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Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*)

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ABSTRACT

The aim of the present study was to assess the physiological response of male zebrafish *Danio rerio* to the fungicide tebuconazole and recovery in fungicide-free water. Acute toxicity tests were carried out and the median lethal concentration (LC_{50}) from 24 to 96 h was calculated. The fish were exposed to a sublethal fungicide concentration of 230 μg/L for 7 or 14 days and allowed to recover for 7 or 14 more days, respectively. Whole-body levels of vitellogenins, triglycerides, cholesterol, glucose, lactate and proteins as well as the activities γ-glutamil transpeptidase (γ-GT), alanin aminotransferase (AlAT), alkaline phosphatase (AP) and lactate dehydrogenase (LDH) were assayed; corpulence factor (*k*) was also calculated. Fish exhibited significant increase of vitellogenins (Vtg), which continued to increase after 14 days of recovery. Levels of glucose, lactate, cholesterol and triglycerides increased after 7 and 14 days of exposure. Finally, cholesterol and glucose recovered after 14 days of recovery whereas triglycerides and lactate continued to be elevated. Proteins and *k* remained unaltered the entire experiments. AAT, AlAT and AP enhanced during exposure and did not recover at the end (except AlAT). A longer recovery period should be necessary to re-establish fish physiology. These results alert about the multiple disruptive physiological actions that tebuconazole may have on fish.

1. Introduction

Current-used pesticides (CUPs) can be defined as those modern pesticides that are currently registered for use, generally developed from chemical synthesis, and typically used in the agriculture or lawn care sector (Konwick et al., 2006). Tebuconazole is widely used as fungicide in paddy fields from Eastern Spain among the Spanish Mediterranean wetlands to avoid and treat rice blast disease caused by *Pyricularia oryzae*. Tebuconazole (commercial name Folicur®) is classified as toxic to aquatic organisms that may cause long-term adverse effects in the aquatic environment (Bayer CropScience Limited, 2005). It is widely used as an alternative to tricyclazole and prochloraz treatments in paddy fields from Eastern Spain among the Spanish Mediterranean wetlands to avoid and treat rice blast disease during July and early August. Information about the environmental fate of tebuconazole is scarce. Konwick et al. (2006) estimated a half-life of one day for tebuconazole in rainbow trout (*Oncorhynchus mykiss*) under laboratory conditions joint to a time to 95% of elimination (t_{95}) for this fungicide of 4.5 days. However, half-lives of 24 and 22 days have been determined in zebrafish (*Danio rerio*) and water, respectively (Andreu et al., under review). During the spraying operations with the commercial Folicur® (25EW) residual concentrations of tebuconazole, while not acutely toxic, had been detected for more than 5 days after the end of treatments (Andreu et al., under review).

The literature indicates that triazole fungicides as well as related imidazoles are used for protection of cereals. Their fungicidal effect is a result of inhibition of cytochrome P450 (CYP450) dependent C14 demethylation of lanosterol, an intermediate in ergosterol biosynthesis and interfering with the synthesis of sterols, which are essential for the construction of normal cell membranes. In fish, CYP-mediated steroid metabolism, in addition to xenobiotic metabolism, can be altered (Konwick et al., 2006).

Fish are particularly sensitive to the influence of pesticides because they are able to uptake and retain dissolved xenobiotic in water via active or passive processes. The physiological changes associated with fish response to environmental low pesticide levels not only provide a mean to understand environmental levels of pollutants in biological terms, but also can be used as model for vertebrate toxicity including man.

The dynamics of intermediary metabolism are greatly influenced by any sort of stressors (any sort of change altering the homeostasis of the animal), which can lead to a group of responses referred to as stress response (Heath, 1995). The stress responses are adaptative and usually help animals to cope with changes in their environment. However, sometimes, modifications

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of stress response or harmful effects derived of it can have serious consequences at the individual level and finally at the population level.

Chemicals that mimic endocrine functional molecules have caused great concern because of their effects on endocrine systems in man and wildlife. Prochloraz, an imidazole fungicide, proved to possess anti-estrogenic effect on *D. rerio* (Kinnberg et al., 2007). In mammals a similar pattern of toxicity for prochloraz and tebuconazole as pro-feminization effects has been demonstrated (Taxvig et al., 2007). Recently, tricyclazole has proved its endocrine reproduction effect among other disruptive effects on fish physiology in zebrafish (*D. rerio*) as investigated in our laboratory (Sancho et al, 2009a). Therefore, it appears promising to study the effect of this relevant triazole in fish physiology during a short-term exposure in order to elucidate harmful effects on health of the individuals and possible consequences.

Zebrafish (*D. rerio*) was selected as test organisms in this study because it is considered as one alternative species for the evaluation of anthropogenic chemicals and widespread use in biological studies. It has been also proposed as a test organism by the OECD (1998).

The purpose of the present research was to study the impact of a short and continuous exposure of a sublethal concentration of tebuconazole for *D. rerio*. Investigated were key biomarkers involved in different physiological pathways as biomarkers of fungicide poisoning and their recovery after 7 or 14 days of exposure of the fish in fungicide-free water. Some biomarkers of intermediary metabolism and detoxification induction, joint to reproductive disruption were used as key indicators of fungicide exposure.

2. Material and methods

2.1. Ethical statement

The University of Valencia (Spain) guaranties that all the experimental fish used in the present study were maintained in the laboratory following national and institutional guidelines for the protection of animal welfare in accordance with our Institutional Animal Care and Use Committee.

2.2. Test animals

Zebrafish (*D. rerio*) stocks were purchased from a commercial fish supplier (*Fauna Acuatica*, Valencia, Spain). Fish were acclimatized for two weeks to laboratory conditions in 300-L glass tanks before the start of the experiments following the guideline of OECD (1998). During the acclimatization period, the fish were fed twice a day with a semi-synthetic diet of fish food (Tetramin). Adult male fish (Mean $\pm$ SEM length and weight 3.20 ± 0.28 cm and 0.50 ± 0.09 g, respectively) were used for this study. All the animals were healthy; a mortality of less than 3% was observed in the stocks used during the acclimatization period.

2.3. Dilution water

The tanks were supplied with a continuous flow of dechlorinated tap water (total hardness: 240 ± 10 mg/L as CaCO3 according to the Merck classification, Aquamerck 8039, Germany; pH 7.9 ± 0.2 using a Crison pHmeter; alkalinity 4.0 ± 0.5 mmol/L, Aquamerck 11109, Germany). The temperature was 25 ± 1 °C and the light regime was 12 h light:12 h dark (Roex et al., 2003).

2.4. Test chemical

Stock solutions were prepared by dissolving technical grade tebuconazole (chemical name IUPAC: (RS)-1-*p*-chlorophenyl)-4,4-dimethyl-3-3(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol) with a purity of 96% in acetone (Pesticide Residue Analysis Grade). Pesticide was obtained from Bayer CropScience Limited. Solid phase extraction-gas chromatography analysis (SPE-GC/MSMS) confirmed the presence of tebuconazole in the water (90%) over the entire exposure time. Water analysis determined that the tebuconazole-measured concentrations were very close to nominal concentrations (217.0 ± 7.6 μg/L, Andreu et al., under review).

2.5. Toxicity testing protocol

Four-day static acute toxicity tests were performed in our laboratory to determine the LC_{50} values of tebuconazole in *D. rerio* (OECD guidelines, 1998). Fungicide concentrations of 15, 18, 20, 23 and 25 mg/L were used. After being acclimatized to laboratory conditions adult male fish were randomly distributed in each test aquarium. For each concentration, a group of 10 randomly selected fish was placed in the glass tanks (10 L per tank of continuous aerated water). This procedure was repeated three times for each concentration. The control zebrafish groups were kept in clean water as in the experimental sets. During the acute toxicity test (96 h) animals were not fed. The number of dead fish was recorded at 24, 48, 72 and 96 h. Dead fish were removed from the tanks.

2.6. Sub-acute short-term test

A sub-lethal test concentration of 1/100 LC_{50}-24 h (230 μg/L) was used for the assessment of tebuconazole physiological and biochemical effects on fish. The experiments were carried out in a continuous flow-through system, based on OECD guidelines (1998). For the exposure period, tebuconazole was dissolved in acetone and the solution was supplied to a glass mixing chamber with tap water and connected to a perfusor pump (Gilson, Minipulse 3) that generated a constant solution flow. The outlet was connected to a 100-L glass test aquarium, diluting the pesticide to the desired concentration (230 μg/L) by a constant water flow. Flow rates were checked once daily, and there were approximately three volume changes per 24 h (Sancho et al., 2009a). This system was connected 48 h before the start of the experiments to reach a balance of tebuconazole contaminated water in the test aquaria. Final concentration of solvent (acetone) in the test aquarium was < 17 μL/L.

Previous studies carried out in our laboratory (unpublished data) indicated that the amount of acetone (17 μL/L) used as solvent during 14 days did not disturb either enzyme activity or biochemical–physiological parameters measured in zebrafish.

Fish were fed twice a day during the entire experiments with commercial fish food (Tetramin) at a level of approximately 1% of their body weight per day (Roex et al., 2003). Based on the conditions published in Sancho et al. (2009a), two different experiments were carried out as follows:

Experiment 1: Zebrafish individuals (total number=12) were exposed in 2 different tanks (100 L) for one week to 230 μg/L of fungicide. After 7 days of exposure, six zebrafish were randomly removed, rinsed with tap water, euthanized with MS-222 (Kinnberg et al., 2007), weighed, measured and stored frozen (-80 °C). In a second part of the experiment, the other six zebrafish pre-exposed for 7 days to tebuconazole were transferred to clean water in a 100 L glass aquaria with the same flow-through system under the above-described conditions but without fungicide (recovery period). Fish were removed after 7 days in these conditions, total length (l) and total weigh (w) pointed out and stored frozen (-80 °C) until analysis. Control groups with the same amount of acetone were kept in 100 L test aquaria in the same experimental conditions but without fungicide.

Experiment 2: Zebrafish individuals (total number=18) in 3 different tanks (100 L) were exposed for two weeks to 230 μg/L of tebuconazole. After 14 days of fungicide exposure, six zebrafish were removed, rinsed with tap water, euthanized with MS222 (Kinnberg et al., 2007), weighed and measured. At the 14th day, an exposed group of fish was transferred to a different aquaria with clean water for a recovery period in the same conditions described for experiment 1. At the 7th and the 14th day of that recovery period, six extra zebrafish were removed, rinsed with dilution water, euthanized, weighed, measured and stored frozen (-80 °C) until further analysis. A control group with the same amount of acetone was kept in 100 L test aquaria in the same experimental conditions but without fungicide and they were sampled at the same experimental times.

2.7. Analysis procedures

2.7.1. Tebuconazole determination

Tebuconazole concentration in the experimental water was determined in the Agrarian Laboratory of the Generalitat Valenciana (Spain) by Gas-chromatography/MassSpectrophotometry techniques (GC–MS/MS) as in Andreu et al., under review.

Water samples were collected from the tank and stored in amber glass bottles (250 mL). GF/F Whatman glass microfiber filters were used to eliminate organic matter and fish detritus in the water samples. The samples were kept frozen until the analysis. In order to extract the residues of tebuconazole from water samples, extraction cartridges (500 mg of C18-phase) were used. The solid phase was conditioned with three portions of methanol (3 mL) and three portions of