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A metabolomic study of fipronil for the anxiety-like behavior in zebrafish larvae at environmentally relevant levels☆

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ABSTRACT

Field residue of fipronil can interfere with the physiological characters of the domesticated fish; thus, lethal dose test and the general biomarker cannot delineate the low-level situation. Manipulating by video track, we observed an anxiety-like behavior including high speed and abnormal photoperiod accommodation after exposure to fipronil at environmental typical dose in zebrafish larvae. Examining the unbiased metabolomic profiles, we found perturbation in several metabolic pathways, including the increased contents of fatty acids and glycerol and the decreased levels of the glycine, serine, and branched amino acid. We presumed that observed enhanced fatty acid utility was in response to increase energy demands caused by anxiety like behavior. Additionally, the body burden of neurotransmitter such as glycine and L-glutamate may concurrently stimulate the swimming behavior. The insight of this study showed that integral perturbation such as metabolism helps us to further understand the risk to aquatic fish at the environmentally relevant levels.

1. Introduction

Entering the 'new generation' of phenylpyrazole pesticide, fipronil came at the cost of wide occurrence in the ecosystem and its abuse became an inevitable disaster to many non-targeted organisms. The residue level of fipronil which ranged from 1 to 70 μg/L in surface water threatened the crawfish industry and many invertebrates (Bedienta et al., 2005). Inhabitant fishes have also been suffering from the biomass loss as a result of fipronil application (Hayasaka et al., 2012; Clasen et al., 2012; Bencic et al., 2013). Repetitive applications of fipronil significantly altered physiological and biochemical indexes to both adult fishes and their descendants (Hayasaka et al., 2012; Clasen et al., 2012). To no one's surprise, early life stage of fish (embryonic and larval) is extremely vulnerable to the toxicity of fipronil (Beggel et al., 2010; Floyd et al., 2008; Jin et al., 2010), which could lead to defects in individual performance and eventually changes the in fish population health (Little and Finger, 1990).

As a neuronal toxicant, fipronil is widely known to target on gamma-aminobutyric acid (γGABA)-gated chloride channel. GABA inhibition in the central nervous system is the binding mode of fipronil in the poisoning of pests. However, the lower binding affinity to vertebrate GABA compared to invertebrate GABA accounted for its mild toxicity to mammals and fish (Chandler et al., 2004). Nevertheless, researches over the past few decades have led to an increasing interest in fish neuron-development and neuron-behaviors in the early life stage, particularly locomotor behavior, such as escaping or rhythmic swimming. In contrast to the acute exposure testing, sub-lethal dose administration of fipronil should be high enough to induce notochord degeneration and subsequently the locomotor defects in zebrafish (Stehr et al., 2006).

Generally speaking, inhibition of GABA receptor is known as a primary neuron toxic mechanism of fipronil and is the mode of action responsible for killing invertebrate. However, it is not adequate in assessing fipronil toxicity in vertebrates, especially at sub-lethal or chronic low levels. Striving to clarify mechanism related to fipronil-induced defects on fish, researchers conclude that a glycine receptor sub-type rather than a γ-GABA receptor

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inhibition could contribute to the impaired development of spinal locomotor pathway (Stehr et al., 2006). Additionally, upregulation of metallothionein (*mt*) and aspartoacylase (*aspa*) genes may be the potential contributors to impairing neurological functions due to sub-lethal exposure to fipronil (31 μg/L) (Beggel et al., 2012). These studies have suggested that potential novel mechanisms may be associated with low level chronic exposure to fipronil.

Environmental metabolomics, an established part of the omics' movement, can be an ideal tool to discover the association of effects and mechanisms with low levels of chemical exposure. Although studies concerning identification of metabolic pathway perturbations relevant to environmental metals or organic chemicals exposure have been explored (Bundy et al., 2009; Gao et al., 2014), biomarker identification and toxic endpoint under environmentally relevant concentrations are limited. Its successful application in the field of environmental health would make an exceptional contribution to the application of metabolomics in exposure science and risk assessment.

This study investigated the metabolomics associated with locomotor behavior by exposure of fipronil at the environmentally relevant concentrations. The metabolomic profiling by gas chromatography mass spectrometry (GC/MS) was used to investigate the metabolic phenotypes. This study not only identifies perturbations in metabolic pathways modified by low level of fipronil exposure but also extends our current understanding of fipronil effects in vertebrates beyond the inhibition of GABA.

2. Experimental procedures

2.1. Zebrafish husbandry and embryo collection

Adult AB strain zebrafish (*Danio rerio*) were maintained in a recirculating system according to standard husbandry procedures (Westerfield, 1995) at 28 °C for a 14:10-hr dark/light photoperiod. System water was obtained by passing tap-water through a reverse osmosis system (pH 7.0–7.5) and adding instant ocean salt to obtain water conductivity between 450 and 1500 μs/cm. The fish were fed three times daily with either a zebrafish diet (Zeigler, Aquatic Habitats, Apopka, FL, USA) or live *Artemia* (Jiahong Feed Co., Tianjin, China).

Zebrafish embryos were obtained from spawning adult fish with a female to male sex ratio of 1:2. Spawning was induced in the morning when the light was turned on. Fertilized embryos were collected within 1 h of spawning, washed with essential medium (EM), staged and incubated in Petri dishes at 28 ± 1 °C with EM. Normal embryos at 6 h post fertilization (hpf) were selected using a stereomicroscope (Nikon, Japan), according to the procedure of Kimmel (Kimmel et al., 1995) for use in exposure assays.

2.2. Locomotor behavior assessment

Two trials were conducted to evaluate the effects of fipronil on locomotor behavior. In the first trial, zebrafish embryos were exposed to negative control (<0.1% ethanol), 0, 10, 20, 40, or 80 μg/L of fipronil from 6 to 96 hpf. In each six-well plate, 10 normal embryos were randomly distributed into each well with 5 ml of control or test solutions. Solutions were renewed by half every 24 h, and each treatment was performed in triplicate. The larvae were washed several times with EM at 96 hpf and individually transferred to one well of 24-well plates with 2 ml of EM per well. Then, larvae were cultured in the 24-well plates until 120 hpf for the detection of the locomotor activities. The quantification of larvae locomotor activity was achieved using video tracking of zebrafish TM (version 3.5 with background subtraction; Viewpoint). When monitoring the swimming speed, all treatment groups were prepared with four larvae in each group on each plate, and altogether eight plates were used. The swimming speed at 120 hpf was monitored for 20 min, and data from the second 10 min interval were used for observance because larvae require the first 10 min to adapt to a new environment.

2.3. Light/dark paradigm

When monitoring the response to dark-to-light transition stimulation, three concentrations of fipronil (0, 40, 80 μg/L) and the negative control (<0.1% ethanol), were selected. Each plate contained six larvae for each treatment, and 5 plates were used. The lighting parameters consisted of an interval of 10 min of light followed by 10 min of dark repeated for 90 min. The data from the final 70 min were used, and three replicates were performed. The speed at each interval was recorded as mentioned above. After finishing the locomotor testing, all larvae were collected and stored at −80 °C for metabolomic analysis.

2.4. Samples preparation

The overt speed increase occurred at or above 10 μg/L and reached a peak at 20 and 40 μg/L and the light: dark photoperiod accommodation was not significantly altered below 40 μg/L of fipronil (data not shown). Taken together, we chose 40 μg/L groups for metabolomics assessment. Since there were no significant difference between the negative control and the blank, fish larvae in these two groups were pooled as one group. The whole larval body was extracted by a solution of CH_3Cl: MeOH: H_2O = 20:50:20. Generally, 50 mg of freeze-dried sample was sonicated in 2 ml of solution and vortexed for 30 s at 4 °C. The sonicated extracts were then centrifuged at 16,000 × g for 15 min. A 160 μL upper phase was recovered and dried prior to GC/MS analysis. The samples were derivatives prior to analysis.

2.5. GC/MS performance and metabolite analysis

A 1.0 μL aliquot sample of each group was injected into Thermo Focus DSQ GC/MS equipped with a DB-5MS (30 m × 0.25 mm, 0.25 um) column by the splitless mode. The optimal parameters were set as follows: injection temperature, ion source temperature and transmission lines temperature were all 250 °C. The flow of helium carrier gas through the column was maintained constant at 1 mL/min. The initial temperature was held at 80 °C for 2 min. The temperature was then increased at a rate of 15 °C/min to 300 °C and maintained for 6 min. Detection was achieved using MS in the electron impact mode and full scan monitoring with the detection slope of *m*/*z* 50–650. Quality control samples were prepared as pooled samples consisting of 10 μL pretreated samples from each group to ensure consistent method of performance and analysis. Briefly, after every 8 samples injection, quality control samples were injected to the system to ensure it is consistent.

For the auto-acquisition of GC total ion chromatograms and fragmentation patterns we used the GC/MSD ChemStation (Agilent, Shanghai, China) software. And the mass charge ratios as well as the abundance were compared with a standard mass chromatogram in the NIST (National Institute of Standards and Technology) mass spectra library by the ChemStation Software. For each peak, the software would generate a list of similarities comparing with every substance within the NIST library. Peaks would assign a compound name with a similarity index >70% and those who <70% similarity were assigned as unknown metabolites (Chan et al., 2009).