

Research article

3,6-Dichlorocarbazole induces metabolic disturbances and activates drug detoxification processes in the liver of zebrafish (*Danio rerio*)



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ABSTRACT

Polyhalogenated carbazoles (PHCZs) are widely recognized for their persistence and widespread presence in aquatic environments. Although PHCZ accumulation in aquatic organisms induces various forms of biological toxicity, the effects on liver metabolism are not yet fully understood. This study investigated the biological concentration factor (BCF), liver histopathology, metabolomics, and responses of key metabolic genes in the liver tissues of male zebrafish exposed to 20 and 100 µg/L of 3,6-dichlorocarbazole (3,6-DCCZ) for 21 days. The bioconcentration factor of 3,6-DCCZ was found to reach 109.7, based on measurements of both the exposure concentration in water and its concentration in liver tissue. Histopathological observations revealed that 3,6-DCCZ induced liver damage, including hepatic inflammatory infiltration, hepatocyte swelling, and disorganized hepatic cord arrangement. Metabolomics and metabolic gene analyses found that 3,6-DCCZ significantly reduced intermediates in glycolysis and the tricarboxylic acid cycle, potentially hindering liver glycolysis and glycogen synthesis. Additionally, 3,6-DCCZ increased the accumulation of triglycerides and fatty acids in zebrafish, potentially leading to liver lipid metabolism disorders. The increased expression of the *alt* gene further confirms liver damage. Furthermore, the expression of phase I and II detoxification genes (*cyp1a*, *cyp3a65*, *ugt1ab*, and *ugt2a1*) was dramatically upregulated, promoting the hepatic drug detoxification process. Overall, this study emphasizes that exposure to 3,6-DCCZ induces metabolic disorders and liver damage in zebrafish, providing a basis for understanding its potential ecological and health risks.

1. Introduction

Polyhalogenated carbazoles (PHCZs) are emerging pollutants that share similarities with polychlorinated dibenzofurans (PCDFs) in both molecular structure and toxicity (Sun et al., 2022a). PHCZs are formed as by-products during the production of halogenated dyes, photoelectric materials, and chlorination disinfection and are subsequently released into the environment through the use of these products (Chen et al., 2018; Ji et al., 2021). Additionally, PHCZs may also be generated in the environment through enzymatic synthesis and natural disasters (Karon et al., 2014; Parette et al., 2015; Wang et al., 2019a; Zhang and Lin, 2024).

PHCZs have been detected in various environmental media,

including water, air, soil, and sediment (Ji et al., 2021). Notably, PHCZs exhibit high detection rates and concentrations in aquatic environments. Total PHCZ accumulation exceeding 3000 tons has been detected in the sediments of the Great Lakes in North America, several orders of magnitude higher than that of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) (Guo et al., 2017). Furthermore, PHCZs exhibited high persistence in Wuhan drinking water, with concentrations remaining stable even after boiling (Wang et al., 2021). PHCZs may accumulate in organisms due to their persistence and widespread environmental distribution. Wu et al. (2017) found that the median toxic equivalent of PHCZs in organisms ranged from 4.8 to 19.5 pgTEQ/g in San Francisco Bay, driving biomagnification through the food web. Moreover, PHCZs were detected in human urine at a

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concentration of ≤ 16 ng/mL in Quzhou City, China (Mao et al., 2024).

Organisms can accumulate PHCZs, which have the potential to cause harm. Current studies on PHCZ biotoxicity primarily focus on dioxin toxicity resulting from AhR activation (Ji et al., 2019; Ma et al., 2019). Furthermore, reports indicate that PHCZs exhibit a range of toxicities in organisms, including developmental toxicity, neurotoxicity, cardiovascular toxicity, and endocrine disorders (Du et al., 2022; Xu et al., 2024; Yue et al., 2020). Ji et al. (2023) discovered that PHCZs can alter intestinal microbiota in mice, leading to metabolic disorders. In contrast to traditional toxicological methods, metabolomics not only enables high-throughput characterization of small molecular metabolites but also reveals potential toxic effects (Chen et al., 2021a; Zhang et al., 2014). Metabolomics has been employed to investigate the toxic effects of PHCZs. Xu et al. (2024) combined serum metabolic profiling with an in vitro mechanism to study the cardiovascular effects of 1,3,6,8-tetra-bromocarbazole (1368-TBCZ) in mice and found that 1368-TBCZ exposure disrupted energy metabolism and exacerbated atherosclerosis. These studies have demonstrated that PHCZs induce metabolic disorders in various animals and cells at the individual level. The liver plays a critical role in substance metabolism and detoxification in vertebrates (Chen et al., 2017; Wang et al., 2016). However, recent research on the hepatic metabolic toxicity of PHCZs is limited.

Histopathology has been recognized as an effective and sensitive tool for monitoring fish health (Lang et al., 2006). Zebrafish share similarities with humans in terms of hepatic genes, structure, and response mechanisms to liver diseases, making them an important model organism for studying drug-induced liver injury (Goessling and Sadler, 2015; Vliegenthart et al., 2014). This study selected 3,6-DCCZ, a compound with a high environmental detection rate, to investigate its effects on liver toxicity, metabolism, and detoxification. First, tissue sections and hematoxylin and eosin staining were employed to observe pathological changes in the zebrafish liver. Second, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) metabolomics was used to assess changes in small molecular metabolite abundance and identify metabolic markers of hepatotoxicity in zebrafish liver following exposure to 3,6-DCCZ. Third, the impact of 3,6-DCCZ on the regular metabolic and detoxification processes of zebrafish liver was evaluated by examining key metabolism and detoxification genes.

2. Materials and methods

2.1. Fish Care and exposure

To avoid the influence of liver metabolism fluctuations in female zebrafish during different reproductive cycles, male zebrafish were selected for this study (Ladisa et al., 2022; Li et al., 2019). Adult, wild-type male zebrafish (*Danio rerio*), 5 months of age, were obtained from the Haitian Aquarium (Taian, China). The fish were acclimated in chlorine-free tap water for at least 2 weeks under conditions of 26 ± 1 °C, pH 7.4 ± 0.5 , DO ≥ 80 %, and a 14:10 h light/dark cycle before exposure. The zebrafish were fed twice daily with dry flakes. 3,6-DCCZ was obtained from Jaime K Scientific Ltd. (Beijing, China). Due to the poor solubility of 3,6-DCCZ, 0.05 % dimethyl sulfoxide (DMSO, chromatographic grade, purchased from McLean Biotechnology Co., Ltd., Shanghai, China) was used as a cosolvent and solvent control. Based on previous studies, zebrafish were exposed to 20 and 100 µg/L of 3,6-DCCZ for 21 days (Bai et al., 2024; Hou et al., 2024). Previous studies have shown that DMSO has no significant effect on zebrafish at this concentration, so a DMSO solvent control treatment group was not included (Bai et al., 2024; Hoyberghs et al., 2021; David et al., 2012). Each tank contained 120 fish, and each treatment group consisted of three parallel tanks. The exposure experiment was conducted using a semi-static method, with the exposure solution replaced by half every 24 h. Other conditions remained consistent with those during acclimation. To validate changes in the concentration of 3,6-DCCZ in water under semi-static exposure conditions and its accumulation in fish liver

at 21 days, actual exposure concentrations in water were determined using GC-MS/MS. The measured concentrations on day 21 were 12.35 ± 0.64 µg/L (61.75 %) and 83.65 ± 3.71 µg/L (83.65 %), respectively. No mortality was observed during the exposure. Zebrafish were collected after 21 days of exposure, and their livers were dissected. The livers were fixed in 10 % formalin or rapidly frozen in liquid nitrogen for subsequent analysis. The extraction procedures for 3,6-DCCZ from water and fish liver, as well as the GC-MS/MS operating conditions, are detailed in the Supplementary Material (S1). Zebrafish experiments were conducted in accordance with the "Guidelines for the Ethical Review of Laboratory Animal Welfare, People's Republic of China National Standard" (GB/T35892-2018), with animal testing performed after euthanasia (MacArthur Clark and Sun, 2020).

2.2. Histopathological

After 21 days of exposure, 5 zebrafish were randomly collected from three replicates in each treatment group. The livers of zebrafish (one liver per replicate, n = 5) were dissected and fixed in 10 % formalin for 24 h. Liver tissue sections were prepared, and hematoxylin and eosin (H&E) staining was performed by Beijing Zhongke Wanbang Biotechnology Co., Ltd. Briefly, the fixed liver was dehydrated in a gradient ethanol series, embedded in paraffin, and sectioned into 5 µm slices using a microtome. The sections were then stained with H&E. Histological observations were performed using an optical microscope equipped with a digital camera.

2.3. UPLC-MS/MS -based Metabolomic analysis

After 21 days of exposure, 100 zebrafish were randomly collected from three replicates in each treatment group for the extraction of hydrophilic and hydrophobic substances. Hydrophilic and hydrophobic substances were extracted from the liver following the method developed by Wuhan MetWare Biotechnology Co., Ltd. Briefly, (1) Five biological replicates were established for each treatment group. For each replicate, liver samples from 10 fish, each weighing approximately 20 mg, were homogenized in a grinder (30 Hz), and 400 µL of a 70 % methanol internal standard extract (Methanol: Water = 7:3, V/V) was added. The mixture was then centrifuged for 10 min at 4 °C and 12,000 g. The supernatant was further centrifuged at 4 °C and 20,000 g for 3 min. Finally, 200 µL of the supernatant was analyzed by LC-MS/MS. (2) For the extraction of hydrophobic substances, 20 mg of liver samples (dissected from 10 fish, n = 5) were homogenized, and 1 mL of internal standard lipid extract (MTBE: MeOH = 3:1, v/v) was then added. The mixture was centrifuged at 4 °C and 12,000 g for 10 min. The supernatant was concentrated and reconstituted in 200 µL of a solution (ACN: IPA = 1:1, v/v). After centrifuging the mixture at 12,000 g for 3 min, the supernatant was used for LC-MS analysis. Detailed procedures for the extraction of hydrophilic and hydrophobic substances, along with the LC-ESI-MS/MS conditions, are provided in the supplementary materials (S1 and S2).

2.4. Determination of gene expression

After 21 days of exposure, the livers of 3 zebrafish were dissected and placed into 1.5 mL centrifuge tubes. Trizol (CoWin Biosciences Co., Ltd., Beijing, China) was used to extract RNA from the liver. The quality and purity of the RNA were determined using a nucleic acid concentration analyzer (Nanodrop, 2000; Thermo Fisher, USA), and the RNA concentration was adjusted to 0.05 µg/µL with RNase-free water. The RevertAid First Strand cDNA Reverse Transcription Kit was used to synthesize cDNA from the liver. Quantitative RT-PCR was performed using the FastSYBR Green Mixture Kit (Hong Kong Kofite Biotechnology Co., Ltd.) on a StepOnePlus Real-Time PCR Detection Platform (Thermo Fisher Scientific Inc., USA). β-actin was used as the housekeeping gene, and the 2- $\Delta\Delta Ct$ relative quantitative method was applied to calculate

gene expression in zebrafish liver. The transcriptomic (qPCR) data for each treatment group were derived from a single cDNA sample extracted from pooled liver samples of three zebrafish, with four technical replicates per treatment group for RT-PCR experiments. Detailed information about the primers and RT-PCR protocols is provided in the supplementary materials (Tables S1 and S2).

2.5. Statistical analysis

The metabolomics data were based on a self-constructed MWDB database, and the mass spectrometry data were processed using Analyst 1.6.3 software. The R package MetaboAnalystR was used to calculate the VIP value in orthogonal partial least squares discriminant analysis (OPLS-DA). Differentially expressed metabolites were identified by performing multiple comparison corrections using the False Discovery Rate (FDR) method with a threshold of 0.05 to control the Type I error rate. Prior to OPLS-DA, the data were log-transformed (log2) and mean-centered. To avoid overfitting, a permutation test with 200 permutations was performed. The validity of the OPLS-DA model was assessed using R2Y (the explained variance of the Y matrix) and Q2 (the model's predictive ability). For two-group analysis, significantly altered metabolites (SCMs) were identified by VIP ($VIP > 1$, Variable Importance in Projection) and p-value ($p < 0.05$, Student's t-test). The KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>) was used to analyze metabolic pathways, and the p-values from hypergeometric tests were used to determine their significance. Metabolic genes were analyzed using IBM SPSS Statistics 26 software. One-way analysis of variance (ANOVA) was performed, followed by a post-test based on the least significant difference (LSD) to determine the differences between treatment groups.

3. Results

3.1. Hepatic enrichment and hepatic histopathological changes

The bioaccumulation concentrations in the fish liver for the 20 and 100 $\mu\text{g/L}$ 3,6-DCCZ treatment groups were measured at 770.2 and 9171.3 $\mu\text{g/kg}$, respectively, with corresponding biological concentration factors (BCF) of 62.8 and 109.7. Detailed data are provided in the supplementary materials (Fig. S2). Fig. 1 shows the histopathological changes in the livers of zebrafish exposed to different concentrations of 3,6-DCCZ. In the control group, the liver morphology was normal, and

the hepatic cords were well-defined. However, the livers of fish from both the 20 and 100 $\mu\text{g/L}$ 3,6-DCCZ-treated groups exhibited significant inflammatory cell infiltration (indicated by the blue arrow). In contrast to the 20 $\mu\text{g/L}$ group, hepatocyte swelling and disarrangement of liver cords were also observed in the 100 $\mu\text{g/L}$ group.

3.2. Metabolomic alterations

In this study, widely targeted metabolomics based on the UPLC-MS/MS detection platform was used to analyze hydrophilic and hydrophobic substances, resulting in the detection of a total of 2893 metabolites (Fig. S2). The OPLS-DA score plots clearly distinguished between the control group and the groups treated with 3,6-DCCZ, particularly between the control group and the group treated with 100 $\mu\text{g/L}$ of 3,6-DCCZ (Fig. 2A). The results demonstrated a significant alteration in the liver metabolomics of zebrafish following exposure to 3,6-DCCZ, especially in the high-dose exposure group. As shown in Fig. 2B and C, the profiles of samples from the 20 and 100 $\mu\text{g/L}$ 3,6-DCCZ-treated groups were compared separately with those of the control group, and VIP values were used to screen for significantly changed metabolites (SCMs). Two hundred permutation tests of the OPLS-DA model quality showed that the R2Y values (0.994, 0.998, 0.998) were close to 1.0, and the Q2 values (0.673, 0.591, 0.773) were greater than 0.5. Furthermore, the p-values of Q2 (0.005, 0.035, 0.005) and R2Y (0.015, 0.045, 0.005) were all < 0.05 (Fig. S3). These results indicated that the models were stable, reliable, and not overfitted.

SCMs were screened based on the following criteria: $VIP > 1$ in the OPLS-DA models (Fig. 2B and C) and $p < 0.05$ in t-tests. A total of 265 SCMs were upregulated, and 70 SCMs were downregulated in the 20 $\mu\text{g/L}$ 3,6-DCCZ-treated group compared to the control group. Furthermore, 522 SCMs were upregulated, and 91 SCMs were downregulated in the 100 $\mu\text{g/L}$ 3,6-DCCZ-treated group compared to the control group (Fig. S4). Most of these SCMs were involved in lipid and amino acid metabolism (Fig. 3A and B). Representative lipid metabolites include triglycerides (TG), diglycerides (DG), free fatty acids (FFA), acylcarnitines (CAR), oxidized lipids, lyso-phosphatidylcholine (LPC), lyso-phosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin (SM), and ceramides (Cer) (Fig. 3A and B). Representative amino acids include alanine, valine, ornithine, lysine, indole-lactic acid, and reduced glutathione (Table S3). Differential carbohydrates were screened based on $VIP > 1$

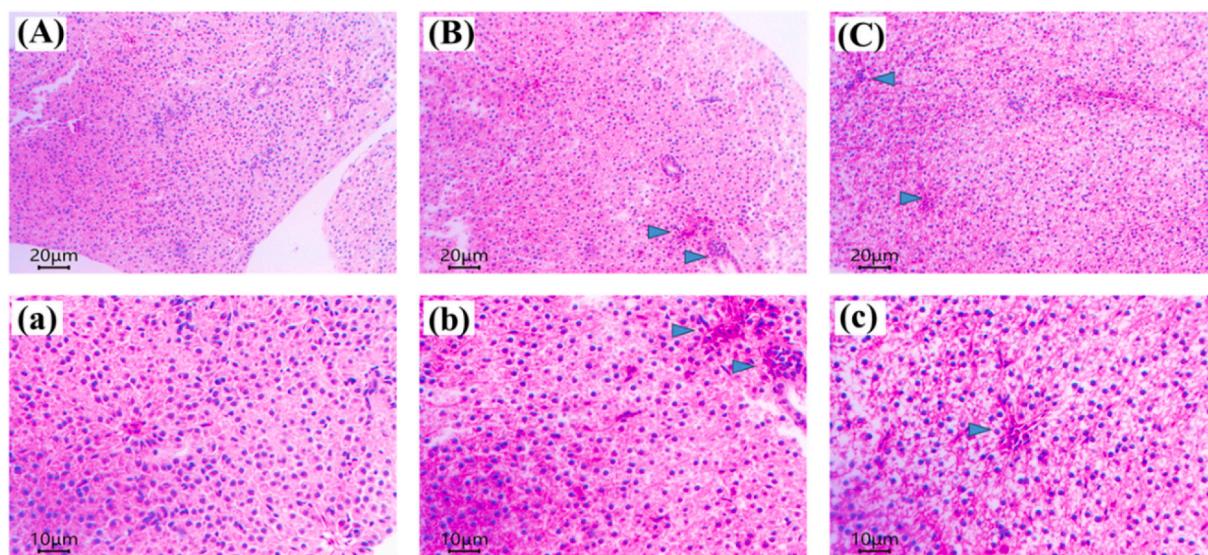


Fig. 1. Representative micrographs of liver sections from zebrafish exposed to different concentrations of 3,6-DCCZ for 21 days. Control group (A) (a), 20 $\mu\text{g/L}$ 3,6-DCCZ-treated group (B) (b), and 100 $\mu\text{g/L}$ 3,6-DCCZ-treated group (C) (c). The blue arrow indicates inflammatory cell infiltration.

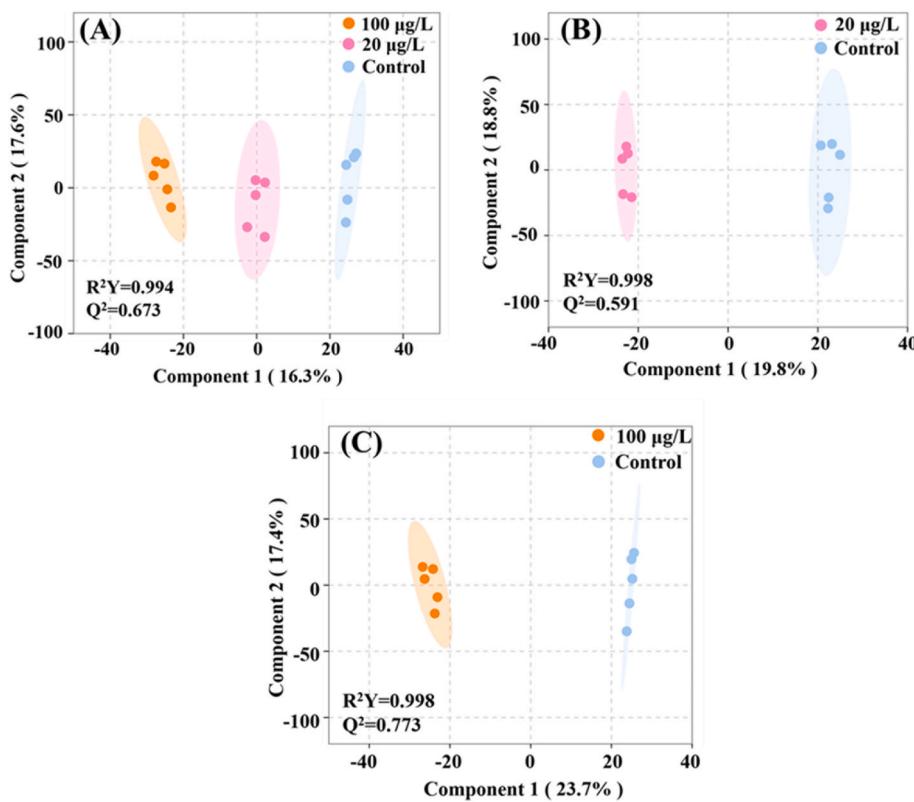


Fig. 2. OPLS-DA score plots: 100 µg/L vs 20 µg/L vs Control treatment group (A); 20 µg/L vs Control treatment group (B); and 100 µg/L vs Control treatment group (C).

and $p < 0.1$. The representative differential carbohydrates identified include succinic acid, D-glucosaminic acid, glucose, D-fructose-6-disodium phosphate, D-glucose-6-phosphate, α -ketoglutaric acid, and glucuronic acid (Table S4). These altered metabolites suggest that 3,6-DCCZ induces metabolic disorders in the zebrafish liver.

To gain a better understanding of the identified SCMs, KEGG pathway enrichment analysis was performed. Fig. 4 shows the top 20 pathways, ranked by increasing p-value. Four significantly enriched pathways were identified based on p-value, enrichment number, and enrichment factors, including glycerolipid metabolism, inositol phosphate metabolism, phosphatidylinositol signaling system, and glycerophospholipid metabolism.

3.3. Expressions of genes

Changes in the transcription of genes involved in carbohydrate metabolism (*pfk1b* and *gys2*), fat metabolism (*lipc*, *acc1*, *cpt1*, and *ppara*), amino acid metabolism (*alt*), and drug detoxification (*cyp1a*, *cyp3a65*, *ugt1ab*, and *ugt2a1*) are shown in Fig. 5. Compared to the control group, the expression of *pfk1b*, *gys2*, *lipc*, *cpt1*, and *ppara* genes was significantly downregulated in the 20 and 100 µg/L 3,6-DCCZ-treated groups ($p < 0.05$). Conversely, compared to the control group, the expression of *alt*, *cyp1a*, *cyp3a65*, *ugt1ab*, and *ugt2a1* genes was significantly upregulated under both concentrations of exposure ($p < 0.05$). Additionally, *acc1* gene expression was significantly increased in the 100 µg/L 3,6-DCCZ-treated group compared to the control group ($p < 0.05$), while no significant difference was observed in the 20 µg/L 3,6-DCCZ-treated group ($p > 0.05$). The changes in gene expression further support the conclusion that 3,6-DCCZ induces a complex metabolic disorder in the liver (Fig. 6).

4. Discussion

PHCZs have been widely detected in various environmental matrices, including drinking water. The human body also detects PHCZs, posing a significant threat to individual health and well-being. Current data suggest that PHCZs, a novel pollutant with dioxin-like toxicity, exhibit a significant trend of bioaccumulation and biomagnification through the food chain. PHCZs can cause developmental toxicity, cardiovascular toxicity, and endocrine and metabolic disorders. Different organs *in vivo* perform distinct functions, and studying individual organs facilitates a better understanding of the biological toxic effects of PHCZs. However, there is currently a lack of research on the hepatic metabolic toxicity of PHCZs. In this study, we investigated the metabolic damage caused by 3,6-DCCZ in zebrafish liver by combining histopathology, metabolomics, and the analysis of key metabolic genes.

The high octanol-water partition coefficient ($\text{Log K}_{\text{o/w}} = 4.97$) of 3,6-DCCZ suggests its strong lipophilic nature and potential for bioaccumulation in fish liver (Bai et al., 2024). In the present study, the BCF of 3,6-DCCZ in fish liver was found to be 62.8 and 109.7 in the 20 and 100 µg/L 3,6-DCCZ treatment groups, respectively, indicating that zebrafish have a high capacity to accumulate 3,6-DCCZ. The properties of difenoconazole ($\text{Log K}_{\text{o/w}} = 4.3$) are similar to those of 3,6-DCCZ. The BCF of difenoconazole in marine medaka ranges from 24.7 to 149.0, which is similar to the result of this study (Zhang et al., 2017). In the low concentration 3,6-DCCZ treatment group, more 3,6-DCCZ was adsorbed to the fish tanks and fish excreta, whereas the high concentration 3,6-DCCZ treatment group may have exceeded the metabolizing capacity of the fish, resulting in the accumulation of 3,6-DCCZ in the liver.

In terms of carbohydrate metabolism, low concentration exposure to 3,6-DCCZ in zebrafish causes hepatic accumulation of glucose, suggesting that the conversion of glucose to energy may be impaired. In a previous study, the common response of fish to stress was to maintain balance by increasing glucose (Panetto et al., 2019). Glycolysis and the

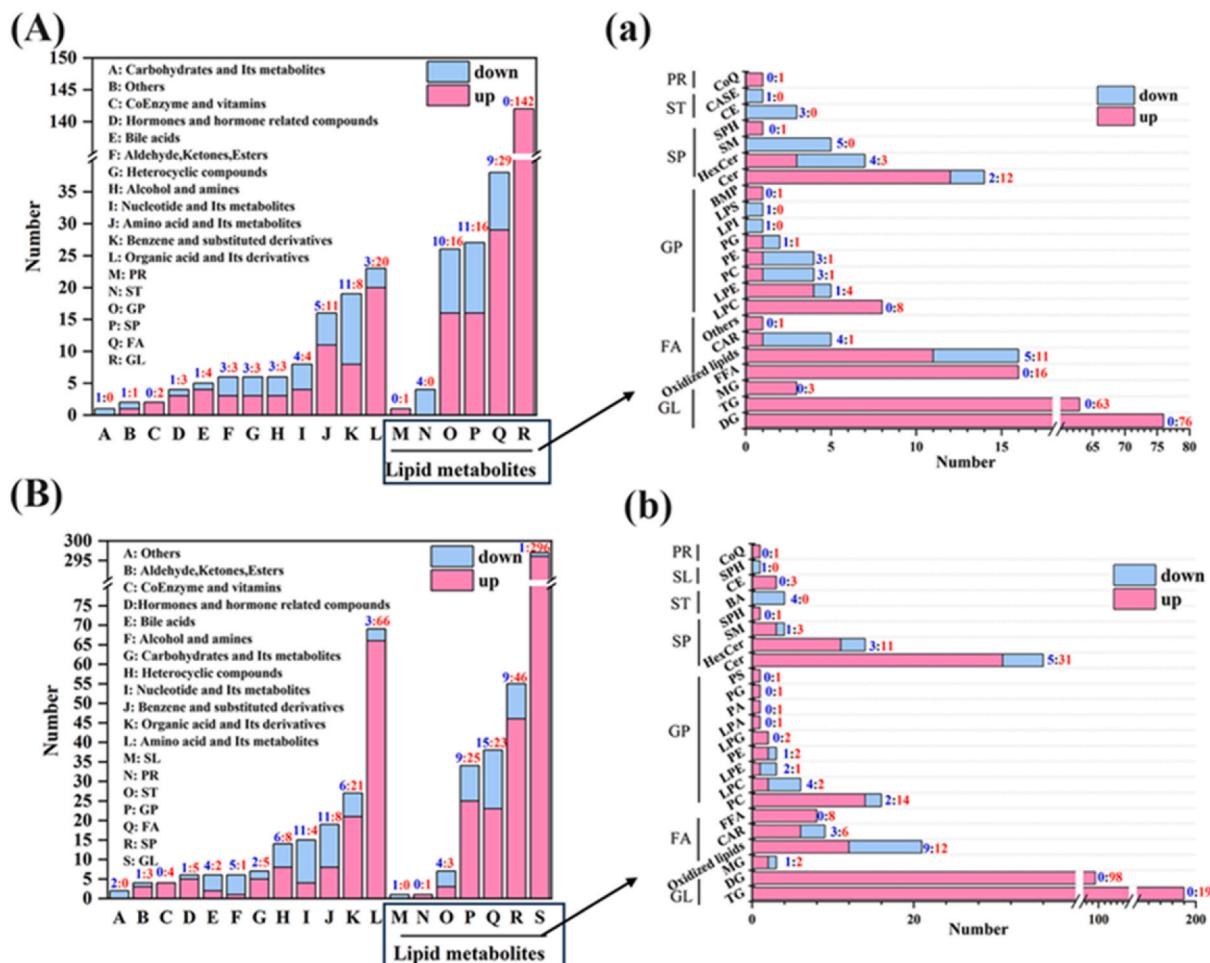


Fig. 3. Histogram of differential metabolite accumulation: the classification and the number of upregulated and downregulated metabolites in the 20 µg/L vs Control (A) and 100 µg/L vs Control treatment groups (B), as well as the classification and up-down regulation of lipid metabolites in these treatment groups (a) and (b). In the figure, the ratio represents the number of downregulations and upregulations, with blue numbers indicating downregulations and red numbers indicating upregulations.

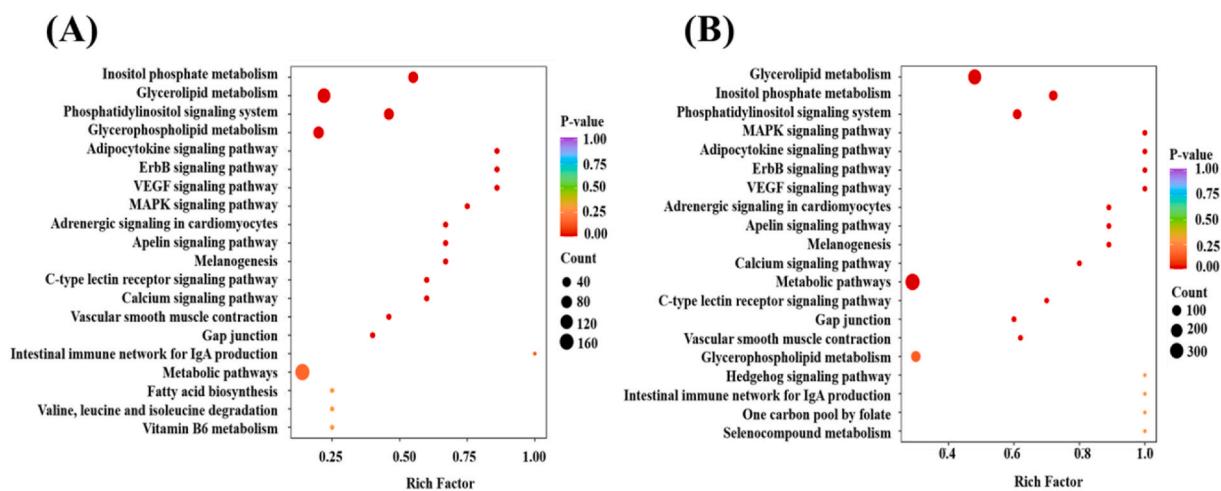


Fig. 4. KEGG enrichment map of differential metabolites (top 20 pathways). Analysis of the 20 µg/L vs Control treatment group (A) and the 100 µg/L vs Control treatment group (B).

tricarboxylic acid cycle break down glucose in the liver, often producing large amounts of ATP (Polakof et al., 2012). The levels of D-glucose-6-phosphate and D-fructose-6-disodium phosphate (glycolysis intermediates) were significantly reduced in the high concentration 3,

6-DCCZ-treated group, suggesting that the zebrafish glycolytic process may be impaired. This study also observed the accumulation of α -ketoglutaric acid (at low concentrations) and succinic acid (at high concentrations), intermediates of the tricarboxylic acid (TCA) cycle

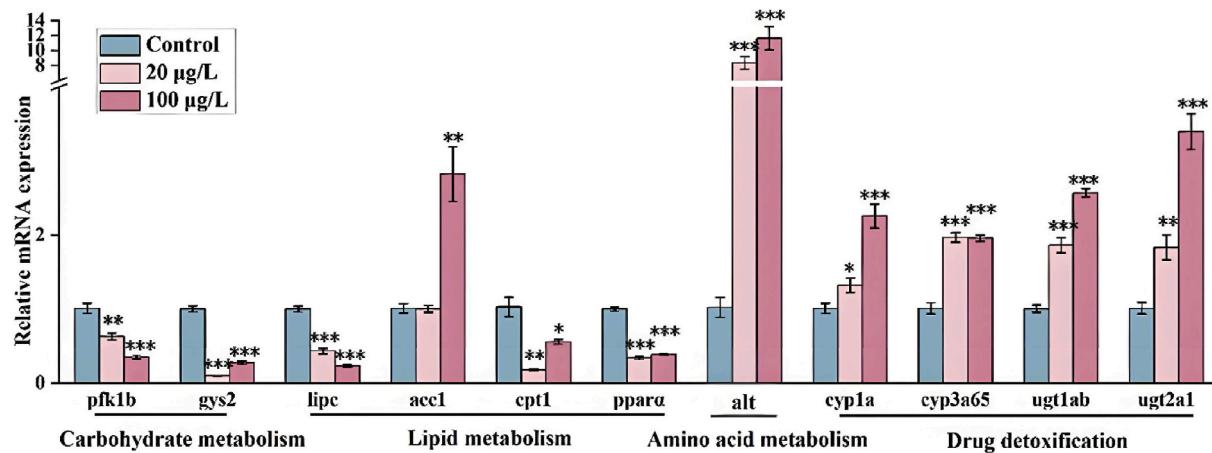


Fig. 5. Effect of 3,6-DCCZ on the expression of key metabolic genes in zebrafish liver. The standard error of the four samples is represented by the error bars. * indicates $p < 0.05$ versus control, ** indicates $p < 0.01$ versus control, and *** indicates $p < 0.001$ versus control.

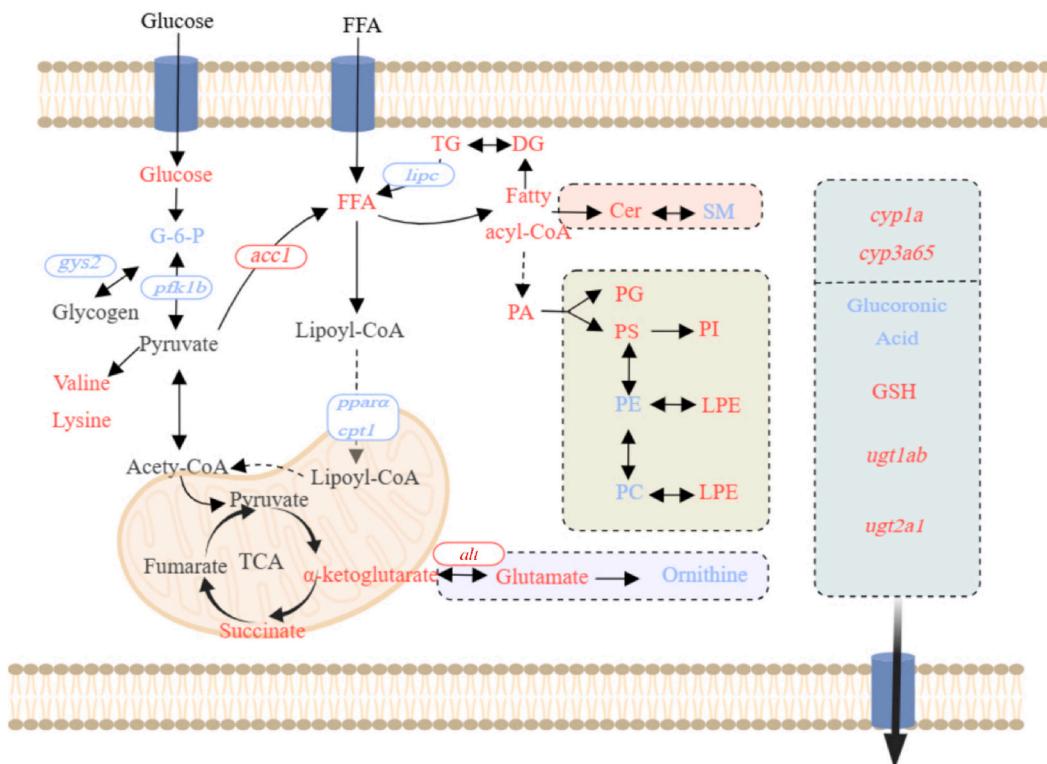


Fig. 6. Exposure to 3,6-DCCZ interferes with liver metabolism and detoxification in zebrafish. Red and blue represent upregulation and downregulation, respectively.

related to energy conversion in fish liver, caused by exposure to 3,6-DCCZ. To further evaluate the effect of 3,6-DCCZ on carbohydrate metabolism, the genes *pfk1b* and *gys2*, which are involved in glycolysis and glycogen synthesis, were analyzed. *pfk1b*, which encodes phosphofructokinase, catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate and is the primary rate-limiting enzyme in glycolysis. Downregulation of *pfk1b* expression leads to decreased glucose accumulation and reduced downstream glycolytic products (Wang et al., 2019c). *gys2*, which encodes glycogen synthase, converts excess glucose into glycogen. Downregulation of *gys2* expression may result in further accumulation of glucose (Chen et al., 2024). In this study, compared to the control group, the expression levels of *pfk1b* and *gys2* in the liver of zebrafish exposed to 3,6-DCCZ were significantly downregulated, indicating that the zebrafish energy metabolism system

was significantly impaired. This is consistent with previous in vitro studies showing that 1,3,6,8-BCZ can disrupt the energy metabolism of MOVAS cells and inhibit ATP synthesis (Xu et al., 2024). Previous studies have shown that d-glucosamine can normalize liver glycogen synthesis function and also possesses strong antioxidant capacity (Yan et al., 2006, 2007). In this study, the depletion of d-glucosamine in the liver of zebrafish treated with high concentration 3,6-DCCZ may impair normal glycogen synthesis.

Lipid content regulation in the liver is a dynamic process that can lead to lipid metabolism disorders when there is an imbalance between lipid acquisition (fatty acid uptake and de novo lipogenesis) and lipid breakdown (fatty acid β -oxidation and lipid export) (Bechmann et al., 2012; Sun et al., 2022b). The accumulation of triglycerides (TG) in the liver of zebrafish exposed to 3,6-DCCZ may result from lipid homeostasis

imbalance, as suggested by this study. Huang et al. (2024) found that exposure to 3,6-DCCZ could lead to liver lipid accumulation and liver injury in adult zebrafish. *acc1* encodes the enzyme acetyl-CoA carboxylase, which plays a role in the production of fatty acids (Lin et al., 2024). De novo lipogenesis is defined as the synthesis of new endogenous fatty acids from acetyl-CoA in the liver, with the rate of synthesis regulated by acetyl-CoA carboxylase (Wang et al., 2018). Thus, the upregulation of *acc1* gene expression in the high-dose exposure group may promote the formation of de novo adipose tissue and increase the production of TG (Xu et al., 2023). Furthermore, we analyzed several key genes involved in lipid breakdown (*lipc*, *cpt1*, and *ppara*). Exposure to 3,6-DCCZ significantly reduced the expression levels of these three genes compared to the control group, suggesting a restriction in lipid breakdown in zebrafish liver. The *lipc* gene, which encodes lipase, hydrolyzes TG into glycerol and free fatty acids (FA). Decreases in its expression levels can lead to a buildup of TG (Feng et al., 2014; Yao et al., 2018). Previous studies have shown that peroxisome proliferator-activated receptor α (*ppara*) is an important nuclear receptor in lipid metabolism (La Cour Poulsen et al., 2012). Free fatty acids (FFA) can bind to and activate *ppara* in the liver (Chen et al., 2020). *ppara* regulates the downstream target gene *cpt1* to transport FFA to mitochondria for oxidation, thereby reducing lipid accumulation in the liver (Sun et al., 2022b). Carnitine (CAR) plays a crucial role in liver lipid transfer and utilization, and a reduction in its levels may lead to lipid droplet formation and TG accumulation (Du et al., 2016). Therefore, the buildup of FFA, the reduction of acylcarnitine, and the suppression of *cpt1* gene expression in zebrafish liver suggest that exposure to 3,6-DCCZ inhibits fatty acid β -oxidation. The same phenomenon was observed in the liver exposed to dioxins. Previous studies have shown that dioxins induce liver steatosis by activating AhR to inhibit fatty acid β -oxidation, resulting in triglyceride accumulation (Lee et al., 2010). Collectively, our results demonstrate that 3,6-DCCZ exposure increased the production of fatty acids in the liver of zebrafish and decreased the breakdown of TG and the oxidation of fatty acids.

Exposure to 3,6-DCCZ significantly affected lipid metabolic pathways in the liver, including glycerol metabolism, glycerophospholipid metabolism, inositol phosphate metabolism, and the phosphatidylinositol signaling system, as indicated by KEGG pathway analysis of significantly altered metabolites. Glycerophospholipids are the main components of biological membranes, participating in cell signal transduction by interacting with proteins or providing signaling molecular precursors (Gong et al., 2023; Wang et al., 2019b). Inositol phosphate metabolism and the phosphatidylinositol signaling system are involved in diglyceride (DG) synthesis. Inositol phosphate is a critical component of the phosphatidylinositol signaling system, and its metabolism directly affects the function of this signaling system (Xiao et al., 2022). In the phosphatidylinositol signaling system, phosphatidylinositol (PI) can be hydrolyzed into inositol 1,4,5-triphosphate (IP3) and DG by phospholipase C, which transmits extracellular signals to cells (Li et al., 2023a; Wei et al., 2023). This transmits signals from the outside of cells to the inside. In this study, exposure to 3,6-DCCZ caused hepatic DG accumulation in zebrafish. Hepatic DG accumulation may result in lipotoxicity and non-alcoholic fatty liver disease (Gorden et al., 2011). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids in biological membranes and play a crucial role in lipid metabolism and biological health. Previous studies have found that low PC content may lead to lipid droplet accumulation, liver lipid accumulation, and fatty liver disease (Walker et al., 2011). A decrease in PE content in the liver can impair hepatocyte autophagy, which can subsequently lead to morphological damage (Li et al., 2021). In this study, exposure to a low dose of 3,6-DCCZ significantly reduced the levels of PC and PE glycerophospholipids in zebrafish liver, leading to increased DG and TG accumulation. When the production of PC or PE is hindered, excess DG can be converted into TG by DG acyltransferase (Sun et al., 2022b). Lyso-PC (LPC) can trigger the production of mitochondrial reactive oxygen species (mtROS), resulting in inflammation,

mitochondrial damage, and apoptosis (Zhao et al., 2021). Previous studies have shown that the upregulation of hemolytic phospholipids (LPC, LPE) can promote the production of mtROS and induce inflammation, contributing to oxidative stress in lipid metabolism disorders (Li et al., 2021; Liu et al., 2023). In this study, exposure to 3,6-DCCZ significantly upregulated lyso-phospholipids (LPC and LPE) in zebrafish liver, consistent with the pathological changes of inflammatory cell infiltration and hepatocyte swelling. Oxidized lipids are a series of oxidative metabolites produced by spontaneous oxidation or specific enzymes of polyunsaturated fatty acids, playing a critical role in inflammatory response, immune defense, and oxidative stress (Alba et al., 2023). For example, hydroxy eicosatetraenoic acid (HETEs) produced by arachidonic acid (AA) typically has pro-inflammatory effects, and its level is positively correlated with liver function parameters such as alanine aminotransferase (Jurado-Fasoli et al., 2023). Thus, the accumulation of some oxidized lipids in the liver in this study, such as (\pm) 17-HETE and (\pm) 15-HETE, may exacerbate the inflammatory response. Previous studies have shown that exposure to 1.2 mg/L of 3,6-DCCZ could induce excessive ROS production in yellow catfish and lead to histological lesions, such as liver inflammatory infiltration and hepatocyte disarrangement (Li et al., 2023b). Our previous study also demonstrated that exposure to 3,6-DCCZ significantly increased ROS levels in the liver of red crucian carp, leading to lipid peroxidation and DNA damage (Bai et al., 2024).

Sphingolipids are not only the main components of biological membranes but also play an important role in regulating cell division, differentiation, and cell death (Francis et al., 2020). Ceramide (Cer) is an important bioactive sphingolipid that regulates cell differentiation, apoptosis, and autophagy (Hannun and Obeid, 2008). Sphingomyelin (SM) participates in cell proliferation and survival by influencing receptor-mediated signal transduction. The "sphingolipid rheostat" refers to the balance between specific metabolites, including Cer, sphingosine (SPH), and S1P, in sphingolipid metabolism, which controls cellular responses (McGowan et al., 2015). However, the accumulation of Cer and SPH in the livers of zebrafish exposed to 3,6-DCCZ may result from dysregulation of the sphingolipid rheostat. The conversion of SM to Cer is considered a key signaling pathway that promotes and contributes to apoptosis (Liu et al., 2023). In this study, the content of Cer in zebrafish liver increased significantly, while the content of SM decreased significantly after exposure to 3,6-DCCZ, potentially leading to hepatocyte apoptosis. Our previous study observed nuclear pyknosis in the liver of red crucian carp exposed to 3,6-DCCZ, potentially leading to necrosis and apoptosis of fish hepatocytes (Bai et al., 2024). Increasing evidence suggests that sphingolipids and glycerophospholipids maintain a "yin-yang" balance in the liver (Rodriguez-Cuenca et al., 2017). For example, sphingolipids can interfere with phospholipid-mediated signal transduction. Alternatively, sphingolipids regulate phospholipid composition and signal transduction by modulating phospholipase activity. Moreover, SM in sphingolipids and PC in glycerophospholipids interfere with each other to maintain homeostasis. PC depletion can impair SM synthesis and increase Cer content, while SM depletion stimulates PC hydrolysis (Rodriguez-Cuenca et al., 2017). In this study, the decrease in SM and PC may result from crosstalk following exposure to a low concentration of 3,6-DCCZ. However, after exposure to 3,6-DCCZ, there was considerable buildup of glycerophospholipids (LPE, LPC, PA, PG, and PS) and sphingolipids (Cer) in the liver, indicating that their crosstalk disorders led to glycerophospholipid and sphingolipid metabolism dysregulation (Lordan and Blesso, 2023). Consistent with the results of this study, dioxin exposure inhibited fatty acid oxidation in the liver and disrupted the metabolism of glycerophospholipids and sphingolipids, leading to inflammation in humans (Liang et al., 2020).

Amino acids are key building blocks for peptide and protein synthesis in the liver, and changes in their metabolism can serve as valuable indicators for disease detection (Wu et al., 2023). Alanine plays a role in the liver's nitrogen cycle and glucose production (Wang et al., 2017). In

this study, the increase in alanine in the high-dose exposure group may be a response to insufficient ATP levels. Previous studies have shown that branched-chain amino acids (BCAAs) regulate glucose metabolism to provide energy for the body, and their increased content is considered an indicator of tissue damage (Chen et al., 2021b). An increase in valine (a BCAA) was observed in the liver of zebrafish in this study. This may be related to the damage caused by 3,6-DCCZ to the liver, which also supports the idea that energy metabolism was altered. Ornithine has been shown to stimulate the liver urea cycle and tissue protein synthesis (Kokubo et al., 2015). However, the decrease in ornithine content in the liver may cause a disturbance in the urea cycle and lead to liver failure, liver cancer, and other diseases (Bigot et al., 2017). Lysine in the liver can be converted into acetylcarnitine and promote fatty acid oxidation (Jia et al., 2019). However, the increased lysine in the low-dose exposure group indicated that this process was disrupted, which was consistent with the decrease in acylcarnitine content. In the present study, a significant increase in the content of indole-lactic acid was observed in the liver of zebrafish exposed to 3,6-DCCZ. Similar findings have been reported in humans exposed to dioxins (Liang et al., 2020). Indole-lactic acid, a tryptophan metabolite, is considered an AhR ligand that can upregulate eight genes, including *CYP1A1* and *CYP1B1* (Noakes, 2015). *alt*, which encodes alanine aminotransferase, plays a crucial role in the synthesis and degradation of amino acids. As a link between carbohydrate and amino acid metabolism, *alt* has been used as an important indicator of hepatotoxicity (Ma et al., 2023). Furthermore, previous studies have found that an increase in *alt* may serve as a compensatory mechanism for organisms to mitigate drug-induced stress (Mog et al., 2021; Saravanan et al., 2011). The results of this study's upregulation of *alt* gene expression were consistent with liver histopathological changes, further indicating that 3,6-DCCZ could cause liver injury in zebrafish.

The study's data also demonstrated significant activation of phase I (*cyp1a*, *cyp3a65*) and phase II (*ugt1ab*) detoxification genes in the liver of zebrafish following exposure to 3,6-DCCZ. Previous studies have shown that PHCZs activate the aromatic hydrocarbon receptor (AhR) and upregulate the expression of its downstream gene *cyp1a*, thereby inducing dioxin-like toxicity (Ji et al., 2019; Ma et al., 2019). Previous research has shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other AhR activators can effectively activate the *cyp3a65* gene through an AhR2-dependent pathway (Chang et al., 2013; Tseng et al., 2005). Previous studies have shown that 3,6-DCCZ exerts an AhR effect (Ji et al., 2019). Thus, these findings suggest that the toxicity of 3,6-DCCZ in zebrafish liver is similar to that of other dioxin-like compounds (DLCs). Moreover, as phase I detoxification genes, *cyp1a* and *cyp3a65* are also involved in drug metabolism and detoxification (Kubota et al., 2015). Phase I enzymes in the liver metabolize toxic substances upon entry into the organism, forming intermediate products with higher polarity for easier excretion. Phase II enzymes then catalyze the excretion of intermediate products with glucuronic acid or glutathione, thereby achieving detoxification (Wang et al., 2016). Fish accumulate GSH, an important antioxidant, to protect the liver from further damage caused by excessive ROS. The level of ROS in fish liver increases in a dose-dependent manner, activating the expression of oxidative stress markers such as SOD, GSH, and GPX (Liu et al., 2023). Glucuronic acid, an important substrate of UDP-glucuronosyltransferase, and its depletion confirm that 3,6-DCCZ can detoxify the liver by activating the phase II detoxification gene. Therefore, we speculate that stimulation of the zebrafish liver by 3,6-DCCZ activates the liver's self-protection and detoxification mechanisms. Our previous study, which found that the accumulation of 3,6-DCCZ in the liver of golden crucian carp activated GST activity to eliminate excessive ROS and reduce oxidative damage, is consistent with these findings (Bai et al., 2024). Moreover, other DLCs, such as TCDD (Hanioka et al., 1994) and PCBs (Grimm et al., 2015), have also reported similar results.

5. Conclusions

In summary, this study systematically examined the metabolic toxicity of 3,6-DCCZ in the zebrafish liver. The results showed that the BCF in zebrafish liver reached 109.7 after exposure to 3,6-DCCZ, leading to liver damage, including inflammatory cell infiltration, hepatocyte swelling, and disordered liver cord arrangement. Combining metabolomics with the analysis of key metabolic genes, we found that 3,6-DCCZ exposure disturbed the metabolism of carbohydrates, lipids, and amino acids in zebrafish liver. The disruption of lipid metabolism may be the main cause of liver inflammation. Moreover, the activation of detoxification genes in zebrafish liver helped alleviate the damage caused by 3,6-DCCZ. In the future, we plan to analyze the metabolites of PHCZs in zebrafish and infer the main metabolic pathways involved.

CRediT authorship contribution statement

Haoran Meng: Writing – original draft, Formal analysis, Data curation. **Jie Zhang:** Validation, Funding acquisition, Data curation. **Yangzhe Zhu:** Writing – review & editing, Validation. **Yao Bai:** Writing – review & editing, Validation. **Lusheng Zhu:** Writing – review & editing, Resources, Funding acquisition. **Bing Li:** Writing – review & editing, Methodology. **Zhongkun Du:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Jinhua Wang:** Writing – review & editing, Methodology. **Jun Wang:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2025.126739>.

Data availability

Data will be made available on request.

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