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Comparison of social behavior responses of Japanese medaka (*Oryzias latipes*) to lethal and sublethal chlorpyrifos concentrations at different exposure times

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ABSTRACT

Chlorpyrifos (CPF) is one of the most widely used insecticides and has been found in both urban and rural water bodies. In this study, we studied variations in the social behavioral patterns, swimming behavior, and brain acetylcholinesterase (AChE) activity of Japanese medaka exposed to lethal (0.12 mg/L) and sublethal (0.012 mg/L) concentrations of CPF after different exposure times. Group behavior performance (schooling, shoaling, and solitary) was determined on day 4 of lethal exposure and on days 4, 8, and 12 of sublethal exposure. Swimming speed and brain AChE activity were measured on days 4, 8, 12, 16, and 20 of sublethal CPF exposure. We observed significant decreases in social behavior and swimming speed (i.e., hypoactivity) in fish exposed to lethal CPF concentrations for 4 days. At the sublethal concentration, there was increased schooling duration and hyperactivity of fish on day 8 but not on day 4. In contrast, 12 days of sublethal CPF exposure resulted in social behavior responses similar to those after 4 days' lethal exposure, i.e., significant decreases in schooling frequency and duration with a notable increase in duration of solitary behavior. Brain AChE activity was inhibited in a time-dependent manner. Altered fish behavior in response to organophosphorus pesticides such as CPF may be mediated by more than AChE inhibition alone.

1. Introduction

The environment contains many contaminants that pose a serious risk to organisms. These various contaminants include the widely used organophosphorus pesticides (OPs), which can ultimately reach surface waters and may kill, or at least adversely affect, aquatic organisms (Köprücü and Aydın, 2004; Saunders et al., 2012; Williams et al., 2014). Chlorpyrifos (CPF) is one of the most widely used broad-spectrum OPs for pest control (Giddings et al., 2014; Levin et al., 2003; Saunders et al., 2012; Williams et al., 2014). Because of its long half-life in water and its toxicity to non-target organisms, there is great concern about the potential ecotoxicological impacts of CPF on aquatic ecosystems at different trophic levels (Asselborn et al., 2015; Bonansea et al., 2016; Giddings et al., 2014; Khalil, 2015). Although the concentrations of CPF in the aquatic systems of North America and Europe are generally at very low levels (López-Doval et al., 2012; Williams et al., 2014), water contaminations by CPF have been detected to reach the levels up to 17 μg/L (2005–2007) in various regions of Argentina (Mugni et al., 2011) and to reach the range of 28–44 μg/L (2010–2011) in canal waters of Malaysia (Ismail et al., 2012).

The impacts of these contaminants on aquatic organisms can be assessed at their death, but are also observable at the biochemical, organism, and population level (Weis et al., 2001). Behavior is one kind of response at the organism level that has clear links to the biochemical level and close connections to effects at higher levels of organization (e.g. population and community), with ecological relevance (Weis et al., 2001). At the biochemical level, the mechanism of CPF toxic action is through inhibition of acetylcholinesterase (AChE) by the active metabolite chlorpyrifos oxon (CPF-oxon). Inhibition of AChE results in an accumulation of acetylcholine (a key muscle

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neurotransmitter) in the central and peripheral synapses, which modifies physiological and neuroendocrine processes (Behra et al., 2002; Sandahl et al., 2005; Tilton et al., 2011). Such modifications can directly cause death, or may induce alterations in behaviors of fishes, e.g. abnormal swimming performance (Tilton et al., 2011; Yang et al., 2011), reduced foraging (Sandahl et al., 2005), and altered social behavior (Khalil et al., 2013). Moreover, changes in behavior due to exposure to a toxicant are often 10–100 times more sensitive when compared to mortality (Gerhardt, 2007). Therefore, behavioral response has been considered as ideal endpoint for discerning the effects of exposure to environmental contaminants, as well as their potential ecotoxicological impacts (Hellou, 2011; Kane et al., 2005; Scott and Sloman, 2004; Weis et al., 2001).

The Japanese medaka (*Oryzias latipes*) has been proposed as the standard fish for toxicology tests by the Organization for Economic Cooperation and Development (OECD, 1998). There are significant variations in behavioral responses of medaka exposed to various contaminants (Kang et al., 2009; Khalil et al., 2013; Nakayama et al., 2005; Oshima et al., 2003; Ren et al., 2012). We have reported previously that medaka exposed to lethal and sublethal concentrations of CPF exhibit different behavioral responses, and these behavioral disturbances may pose some risks at the population level (Khalil et al., 2013). In this study, we examined time-dependent variations in social behaviors, swimming speed, and brain AChE activity of medaka during long-term (20 days) sublethal exposure to CPF. We also compared short- and long-term effects of CPF exposure on these responses of medaka, with the goal of investigating the effects of long-term sublethal CPF exposure on the complex group behaviors of aquatic animals.

2. Materials and methods

2.1. Test chemicals

Acetylthiocholine iodide (AChI) was obtained from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate), 5,5′-dithiobis (2-nitrobenzoic acid), and other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). All reagents were of analytical grade.

2.2. Test organisms

Medaka fish were collected from broodstock maintained at the Laboratory of Marine Environmental Science of Kyushu University (Fukuoka, Japan). The test fish (3 months after hatching, 0.25 ± 0.03 g body weight and 2.3 ± 0.03 cm body length [mean ± SD]) were held in a 56-L glass aquarium (60 cm long × 30 cm wide × 36 cm high) containing artificial seawater at a salinity of 0.01%. The aquarium was equipped with an aerating filter system, and the water temperature was maintained at 25 ± 1 °C. The fish were kept under a 16:8 h light: dark regime and were fed *Artemia* nauplii (< 24 h after hatching) twice a day. Half of the water was replaced every week.

2.3. CPF exposure test

A stock CPF solution was prepared by dissolving CPF in ethanol at 12 mg/L. The test fish were starved 24 h prior to CPF exposure. For each replicate of the exposure tests, 13 fish were placed in a glass chamber (2.5 L) containing 2 L of test solution. We used four replicates for the control group and for each of two exposure groups for a total of 156 medaka. Based on the median lethal concentration (LC_{50}) at 96 h of CPF for Japanese medaka (Khalil et al., 2013), medaka were exposed to CPF at 0 mg/L (control), 0.012 mg/L (10% of the 96 h LC_{50}; "sub-lethal" level), and 0.12 mg/L (96 h LC_{50}; "lethal" level). For the control, 100 μL of ethanol was added to 2 L of test water. The final ethanol concentrations in the exposure test water were < 0.01% by volume. Test water was renewed daily.

2.4. Behavior measurements

Group behavior of medaka was recorded on days 4, 8, and 12 of sub-lethal exposure and on day 4 of lethal exposure following the method of Khalil et al. (2013). Briefly, 6 fish were randomly selected from each chamber and placed in a circular glass aquarium (22 cm diameter and 10 cm height) containing 0.8 L of water. After a 10-min acclimation, their behavior was recorded for 10 min using a digital video camera (CCD, model GE60, Library, Tokyo, Japan) that was installed 30 cm above the water surface. The group behavior patterns and average swimming speed of fish were determined using motion analyzer software (Move-Tr 32/2D; Library), as described by Khalil et al. (2013). Group behavior was classified into three patterns: schooling (4–6 fish swimming together in the same direction, Fig. S1A-C); shoaling (3 fish swimming together, even if in different directions, Fig. S1D); and solitary (the 6 fish were scattered, Fig. S1E). The frequency and duration of each social pattern were recorded, and the swimming speed for each replicate was calculated as the arithmetic mean of the 6 fish. On day 16 and 20 of exposure to 0.012 mg/L CPF, number of medaka was not sufficient for social behavior recording. Therefore, only swimming speed and AChE activities were measured. To determine the swimming speed of medaka on day 16 and 20, individual fish from each replicate was placed in a circular glass aquarium (14 cm diameter, 11 cm height). After a 10-min acclimation, fish behavior was recorded for 5 min and the swimming speed of each treatment was calculated as the arithmetic mean of the 4 fish (1 fish per chamber).

2.5. Brain AChE activity of medaka

Brain AChE activity of medaka was determined on days 4, 8, 12, 16 and 20 of sub-lethal exposure and on day 4 of lethal exposure. For sample preparation, the whole brains of 3 fish were extracted and placed in a pre-weighed 1.5 ml tube. The brains were homogenized using a plastic homogenizer after addition of 0.1 M phosphate buffer (pH 8.0). Homogenate was centrifuged at 10.000 × *g* for 15 min at 4 °C. The obtained supernatant was kept frozen at –30 °C until the AChE assay. The AChE activity of medaka was assayed as described by (Ellman et al., 1961) and modified by Khalil et al. (2013). The enzyme activity is expressed as μmol of substrate hydrolyzed per milligram of wet tissue per minute (μmol/min/mg-wet tissue).

2.6. Statistical analysis

Experimental data were checked for assumptions of homogeneity of variance across treatments using Levene's test. If the variances were homogeneous, we used one-way analysis of variance (ANOVA) with Dunnett's test to test for differences between the exposures and control, and with post-hoc Tukey HSD to test for differences in each endpoint of controls (among time points). When there was no proof of data homoscedasticity, we used the Mann-Whitney *U*-test for nonparametric data to compare the exposure and control, and to test for differences in each endpoint of controls (among time points) (the *p* value was adjusted by Bonferroni correction, if necessary). We used Spearman's rank correlation to examine the relationship between brain AChE activity and exposure time for medaka exposed to sub-lethal CPF concentrations. All statistical analyses were performed using SPSS Advanced Models 11.0J software (SPSS Japan, Tokyo, Japan).

3. Results

In the toxicity tests, lethal concentrations of CPF (0.12 mg/L) caused 29% mortality of exposed medaka after 4 days, whereas sub-lethal concentrations of CPF (0.012 mg/L) induced no mortality during the 20 days exposure. After exposure to lethal concentrations (0.12 mg/