Impacts of Diazepam on the Locomotor Activity and Brain Gene Expression of Female Japanese Medaka (Oryzias latipes)

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Impacts of Diazepam on the Locomotor Activity and Brain Gene Expression of Female Japanese Medaka (*Oryzias latipes*)

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Due to its extensive use and high stability in water–sediment systems, diazepam (DZP) has become an emerging pollutant of great concern. The adverse effects of DZP on fish have been well documented, but information on the molecular mechanisms underlying these toxic effects is still lacking. In this study, female Japanese medaka (*Oryzias latipes*) were exposed to DZP (800 ng/L) for 21 days, and responses in their behavior and brain transcriptomic profiles were investigated. With prolonged exposure, DZP significantly reduced the average swimming velocity (on day 21), frequency of active mobility (on day 14 and 21), and cumulative duration of active mobility (on day 14 and 21) in Japanese medaka, suggesting a typical sedative effect. The transcriptome analysis identified 913 differentially expressed genes (DEGs, 374 up–regulated and 539 down–regulated) in Japanese medaka exposed to DZP at 800 ng/L. The enrichment analysis revealed that the DEGs, which have molecular functions related to hormone activity and receptor functions, were significantly enriched in the pathways belonging to environmental information processing, genetic information processing, and cellular processes. Based on the protein-protein interaction (PPI) network, five DEGs were predicted as key driver genes, which may play critical roles in medicating the molecular networks of neuroactive ligand–receptor interaction (*prl*), histidine metabolism (*suox*), and estrogen signaling pathway (*pgr; hsp90aa1.2,* and *foxa3*).

Key words: Diazepam, Behavior, Japanese medaka, RNA sequencing

INTRODUCTION

Diazepam (DZP), which can enhance the activity of gamma–aminobutyric acid (GABA) in the central nervous system, has been widely used to treat anxiety disorders, insomnia, and seizures (Edinoff *et al*., 2021; Riss *et al*., 2008). Due to its extensive use and high stability in water–sediment systems, DZP has become an emerging pollutant of great concern (Cunha *et al*., 2019). Nowadays, DZP contamination has been confirmed in various aquatic ecosystems, including surface water (Davey *et al*., 2022), groundwater (Candela *et al*., 2016), and even drinking water (Zhang *et al*., 2022). Recently, Wilkinson *et al*. (2022) investigated drug contamination in 258 rivers across 104 countries and found that the maximum DZP concentration could reach 850 ng/L.

Previous studies have shown that DZP exposure could induce various adverse effects on the behavior, growth, reproduction, and gene expression in fish (Brandão *et al*., 2013; Wang *et al*., 2024). For instance, Qiu *et al*. (2023) reported that DZP exposure impaired locomotor performance and social behavior of Japanese medaka, and Lorenzi *et al*. (2016) found that exposure to environmental concentrations of DZP had an impact on the number of eggs in the fathead minnow (*Pimephales promelas*). As a typical neuroactive substance, DZP could induce various behavioral abnormalities in fish, even at relatively low concentrations (Chen *et al*., 2021; Qiu *et al*., 2023; Wang *et al*., 2024; Wu *et al*., 2020). Furthermore, it has been reported that there is a difference in the toxic effects of DZP on male and female fish (Genario *et al*., 2020; Qiu *et al*., 2023). For example, female zebrafish have been found to be more sensitive to DZP exposure (Chen *et al*., 2021). Additionally, Vossen *et al*. (2020) reported that female zebrafish showed reduced diving responses to conspecific alarm pheromone after 7 days, while no such effect was observed in males. However, insights into the molecular mechanisms underlying the neurobehavioral toxic effects of DZP on fish are still limited.

Japanese medaka (*Oryzias latipes*) is a widely used model organism for aquatic toxicology (Hong and J. Zha, 2019; Matsumoto *et al*., 2009), and its high–quality genome sequence has been reported (Kasahara *et al*., 2007). In this study, we exposed female Japanese medaka to DZP at 800 ng/L for 21 days and investigated variations in their behavior and brain transcriptomic profile. This study aimed to explore the potential mechanism of neurobehavioral toxic effects of DZP on fish.

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MATERIALS AND METHODS

Test chemicals

DZP (DZP, CAS: 439–14–5; purity: ≥99.8%) was purchased from the Laiyao Biotechnology Co., Ltd. (Beijing, China). Methanol and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Test fish

Japanese medaka (*Oryzias latipes*; 6–month–old; body length = 2.6 ± 0.1 cm; body weight = 0.17 ± 0.02 g.) were obtained from the Aquatic Toxicology Laboratory of *Jiangsu University* (Jiangsu Province, China). The fish were raised in glass tanks with artificial seawater (salinity of 1 ‰; 25 ± 1°C; 14 h–Light :10 h–Dark). The culture water was renewed every 3 days. The fish were fed with Artemia nauplii twice a day at a dosage of 2% of Japanese medaka body weight.

Experimental design

In order to avoid the possible influence of sexual dimorphic responses to DZP exposure (Chen *et al.*, 2021; Qiu *et al.*, 2023), female individuals were used in this study. The exposure concentration of DZP in this study was set at 800 ng/L, which is close to the maximum concentration of DZP detected in river waters (Wilkinson *et al.*, 2022). For the control group, only the artificial seawater was used as the test solution. For each treatment, four female Japanese medaka were gently introduced into 3–L glass beakers containing 2 L of a test solution, with three replicates. The exposure test was conducted for 21 days under the same conditions as described above, except that the DZP solution was renewed every 3 days.

Behavioral assay

On days 7, 14, and 21 of the exposure, the behavioral assay was conducted to test the effects of DZP exposure on fish locomotor activity. For this assay, one fish was introduced into the test box (20 cm × 9 cm × 10 cm) containing 1000 ml artificial seawater ($n = 3$ for each treatment). After a 10–minute adaptation period, the fish locomotion was tracked for 10 minutes using a DanioVision system (Noldus, Wageningen, Netherlands). The behavioral traits were analyzed using EthoVision XT software (Vison 11.5; Noldus), following the method described in our previous study (Qiu *et al.*, 2023). Briefly, the average swimming velocity (ASV, cm/s), the frequency of active mobility (FAM, number/min), and the cumulative duration of active mobility (DAM, percentage of total observation time) was used to measure the locomotor activity of the fish.

Transcriptome analysis

After 21 days of exposure, the fish were placed into an ice water bath (0–4°C, 10 min) for euthanasia (Shi *et al.*, 2023). After measuring body weight and length, fish brains were dissected and used for RNA sequencing (RNA–Seq). The transcriptome analysis was conducted following the method described by Qiu *et al.* (2022), with some modifications. Briefly, the whole brains of four Japanese medaka were pooled as one sample for RNA–Seq analysis ($n = 1$). The cDNA library was constructed following a standardized procedure at the Beijing Genome Institute (BGI; Shenzhen, China) and was sequenced on a BGISEQ–500 platform. Differentially expressed genes (DEGs) were identified using the DEGseq program with criteria of a fold–change ≥ 2 and Q–value ≤ 0.001. The obtained DEGs were used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis. Moreover, the key driver analysis (KDA) was performed to predict key driver genes involved in the protein–protein interaction (PPI) of DEGs by Cytoscape software (v3.9.1).

Statistical analysis

A generalized linear model (GzLM) was used to examine the statistical significance of the effects of DZP at different exposure times on the locomotor behavior of Japanese medaka. All statistical analyses were performed with SPSS Statistics 16.0.

RESULTS

Impacts of DZP exposure on the locomotor activity of Japanese medaka

No mortality was observed during the experiment. Variations in the behavioral traits related to the locomotor activity of Japanese medaka are shown in Fig. 1. As the exposure time prolonged, DZP tended to decrease the locomotor activity in Japanese medaka. Based on the GzLM analysis, only the DZP exposure exhibited significant main effects on the locomotor activity of Japanese medaka (Table 1). After being exposed to DZP for 14 days, Japanese medaka exhibited significantly decreased FAM (to 80% of control, Fig. 1B) and DAM (to 63% of control, Fig. 1C). After being exposed to DZP for 21 days, Japanese medaka exhibited significantly reduced ASV (to 50% of control, Fig. 1A), FAM (to 36% of control, Fig. 1B), and DAM (to 21% of control, Fig. 1C). These results suggest that DZP has a sedative effect on female Japanese medaka.

Brain transcriptional responses in Japanese medaka exposed to 800 ng/L-DZP

Compared with the control group, 913 differentially expressed genes (DEGs) were identified in the 800 ng/L-DZP exposure group. Among the DEGs, the expression of 374 genes was up-regulated, and that of 539 genes was down–regulated. As shown in Fig. 2, the KEGG analysis showed that the DEGs were significantly enriched in the pathways belonging to environmental information processing (i.e., ABC transporters, ECM–receptor interaction, Neuroactive ligand–receptor interaction, PI3K–Akt signaling pathway, and Notch signaling pathway), genetic information processing (i.e., Base excision repair, DNA replication, and Nucleotide excision repair), and cellular processes (i.e., Focal adhesion).

As shown in Fig. 3, the GO analysis showed that the enrichment of DEGs in the molecular function related to hormone activity and receptor functions (i.e., signaling receptor activator activity, receptor ligand activity, receptor regulator activity, and signaling receptor binding).

Based on the PPI network, five DEGs (i.e., *prl, suox, pgr; hsp90aa1.2,* and *foxa3*) were predicted as key driver genes (Fig. 4). Among them, *prl* is involved in the molecular network of neuroactive ligand–receptor interaction; *suox* is involved in the molecular network of histidine metabolism; and *pgr; hsp90aa1.2*, and *foxa3* genes are involved in the molecular network of estrogen signaling pathway. The expression of *prl, suox, hsp90aa1.2*, and *foxa3* was down–regulated, but the expression of *pgr* was significantly up–regulated.

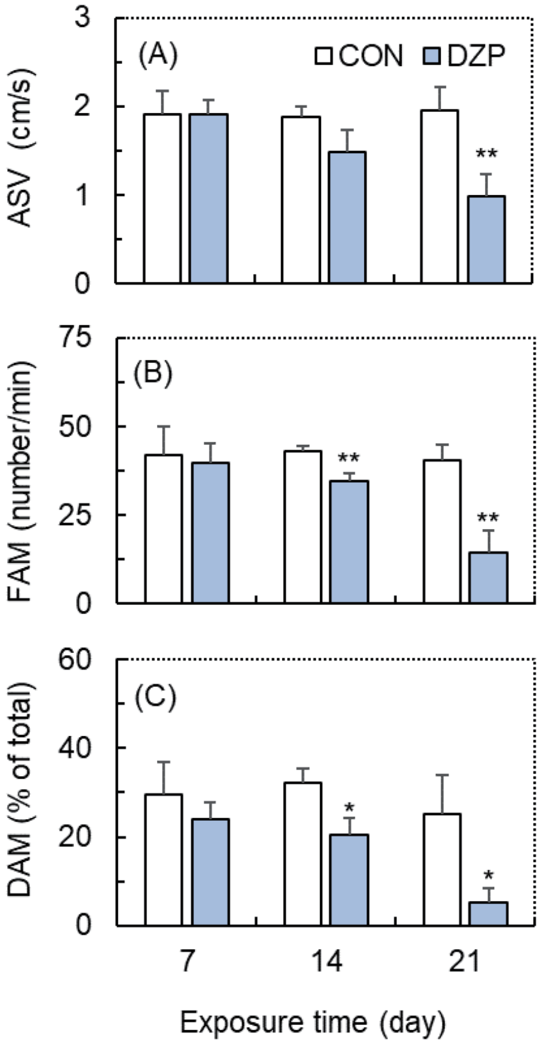

Fig. 1. Variations in the average swimming velocity (ASV, A), frequency of active mobility (FAM, B), and cumulative duration of active mobility (DAM, C) of Japanese medaka (*Oryzias latipes*) exposed to DZP at 0 (control) and 800 ng/L. Data are shown as mean ± SD (n = 3). Statistical significance was in comparison with the respective control (*: $p < 0.05$; **: $p < 0.01$).

Table 1. Summary of a generalized linear model testing the statistical significance of the effects of DZP, exposure time, and their interaction on the locomotor activity of adult female Japanese medaka (*Oryzias latipes*)

Wald Chi–Square	Behavioral traits		
	ASV	FAM	DAM
DZP ($df = 1$)	6.144*	8.081**	8.031**
TIME ($df = 2$)	3.909	5.442	5.649
DZP × TIME ($df = 2$)	5.015	3.102	1.186

*: $p < 0.05$; **: $p < 0.01$

DISCUSSION

Our results demonstrated that chronic exposure to DZP exposure could induce a typical sedative effect on Japanese medaka, as indicated by the significantly reduced ASV, FAM, and DAM with prolonged exposure. Consistent with our findings, the sedative effects of benzodiazepines (BZDs, including DZP) have been well demonstrated in Japanese medaka (Qiu *et al*., 2023; Wang *et al*., 2024) and zebrafish (Chen *et al*., 2021; Pieróg *et al*., 2021; Wu *et al*., 2020). For instance, chronic exposure to DZP at 12 μg/L for 21 days resulted in significant decreases in the ASV and duration of high mobility of female zebrafish (Chen *et al*., 2021), and exposure to DZP at 8.0 μg/L tended to decrease the locomotor activity in adult Japanese medaka Qiu *et al*. (2023). In natural ecosystems, the decreased locomotor activity may minimize encounter rates with predators but often means less feeding, growth, and reproductive chance (Anwar *et al*., 2016; Domenici, 2010).

The transcriptome analysis identified 913 DEGs (374 up–regulated and 539 down–regulated), suggesting a global change in gene expression in female Japanese medaka exposed to DZP. Furthermore, the enrichment analysis revealed that dysregulation in the pathways belonging to environmental information processing, genetic information processing, and cellular processes may be associated with the behavioral impairments induced by DZP.

Our KEGG analysis revealed that the pathways related to nerve signaling, i.e., ECM–receptor interaction, PI3K–Akt signaling pathway, and neuroactive ligand–receptor interaction, were dysregulated by DZP exposure. The extracellular matrix (ECM) is a noncellular component of tissue that biochemically and structurally supports cells, and the ECM–receptor interaction plays various critical roles in regulating neuronal structure, synaptic plasticity, and behavior (Meghan *et al*., 2014). The PI3K–Akt signaling pathway is also pivotal in governing neuronal survival, differentiation, and plasticity (Zhou *et al*., 2014). Furthermore, the pathway of neuroactive ligand–receptor interaction, which plays an important role in the regulation of neuron function through modulating transcription factors and gene expression (Xu *et al*., 2012), was dysregulated by DZP exposure. The *prl*, which encodes prolactin, was predicted as the key driver gene involved in the network of neuroactive ligand–receptor interaction. Prolactin plays an important role in many brain functions, including the regulation of complex behaviors (Grattan, 2001).

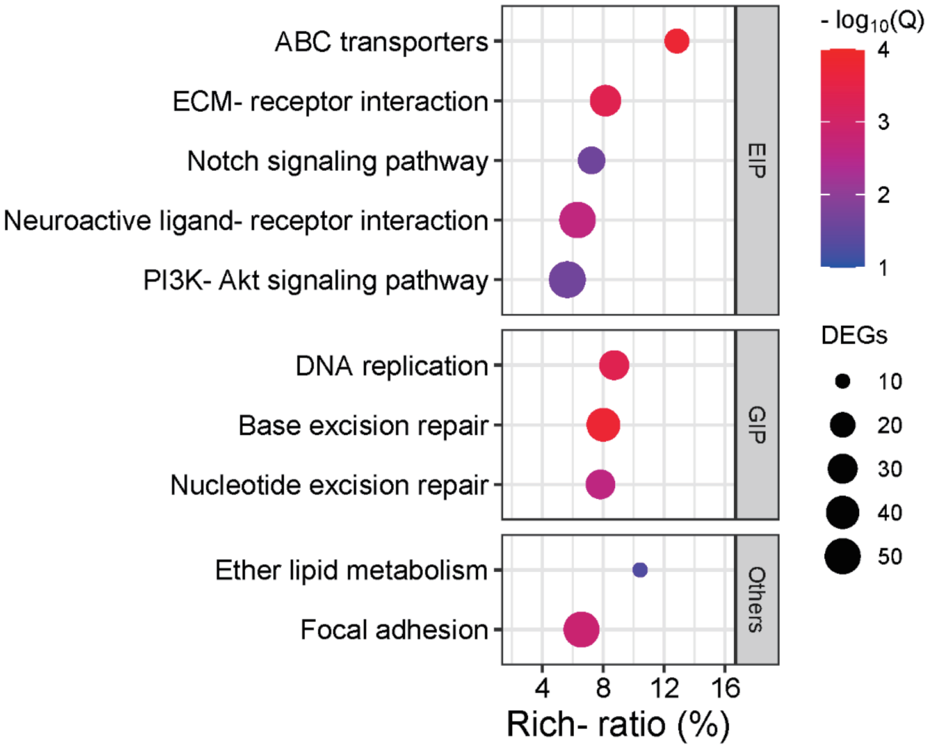

Fig. 2. KEGG enrichment analysis of differentially expressed genes (DEGs). The top 10 (a ranking by Q–value) enriched KEGG terms involved in Environmental Information Processing (EIP), Genetic Information Processing (GIP), and Other categories (Others; Cellular Processes and Metabolism) were displayed, respectively.

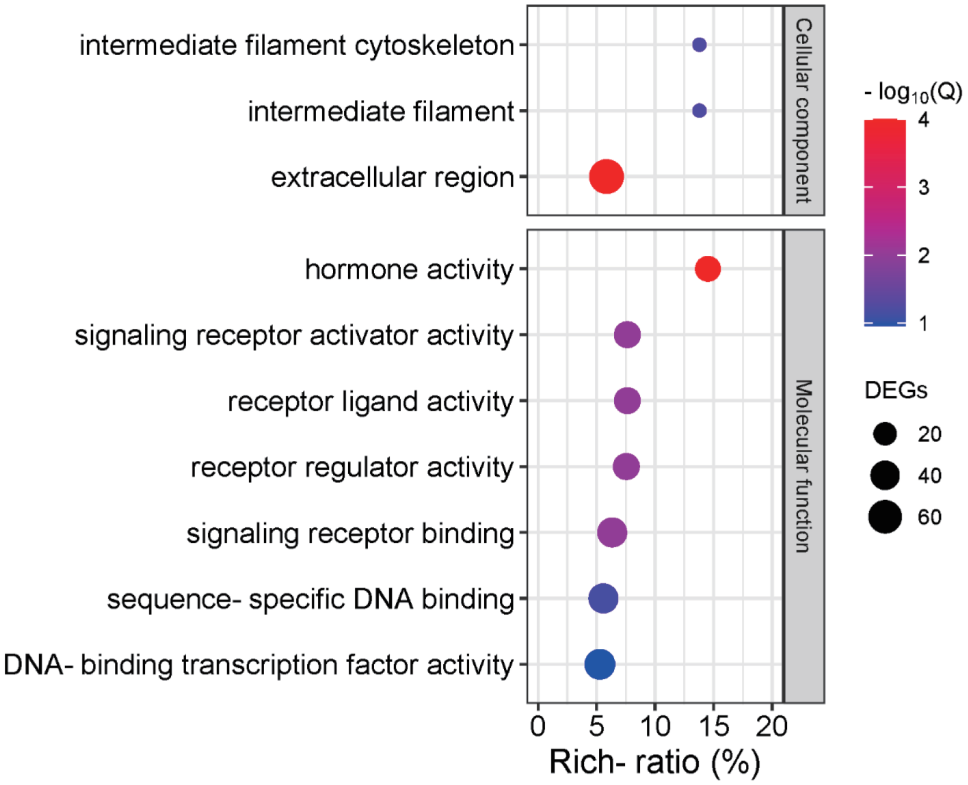

Fig. 3. GO enrichment analysis of differentially expressed genes (DEGs). The top 10 (a ranking by Q–value) enriched GO terms were displayed, respectively.

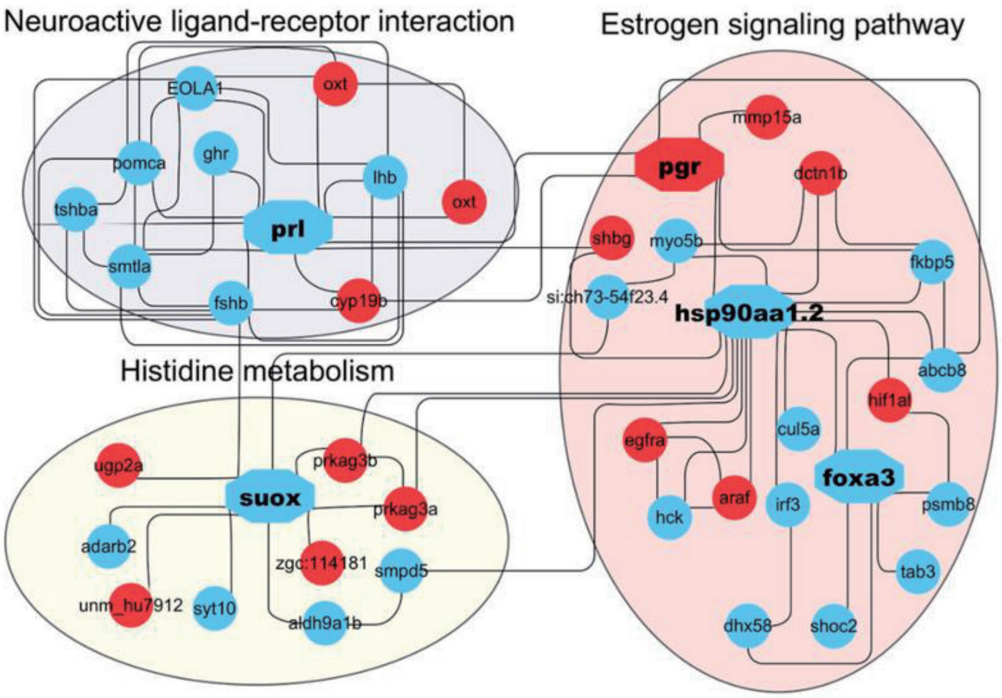

Fig. 4. The protein–protein interaction network of key driver genes (octagon symbols) and related DEGs (red color: up–regulated; blue color: down–regulated).

On the other hand, DZP exposure might affect the molecular network related to histidine metabolism, with *suox* (encoding sulfite oxidase) as the key driver gene. Under the catalysis of histidine decarboxylase, histidine can be decarboxylated to histamine, which plays an essential role in the growth and neuron development of fish species (Brosnan and Brosnan, 2020; Panula *et al.*, 2022). Thus, we infer that the dysregulation of those pathways induced by DZP exposure may affect the neuronal functions in Japanese medaka and thereby impair their locomotor behaviors.

Our results also revealed that DZP exposure could affect the molecular network of the estrogen signaling pathway, which can mediate many physiological processes by regulating transcriptional processes (Björnström and Sjöberg, 2005). As a category of sex hormone, estrogens play important roles in fish sexual plasticity, sex differentiation, and various aspects of reproduction (Nelson and Habibi, 2013). Thus, our findings implied that DZP may exhibit endocrine disrupting effects on Japanese medaka to some extent. Moreover, the estrogen signaling pathway also plays an important role in mediating the functions of nervous and sensory systems in fish (Brann *et al.*, 2022; Cohen *et al.*, 2022; Coumailleau *et al.*, 2015).

Three DEGs, *pgr* (up–regulated), *hsp90aa1.2* (down–regulated), and *foxa3* (down–regulated), were predicted as the key driver genes that may be involved in the estrogen signaling pathway. As a member of the steroid hormone receptor family (Grimm *et al.*, 2016), the progesterone receptor (encoded by *pgr*) plays a central role in reproductive processes associated with the establishment and maintenance of pregnancy (Fang *et al.*, 2018). As a member of the HSP90 family (Chen *et al.*, 2005), heat shock protein 90 alpha family class A member 1.2 (encoded by *hsp90aa1.2*) plays a key role in cell cycle progression, cell polarization, and cell migration (Pfeiffer *et al.*, 2018). Meanwhile, the hepatocyte nuclear factor 3–gamma (encoded by *foxa3*) is expressed early in the embryonic endoderm and plays an important role in regulating gene expression in the liver (Burtscher and Heiko, 2009; Friedman and Kaestner, 2006).

In summary, our results demonstrated that chronic exposure to DZP exhibited a typical sedative effect on female Japanese medaka. Based on the results of transcriptome analysis, DZP exposure could dysregulate the expression of genes involved in the pathways associated with neurodevelopment and neurotransmission, which may contribute to its neurobehavioral toxicity to Japanese medaka. Moreover, our findings also suggested that exposure to DZP has the potential to induce endocrine disrupting effects on Japanese medaka, which may further adversely affect their reproduction. Therefore, future studies are needed to examine the reproductive and multi–generational toxic effects of DZP on fish to assess its ecological risk in aquatic ecosystems better.

AUTHOR CONTRIBUTIONS

X. QIU designed the study, wrote the paper, and provided facilities and resources. M. ZHUO and Y. SHI performed the exposure test, analyzed the data, and wrote the paper. K. CHEN designed the study, wrote the paper, and provided facilities and resources. Y. TAKAI,

Y. SHIMASAKI, and Y. OSHIMA designed the study, wrote the paper, and provided resources. All authors assisted in editing the manuscript and approved the final version.

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