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<https://doi.org/10.5109/6796256>

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出版情報：九州大学大学院農学研究院紀要. 68 (2), pp.135-141, 2023-09. 九州大学大学院農学研究院  
バージョン：  
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## Bioconcentration and Behavioral Interference Effect of Diazepam on Adult Japanese Medaka (*Oryzias latipes*)

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(Received May 9, 2023 and accepted May 18, 2023)

As one of the most commonly detected polluting drugs in the environment, diazepam (DZP) has been widely detected in rivers, soil, and organisms. To better understand the bioconcentration of DZP in fish and its impact on fish behaviors, we exposed both female and male medaka to a DZP (120 µg/L) for 7 days, and then transformed them to clean dechlorinated water for 3 days of depuration. Regardless of gender, DZP can rapidly accumulate in the brain and liver tissue and reach a stable phase within 24 hours of exposure. When the fish was transferred to purified water for purification, the concentration of DZP in the tissue was rapidly discharged, decreasing by one order of magnitude within 24 hours. The bioconcentration factors (BCF) of DZP in the brain and liver of males were calculated to be 10.47 and 19.58 L/kg, respectively, and those in females were calculated to be 6.12 and 6.03 L/kg. Compared with females, DZP accumulated more in the tissue of male fish, which exhibited a higher mortality rate during exposure. In addition, DZP can be significantly enriched in the ovaries of females, with a BCF of 10.54 L/kg. Furthermore, DZP exposure could significantly interfere with the interactive behaviors between males and females during the courtship, as indicated by the reduced duration of body contact and increased inter-individual distance. These findings highlight the need to study the transgenerational toxicity of DZP in Japanese medaka to assess its potential risk to fish populations accurately.

**Key words:** Behavior, Bioconcentration coefficient (BCF), Diazepam, Sex-dependent impacts, Japanese medaka

### INTRODUCTION

Due to the long treatment time and applicability of psychiatric diseases to patients of all ages, the production and consumption of psychotropic drugs are very large (Calisto and Esteves, 2009). Correspondingly, the environmental pollution caused by the widespread use of psychotropic drugs has attracted widespread attention (Argaluz et al., 2021). Among them, benzodiazepines (BZDs) are typical antianxiety and sedative drugs that can selectively act on the limbic system of the brain, enhancing the efficacy of the main inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA) (Fraser, 1998). Due to their fast efficacy, low adverse reactions and high safety, BZDs have been widely used in clinical practice and also be detected in wastewater, surface water, drinking waters, and tissues of organisms in many countries (Ma et al., 2018).

As a typical long-acting BZD prescription, diazepam (DZP; C<sub>16</sub>H<sub>13</sub>ClN<sub>2</sub>O) is one of the world's largest sedative

drugs (Puia, 2001). Moreover, its metabolites (e.g., oxazepam, temazepam, and nordiazepam) can still inhibit the activity of the central nervous system, also as a common benzodiazepine drug used in clinical practice (Eghbali et al., 1997). The pollution of DZP and its metabolites has become very severe in various water ecosystems. For example, Yuan et al. (2012) detected the residues of oxazepam in medical and municipal wastewater in Beijing, China, and found that the highest concentrations detected in the inlet and outlet were 942 ng/L and 752 ng/L, respectively. John et al. (2022) collected surface water samples from 1052 regions along 258 rivers in 104 countries to detect pharmaceutical pollution and found that the highest concentration of DZP was 851 ng/L.

Previous studies have demonstrated that DZP belongs to the highly persistent drug pollutants, with a half-life in natural ecosystems of  $311 \pm 26$  days (West and Rowland, 2012). According to its Log-Kow value ( $=2.86$ ), the bioconcentration coefficient (BCF) of DZP in aquatic organisms can be estimated to be approximately 33, i.e., it has a moderate bioaccumulation ability (Overturf et al., 2016; Wang et al., 2019). Overturf et al. (2016) reported that DZP could enrich in various tissues of channel catfish (*Ictalurus punctatus*) with kinetic BCFs of 146, 45, 15, 9.8, and 2.1 in plasma, gonads, brain, liver, and muscle, respectively. The investigation of wild fish and aquatic products also indirectly proves the bioaccumulation of DZP. For example, Kwon et al. (2009) reported that the concentration of DZP in the livers of wild-caught hornyhead turbot (*Pleuronichthys verticalis*) ranged from 23 to 110 ng/g, and Mottaleb et

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*al.* (2016) reported that the concentrations of DZP in the muscles of several commercially available fish species ranged from 1.99 to 16.57 ng/g. Therefore, estimating the accumulation of DZP in aquatic species and its ecological consequences is essential to assess the potential risk of DZP pollution.

As a typical psychotropic drug, DZP may directly interfere with the function of fish brains and adversely affect their behavioral traits, closely related to socializing, foraging, and survival (Brodin *et al.*, 2014; Chen *et al.*, 2021a). For example, exposure to sublethal concentrations of DZP (1200, 120, 12  $\mu$ g/L) for 4 days can disturb juvenile zebrafish's swimming activity and social behaviors by affecting their brain GABA levels (Wu *et al.*, 2020). The metabolite of DZP, oxazepam, has also been shown to significantly alter the social behavior of wild bass at environmental concentrations, thereby increasing their risk of being preyed on by pike fish, reducing their population size, and disrupting the ecological balance of the entire water body (Brodin *et al.*, 2013). Several studies have demonstrated that DZP and its metabolites to aquatic organisms exhibited gender-specific toxic effects on aquatic organisms. For example, Chen *et al.* (2020) exposed adult zebrafish to a sublethal dose of DZP for 21 days and found gender differences in neurotoxicity and behavioral toxicity of DZP to zebrafish, with females being more sensitive to DZP. On the contrary, Genario *et al.* (2020) found that only males showed anxiolytic responses to diazepam exposure (16  $\mu$ g/L for 24 h). In mammals, the sex difference in hormones and pharmacokinetics and pharmacodynamics between females and males may influence their sensitivity to the activity of drugs (Lynch *et al.*, 2002; Ravenelle *et al.*, 2014). However, the mechanisms underlying the sex-specific toxic effects of diazepam on fish are still unclear.

Japanese medaka (*Oryzias latipes*) is a model organism for environmental monitoring and chemical risk assessment (OECD, 2012; Shima and Mitani, 2004). Due to the similarity in the nervous system development to mammals, Japanese medaka has also been widely used in studying the pathogenic mechanisms of neurological diseases (Matsui, 2017; Wittbrodt *et al.*, 2002). Furthermore, many studies also use the behavioral changes in Japanese medaka to evaluate the ecological risks associated with sublethal exposure to neurotoxic pollutants (Chen *et al.*, 2021b; Gerhardt, 2007; Qiu *et al.*, 2017; Qiu *et al.*, 2020a; Qiu *et al.*, 2020b). In this study, we determined the biological concentration factors of DZP in different tissues in both male and female Japanese medaka, according to the (OECD, 2012). Moreover, time-series variations in the behavioral traits of fish were also determined. This study aimed to investigate the tissue-specific and gender-specific differences in the bioconcentration of DZP in Japanese medaka and its impact on fish behaviors during the countship.

## MATERIALS AND METHODS

### Chemicals

Diazepam (CAS No. 439-14-5) was purchased from

the Laiyao Biotechnology Co., Ltd. (Beijing, China). Methanol (analytical grade) was purchased from Kemei Biotechnology Co., Ltd. (Zhenjiang, Jiangsu, China). The enzyme-linked immunosorbent assay (ELISA) kit for assaying diazepam was purchased from Yanjin Biotechnology Co., Ltd (Shanghai, China).

### Organisms

Japanese medaka (nine-month-old, body length: 2.3–3.1 cm) used in this study was obtained from blood-stock maintained in our laboratory, Jiangsu University (Zhenjiang, Jiangsu, China). Adult Japanese medaka was held in 16-L circular glass aquariums (28 cm diameter, 30 cm height) containing dechlorinated artificial seawater (with a salinity of 1‰; conductivity at 0.50–0.53). The water temperature was maintained at  $25 \pm 1^\circ\text{C}$ , and half of the water was changed every two days. The fish were kept under the light: dark cycle of 14:10 h and fed with *Artemia* nauplii (<24 h after hatching) twice daily. The feeding dose is approximately 2% of body weight.

### Exposure and depuration test

The bioconcentration test consisted of a 7-day uptake and 3-day depuration phases, following an abbreviated OECD 305 guideline (Nallani *et al.*, 2011). The test solutions were prepared by pipetting calculated amounts of the DZP stock solution (1 mg/mL in methanol) into dechlorinated tap water to obtain final concentrations of 120  $\mu$ g/L, approximately equal to 1/10 of its 96-h  $\text{LC}_{50}$  for medaka (Takai *et al.*, 2022). A total of 48 healthy fish (half male and half female) were randomly selected and transferred into square glass tanks (25×25×50 cm) that contained 8 L of test solution of DZP (females and males were exposed separately). The exposure and depuration test was conducted under the same condition mentioned before ( $25 \pm 1^\circ\text{C}$ ; light: dark cycle = 14:10 h), except the test solution in each tank was renewed regularly. During the experiment, periodic observations were conducted, and dead individuals were promptly removed after recording their death time.

On days 1, 2, 4, 7, 8 (i.e., day 1 after depuration), and 10 (i.e., day 3 after depuration) of the experiment, six fish (half male and half female) were sampled and anesthetized in an ice-water bath. After measurement of body weight and standard body length, the fish brain, liver and gonad (female only) were excised and used to detect DZP concentration in tissues. In addition, on days 1, 7, and 10 of the experiment, water samples were collected to detect the concentration of DZP in the experimental system.

### Detection of DZP content

The concentration of DZP in tissues and water samples was detected by a diazepam ELISA assay kit (Yanjin Biotechnology Co., Ltd), according to the instruction manual. Briefly, an appropriate amount of weighted tissue was homogenized and sampled into a 2-ml centrifuge tube. After adding sodium hydroxide solution at a ratio of 2:8, the centrifuge tube was shaken for 3 minutes and centrifuged at 4,000×g for 10 min. Subsequently,

0.1 ml of the supernatant was transferred into a new tube, and 1 ml of *n*-hexane was added. After a 5-min shake, the tube was centrifuged at  $4,000\times g$  for 10 min, and then 0.5 ml of the upper *n*-hexane was transferred into a new tube and blown dry. Finally, the sample was re-dissolved in 0.3 ml reconstitution solution and used for analysis. The bioconcentration factor (BCF; L/kg) was calculated using the following formula (OECD, 2012):

$$BCF = \frac{C_f}{C_w}$$

The  $C_f$  and  $C_w$  are the DZP concentration in the tissue of Japanese medaka ( $\mu\text{g/kg}$ -wet weight) and water ( $\mu\text{g/L}$ ) samples, respectively.

### Measurement of behavioral responses during courtship

Behavioral tests were conducted before exposure (i.e., day 0) and on days 4 and 7 after exposure, following the methods described by Chen *et al.* (2020). In short, one female and one male fish were placed in a spawning box containing 1000 mL of artificial seawater ( $20 \times 9 \times 10$  cm), with a transparent baffle in the middle of the box to separate them ( $n = 4$ ). After a 30-min acclimation, the transparent baffle was removed, and the fish motion trajectory was tracked for 10 min using a DanioVision system (Noldus, Wageningen, Netherlands). The behavioral parameters were analyzed by EthoVision XT software (Vison 11.5; Noldus) and were roughly classified into two kinds of behavioral patterns. The average swimming velocity (ASV, cm/s), maximum swimming velocity (MSV, cm/s), and cumulative duration of high mobility (DHM% of the total observation period) were used to indicate the behavioral patterns of locomotor activity; the cumulative duration of chasing behavior (DCH), cumulative duration of body contact (DBC), and the average distance between individuals (ADI, cm) were used to indicate the behavioral patterns of courtship between female and male fish.

### Statistical analysis

Generalized Linear Model (GzLM) was used to analyze the behavioral parameters of medaka during the courtship, and SPSS 16.0 (SPSS Inc., Chicago, IL) was used for the above statistical analysis.

## RESULTS

### Bioconcentration of DZP

The average body length (Mean  $\pm$  S.E.) of female and male fish were  $2.74 \pm 0.23$  and  $2.80 \pm 0.25$  cm, and the average body weight was  $14.50 \pm 4.58$  and  $16.03 \pm 4.26$  mg, respectively. The survival curves of Japanese medaka are shown in Fig. 1. Compared with the females, males exhibited a higher mortality rate during the 7-day exposure period. At the end of the exposure period, the survival rate of female and male fish was 95.83% and 66.67%, respectively (Fig. 1).

The temporal variation of the measured concentration of DZP in Japanese medaka is shown in Fig. 2.

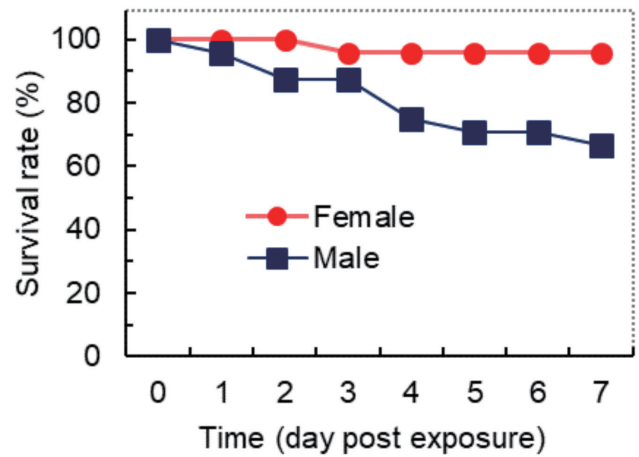


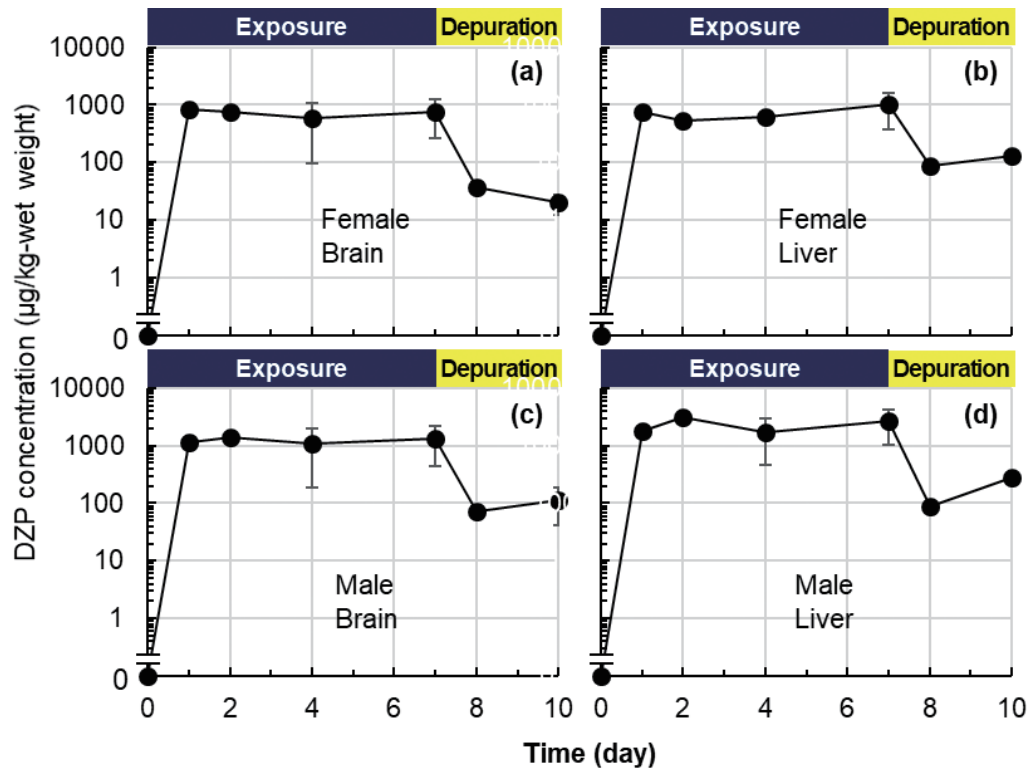
Fig. 1. Survival curves of Japanese medaka exposed to DZP ( $120 \mu\text{g/L}$ ) for 7 days.

Regardless of gender, DZP can rapidly accumulate in the brain and liver tissue and reach a stable phase within 24 hours of exposure. When the fish was transferred to purified water for purification, the concentration of DZP in the tissue was rapidly discharged, decreasing by one order of magnitude within 24 hours (Fig. 2). For females, the concentration of DZP in the brain and liver maintains a stable range of  $734.9 \pm 79.2$  and  $724.1 \pm 81.5 \mu\text{g/kg}$ -wet weight during days 2 to 7 of exposure (Fig. 2 a and b). For males, the concentration of DZP in the brain and liver were maintained at  $1256.8 \pm 141$  and  $2349.4 \pm 268.9 \mu\text{g/kg}$ -wet weight during days 2 to 7 of exposure (Fig. 2 c and d). In addition, the average concentration of DZP in the female ovaries is  $1265 \pm 355.8 \mu\text{g/kg}$ -wet weight.

Based on the above data, the bioconcentration factors (BCF) were calculated according to the OECD TG305 guidelines (OECD, 2012), as shown in Table 1. For males, the BCF value of DZP in the brain and liver is 10.47 and 19.58 L/kg, respectively (Table 1). For females, the BCF of DZP in the brain and liver is 6.12 and 6.03 L/kg (Table 1). In addition, the BCF of DZP in the gonad of female fish is 10.54 L/kg (Table 1).

### Temporal variations in behavioral traits during the courtship

The temporal variations in behavioral traits during the courtship of medaka exposed to DZP are shown in Fig. 3. There was no significant change in the average swimming speed (ASV) during the exposure (Fig. 3a). However, the maximum swimming speed (MSV) on day 4 of exposure was significantly higher than that on day 7 (Fig. 3b). In addition, the duration of high-speed exercise (DHM) on days 4 of exposure was significantly higher than those observed on day 0 and 7 of exposure (Fig. 3c). As the exposure time prolongs, the duration of chasing behavior (DCH) between males and females did not significantly change (Fig. 3d). However, the body contact duration between males and females significantly decreased (Fig. 3e), and the average distance between them was also significantly increased (Fig. 3f).



**Fig. 2.** Temporal variation of DZP concentration ( $\mu\text{g/kg-wet weight}$ ) in the tissue of Japanese medaka. (a) Brain tissue of female; (b) Liver tissue of female; (c) Brain tissue of male; (d) Liver tissue of male.

**Table 1.** Bioconcentration coefficient of DZP in various tissues of Japanese medaka (BCF, L/kg)

	Brain	Liver	Ovary
Female	6.12	6.03	10.54
Male	10.47	19.58	–

## DISCUSSION

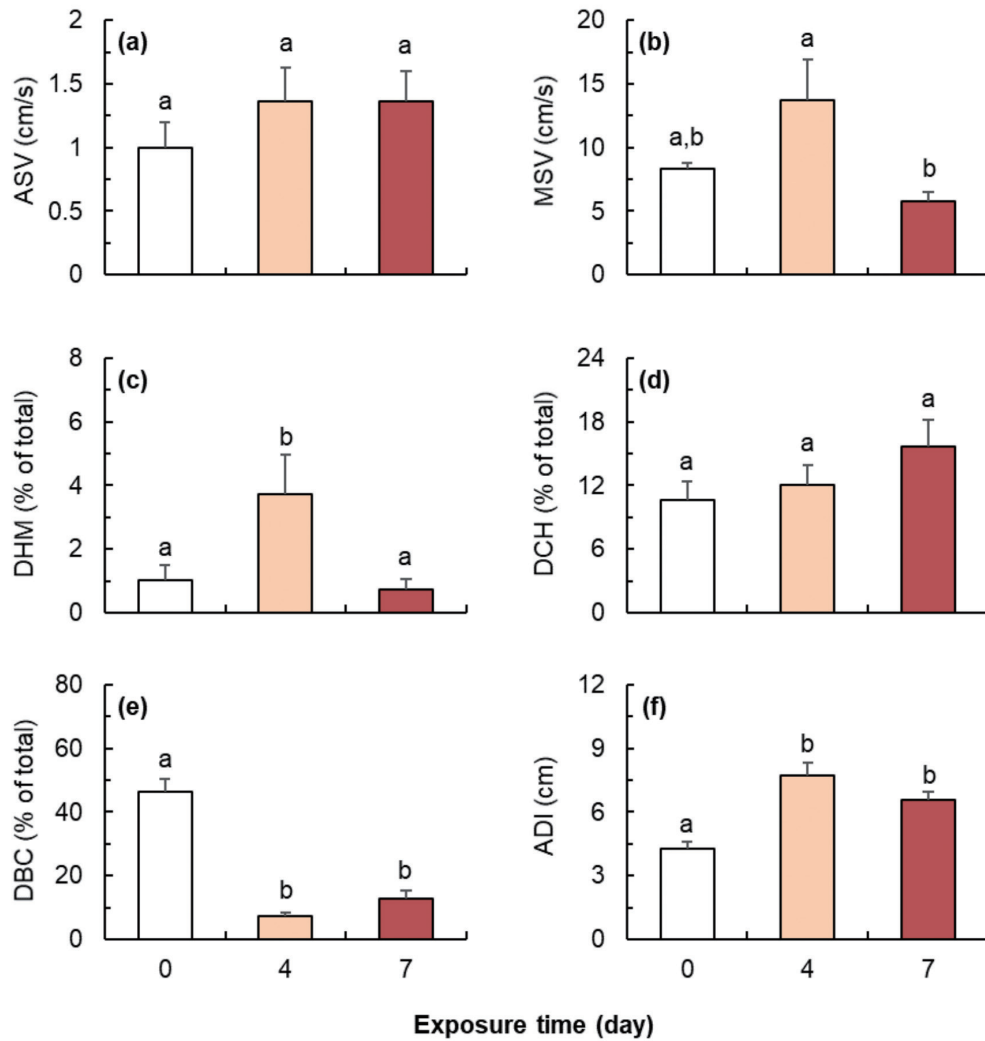
Our results demonstrated that exposure to DZP could rapidly accumulate in the brain, liver, and gonad of Japanese medaka, with tissue-specific and sex-specific bioconcentration factors. Based on the BCF values in various tissues (6.0–19.6 L/kg) calculated in this study, DZP shows a moderate bioaccumulation ability in Japanese medaka. Pharmacokinetic studies have shown that BZDs can be rapidly distributed to the brain, the main areas of action, the liver and the gonad in mammals (Ellison, 2002). DZP has similar tissue distribution characteristics in fish and mammals. Similar to our findings, Overturf *et al.* (2016) found that the enrichment of DZP in the channel catfish (*Ictalurus punctatus*) also showed tissue differences, with BCFs of 45, 15, 9.8, and 2.1 in the gonad, brain, liver, and muscles, respectively.

On the other hand, our results also indicate that DZP was accumulated more in male medaka, which also exhibited a higher mortality rate than females during the exposure period. In mammals, it has been well-documented that age and gender are important factors in the pharmacokinetics of DZP (Wang *et al.*, 2022). Furthermore, those gender-specific pharmacokinetics also affect their

toxicity to organisms. For example, Wilson *et al.* (2004) reported that exposure to DZP at a dose of 1.0 mg/kg could decrease anxiety-like behavior in male but not female rats. On the contrary, 1 mg/kg of DZP improved avoidance behavior in female mice in both the active and passive avoidance paradigms and did not affect the behavior of males in the passive avoidance task (Podhorna *et al.*, 2002). As to zebrafish, Genario *et al.* (2020) found that only males showed anxiolytic responses to diazepam exposure (16  $\mu\text{g/L}$  for 24 h). However, a sublethal dose of DZP for 21 days found gender differences in neurotoxicity and behavioral toxicity of DZP to zebrafish, with females being more sensitive to DZP (Chen *et al.*, 2020). Our study suggested the gender differences in the toxicity of BZDs to medaka fish, with males being more sensitive. In addition, we found that DZP can be significantly accumulated in the ovaries of Japanese medaka, which may transfer to the next generation and impact their offspring. Nyholm *et al.* (2008) reported that the hydrophobic organic pollutants that accumulate in the ovaries of zebrafish could be transferred to their offspring, leading to bioaccumulation in embryos. So further investigation is needed to determine whether DZP will further affect ovarian function and produce transgenerational toxicity in Japanese medaka.

Furthermore, our results also indicated that DZP exposure could significantly interfere with the interaction between female and male Japanese medaka during the courtship. The behavior of organisms is closely related to food intake, mating, and survival (Wong and





**Fig. 3.** The temporal variations in the behavioral traits of Japanese medaka exposed to DZP at 120 µg/L. (a) ASV: average swimming velocity; (b) MSV: maximum swimming velocity; (c) DHM: cumulative duration of high mobility; (d) DCH: cumulative duration of chasing behavior; (e) DBC: cumulative duration of body contact; (f) ADI: average distance between individuals. Data are shown as mean  $\pm$  S.E. (n = 4). The same superscript letter indicates no significant difference, while different letters indicate a significant difference ( $p < 0.05$ ).

Candolin, 2014). Many studies have documented that behavioral abnormalities induced by drug contamination may cause serious ecological consequences. (Chen *et al.*, 2020; Jessa *et al.*, 1996; Salomons *et al.*, 2012; Takai *et al.*, 2022). For example, the decrease in exercise activity may increase their risk be predated and decrease their feeding rate, ultimately weakening their survival ability in nature and facing the risk of a sudden population decline (Ackerly and Ward, 2015; Brodin *et al.*, 2014). Exposure to fluoxetine affected the anti-predator behavior of guppies, with exposed fish remaining stationary for longer after the simulated strike and spending more time under plant cover (Saaristo *et al.*, 2016). Petersen *et al.* (2020) exposed adult zebrafish to environmentally relevant concentrations of different antibiotics (Chlortetracycline, Ciprofloxacin, and Ceftazidime) and found these antibiotics could induce hyperlocomotion, promote cognitive decline, and exacerbate aggressive behavior.

Courtship displays are behaviors aimed at facilitating attraction and mating with the opposite sex and are very important for fish reproduction of many fishes (Mitoyen *et al.*, 2019; Ogino *et al.*, 2023). The increased risk of capture and decreased foraging efficiency of organisms during mating make their courtship behavior more sensitive to environmental changes (Fursdon *et al.*, 2019). For example, Lorenzi *et al.* (2014) found that the DZP could have led to less timid behavior, resulting in less time in the nest, causing a diminished reproductive effort in fathead minnow. Billings *et al.* (2018) found that during starvation stress, male drosophila melanogaster had reduced courtship behaviors with fewer wing bouts per minute and shorter duration of wing bouts. Tang *et al.* (2022) reported that low concentrations of the antidepressant venlafaxine caused significantly lower motor activity and disturbed brain monoamine levels, affecting courtship behavior in adult zebrafish. Therefore, the impact of DZP on the behavior of

Japanese medaka during courtship and its potential impact on offspring is worth exploring in the future.

This study indicates that exposure to DZP may lead to rapid enrichment of DZP in adult Japanese medaka, and its concentration has tissue specificity and gender differences. Compared to females, DZP accumulated more in the tissue of male fish, which may induce higher mortality. Moreover, we found that DZP could significantly accumulate in the ovaries of Japanese medaka and interfere with their behavioral traits during the courtship. These findings highlight the need to study the transgenerational toxicity of DZP in Japanese medaka to assess its potential risk to fish populations accurately.

#### AUTHOR CONTRIBUTIONS

S. WANG and M. ZHUO performed the exposure test, analyzed the data, and wrote the paper. K. CHEN and Y. SHI 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. 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.

#### ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of Jiangsu Province of China (Grant No. BK20191433). Thanks for the support of Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment, Suzhou University of Science and Technology Suzhou 215009, China.

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