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Impacts of Diazepam on the Survival, Development, and Response to Light Stimulation in Early–life stages of Zebrafish (*Danio rerio*)

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Environmental residues of diazepam (DZP) may pose potential risks to aquatic species, especially to fishes. This study exposed zebrafish embryos to DZP (0, 0.4, 4.0, 40, 400, 4000 μg/L) until 120 hours post–fertilization (hpf) and examined their survival, development, hatching, and behavioral responses to light–dark transition. Exposure to DZP at 4000 μg/L significantly reduced the survival of zebrafish, and the 96 h–LC_{50} for DZP was calculated as 3202.4 (3068.2–3555.6) μg/L. Exposure to DZP at 4, 40, and 400 μg/L could induce bradycardia in zebrafish embryos on 48 hpf and significantly delay their hatching time. Under the cycle of light–dark transition, zebrafish larvae (120 hpf) in all groups were more active in the dark period than the illumination period. However, exposure to DZP at ≥0.4 μg/L could evoke an anti–anxiety effect in the illumination period and increase their sensitivity to the conversion of light and dark.

Key words: Behavioral responses, Diazepam, Early–life stages of zebrafish, Time–to–death, Time–to–hatching

INTRODUCTION

Benzodiazepines (BZDs) are the most widely used prescribed psychoactive drugs with anxiolytic, sedative, and hypnotic effects (Calisto & Esteves 2009). The BZDs are designed to induce temporary changes in mood, perception, and behavior via binding to the γ–aminobutyric acid (GABA) receptors in the human central nervous system, which are shared among a wide variety of animals (Haefely *et al*. 1990; Cunha *et al*. 2017; Moore & Mattison 2017). On the other hand, BZDs may be excreted along with their metabolites or liberality disposal as the expired drug, and finally convergent with the sewage (Ebele *et al*. 2017). Although those drugs can be degraded in wastewater treatment plants (WWTP) by biodegradation, photodegradation, and chlorination, they cannot be eliminated entirely and finally be emitted into natural aquatic ecosystems (Kosjek *et al*. 2012; West & Rowland 2012).

Diazepam (DZP), one of the most widely used BZDs, has become a common emerging pollutant detected in various aquatic environments (Wu *et al*. 2015; Cunha *et al*. 2017; Lei *et al*. 2021). It is reported that the residual concentrations of DZP can reach 4.0 ug/L in some WWTP influents or effluents (Cunha *et al*. 2017; Lei *et al*. 2021), and can still be up to 0.88 ug/L in some surface waters (Ternes 1998; Ternes *et al*. 2001). Although the environmental level of BZDs (e.g., DZP and its metabolites) may not cause acute lethal effects on aquatic species, their sublethal toxicity should not be ignored. For example, it has been reported that BZDs could alter various behavioral traits of aquatic organisms, such as feeding behavior (Brodin *et al*. 2013), social behavior (Cerveny *et al*. 2020; Wu *et al*. 2020), and reproduce behavior (Chen *et al*. 2021).

Behavior can reflect the most real changes in the individual level relevant to feeding, growth, and survival (Spence *et al*. 2008; Qiu *et al*. 2020a). Many studies have demonstrated that changes in behavior are represented early responses of mammals exposed to BZDs (Brodin *et al*. 2014). For example, Choleris (2001) reported that mice exposed to DZP tended to be less anxious and reduce stressed behaviors. In recent years, zebrafish (*Danio rerio*), a typical model for toxicology, has also been widely used to screen for the behavioral toxicity of BDZs. For example, Wu *et al*. (2020) found that acute exposure to DZP exhibited concentration–specific impacts on behavioral traits of juvenile zebrafish, and Chen *et al*. (2021) reported that chronic exposure to sublethal DZP had sedative effects on adult zebrafish, with sex–dependent behavioral impacts. However, information about the impacts of DZP on the early–life stages of fishes is still scarce.

In this study, therefore, we exposed zebrafish embryos to a range of concentrations of DZP until 120 hours post–fertilization (hpf) and examined their survival, development, hatching, and behavioral responses to light–dark transition. The purpose of this study is to provide more detailed information about the impacts of DZP on the early–life stages of zebrafish.

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MATERIALS AND METHODS

Chemicals

Diazepam (CAS No. 439–14–5) was purchased from Laiyao Biotechnology Co., Ltd. (Beijing, China). Methanol and other reagents (analytical grade) were purchased from Kemei Biotechnology Co, Ltd. (Zhenjiang, Jiangsu, China).

Test organisms

Zebrafish (AB strain) broodstock has been maintained in our laboratory for more than six months. The female and male zebrafish (9 months post–hatching) were separately cultured in two 16–L circular glass aquarium (28 cm diameter and 30 cm height) containing dechlorinated tap water (conductivity at 0.50–0.53 mS/cm; 27±1°C). The aquariums were kept under a 14L:10D h light:dark cycle, and half the water was replaced every three days. Zebrafish were fed with *Artemia nauplii* (<24 h after hatching) twice a day.

A total of 18 zebrafish pairs were used for producing embryos in the following manner. Briefly, three pairs were introduced in a spawning box the evening before the exposure test, and a baffle was used to separate females and males (AquaSchwarz, Germany). Spawning and fertilization took place within two hours after the light onset (simultaneously remove the baffle) the following morning. All health embryos from each spawning box were transferred to the same Petri dish and maintained in E3 medium until the exposure test.

Exposure experiment

The test solutions were prepared by pipetting calculated amounts of the DZP stock solution (20 mg/mL in methanol) into E3 medium to obtain final concentrations of 0 (control), 0.4, 4.0, 40, 400, and 4000 μg/L. The additional amount of methanol was added to each test solution to ensure its final concentration in all test solutions was equal to 0.02% (v/v).

For each treatment, random 120 embryos were selected and introduced to three Petri dishes (40 embryos per dish, n=3) that contained 10 mL test solution. Embryos were maintained at 28°C in Petri dishes in an incubator with the same photoperiod as adults. The exposure test was conducted for 120 hours post–fertilization (hpf), and the test solutions were renewed every 24 hours. Mortality and abnormal development were confirmed on 2, 4, 8, 24, 48, 72, 96, and 120 hpf, and dead embryos were removed immediately. On 48 hpf, the heart rate (heartbeats per min) of embryos (random ten individuals per group) was recorded. In addition, the hatching time of each successfully hatched larva (i.e., hatched within 120 hpf and without any evidence of abnormal development) was also recorded for assessing the time–to–hatching.

Light–dark locomotion test of newly hatched larval

The Light–dark locomotion test was conducted during the same time interval (09:00 to 12:00 China standard time) to minimize the possible effects of diel periodicity (Qiu *et al*. 2017; 2020b). The behavioral test was conducted in 96–well plates containing 200 μL E3 medium, with a DanioVision system (Noldus, Wageningen, Netherlands). The larvae were carefully transferred to 96–well plates with a single larva in each well (inner diameter=8 mm). The larvae were given a 10 min acclimation period in the dark, followed by an alternating cycle of light–dark transitions (i.e., 10 min dark–10 min light–10 min dark) to examine their responses to light–dark transitions. The cumulative duration of movement (CDM) and average distance to the center point (ADC) of zebrafish larvae were analyzed and calculated by using EthoVision XT software (Vison 11.5; Noldus).

Statistical analysis

The survival (i.e., time–to–death within 96 hpf) and hatching (i.e., time–to–hatching within 120 hpf) data were analyzed using an accelerated failure time (AFT) model by using the eha and survival packages in R version 3.4.4 (Qiu *et al*. 2019; 2020c). Briefly, the AFT model used here was the following:

$$T=\exp(\beta \cdot d_{DZP}) \cdot T_0 \quad (1)$$

$$S(t)=(1+(t \cdot \exp(\mu)^{-1} \cdot \exp(\beta \cdot d_{DZP})^{-1})^{1/\sigma})^{-1} \quad (2)$$

Where T_0 and T are the time–to–event of embryos in the control and DZP exposure groups, respectively. The d_{DZP} is the concentration (at μg/L) of DZP, and the β is an unknown coefficient that shows the effect of DZP exposure. The DZP concentration was assumed to be a continuous variable for fitting the survival data and a categorical variable for fitting hatching data. Because the time–to–event data were assumed to follow a log–logistic distribution, the survival $S(t)$ at the time point (t) can be computed the equation (2), where the μ (intercept) and σ (scale) are unknown parameters estimated by the AFT model.

The effects of DZP on the zebrafish larvae's locomotion were first analyzed using repeated measures of analysis of variance, with the DZP concentration as the between-subjects factor and the 10–min periods as the within–subjects factor. Subsequently, multiple comparisons between each DZP concentration (within each 10–min period) and between each 10–min period (within each DZP concentration) were conducted, using the Bonferroni correction to adjust the p–value. The statistical analyses were performed using SPSS Advanced Models 11.0J software (SPSS Japan, Tokyo, Japan).

RESULTS

Effects on embryonic mortality and heart rate

As shown in Fig. 1A, the survival rate of zebrafish embryos in the control groups was 89.2% at 120 hpf. Although exposure to DZP at the range of 0.4–400 μg/L did not notably affect the survival curve of zebrafish embryos, the highest DZP concentration (4000 μg/L) induced significantly higher mortality (Fig. 1A). The estimated coefficients from AFT model fitting revealed that DZP exhibited significantly negative associations

with embryo survival (Table 1). The 96–h LC_{50} (ranges) for DZP to zebrafish embryos was calculated as 3202.4 (3068.2–3555.6) μg/L.

The average heart rate of zebrafish embryos at 48 hpf was 148±7 beats/min in the control groups (Fig. 1B). Compared with the control, significantly lower heart rates of zebrafish embryos were observed in exposures to DZP at 4.0, 40, and 400 μg/L (Fig. 1B). In the highest DZP concentration (4000 μg/L), no heartbeat of embryos observed.

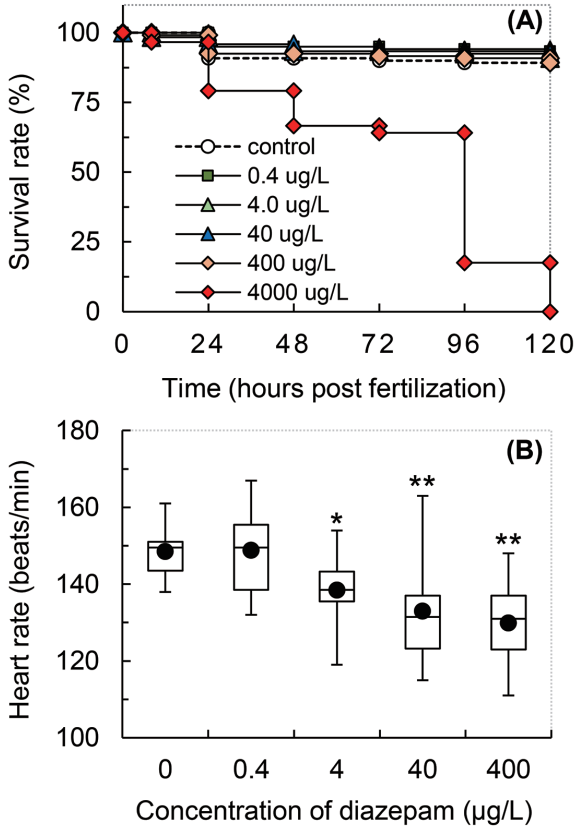

Fig. 1. Survival curves (A) and heart rate (B) of zebrafish (*Danio rerio*) embryos exposed to diazepam at 0.4, 4.0, 40, 400, and 4000 μg/L for 120 hours post fertilization (hpf). The Asterisks following the parameter labels indicates significant between treatment and control (* $p<0.05$; ** $p<0.01$).

Effects on the time–to–hatching of embryos

The average hatching time of zebrafish embryos exposed to DZP at 0 (i.e., control), 0.4, 4.0, 40, and 400 μg/L was calculated as 73.0±1.1, 69.5±0.7, 77.6 ±1.0, 79.2±1.2, and 77.9±1.1 hpf, respectively. Those time–to–hatching data were fitted with the AFT model, and the estimated parameters are listed in Table 2. The estimated coefficients indicated that exposure to DZP at 0.4 μg/L did not significantly affect the hatching time of zebrafish embryos, while those at 4.0, 40, and 400 μg/L could significantly delay the embryos' hatching (Fig. 2).

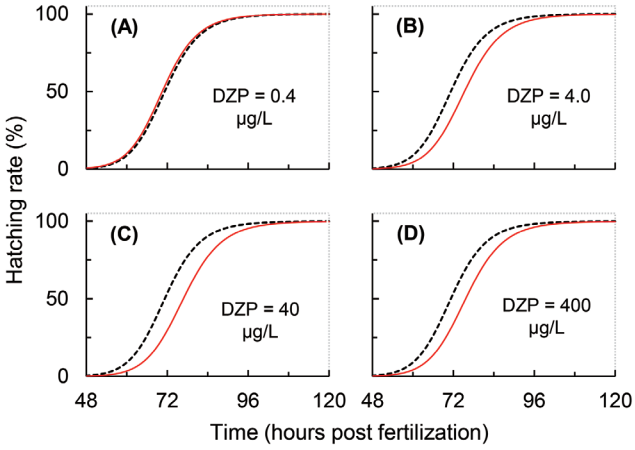

Fig. 2. AFT model fitted effects of diazepam on the time–to–hatching of successfully hatched larvae of zebrafish (*Danio rerio*). The diazepam concentration is listed in each sub–figure, and lines indicate the fitted hatching curves (dotted: controls; red: exposures).

Table 1. Estimated coefficients for an accelerated failure time model describing the effects of diazepam on time–to–death of zebrafish (*Danio rerio*) embryos

Factors	Coefficients (S.E.)[1]	Wald–Z	Pr(>|Z|)
Intercept (μ)	6.13(1.54)	39.7	<0.01
$d_{diazepam}$	−4.56(0.45) ×10^{-4}	−10.1	<0.01
Log(scale)	−4.52(0.75)	−6.03	<0.01

[1] The concentration of diazepam (DZP) was treated as continuous variables when fitting hatching data; S.E.: standard error

Table 2. Estimated coefficients for an accelerated failure time model describing the effects of diazepam on time–to–hatching of zebrafish (*Danio rerio*) embryos

Factors	Diazepam (unit)	Coefficients (S.E.)[1]	Wald–Z	Pr(>|Z|)
Intercept (μ)	0 μg/L	4.26 (0.01)	532.9	<0.01
$d_{0.4}$	0.4 μg/L	−0.01(0.01)	−0.75	0.45
$d_{4.0}$	4.0 μg/L	0.06 (0.01)	3.88	<0.01
d_{40}	40 μg/L	0.07 (0.02)	4.90	<0.01
d_{400}	400 μg/L	0.06 (0.01)	4.05	<0.01
Log(scale)		−2.60 (0.04)	−74.0	<0.01

[1] The concentration of diazepam (DZP) was treated as categorical variables when fitting hatching data; S.E.: standard error

Effects on the behavioral traits of larval during a light–dark locomotion test

As shown in Fig. 3A, exposure to DZP did not change responses in the movement pattern of zebrafish larvae to light–dark transition, which exhibited a relatively high moving state in the first dark, followed by a resting state in the illumination and a much higher moving state in the second dark. However, exposure to DZP did alter the locomotor activity of zebrafish larvae (Fig. 3B). Within the first 10–min dark period, no significant difference between the control group and any exposure group. Within the 10–min illumination period, zebrafish larvae exposed to DZP at 0.4 and 4.0 μg/L exhibited significantly higher locomotor activity than those exposed to DZP at 40 and 400 μg/L. Within the second 10–min dark period, zebrafish larvae in the control group exhibited the lowest locomotor activity, and those exposed to DZP at 0.4 and 4.0 μg/L exhibited the highest locomotor activity.

As shown in Fig. 3C, exposure to DZP significantly changed the average distance between zebrafish larvae and the center point, especially in the light and the second dark periods. Within the first 10–min dark period, no significant difference in the distance between zebrafish larvae and the center point was observed (Fig. 3 D). Within the 10–min illumination period, zebrafish larvae in the control group had the significantly most significant distance to the center point. Within the second 10–min dark period, zebrafish larvae exposed to DZP at 0.4 μg/L exhibited the lowest distance to the center point.

DISCUSSION

Based on the AFT model fitting, the 96–h LC_{50} for DZP to early-life stages of zebrafish was calculated as 3202.4 (3068.2–3555.6) μg/L. Previously, Nunes *et al.* (2005) reported that the 96–h LC_{50} for DZP to mosquitofish (*Gambusia holbrooki*) was 12.7 (12.57–12.85) mg/L. Generally, the maximum concentrations of DZP and other BZDs in aquatic environments are at the level of several ug/L (Ternes 1998; Cunha *et al.* 2017). Thus, the residue of DZP may exhibit limited lethal effects on fishes in natural aquatic systems. However, our results also demonstrate that exposure to DZP could disturb the development and/or behaviors in the early–life stages of zebrafish, even at the environmentally relevant concentrations (i.e., 0.4 and 4.0 μg/L).

The significantly decreased heart rate of embryos in exposure groups (4, 40, and 400 μg/L) suggests that DZP can disrupt heart development in the early–life stages of fish. Generally, reduced heart rate is closely

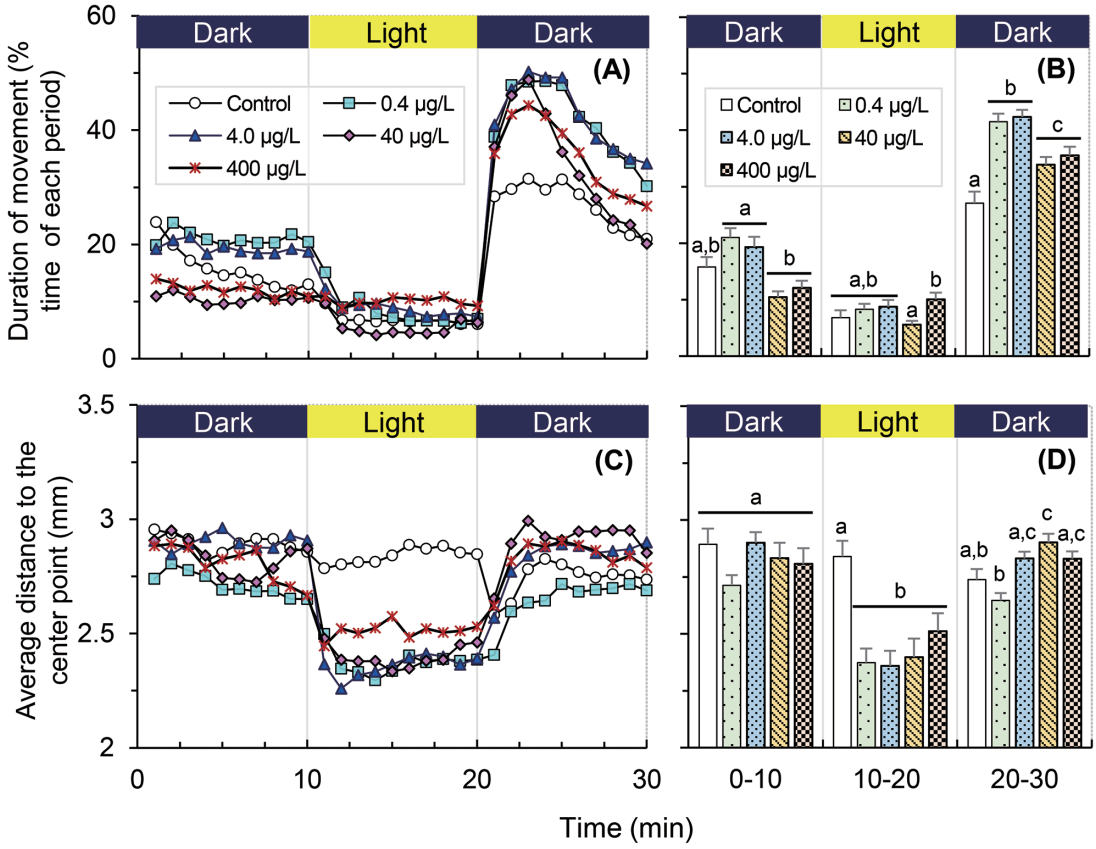

Fig. 3. The cumulative duration of movement (A and B) and average distance to the center point (C and D) in larval zebrafish (*Danio rerio*) during a light–dark locomotion test. A 10–min period of darkness was followed by an alternating cycle of 10–min light and 10–min dark. The blue and yellow bars at the top signify dark and illumination conditions, respectively. Data are presented as means ± SE (n=80) in 1–min intervals (A and C) and within each 10–min period (B and D). Values that do not share a common letter are significantly different at p<0.05.

related to the underdevelopment of the heart and pericardium (Yamauchi *et al*. 2005) or the high level of apoptotic cells in the heart (Deng *et al*. 2009). The bradycardia induced by DZP may further lead to other adverse effects. Indeed, the hatching time of embryos exposed to DZP at 4.0, 40, 400 μg/L was also significantly delayed.

The light–dark locomotion test is a standard method to reflect the brain function and nervous system development of fish larvae (Basnet *et al*. 2019). In this study, zebrafish larvae (120 hpf) in both control and exposure groups were more active in the dark period than the illumination period. Similarly, Leuthold *et al*. (2019) reported that zebrafish larvae exposed to neuroactive chemicals tended to be more active in the dark and turn to be hypoactive in the light period. Moreover, exposure to DZP significantly increased the locomotor activity of zebrafish larvae, especially in the second dark period. In a previous study, hyperactivity was also demonstrated in juvenile zebrafish exposed to DZP at 12 and 120 μg/L (Wu *et al*. 2020). In natural water bodies, hyperactivity in fish may attract a predator's attention or increase metabolic activity (Zhou & Weis 1998; Chen *et al*. 2021), and thereby cause potential ecological risks.

Regardless of the exposure concentration, exposure to DZP significantly reduced the thigmotaxis in zebrafish larvae in the 10–min illumination period, suggesting that DZP exhibited an anti–anxiety effect early–life stages of zebrafish. Anti–anxiety is a typical effect of BZDs that has been well documented in mammals (Argyropoulos & Nutt 1999; Egan *et al*. 2009). Furthermore, an organism with the thigmotaxis tends to avoid the center and stay in the boundaries, which can help them adapt to a novel space and search for shelter or escape routes (Treit & Fundytus 1988; Qiu *et al*. 2017). Thus, our results suggest that DZP might decrease the sensitivity and slow down the response to stress, thus causing them to fail to escape and reduce survival when facing dangers in a natural environment (Champagne *et al*. 2010; Miller & Gerlai 2012).

In conclusion, our results demonstrate that exposure to DZP at environmentally relevant concentrations could disturb the development and behavioral response to light stimulation in the early–life stages of zebrafish, which may induce some potential ecological consequences in natural ecosystems. Detailed mechanisms involved in the developmental and behavioral toxicity of DZP to fishes should be addressed in future studies to understand its ecological risks better.

AUTHOR CONTRIBUTIONS

C. Chen performed the experiments, analyzed the data and wrote the paper. L. Li and M. Li performed the behavioral experiments and data analysis. M. Wu, W. Liang, and Y. Takai performed the experiments, and participated in the Formal 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 of the manuscript and approved the final version.

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