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Tributyltin affects shoaling and anxiety behavior in female rare minnow (*Gobiocypris rarus*)

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ABSTRACT

Effects of tributyltin (TBT) on reproduction are well established in many fish species. However, few studies report the effects of TBT on non-reproductive behaviors, which is a novel aspect of endocrine disruption in fish. Thus, the present study used rare minnow (*Gobiocypris rarus*) to investigate the effects of TBT, at environmental concentrations of 1, 10 and 100 ng/L, on shoaling and anxiety behaviors. The results showed that fish exposed to TBT had less group cohesion during the course of the 10-min observation period as compared with the control fish. Further, TBT altered the shoaling in the Novel tank test, where shoaling is determined as the tendency to leave a shoal of littermates trapped behind a Plexiglas barrier at one end of the test tank. Fish exposed to TBT had shorter latency before leaving shoal mates and spent more time away from shoal than control fish. In addition, we also used Novel tanks to study the anxiety behavior as the tendency to stay at the bottom when introduced into an unfamiliar environment. The fish exposed to TBT showed increased anxiety, manifested as increased latency to enter the upper half and decreased time in upper half when compared with the control fish. TBT exposure increased the levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid, and decreased the levels of 5-hydroxytryptamine and its metabolite 5-hydroxy indole acetic acid in the brain. Thus, the hypofunction of the dopaminergic system or of the serotoninergic system or the combination of the two may underlie the observed behavioral change, which might affect the fitness of fish in their natural environment.

1. Introduction

Tributyltin (TBT), one of the organotin compounds, possesses broad-spectrum biocidal properties. It is used for various industrial purposes such as the production of PVC, agrochemicals, textile, etc. The most relevant application of TBT has been its use as antifouling agent for the undersides of ships (Antizar-Ladislao, 2008). Aquatic pollution resulting from its usage is of great concern due to its highly toxic effects on nontarget aquatic life (Alzieu, 2000) and persistence in sediments for a long time (Antizar-Ladislao, 2008). The use of TBT has been progressively restricted because of its deleterious effects. Ultimately, a global ban on the use of TBT by the IMO International Convention on the Control of Harmful Antifouling Systems on Ships entered into force from September 2008 (IMO, 2001). Despite such restrictions, continuing high concentrations of TBT are still detected in marine and freshwater ecosystems exceeding toxicity levels. It is reported that the total butyltins in water samples vary between ~1.7 and 342 ng/L as Sn from various ports of India (Garg et al., 2011). The butyltins range from 0.46 to 27.98 ng/L as Sn in seawater along the Croatian Adriatic Coast (Furdek et al., 2012). The TBT levels are in the range of non-detected to 23.9 ng/L as Sn in seawater from coastal areas of South Korea (Kim et al., 2014). In mainland China, published TBT concentrations from water samples range from below the detection limit to 977 ng/L as Sn (Reviewed by Cao et al., 2009). In fishes, the concentrations of TBT range from 11 to 182 μg/kg wet weight in the muscle tissues of 11 species from Japan (Harino et al., 2000), from 0.161 to 0.847 μg/g dry weight in the livers of eels (*Anguilla anguilla*) from the Thames Estuary (Harino et al., 2002), and from 26.35 to 194.22 ng/g wet weight in the muscle tissues from Kaohsiung Harbor and Kaoping River estuary of Taiwan (Shue et al., 2014).

Effects of TBT on reproduction are well established in many fish species, including disrupting steroidogenesis in zebrafish (*Danio rerio*) and brown trout (*Salmo trutta fario*) (McGinnis and Crivello, 2011; a Marca Pereira et al., 2014), inhibiting gonad development in rockfish (*Sebastiscus marmoratus*) (Zhang et al., 2007,

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2009), changing sex ratio in zebrafish and Japanese flounder (*Paralichthys olivaceus*) (McAllister and Kime, 2003; Shimasaki et al., 2003), and decreasing fertility and fecundity in Japanese whiting (*Sillago japonica*) (Shimasaki et al., 2006). Disruptions in reproductive behavior in male medaka (*Oryzias latipes*) (Nakayama et al., 2004) and male guppies (*Poecilia reticulata*) (Tian et al., 2015) by TBT administration are also observed. Compared with reproductive endpoints, only a few studies report the effects of TBT on non-reproductive behaviors, which is a novel aspect of endocrine disruption in fish with few published studies. It is reported that bis(tributyltin)oxide (TBTO) affects swimming distances, speed and tracks in rainbow trout (*Oncorhynchus mykiss*) (Triebskorn et al., 1994a), causes changes in spatial positions and responses to predator attacks in threespine stickleback (*Gasterosteus aculeatus* L.) (Wibe et al., 2001), influences predatory behaviors in rockfish (Yu et al., 2013), and disrupts feeding in goldfish (*Carassius auratus*) (Zhang et al., 2016). Furthermore, TBT is involved in many aspects of the neuroendocrine system influencing brain structure as well as behavior. TBT exposure induces ultrastructural lesions in the optic tectum and the optic nerve in rainbow trout (Triebskorn et al., 1994b), causes apoptosis in retinal neuronal cells of developing zebrafish (Dong et al., 2006), and results in brain damage (Zhang et al., 2008) and decreases of glutamatergic Nmethyl-D-aspartate receptor expression and its signaling pathway components in rockfish (Zuo et al., 2009).

The non-reproductive behaviors, such as shoaling and anxiety, have high ecological significance in wild fish populations and likely affect fitness by influencing foraging, reactions to predators and opportunities to reproduce. Thus, the present study uses rare minnow (*Gobiocypris rarus*) to investigate the effects of TBT at environmental levels on shoaling and anxiety behaviors. Rare minnow, a freshwater cyprinid, possesses lots of attractive features that make it a suitable model in aquatic toxicity tests to become a standardized aquatic test species in China (Zhou et al., 2002). Earlier studies show that rare minnow is sensitive to many aquatic pollutants including TBT (Zhou et al., 2002; Zhu et al., 2011; Zhu et al., 2013).

2. Materials and methods

2.1. Chemicals

TBT (purity ≥97%) was obtained from Fluka AG (Switzerland). It was dissolved in absolute ethanol to reach stock concentrations of 1, 10 and 100 μg/mL as corresponding to molar concentrations of 8.4, 84 and 840 nmol/mL. For exposure, direct spike of stock solutions of TBT into water of each aquarium was conducted. All other chemicals were of analytical grade and obtained from commercial sources.

2.2. Experimental species and exposure

Rare minnows were purchased from Chinese Academy of Fishery Sciences (Wuhan, China). They were raised in glass tanks with dechlorinated tap water (pH 7.3 ± 0.3 and dissolved oxygen 8.3 ± 1.2 mg/L) at a constant temperature of 25 °C with a photoperiod of 12:12 h (light: dark) and fed with chironomid larvae twice a day. All experiments and handling of the animals were conducted according to the research protocols approved by the Institutional Animal Care and Use Committee, Henan University of Science and Technology.

There were three exposures with four replicates of each, receiving 1, 10 and 100 ng/L nominal concentrations of TBT (8.4, 84 and 840 pmol/L), respectively. The control with four replicates received only equal volume of the solvent (1 μL ethanol/L). After 14-day acclimation, 30 female fish (1.32 ± 0.15 g) per group were randomly selected and exposed to TBT or the control for 60 days in separate 15 L glass tanks containing 12 L dechlorinated tap water at the same conditions described for the acclimation period. The concentrations used in this study were based on environmental levels of TBT and previous publications (Cao et al., 2009). To maintain the effective concentrations of TBT in the exposures, half of the water in of each tank was renewed daily and re-dosed immediately after renewal. To ensure agreement between nominal and actual concentrations of TBT, water samples collected after 12 h of changing the water were analyzed using a gas chromatograph equipped with a flame photometric detector (GC-FPD) based on the method of Jiang et al. (2001). The actual concentrations were 88.3 ± 8.4%, 92.1 ± 10.5% and 92.9 ± 12.1% of the nominal values of 1, 10 and 100 ng/L TBT, respectively. All fish survived the exposure period, with no signs of negative health effects. After exposure for 60 d, behavior studies were performed, after which fish were sampled. The isolated brains and livers were flash frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Butyltin analysis

The quantification of TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) in brain samples of 6 fish from each group was carried out as described by Jiang et al. (2001) and Zhou et al. (2001). The quantification of butyltins involved extraction, derivatization, clean-up steps, and analysis by GC-FPD. The recoveries of TBT, DBT and MBT were 107.9, 98.3 and 88.7%, respectively. The minimum detectable concentrations were 7.5 ng/g for TBT, 6.9 ng/g for DBT, and 8.8 ng/g for MBT, respectively.

2.4. Behavior studies

2.4.1. Cluster scores of shoaling

Based on an earlier study (Parker et al., 2013), a group of 5 fish were placed in an open arena (W × L × H: 40 × 60 × 15 cm) filled with 6 L aquarium treated water and filmed for 10-min following 5-min habituation. The arena was separated into eight equal sections (see Fig. 1A). At each 30-s interval during the 10 min, cluster scores ($Clus_T$) were calculated as follows: $Clus_T = Max_T/Total_T$. Where Max_T is the maximum number of fish in one section, and $Total_T$ is the total number of sections occupied by the fish. Scores were averaged for each shoal over a 10-min observation period. There were three replicates for each of the tanks using different groups of individuals.

2.4.2. Novel tank diving test-shoaling behavior

The procedure of Novel tank diving test was performed as previously described (Hallgren et al., 2011). In Novel tank, the spontaneous behavior response of fish was recorded when they were placed in a novel environment to seek protection by diving to the bottom and keep motionless until they feel safe to explore (Egan et al., 2009). Four Novel tanks for each exposure were run in parallel, and all experiments were performed between 9.00 a.m. and 13.00 p.m.

The glass test tank (W × L × H: 20 × 42 × 20 cm) was fully filled with treated water. In the right end, a vertical zone was created by a transparent Plexiglas screen 6 cm from the short end of the tank, trapping a shoal of 5 female fish that had not been exposed to TBT (Fig. 1C). Visual contact was prevented by a black sheet covering the Plexiglas screen. A vertical midline divided the rest of the tank into right/left halves. When the test fish (12 of each tank) were gently introduced into tank and entered the bottom half, the black sheet was removed from the transparency and the shoaling test was recorded as latency to first vertical midline crossing, total number of midline transitions and time spent away from shoal in the other half.