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Time-Series Responses in Behaviors of Male Zebrafish to Short-Term Cetylpyridinium Chloride Exposure

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Cetylpyridinium chloride (CPC) is a cationic surfactant that has been widely used in pharmaceutical and personal care products. Due to its widespread use, high CPC residues have been widely detected in surface waters, but knowledge of its toxicity to fish remains scarce. Therefore, this study exposed adult male zebrafish to CPC (0, 40, and 80 $\mu\text{g/L}$) for 96 h, then transferred them into CPC-free water to recover for 168 h. Time-Series variations in behavioral traits of zebrafish and neurotransmitter levels in their eyes were investigated. The behavioral assay showed that the 96-h CPC exposure significantly altered the locomotor activity and exploratory behavior, while the subsequent 168-h recovery treatment still could not completely eliminate these adverse effects. The short-term CPC exposure also induced persistent effects on the levels of serotonin (5-HT), dopamine (DA), and norepinephrine (NE) in the eyes of zebrafish. Correlation analysis revealed that those neurotransmitters might play important roles in mediating the time-series behavioral response to CPC. Our findings demonstrated that CPC exposure could cause persistent abnormal behavior in zebrafish *via* disturbing neurotransmitters in their eyes, even at environmentally relevant concentrations, and thus highlighted the necessity for further assessing its potential risks to aquatic ecosystems.

Key words: Behavior; Cetylpyridinium Chloride; Neurotransmitter; Time-Series responses; zebrafish

INTRODUCTION

Cetylpyridinium chloride (CPC, $\text{C}_{21}\text{H}_{38}\text{ClN}$), a cationic surfactant, is widely used as an antibacterial ingredient in mouthwashes, toothpaste, and other personal care products and cosmetics (McDonnell and Russell 1999; Costa *et al.*, 2013). This surfactant is also used as a disinfectant in swimming pools and public places (Imai *et al.*, 2017). In addition, CPC has been accepted for use in food processing to fight against microbial contamination (Saucedo-Alderete *et al.*, 2018). Due to its widespread application, CPC can enter the wastewater treatment plants (WWTPs) or directly be discharged into the natural environment (Shrivastava and Wu 2007; Russo *et al.*, 2019). Furthermore, CPC has also been used to remove reactive dyes, phenols, and other organic solutes from sewage or treated wastewater (Park *et al.*, 2016). Although more than half of CPC could be removed by biodegradation and adsorption, CPC residues could still be detected up to 52 $\mu\text{g/L}$ in surface water samples (Shrivastava and Wu 2007). Therefore, aquatic organisms near the sewage treatment plants may be temporarily exposed to high concentrations of CPC due to periodic emissions from wastewater treatment plants (Vieno *et al.*, 2005; Park *et al.*, 2016).

Previous studies have shown that exposure to CPC

may adversely affect the growth, development, reproduction, and oxidative stress of aquatic species (Bhattacharya *et al.*, 2021; Bhattacharya *et al.*, 2022; Qiu *et al.*, 2022b). For example, exposure to CPC at 1.5 μM significantly reduced the growth of frog embryos (*Bombina orientalis*) and increased developmental abnormalities (Park *et al.*, 2016). Even at much lower concentrations (2 and 6 $\mu\text{g/L}$), CPC adversely affected the oxidative stress and antioxidant enzymes in the liver of the carp, *Cyprinus carpio* (Bhattacharya *et al.*, 2022). Recent studies found that CPC exposure could also strongly affect the behaviors of aquatic organisms. For example, Bhattacharya *et al.* (2019) reported that exposure to CPC at 150 $\mu\text{g/L}$ could induce hyperactivity and decreased clumping tendency in the sludge worm (*T. tubifex*). Our previous study found that CPC at 4 and 40 $\mu\text{g/L}$ could induce oxidative stress and abnormal behavior in larval zebrafish (Qiu *et al.*, 2022b). However, insight into the mechanisms underlying the behavioral effects of CPC on fish is still scarce.

As an integration product of sensory, hormonal, neural, and metabolic systems, behavioral responses are important endpoints when considering the toxicological impacts of aquatic pollutants on organisms (Scott and Sloman 2004; Sismeiro-Vivas *et al.*, 2007). Generally, abnormalities in neurotransmitter release and/or abnormal levels of extracellular neurotransmitter concentrations play important roles in causing abnormal behaviors (Sarter *et al.*, 2007). For example, abnormally low and high levels of cholinergic neurotransmission were associated with dementia and the impairments in filtering irrelevant stimuli in schizophrenia (Mesulam 2004; Sarter and Parikh 2005). The loss of serotonin and norepinephrine was associated with developing depressive behavior

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(Delgado and Moreno 2000). Dopaminergic transmission plays a critical role in developing anxiety (Kacprzak *et al.*, 2017). In teleosts, stress-induced abnormalities in neurotransmitters have been proved to play important roles in mediating their behavioral responses (Qiu *et al.*, 2020; Wu *et al.*, 2020; Chen *et al.*, 2021a). Nevertheless, no previous studies have focused on the behavioral responses to short-term CPC exposure, let alone examined the roles of neurotransmitters in those processes.

Zebrafish (*Danio rerio*) is a model species that has been widely used to assess the toxicological effects of chemicals (Chen *et al.*, 2021b; Qiu *et al.*, 2022a). Therefore, in this study, we exposed zebrafish to sublethal CPC for 96 h and then transferred the fish into clean dechlorinated tap water to recover for 168 h. Time-series variations in behavioral traits of zebrafish and neurotransmitter levels in their eyes were investigated. This study aimed to examine the recovery patterns of behavioral responses in zebrafish to short-term CPC exposure, and investigate the possible links between several eyes neurotransmitters and behavioral responses.

MATERIALS AND METHODS

Chemical

Cetylpyridinium Chloride (CAS No. 123-03-5) was purchased from the Shanghai Yien Chemical Technology Co., Ltd (Shanghai, China). Other used reagents at analytical grade were purchased from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The enzyme-linked immunosorbent assay (ELISA) kits for assaying serotonin (5-HT), dopamine (DA), and norepinephrine (NE) were purchased from the Bomei Biotechnology Co., Ltd (Hefei, Anhui, China).

Test Organisms

Male zebrafish (AB Strain; nine months after hatching) used in this study were maintained in our laboratory for over four months. They were maintained in several 16-L glass aquariums (28 cm diameter and 30 cm height) containing 12-L dechlorinated tap water (conductivity at 0.50–0.53 mS/cm) at $27 \pm 1^\circ\text{C}$ and cultured under light: darkness=14:10 h cycles. Zebrafish were fed with newly hatched *Artemia nauplii* twice a day, and the water was renewed every two days.

Exposure and Recovery Experiment

The test solutions were prepared by pipetting calculated amounts of the CPC stock solution (40 mg/L) into dechlorinated tap water, with the final concentrations of 0 (control), 40, and 80 $\mu\text{g/L}$. For the exposure experiment, 12 healthy zebrafish were selected from each concentration group and placed in a 3-L glass circular aquarium containing 2 L of test solution ($n=3$). After the 96-h exposure, zebrafish were transferred to spawning boxes (20×9×10 cm, Aqua Schwarz GmbH, Goettingen, Germany) containing 1 L of dechlorinated tap water to recover for 168 h. Behavioral traits were assayed at the end of the 96-h exposure (i.e., R-0) and after 24 (R-24), 48 (R-48), 96 (R-96), and 168 (R-168) h of the

recovery treatment. At the end of the exposure treatment (i.e., R-0) and recovery treatment (i.e., R-168), 2 fishes from each tank were randomly selected and placed into an ice bath (0–4°C, 10 min) for euthanasia. Subsequently, the eyes of zebrafish were extracted and stored at -80°C until the neurotransmitter analysis.

Behavioral assay

The behavioral assay was conducted from 09:00 to 12:00 AM (China standard time) to minimize the possible effects of diel periodicity. For this assay, two zebrafish were randomly selected and placed in an aquarium (20×9.5×10 cm) containing 1000 mL of dechlorinated tap water in the exposure experiment. After 10-min acclimation, fish locomotion was tracked for 15 min using the DanioVision system (Noldus, Netherlands). The behavioral traits were then analyzed using EthoVision XT software (Vision 11.5; Noldus). The locomotor activity was judged based on the threshold values reported by Chen *et al.* (2021b). An area with a size of 14×4.8 cm located in the center of the aquarium was defined as the central zone, and the fish behavior was considered exploratory when they swam in the central zone (Qiu *et al.*, 2017).

Eye neurotransmitter levels

The levels of serotonin (5-HT), dopamine (DA), and norepinephrine (NE) in the eyes were measured using the corresponding ELISA kits (Bomei Biotechnology Co., Ltd.). The homogenate and supernatant were prepared following the method described by Qiu *et al.* (2022b). The supernatant of each sample was used for biochemical assays, following the manufacturer's instructions for the corresponding ELISA Kit. The protein concentration in each supernatant was also measured using a BCA Kit (Bomei Biotechnology Co., Ltd.), and brain neurotransmitter levels were normalized to per mg protein (mg-protein).

Statistical Analysis

A generalized linear model (GzLM) was employed to analyze the combined effects of CPC and sampling time on the behavioral traits and the neurotransmitter levels. Following the GzLM analysis, a simple effects analysis was conducted to examine the difference between exposure and control within either time point. For the correlation analysis, data were first normalized to percentages respective to the control, and the correlations of neurotransmitter levels with those of behavioral traits were tested for significance using Spearman's correlation analysis. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Time-series responses in swimming velocity

Time-series responses in the average swimming velocity (ASV) and maximum swimming velocity (MSV) of zebrafish are shown in Fig. 1. The GzLM analysis showed that CPC and recovery time (RT) exhibited sig-

nificant main effects on the ASV of zebrafish, and their interaction exhibited significant effects on both ASV and MSV of zebrafish (Table 1). Compared with the control group, the 96-h CPC exposure to 40 and 80 CPC did not significantly affect the ASV of zebrafish (Fig. 1A). However, in the 40- $\mu\text{g/L}$ CPC group, zebrafish exhibited significantly higher ASV on R-48 and R-168 (vs. control, Fig. 1A). In the 80- $\mu\text{g/L}$ CPC group, zebrafish exhibited significantly higher ASV on R-168 (vs. control, Fig. 1A), and significantly lower MSV on R-0 (vs. control, Fig. 1B).

Time-series responses in locomotor activity

As shown in Fig. 2, the 96-h CPC exposure tended to decrease the frequency of high mobility (FHM) and duration of high mobility (DHM) of zebrafish on R-0, but increased their locomotor activity during the recovery period. The GzLM analysis also showed that CPC, RT,

and their interaction exhibited significant effects on FHM and DHM of zebrafish (Table 1). In the 40- $\mu\text{g/L}$ CPC group, significantly increased FHM and DHM were observed on R-48 and R-168 (vs. control, Fig. 2). In the 80- $\mu\text{g/L}$ CPC group, the FHM and DHM of zebrafish significantly decreased R-0, but significantly increased on R-168 (vs. control, Fig. 2).

Time-series responses in exploratory behavior

As shown in Fig. 3, the recovery patterns of exploratory behavior to the 96-h CPC exposure were complex. The GzLM analysis revealed that the frequency of exploratory behavior (FEX) was significantly affected by CPC and RT, and the duration of exploratory behavior (DEX) was significantly affected by CPC and the interaction (Table 1). In the 40- $\mu\text{g/L}$ CPC group, zebrafish exhibited significantly higher FEX on R-24 and R-168,

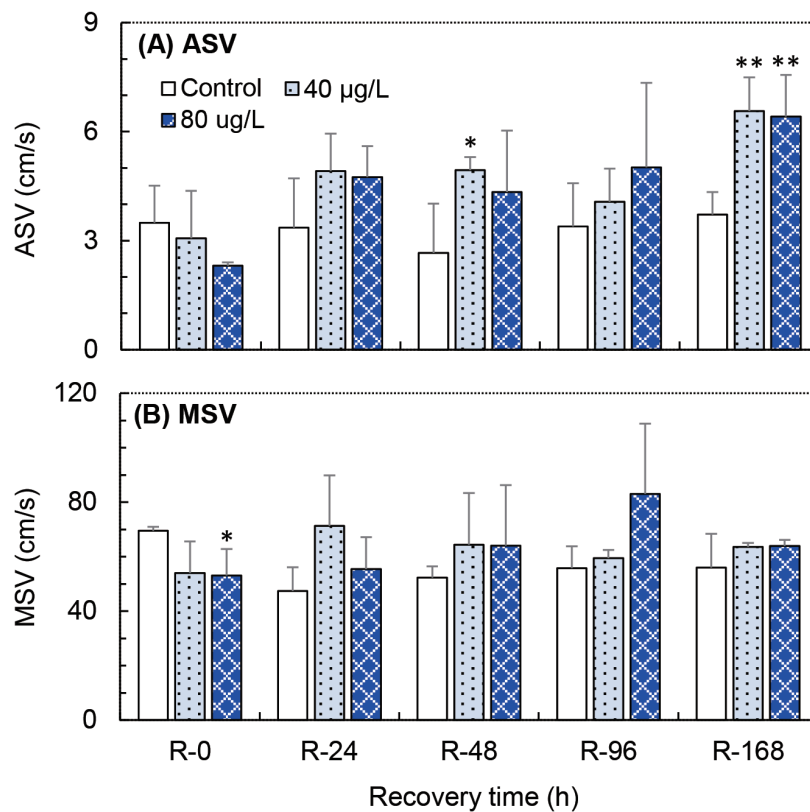


Fig. 1. Time-series responses in the average swimming velocity (ASV, A) and maximum swimming velocity (MSV, B) of zebrafish (*Danio rerio*) pre-exposed to cetylpyridinium chloride at 40 and 80 $\mu\text{g/L}$. Data were obtained immediately after the 96-h exposure (i.e., R-0), and after the subsequent 24, 48, 96, and 168 h recovery period (i.e., R-24, R-48, R-96, and R-168, respectively). Data are mean \pm SE ($n=3$), and asterisks indicate significant differences (vs. the control at the same time point; * $p<0.05$; ** $p<0.01$).

Table 1. Summary of generalized linear model testing the statistical significance for the effect of cetylpyridinium chloride (CPC), sampling time (RT), and their interaction (CPC \times RT) on the behavioral parameters of zebrafish (*Danio rerio*)

Variables (df) ^a	ASV	MSV	FHM	DHM	FEX	DEX
CPC (df=2)	10.5**	3.2	16.6**	13.3**	9.6**	8.7**
RT (df=4)	26.4**	1.4	54.7**	51.9**	32.1**	0.6
CPC \times RT (df=8)	17.2*	17.9*	44.6**	29.6**	15.1	75.0**

^a Wald Chi-Square is listed; df: degree of freedom; asterisks indicate statistical significance (* $p<0.05$; ** $p<0.01$).

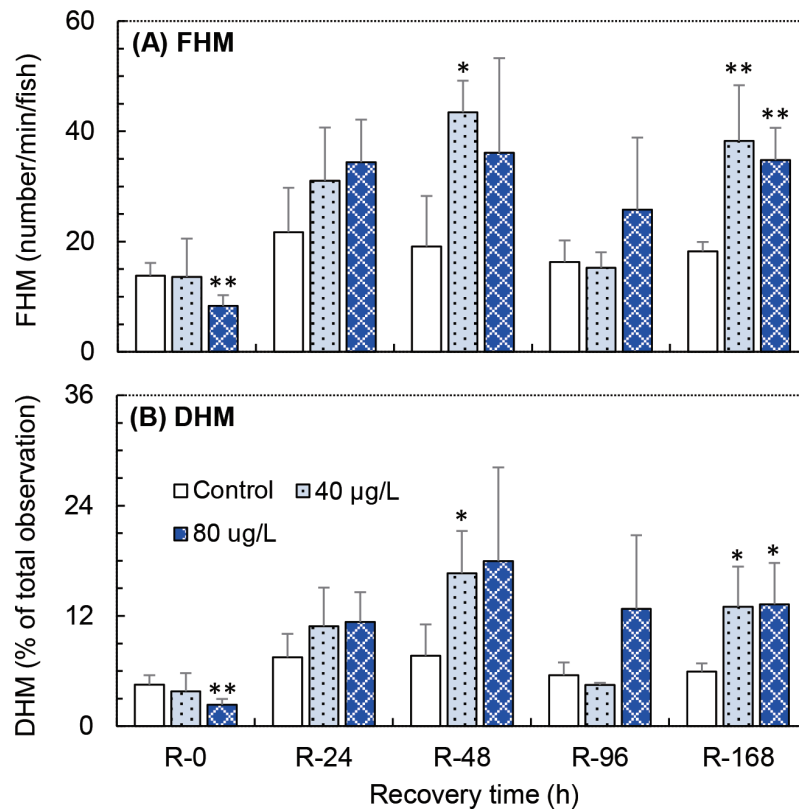


Fig. 2. Time-series responses in the frequency of high mobility (FHM, A), and duration of high mobility (DHM, B) of zebrafish (*Danio rerio*) pre-exposed to cetylpyridinium chloride at 40 and 80 µg/L. Data were obtained immediately after the 96-h exposure (i.e., R-0), and after the subsequent 24, 48, 96, and 168 h recovery period (i.e., R-24, R-48, R-96, and R-168, respectively). Data are mean ± SE ($n=3$), and asterisks indicate significant differences (vs. the control at the same time point; * $p<0.05$; ** $p<0.01$).

Table 2. Spearman correlation coefficients between neurotransmitter levels and behavioral traits of zebrafish (*Danio rerio*) exposed to cetylpyridinium chloride^a

	ASV	MSV	FHM	DHM	FEX	DEX
Serotonin	-0.324	-0.461	-0.437	-0.468	-0.134	0.387
Dopamine	-0.482*	-0.344	-0.507*	-0.493*	-0.187	0.536*
Norepinephrine	-0.673**	-0.455	-0.771**	-0.743**	-0.624**	0.286

^a Data were obtained at the end of the 96-h exposure and the 168-h recovery treatment ($n=18$), and asterisks indicate statistical significance (Spearman's correlation; * $p<0.05$, ** $p<0.01$).

significantly higher DEX on R-0 and R-48, but significantly lower DEX on R-168 (vs. control, Fig. 3). In the 80-µg/L CPC group, zebrafish exhibited significantly higher FEX on R-24 and R-168, significantly higher DEX on R-0 and R-168, but significantly lower DEX on R-96 (vs. control, Fig. 3).

Responses in the eyes levels of neurotransmitters

As shown in Fig. 4, the 96-h CPC exposure tended to increase the neurotransmitter levels in the eyes of zebrafish on R-0 but increased them on R-168. In the 40-µg/L CPC group, zebrafish exhibited significantly higher DA levels on R-0 (vs. control, Fig. 4B), and significantly lower 5-HT and DA levels on R-168 (vs. control, Fig. 4A and B). In the 80-µg/L CPC group, zebrafish

exhibited significantly lower 5-HT, DA, and NE levels on R-168 (vs. control, Fig. 4). The results of Spearman's correlation analysis are shown in Table 2. The levels of 5-HT exhibited no significant correlation with the behavioral traits of zebrafish. The DA level was significantly negatively correlated with the ASV, FHM, and DHM of zebrafish and significantly positively correlated with the DEX of zebrafish. The NE level exhibited significant negative correlations with the ASV, FHM, DHM, and FEX of zebrafish.

DISCUSSION

Our results demonstrated that the 96-h CPC exposure (40 and 80 µg/L) could significantly alter the loco-

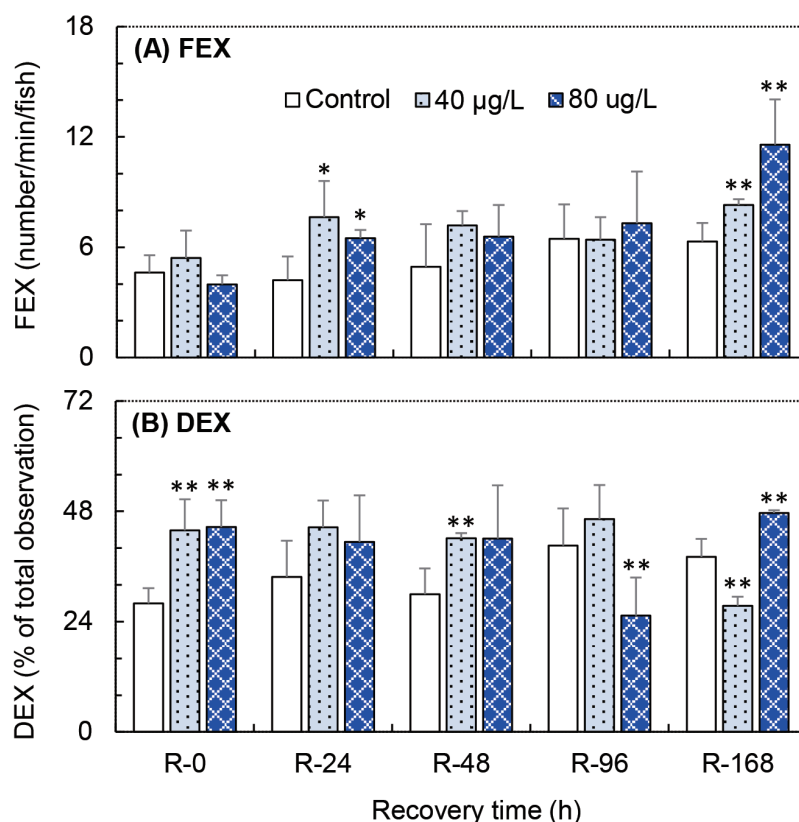


Fig. 3. Time-series responses in the frequency (A) and duration (B) of the exploratory behavior of zebrafish (*Danio rerio*) pre-exposed to cetylpyridinium chloride at 40 and 80 µg/L. Data were obtained immediately after the 96-h exposure (i.e., R-0), and after the subsequent 24, 48, 96, and 168 h recovery period (i.e., R-24, R-48, R-96, and R-168, respectively). Data are mean ± SE ($n=3$), and asterisks indicate significant differences (vs. the control at the same time point; * $p<0.05$; ** $p<0.01$).

motor activity and exploratory behavior, while the subsequent 168-h recovery treatment still could not completely eliminate these adverse effects. Using a similar experimental design, Qiu *et al.* (2022a) found that exposure of zebrafish to AMI (at 40 µg/L) for 7 days reduced the locomotor activity of zebrafish, which still can be observed after 21 days recovery period. However, Qiu *et al.* (2017) reported that acute sublethal exposure to 24 µg/L chlorpyrifos resulted in hyperactivity and anxiety in Japanese medaka (*Oryzias latipes*), but the fish recovered from these abnormal behavioral traits within 7 days of recovery treatment. Floyd *et al.* (2008) also found that abnormal swimming behavior of fathead minnow (*Pimephales promelas*) induced by short-term pyrethroid insecticide exposure larvae could recover to normal within 1–2 days. Thus, the time-series responses in fish behaviors to short-term pollutant exposure seem to be determined by the species, chemical types, exposure time, and other factors.

Nevertheless, the behavioral impairments induced by pollutants may have potential ecological consequences (Brodin *et al.*, 2014; Jolles *et al.*, 2017). For example, locomotor activity integrates physiological abilities that enable the fish to generate and coordinate the energy needed for basic functions such as migrating or avoiding predators (Gaworecki and Klaine 2008; Eisenreich and Szalda-Petree 2015). Increased explora-

tory behavior may increase feeding efficiency, but staying in the area without shelter for a long time may also increase the risk of prey vulnerability (Qiu *et al.*, 2017; Jenkins *et al.*, 2021). Considering that CPC could be detected at up to 52 µg/L in surface water samples which is sufficient to cause behavioral abnormalities in zebrafish, the behavioral impacts of CPC on fish should not be ignored when assessing its potential risks (Shrivastava and Wu 2007). However, insight into the mechanisms underlying the behavioral effects of CPC on fish is still scarce.

Persistent behavioral impairment induced by neurotoxic pollutants to the teleosts has been previously reported, usually accompanied by varied responses in neurotransmitter levels (Qiu *et al.*, 2017; Wu *et al.*, 2020). Our result showed that the short-term CPC exposure also induced persistent effects on 5-HT, DA, and NE levels in zebrafish. These neurotransmitters play an important role in various disorders, including depression and anxiety in the nervous system (Delgado and Moreno 2000; Mohammad-Zadeh *et al.*, 2008; Kacprzak *et al.*, 2017). For example, Li *et al.* (2021) found that at the acceptable daily intake (ADI) levels (0, 1, 10, and 100 mg/L), exploratory profiles of adult zebrafish were remarkably impaired by APM, while the level of 5-HT in the brain decreased and the level of NE increased. Exposure to high concentrations of Se (as

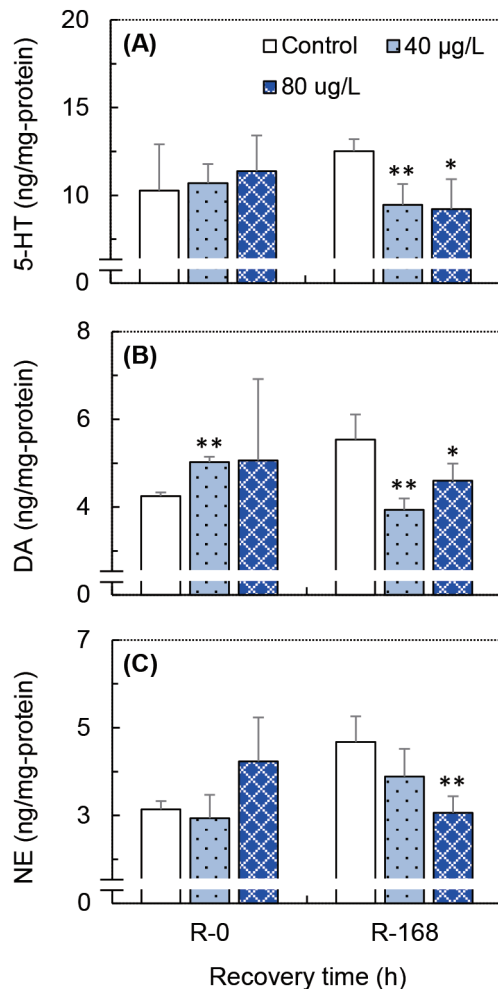


Fig. 4. Responses in the serotonin (5-HT; A), dopamine (DA; B), and norepinephrine (NE; C) in the eyes of zebrafish pre-exposed to cetylpyridinium chloride at 40 and 80 µg/L. Data were obtained immediately after the 96-h exposure (i.e., R-0), and after the subsequent 168 h recovery period (i.e., R-168). Data are mean ± SE ($n=3$), and asterisks indicate significant differences (vs. the control at the same time point; * $p < 0.05$; ** $p < 0.01$).

Selenomethionine) impaired social and anti-predation behaviors of zebrafish, and variations in mRNA expression of important genes associated with serotonin were detected (Attaran *et al.*, 2019). In addition, the increased anxiety behavior and the downregulation of key genes that function in the 5-HT and DA neurotransmitter systems were detected in the brain of adult male zebrafish exposed to tributyltin (Tu *et al.*, 2020). After alcohol exposure, the behavioral response to predators was enhanced, and the levels of 5-HT and DA and their metabolites changed significantly in zebrafish (Gerlai *et al.*, 2008; Chatterjee and Gerlai 2009).

Nevertheless, previous studies on neurotransmitters and behavioral response are mainly focused on brain tissue, while there is less discussion on the link between neurotransmitters and behavioral response in other tissues such as eyes. According to Qiu *et al.* (2017), AChE may be more sensitive to CPF exposure in the eyes than

in the brain. Moreover, the activity of AChE in the eyes of European Eel (*Anguilla anguilla*) exposed to organophosphorus insecticides was higher than that in brain tissue (Ceron *et al.*, 1996). Similarly, the AChE activity in *A. anguilla* eyes exposed to carbamate herbicide was significantly inhibited and did not return to control levels after one week of recovery in clear water (Sancho *et al.*, 2000). Our correlation analysis revealed that the DA and NE levels in fish eyes were associated with CPC-induced hyperactivity and excessive exploratory behavior. CPC may combine with the anionic charge on the cell surface to interfere with neuronal electrical activity as a cationic surfactant and damage synaptic transmission. Therefore, we infer that the eye monoamine neurotransmitters may serve as an important modulator in the behavioral responses in male zebrafish to CPC, but further studies are necessary to establish the exact mechanisms.

AUTHOR CONTRIBUTIONS

L. LIU and C. CHEN performed the exposure test, analyzed the data, and wrote the paper. K. CHEN, Y. SHI, and X. QIU performed the behavioral experiments and participated in the data analysis. X. QIU designed the study, supervised the work, wrote the paper, and provided facilities and resources. 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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