages, regulating reproductive functions [21]. In addition, kisspeptin, an essential reproductive regulator encoded by the *KISS* gene, regulates the secretion of sex hormones and the sexual maturation process [22].

Transcriptomics, as a powerful tool for understanding the mechanism of toxicants, has been extensively employed to study gene expression and molecular pathways in aquatic stress responses [23]. In the present study, turbot were exposed to TCEP for transcriptomic analysis of whole brain samples, and changes in plasma sex steroids (LH, FSH, E2, and 11-KT) were measured, which aimed to reveal the potential mechanism of the neural and reproductive toxicity of TCEP exposure to turbot through transcriptomics.

Materials and methods

Chemicals and experimental fish domestication

Tri (2-chloroethyl) phosphate (TCEP, CAS: 115-96-8; 99% purity) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. All reagents used in the exposure experiments were of analytical grade.

Turbot was purchased from Shandong Kehe Marine High Technology Co., LTD. (Weihai, China), which is the standard species with a consistent genetic profile, including 4-month-old juvenile turbot (body length 17 ± 2 cm, weight 120 ± 5 g) in the early stage of sex differentiation and adult female turbot (body length 35 ± 1 cm, weight 1000 ± 200 g). Before the experiment, the fish were acclimatized to laboratory conditions for a period of 2 weeks in water tanks (72.2 cm × 52.5 cm × 43.3 cm, 110 L) with temperature 16 ± 2 °C, salinity 34.2 ± 0.2, pH 7.8 ± 0.2,

dissolved oxygen 7.5 ± 0.2 mg/L, and light cycle 14 L/10D (light/dark). Additionally, the fish were fed once a day at 15:00 with commercial feed provided by the farm at 3% of their body weight (i.e., 3.6 ± 0.15 g), and the seawater was entirely replaced every day.

TCEP exposure and sample collection

Based on previous laboratory studies, the half-lethal concentration (LC50) of TCEP for 24 h treatment was 190.76 mg/L in juvenile turbots, and the stress response of turbot was more significant at 1/4LC50 treatment [24]. In addition, treatment concentrations exceeding environmental regulations were used in acute stress experiments to produce strong stress responses in organisms and to ensure that the results were meaningful. Therefore, the turbot were exposed to TCEP at an acute toxicity concentration of $1/4LC_{50}$ (47.69 mg/L) for 2 h, 6 h, 12 h, and 24 h as the experimental groups. while fish in clean seawater was used as the control group. To mitigate the influence of circadian rhythm, each group was sampled at 9:00 am. Fig. S1 showed the experimental period, TCEP stress initiation time and sampling time of each group after domestication, which were represented by rectangles, triangles and pentagons respectively. Thirty individuals were included in each group, and three replicates were conducted for all groups. For each time endpoint, brain samples were collected from 3 individuals randomly selected from each replicate after anesthesia with a lethal dose of MS-222 (100 mg/L), and then frozen in liquid nitrogen for 10 min and stored at − 80 °C. These tissue samples were collected for transcriptome analysis. All of the turbot collection and the anatomy experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

In addition, 1-year-old turbots were exposed to 1.91 mg/L of TCEP. The experimental groups were treated with 1 d, 5 d, and 10 d, while the control group fish were kept in clean seawater (Fig. S1B). Each group had three replicates with five fish per replicate. The experimental conditions were consistent with those during domestication except for TCEP concentration. During this period, the fish were fed commercial feed once daily at 15:00, totaling 3% of their body weight. Feeding was stopped 24 h before sampling. Blood samples were collected from the caudal vein via heparinized microcapillary tubes. Blood samples of three individuals per replicate were pooled as one sample and centrifuged at 4000 *rpm* for 20 min at 4 °C to separate supernatants for hormone quantification.

Hormonal measurement

The levels of FSH, LH, E2, and 11-KT in the fish were determined using the corresponding commercial enzyme-linked immunosorbent assay (ELISA) kits (JL17630, L13402, JL46284, and JL17632, respectively; Shanghai Jianglai Biotechnology Co., Ltd., China) according to the instructions. Serum from the control and TCEP exposure groups was used for testing and each experiment was replicated three times.

RNA extraction and transcriptome analysis

All tissue samples for testing were packed in dry ice and sent to Novogene Biotech Co., Ltd. (Beijing, China). Total RNA was extracted from brain tissues of each group using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The integrity and concentration of RNA were determined by the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The NEBNext® Ultra™ RNA Library Prep Kit for Illumina® was used to prepare cDNA library. After library qualification, sequencing was performed on the Illumina NovaSeq 6000 (Illumina, USA), generating 150 bp paired-end reads.

After reads containing adapters, reads containing N bases, and low-quality reads were filtered out, the clean reads were obtained, which were mapped to the reference genome (GCF_022379125.1) obtained from NCBI for comparison using HISAT2 v2.0.5 [25]. The FPKM (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced) of each gene was used to assess the level of gene expression [26]. Differentially expression analysis was performed using the DESeq2 R package v1.10.1 [27]. Differentially expressed genes (DEGs) were screened with a threshold of adjusted P value < 0.05. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis for DEGs were performed using Cluster Profiler (3.8.1).

Weighted gene co-expression network analysis (WGCNA)

After filtering genes, the expression values of the screened genes (FPKM > 1) were used to construct co-expression modules comprising 15 transcriptomes. The biological modules were classified according to the expression levels of genes and the similarity of module eigengenes (ME). Pearson correlation coefficients of relationships between the module and the sample trait were calculated according to the ME value of the module. Modules with Pearson correlation coefficients > 0.5 and $P < 0.05$ were considered to be associated with TCEP exposure, and GO and KEGG analyses were performed to clarify the biological functions of these response modules [28].

Reproductive related gene analysis

The total RNA used in qRT-PCR assays was consistent with that for transcriptome sequencing. And reverse transcription was performed using PrimeScript RT®

reagent kit with gDNA Eraser (Takara, China), and then test the expression of target genes (*Gt*, *GnRH*, *FSH*, *LH*, *KISS*, and *KISSr*) using qPCR SYBR Green Master Mix kit (Takara, Japan) in a LightCycler® 96 Instrument (Roche, USA) with the 20-μl reaction system including 10 μl PCR Premix (SYBR), 0.5 μl PCR forward primer, 0.5 μl PCR reverse primer, 2.0 μl cDNA, and 7.0 μl dH_2O. Specific primers and annealing temperature were shown in Table S1. The templates were amplified in triplicate with 35 cycles of 95 ℃ for 10 s, annealing for 30 s, and 72 ℃ for 30 s. As a reference gene, the β-actin was used to normalize the cycle threshold (Ct) value. To ensure the accuracy and reliability of the results, standard samples of different concentrations were used to draw standard curves. By comparing with the Ct value of the sample, the relative expression of the target gene was calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method [29].

Statistical data analysis

Statistical analysis and visualization were performed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). The mean ± standard deviation (SD) was used to express experimental data. Hormone levels and relative gene expression at different time points were examined using one-way analysis of variance (ANOVA), after evaluating the normality and homogeneity of variance. Comparisons between the TCEP group and the control group were specifically assessed using the Dunnett test to evaluate significant differences. The significance levels were denoted by asterisks (*) ($*P<0.05$, $**P<0.01$).

Results

Effect of TCEP on reproductive hormone content

The results showed that with increasing TCEP exposure time, the hormonal contents of 1-year-old turbot plasma changed (Fig. 1). Compared to the control, the contents of FSH, LH, E2, and 11-KT showed a decreasing trend. After 10 days, the lowest values occurred with a decrease of 3–7%. The decrease in 11-kT was the smallest. Therefore, continuous exposure to low concentrations of TCEP caused a gradual decrease in reproductive hormones in turbot.

Library construction and sequencing analysis

We evaluated the quality of transcriptome sequencing data from 15 samples (Table S2). After quality trimming, 653,379,440 clean reads were generated from 683,482,810 raw reads, with an error rate of less than 0.01%. The Q20 and Q30 values of each sample were no less than 98.39% and 95.66%, respectively, and the GC content ranged from 46.11 to 50.63%. A comparison of the clean reads with the reference genome revealed that the concordance rate of each sample was greater than 83.95% (Table S3). These date suggested that the transcriptome data were of high quality.

Identification and analysis of DEGs

We further evaluated the overall gene expression profile by examining DEGs associated with TCEP exposure in whole brain tissue of turbot. There were 2,128 (1,075 up- and 1,053 down-regulated), 3,307 (1,599 up- and 1,708 down-regulated), 2,069 (1,014 up- and 1,055 down-regulated), and 3,571 (1,819 up- and 1,752 down-regulated) DEGs in the N_2h vs. N_Ctrl, N_6h vs. N_Ctrl, N_12h vs. N_Ctrl, and N_24h vs. N_Ctrl groups, respectively (Fig. 2A), which were also illustrated in volcano plots (Fig. 2E-H). Venn diagram showed that 1157 DEGs were shared among the four TCEP-exposed groups (Fig. 2B). The square of Pearson's correlation coefficient (R2) exceeded 0.961, indicating high similarity in expression patterns between samples (Fig. 2C). Principal component analysis (PCA) plot revealed complex sample composition relationships for two eigenvalues (PC 1:30.13%, PC 2:11.73%) (Fig. 2D). The above analysis indicated that a large number of genes responsded to TCEP stress.

GO functional enrichment analysis was conducted on the DEGs obtained from different stress groups. Thirty enriched GO terms were displayed, including 10 items each for biological process (BP), cellular component

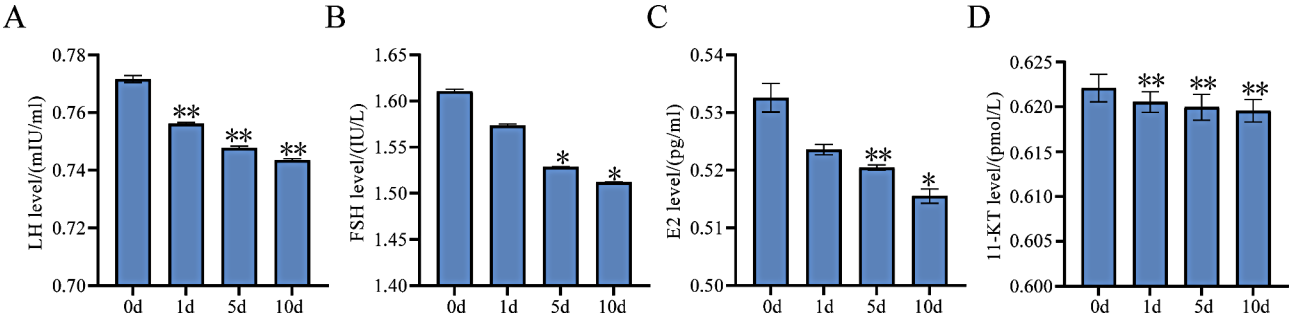

Fig. 1 Changes in reproductive hormones levels after exposure to TCEP (1.91 mg/L) exposure for 1, 5, and 10 days. **(A-D)** The levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), and 11-ketone testosterone (11-KT). Values represent the mean ± standard deviation ($n=3$). Asterisks denote significant differences between TCEP and control ($*p<0.05$, $**p<0.01$)

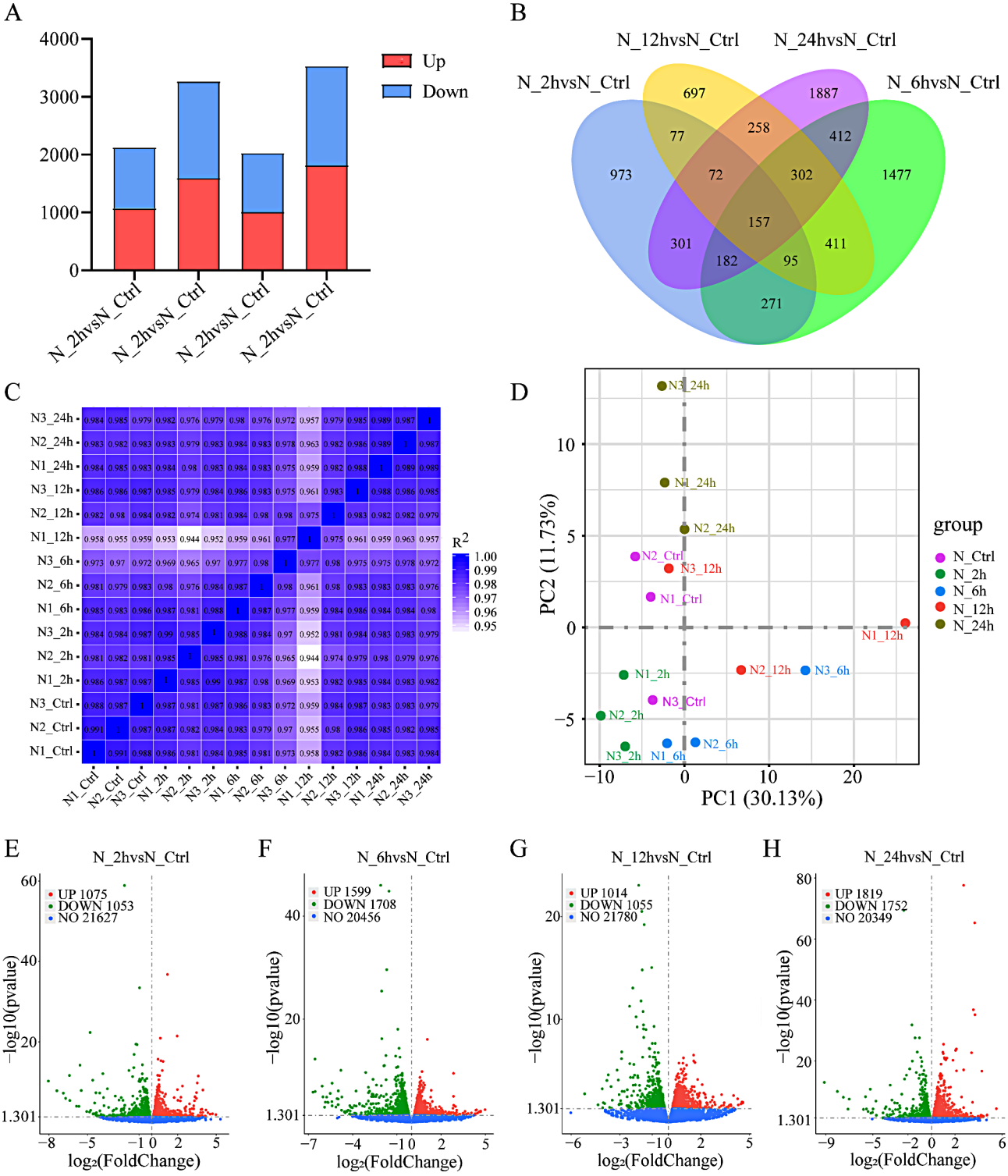

Fig. 2 Transcriptome analysis of turbot exposed to nitrate. **(A)** DEGs between CK and different treatment groups. **(B)** Venn diagram showed the number of shared and unique DEGs between different groups. **(C)** Cluster heat map analysis. **(D)** Principal component analysis. **(E-H)** represented volcano plots of the DEGs between Ctrl and different treatment groups (N_2h, N_6h, N_12h, and N_24h)

(CC), and molecular function (MF) (Fig. S2). For example, cell signal transduction, protein biosynthesis and folding, nucleic acid metabolism, and organelle function regulation were enriched in BP, extracellular structure and space, cell membrane-related components, and organelles in CC, and gene transcription regulation, protein binding and catalytic activity, ion transport, and biomolecular binding and catalytic activity in MF.

To gain a better understanding of the cellular metabolic pathways involved in DEGs, the top 20 enriched KEGG pathways with the most abundant DEGs for four exposure groups were displayed (Fig. 3A-D). In addition to some metabolism-related pathways, "Oxidative stress, oxidative damage and apoptosis" and "Signal transduction" were common enrichment pathways, suggesting that acute exposure to TCEP could initially induce the responses of these pathways in turbot. In addition, the metabolic pathways associated with the endocrine system and oxidative stress were further investigated in this study (Table 1).

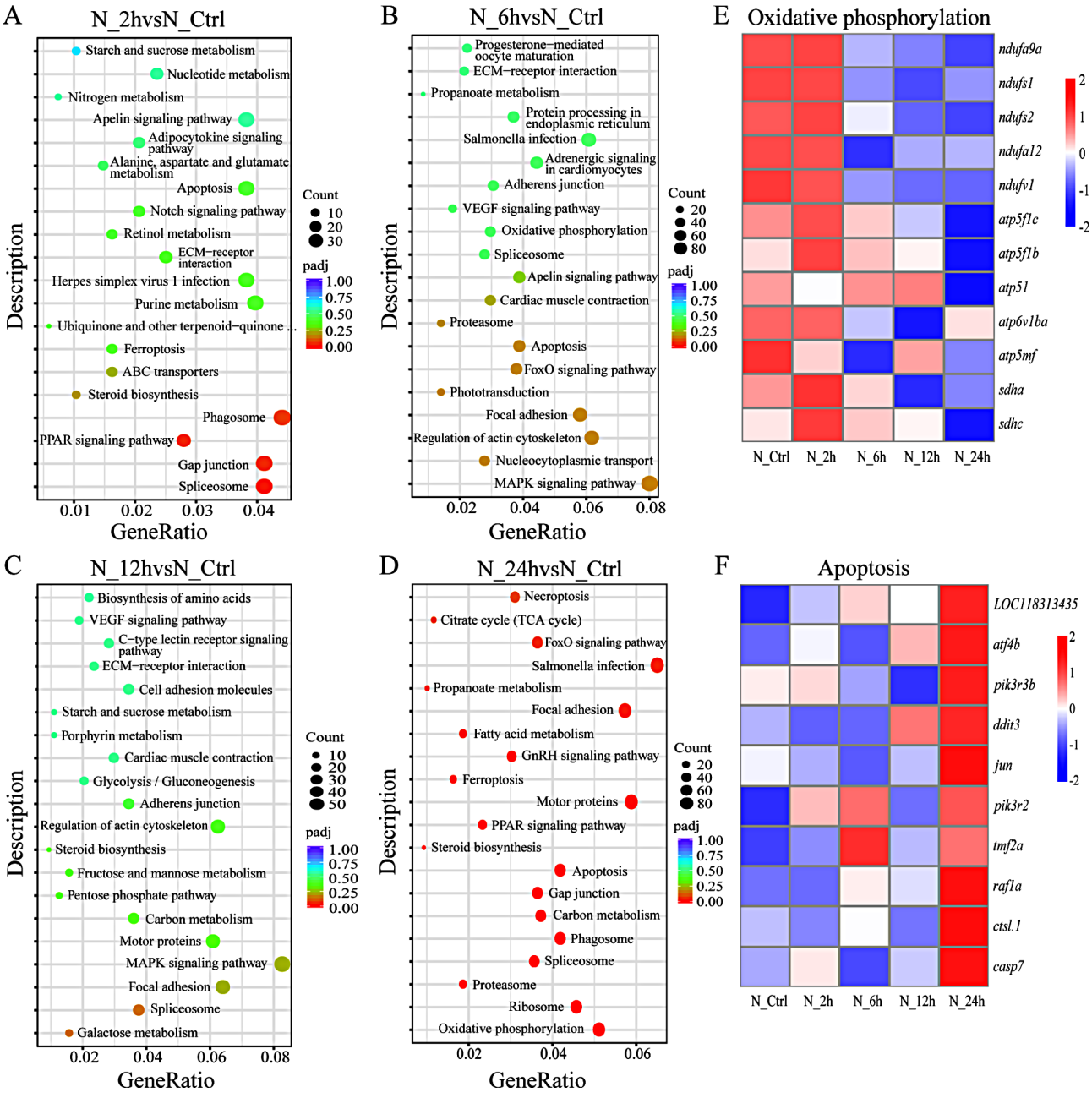

Fig. 3 Enrichment analysis of differentially expressed genes (DEGs) in KEGG pathways. (**A-D**) The top 20 pathways after 2, 6, 12, and 24 h of acute TCEP exposure, respectively. **(E, F)** Heat maps of genes related to oxidative phosphorylation and apoptosis pathway

Table 1 Enrichment profiles of pathways related to the endocrine system and oxidative stress under TCEP exposure

	Pathway	N_2h		N_6h		N_12h		N_24h	
		p value	Ratio (up-regulated DEGs/total DEGs)	p value	Ratio (up-regulated DEGs/total DEGs)	p value	Ratio (up-regulated DEGs/total DEGs)	p value	Ratio (up-regulated DEGs/total DEGs)
Endocrine system	GnRH signaling pathway	0.304	8/15	0.168	21/25	0.226	8/15	0.001	22/39
	Steroid biosynthesis	0.007	3/7	0.533	0/4	0.019	1/6	4.7E-4	1/12
	PPAR signaling pathway	0.001	13/19	0.559	5/14	0.305	3/10	5.49E-4	11/30
	Notch signaling pathway	0.036	7/14	0.995	3/6	0.385	6/9	0.630	9/15
	Adipocytokine signaling pathway	0.055	4/14	0.173	5/18	0.425	1/9	0.036	10/24
Oxidative stress	Oxidative phosphorylation	0.323	14/17	0.042	14/32	0.999	2/5	5.51E-13	1/66
	Ferroptosis	0.024	4/11	0.112	6/13	0.162	4/8	0.001	7/21
	Apoptosis	0.038	13/26	0.008	17/42	0.884	3/13	2.85E-4	21/54
	Phagosome	0.002	19/30	0.443	9/29	0.277	4/19	9.6E-5	13/54
	MAPK signaling pathway	0.133	25/47	0.001	52/87	0.006	19/53	0.230	52/82
	FoxO signaling pathway	0.387	8/19	0.007	24/41	0.359	6/18	0.006	27/47

WGCNA analysis under TCEP exposure

WGCNA was performed to identify gene co-expression networks responding to TCEP stress. Based on 14,543 genes with FPKM > 1, a weighted gene co-expression network was constructed with an empirical soft threshold of 18 and an average connectivity of 36 (Fig. S3A, B). On the basis of the similarity of the gene expression levels, a gene clustering tree was constructed, and 17 different co-expression modules were obtained (Fig. 4A). We used module eigengenes (MEs) to represent the expression level of genes in each module, and analyzed the interactions between these modules (Fig. 4B). The results of the module-trait relationship indicated that six modules were significantly associated with TCEP exposure traits, including purple and green corresponding to TCEP for 2 h, turquoise, magenta, and tan corresponding to TCEP for 12 h, and midnight blue corresponding to TCEP for 24 h. The ME. pink module had the strongest correlation with the Ctrl group (Fig. 4C). The top 20 DEGs with weight values in the seven modules were shown in Table S4. DEGs in the above seven modules were subjected to KEGG enrichment analysis (Fig. 4D), and the enrichment results were consistent with those of four TCEP-treated groups.

Expression of genes associated with reproduction

The changes in the expression of reproductive-related genes in turbot after TCEP exposure were shown in Fig. 5. With prolonged TCEP stress, the expression levels of *Gt* and *GnRH* genes encoding gonadotropin-releasing hormone generally increased and then decreased. At 6 h, the response was 50 times and 150 times that of the control group, respectively. The highest levels of *LH* and *FSH* also occurred in the 6 h treatment groups. The expression of *FSH* gene responding to TCEP was lower than that of *LH*. The expression of *KISS* and *KISSr* in the treatment groups changed significantly, and the expression of *KISS* decreased significantly at 12 h and 24 h.

Discussion

TCEP is an environmental endocrine disruptor, and due to its widespread presence in the natural environment, an increasing number of studies have evaluated its toxic effects. However, limited attention has been paid to its neurological and reproductive effects on aquatic organisms. With the increasing use of TCEP, it has been frequently detected in water environments because of its high water solubility [30, 31]. Therefore, TCEP pollution and its toxic effects on aquatic organisms cannot be overlooked. According to the physical properties of TCEP and the results of previous experiments, $1/4LC_{50}$ (49.67 mg/L) TCEP was used as the stress condition. Through high-throughput sequencing technology and WGCNA, 2069–3571 DEGs (relatively more DEGs in the N_6h and N_24h groups) and seven modules related to TCEP stress in turbot brains were screened to investigate the molecular mechanism of the turbot response to TCEP exposure.

Effects of TCEP exposure on neurotoxicity

Oxidative stress is the most common mechanism by which aquatic organisms respond to various environmental pollutants [32]. TCEP exposure can lead to the generation of reactive oxygen species (ROS), thereby causing an imbalance in oxidation-antioxidant processes and undermining the cellular structure and function [33, 34]. Life-cycle exposure to TCEP activated the *Nrf2-Keap1* antioxidant pathway in response to TCEP-induced oxidative stress in zebrafish gills [35]. KEGG analysis showed that several significantly enriched pathways were associated with neuropathy, particularly oxidative phosphorylation pathway, and similarly, GO term also revealed that

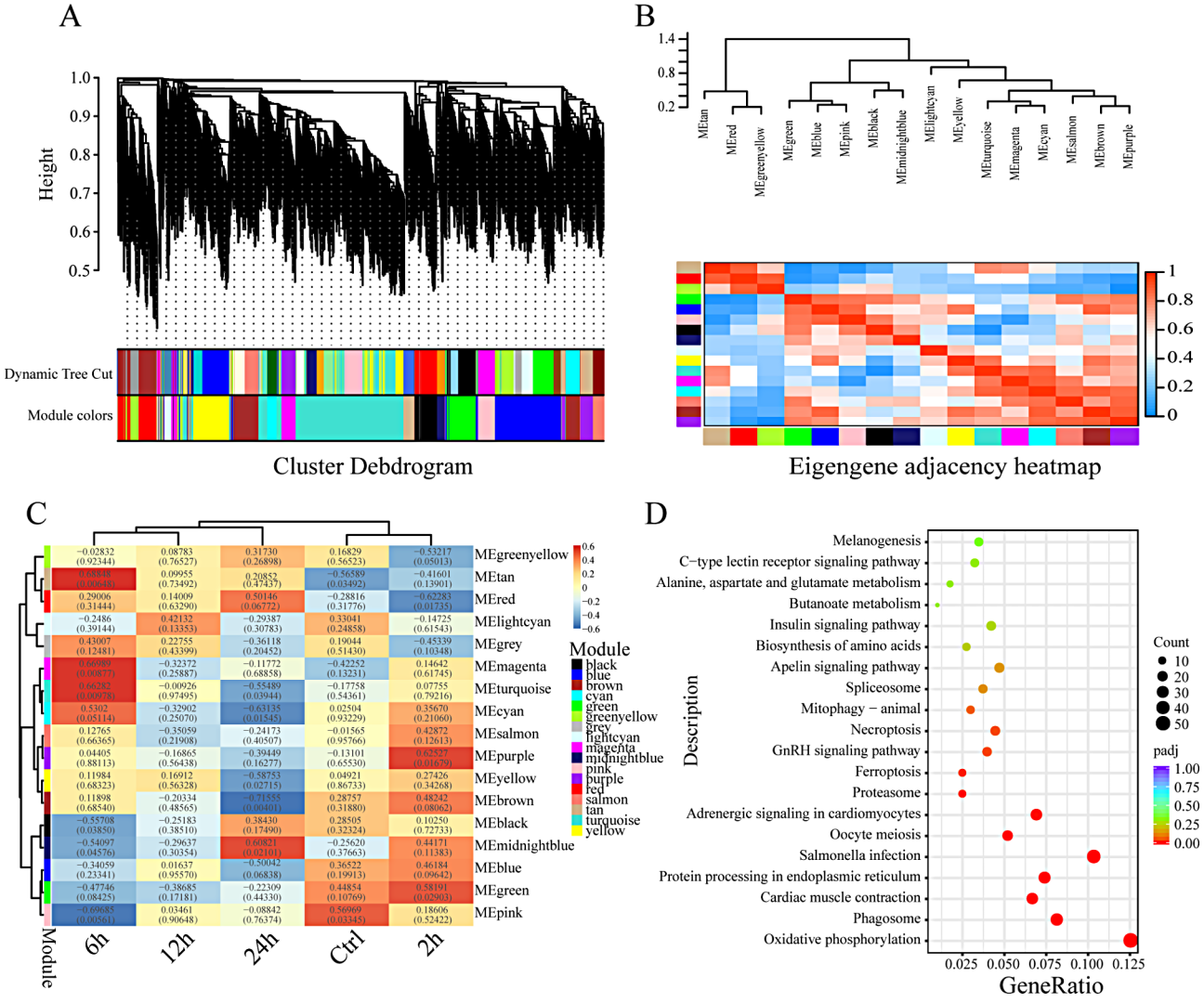

Fig. 4 Weighted gene co-expression network analysis (WGCNA) of acute TCEP stress. (**A**) The hierarchical clustering tree represents the co-expressed modules determined by WGCNA. (**B**) Heatmap of eigengene adjacency. (**C**) Heatmap of correlation between traits and modules. (**D**) Enrichment pathway of seven modules significantly associated with TCEP-exposure traits

TCEP affected cell signaling and redox processes in mitochondria, which were critical for mitochondrial function and neurodevelopment. Oxidative phosphorylation normally occurs in the mitochondria via the respiratory chain and ATP synthase, and it is one of the major cellular pathways used to generate adenosine triphosphate (ATP). The expression of genes encoding mitochondrial electron transport chain components, including NADH dehydrogenase (complex I), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V), was altered. *Nduf* encodes multiple subunits of complex I, a 45-subunit enzyme complex that uses non-covalently bound prosthetic groups to facilitate the initial step of the electron transport chain [36]. *atp5f1c*, *atp5f1d*, *atp5l*, and *atp5mf* genes encode subunits of complex V, which work together to synthesize ATP at the inner mitochondrial membrane to provide energy required for the cell [37]. The downregulation of related genes led to impaired function of the electron transport chain, eventually resulting in neuronal damage, cell apoptosis, and even reproductive toxicity [38]. Multiple studies have shown that exposure to certain refractory and readily accumulating pollutants during the critical developmental period of the brain may permanently affect some signaling pathways in the nervous system [39]. Our findings indicated that impaired mitochondrial function with TCEP exposure may result in an inability to meet the high energy demand of brain neurons, potentially leading to impaired brain cell function or cell death. Apoptosis is an important biological mechanism for the body's resistance to pathogen infection or environmental stress, which helps eliminate damaged

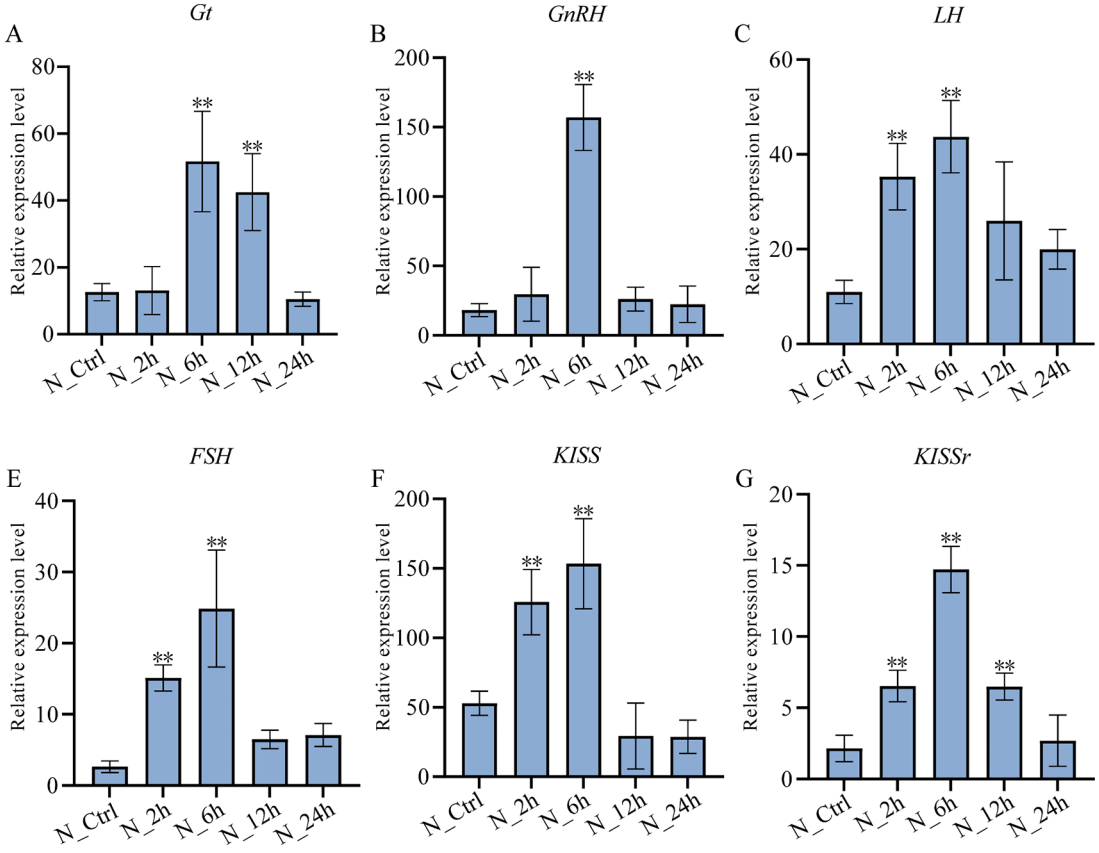

Fig. 5 (A-F) The relative expression levels of reproductive genes (*Gt*, *GnRH*, *LH*, *FSH*, *KISS*, and *KISSr*). * represents $p < 0.05$ and ** represent $p < 0.01$, indicating significant differences between the exposure groups and the control group

cells and maintains tissue homeostasis [40]. In this study, several genes associated with apoptosis, such as *c-Jun*, *caspase-7*, and *ctsl.1*, showed significant expression after TCEP stress. When an apoptotic signal is received from a transmembrane receptor, the extrinsic pathway is activated, which leads to the cascade activation of *caspase-7* [41]. *Caspase-7* is involved in the execution phase of apoptosis, which can be induced by activating other apoptosis-related proteins, such as nucleases. *ATF4B* is a transcription factor that can influence the activation and execution of the apoptotic pathway, thereby promoting cell death. In addition, the expression of *c-Jun* was upregulated, which also promotes cell apoptosis [42]. The expression levels of pro-apoptotic genes were increased, thus inducing apoptosis. This inference is consistent with the adverse effects of TCEP on neurodevelopment [11]. We concluded that after TCEP stress, the energy metabolism of fish brain cells was blocked and accompanied by apoptosis, which may affect the nervous system and further affect the regulation of reproduction.

Effects of TCEP exposure on reproductive toxicity

Toxicity of TCEP on gonadotropins and sex hormones

The hypothalamic-pituitary-gonadal axis is a complex endocrine system that plays a key role in the development and regulation of the reproductive system in fish and other animals. The main regulators of the HPG axis are several hormones, such as GnRH, LH, FSH, E2,11-KT, and their specific receptors [43]. During the gonadal development cycle, the GnRH level changes continuously. The pituitary gland synthesizes and releases gonadotropins (GtHs) into the blood in response to these changes. GnRH has been found to regulate the secretion and synthesis of GtHs in salmonids and rainbow trout (*Oncorhynchus mykiss*) [44]. GtHs are essential regulators of HPG axis. Initially, only one type of GtH was thought to act in fish, but later two types of GtH were isolated from the pituitary glands of various fish, such as salmonids [45], zebrafish [46], and goldfish [47]. Thus, LH and FSH, formerly called GtH I and GtH II, respectively, are the two major gonadotropins in teleost fish, which were present in turbot. 1-year-old female turbot whose oocytes are usually in early growth or early maturation. At this point, the oocyte has begun to grow and accumulate nutrients in preparation for subsequent maturation

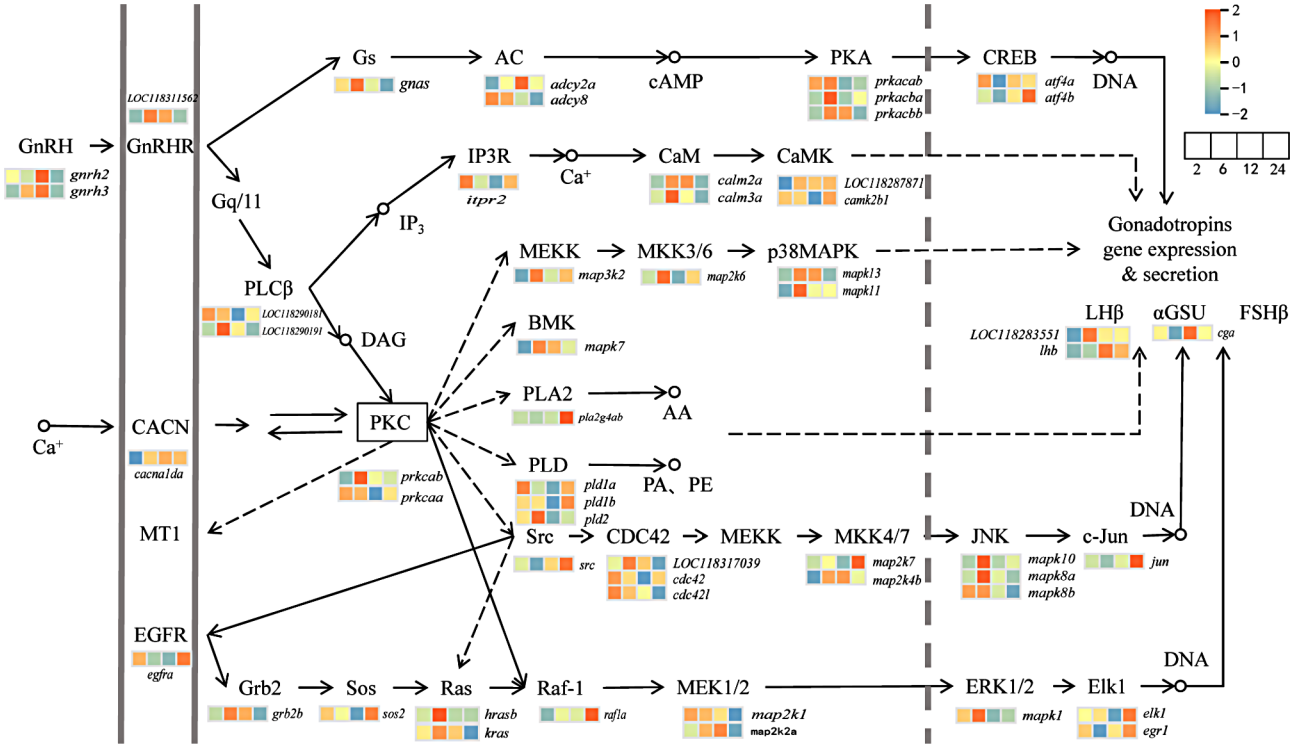

Fig. 6 Expression changes of genes related to GnRH signaling pathway of turbot upon TCEP exposure

and ovulation. In our study, we observed that the levels of FSH and LH were decreased following exposure to TCEP. FSH plays a significant role in the vitelline production and gametogenesis stages of fish gonad development [48]. LH plays an important role in germ cell maturation and release [49]. Our results were consistent with those previously shown for other endocrine disruptors. Bisphenol A (BPA) exposure decreased LH and FSH levels and reduced sperm quality in Wistar rats [50]. Exposure of zebrafish to TBBPA for 40 days resulted in significantly lower levels of LH and FSH [20]. Sex steroid hormones are divided into estrogen, androgen, and progesterone, and all types of sex steroid hormones are synthesized de novo from the precursor cholesterol, and the synthesis process is a cascade reaction involving the nervous and endocrine systems [51]. In our study, the levels of E2 and 11-KT decreased with the duration of TCEP stress, which might be related to the fact that TCEP may cause damage to nerve cells. The continuous reduction in E2 and 11-KT would directly affect the normal maturation and secretion of eggs, delay the production of young turbots, and cause great hindrance to the breeding work of turbot. TCEP induced pathological changes and inhibited gonadal maturation by inhibiting the plasma sex hormone concentration in zebrafish [52]. A decrease in FSH and LH might lead to a decrease in E2 and 11-KT, resulting in lower sperm quality and quantity and an increase in sperm malformations. Lifetime exposure to TCEP interfered with biosynthesis of steroid hormones through multiple pathways, and impaired testicular development and spermatogenesis of zebrafish [30]. In vitro, TCEP stress increased estradiol and testosterone concentrations in human adrenal cortical cancer cells [53], whereas in vivo, TCEP stress reduced hormone levels in the testes of mice [54]. In our results, chronic exposure to TCEP induced the contents of LH, FSH, E2, and 11-KT in turbot, which was consistent with the findings in mammals. It is possible that under the environment of unlimited growth in vitro, the hormone content may continue to rise, while the feedback regulation mechanism in vivo will reduce the hormone content. These results provide evidence for specific reproductive toxicity studies of TCEP.

Effects of TCEP on reproductive pathways

TCEP may induce reproductive toxicity and cytotoxicity in turbot by affecting signaling to modulate the regulation of gonadotropins by GnRH, and the genes involved in the regulation were altered (Fig. 6). The GnRH signaling pathway is a key system that regulates reproductive function. After GnRH binds to its receptor (GnRHR), it can activate a variety of downstream pathways, such as Ca^{2+} signaling pathway, cAMP signaling pathway, and MAPK signaling pathway, which affect the function of the reproductive system [55]. GnRHR couples to Gs, activating adenylate cyclase (AC) and increasing

cyclic adenosine monophosphate (cAMP) levels, which activates protein kinase A (PKA). PKA phosphorylates CREB, enabling its binding to cAMP response elements (CRE) in DNA to regulate gene transcription [56]. *gnas* is a gene encoding the alpha subunit of Gs that interacts with other signaling molecules to regulate signaling pathways within cells [57]. The conversion of ATP to cAMP is catalyzed by AC, *adcy2*a, and *adcy8* genes encode this enzyme. cAMP binds to PKA, activating it to phosphorylate various substrate proteins, thus regulating their activity. *prkacab*, *prkacba*, and *prkacbb* encode the PKA complex. Ethylhexyl salicylate induced abnormal melanin in F0 via cAMP, MAPK, and Wnt signaling pathways. with the UV-regulated cAMP signaling pathway being the main pathway [58]. In the mouse LβT2 cell line, exogenous SN activates PKA and protein kinase C (PKC)-dependent signaling pathways, thereby promoting LH expression [59]. In addition, mitogen-activated protein kinase (MAPK) and Ca/calmodulin dependent kinase II (Ca/CaMKII) pathway are also involved in GnRH-induced GTH subunit gene expression [60]. In this pathway, *mapk11*, and *mapk13* encoding p38MAPK, *calm2a* and *calm3a* encoding calmodulin, and *LOC118287871* and *camk2b1* encoding Ca^{2+}/calmodulin-dependent protein kinase (CaMK) were generally upregulated and then downregulated with TCEP stress. In a goldfish study, decreased levels of PKC resulted in decreased gonadotropin secretion in response to GnRH stimulation [61]. CaMK II plays a key role in ERK activation at slow pulse frequencies, regulating the expression of *LHβ* and *FSHB* genes [62]. Altered expression of genes in the GnRH signaling pathway led to a rapid increase in the expression of genes related to reproduction (*GnRH*, *LH*, and *FSH*) in turbot, which led to an increase in the corresponding hormone expression, resulting in advanced sexual maturity. However, after 24 h of TCEP stress, the expression of related genes decreased, which might be caused by the failure of turbot to recover in time after the treatment time exceeded 12 h. Combined with the effects of TCEP on the nervous system, it indicated that TCEP exerted neuroendocrine disturbance on turbot. Studies have shown that the regulation mechanism of the HPG axis was disrupted, and sex hormone secretion was severely disrupted in adult zebrafish after exposure to OPEs, which eventually led to the destruction of fish reproductive ability, which was similar to the effect of TCEP on turbot.

Effects of TCEP on reproductive gene expression

Genes on the HPG axis were also examined in this study, and the results not only validated the accuracy of the transcriptomic results, but also indicated that TCEP may cause reproductive toxicity by affecting the expression levels of *Gt*, *GnRH*, *LH*, and *FSH* in turbot. *GnRH* encodes gonadotropin-releasing hormone, which regulates the synthesis and release of gonadotropins in the anterior pituitary. *FSH* and *LH* encode two types of gonadotropins respectively, which regulate the function of the reproductive system and play important roles in follicle development, sperm formation, and sex hormone synthesis. In our study, the expression levels of reproductive genes were up-regulated and reached a peak at 6 h of TCEP exposure, and then decreased, which was consistent with the transcriptome levels. The data supported that the contents of reproductive hormones (FSH, LH, E2, and 11-KT) decreased with long-term TCEP stress. Kisspeptin, a class of neuropeptides encoded by the *Kiss1*, is produced mainly by specific hypothalamic neuronal clusters, whose expression is regulated by GPR54 [63]. This receptor is associated with a variety of hormonal signaling pathways, including those involved in sexual maturation and reproduction [64]. Based on our results, it was speculated that TCEP exposure influenced the change in hormone levels by altering the expression of *KISS* and HPG axis-related genes, thereby regulating the timing of egg maturation.

WGCNA analysis under TCEP stress

While many studies have investigated gene expression under environmental stress, few have done so by constructing gene co-expression networks. In our WGCNA, 17 co-expression network modules were obtained. Six modules (purple, green, turquoise, magenta, tan, and midnight blue) were significantly associated with TCEP exposure traits, and one (pink) was significantly correlated in the Ctrl group, which suggested that ME. pink module genes play key roles in maintaining normal physiological functions under stress. KEGG enrichment analysis of the top 20 weighted genes showed that oxidative stress and reproduction were significantly enriched after TCEP exposure, including oxidative phosphorylation, phagosome, ferroptosis, GnRH signaling pathway, etc., which were also enriched in the four treatment groups. The enriched genes related to oxidative phosphorylation, such as *LO118302460*, *atp6v1ba*, and *ndufa12*, were considered key genes that play important roles in encoding the mitochondrial respiratory chain complex. Once mitochondrial dysfunction occurs, it may lead to energy metabolism disorder, which in turn affects the normal physiological function and health of the organism. TCEP stress caused decreased transcript levels of these key genes, indicating that oxidative phosphorylation pathway was closely related to stress, and turbots were in an unhealthy state. Moreover, phagocytosis is an actin-based cell internalization process that removes host cells undergoing apoptosis or necrosis, the activation of which is an important immune mechanism [65, 66]. Our study showed that some phagosome genes were

up-regulated, which may enhance the phagocytosis activity of cells and clear damaged cells (Fig. S4). These findings proved that insufficient energy metabolism led to cell damage and triggered phagocytosis of immune cells. Ferroptosis appeared to be sensitive to TCEP exposure, as it was enriched in all the groups and the seven modules group. Experimental compounds and some clinical drugs have been reported to induce ferroptosis in normal and cancer cells, which has become a hot topic in life science research [67]. Low temperature stress caused renal dysfunction and the downregulation of immune-related pathways as well as iron deficiency in tilapia [68]. Iron-based nanomaterials enlarged the ventricles or atria of zebrafish, leading to reproductive toxicity [69]. WGCNA analysis found that TCEP exposure led to ferroptosis, but the exact mechanism remains unclear, which should be further investigated in the future.

Conclusions

In this study, we conducted a comprehensive analysis of the effects of TCEP stress on the turbot at different time points at the nucleic acid and protein levels using the determination of hormone content and transcriptomics analysis. Under TCEP stress, the expression of *GnRH*, *LH*, *FSH*, *KISS*, and *KISSr* genes was altered, whereas the levels of four reproductive hormones (LH, FSH, E2, and 11-KT) levels were significantly decreased. These changes may affect the earlier sexual maturity of turbot, and may also cause the inability of adult fish to release eggs properly and reproduce the second generation of fish, which will lead to a serious decline in production and cause economic losses. Transcription profile analysis showed that DEGs were enriched in pathways related to oxidative stress and reproductive regulation, including oxidative phosphorylation and apoptosis, GnRH signaling pathway and MAPK signaling pathway, respectively. Furthermore, WGCNA analysis revealed the modules and key pathways, among which oxidative phosphorylation was the most significant, while we also found that phagosome and ferroptosis pathways were enriched. Our results revealed that TCEP affected mitochondrial function and energy metabolism, indicating that TCEP may cause long-term toxicity, but the specific mechanism needs further investigation. Our study may contribute to the understanding of the neuroendocrine-disrupting effect of TCEP and provide guidance for turbot cultivation.

Abbreviations

AC	Adenylate cyclase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BP	Biological process
BPA	Bisphenol A
Ca/CaMKII	Ca/ Calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
CC	Cellular component
complex I	NADH dehydrogenase
complex III	Cytochrome c reductase
complex V	ATP synthase
complex IV	Cytochrome c oxidase
CRE	cAMP response element
CREB	cAMP response element binding protein
DEGs	Differentially expressed genes
E2	Estradiol
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GO	Gene Ontology
GtHs	Gonadotropins
HPG	Hypothalamic-pituitary-gonadal
KEGG	Kyoto Encyclopedia of Genes and Genomes
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
ME	Module eigengene
MF	Molecular function
OPEs	Organophosphate esters
OPFRs	Organophosphorus flame retardants
PBDEs	Polybrominated diphenyl ethers
PCA	Principal component analysis
PKA	Protein kinase A
PKC	Protein kinase C
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
T	Testosterone
TCEP	Tris (2-chloroethyl) phosphate
WGCNA	Weighted gene co-expression network analysis
11-KT	11-ketotestosterone

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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Not applicable.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by ZXQ and CBL. Data Visualization was conducted by DAF and LJ. The first draft of the manuscript was written by ZXQ, then edited by LSH and ZLL. LWF was responsible for funding acquisition. All authors read and approved the final manuscript.

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Data availability
The raw sequence data reported in this paper have been uploaded to the NCBI database (accession: PRJNA1113711, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1113711).

Declarations

Ethics approval and consent to participate
All of the turbot collections and the anatomy experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, which was approved and supervised by the Ethic Committee in First Institute of Oceanography, MNR. All efforts were made to minimize the suffering of the animals. The supporting document was shown in the supplementary material.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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