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Impairment of Cortisol Response to Stress in Zebrafish Acutely Exposed to Methyl-parathion

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ABSTRACT

The current uses of the organophosphorous methyl-parathion (MP) easily allow the pesticide to reach non-target organisms like fish. This substance was previously found as an endocrine disruptor of the Hypothalamus-Pituitary-Interrenal (HPI) axis. Thus, the cortisol response has been investigated in waterborne MP (5.2 mg L^{-1}) exposed adult zebrafish (*Danio rerio*) sampled 96 h after exposure. Stressed fish exposed to MP contaminated water showed lower levels of whole-body cortisol. Data demonstrated that MP produced impairment in the cortisol response to stress in zebrafish. This impairment may reduce the ability of the organism to promote metabolic and ionic adjustments necessary for the stress response. Fish that are incapable of mounting a normal cortisol response are likely to have a reduced ability to respond to the continuous challenges imposed on their homeostatic systems, either by aquaculture practices or by environmental changes.

Key words: Methyl-parathion, HPI disrupten, cortisol response daniorerio, metabolic disturbance

INTRODUCTION

Zebrafish is a good model system to research stress response and its Hypothalamus-Pituitary-Interrenal (HPI) axis has already been characterized (Alsop and Vijayan, 2009; Fuzzen *et al*., 2010). Considering this, it is also useful to aquatic toxicology studies on endocrine disruption of this axis, which coordinates the stress response in fish (Wendelaar Bonga, 1997). HPI and its peripheral product, cortisol, play a key role in the metabolic, ionic and physiologic adjustments necessary for coping with stress. Consequently, any adverse effect on the functioning of the HPI axis would compromise the ability of the animal to mount an adequate response to stressors (Hontela, 1998, 2005).

Methyl-Parathion (MP) is an organophosphorous pesticide largely used in Brazil to avoid agriculture losses due to insect attacks. In addition, MP has also been used in fish-culture ponds to kill the aquatic larvae of the predatory insects (Szarek *et al*., 2000; Luvizotto-Santos *et al*., 2009). MP, commercially named as Folidol 600® is a "less-persistent" organophosphate insecticide which

is moderately soluble in water and acutely toxic to fishes (Walton *et al.*, 1997). MP has been shown to interfere with and disrupt the functioning of the HPI axis in fish such as *Rhamdia quelen* (Cericato *et al.*, 2008).

Thus, the aim of the present work was to verify the effects of an acute exposure to sublethal concentrations of methyl-parathion on the cortisol response to an acute stressor in adult zebrafish.

MATERIALS AND METHODS

Fish and housing conditions: Two hundred and forty adult male "wild type" zebrafish (*Danio rerio*) were obtained from a commercial supplier (Delphis, Porto Alegre, RS) and acclimated for three days before the tests in the experimental aquaria (40 L, with constant aerated dechlorinated tap water), housed in groups of 20 fish, kept under 14-10 h day/night cycle and fed three times a day with commercial flakes (TetraMin®). All protocols were approved by the Institutional Animal Care Committee (CEUA-UPF, number 03/2011). Throughout the experiment, the water temperature was 28±2 °C, pH ranged from 6.6 to 7.2 and dissolved oxygen from 5.2 to 7.1 mg L^{-1}. Total ammonia was lower than 0.5 mg L^{-1}.

Experimental design: After acclimation, fish were divided into four experimental groups, each one housed in six aquaria in a static test design. In the first group fish were kept without any stressor or Contaminant (C). In the second group (MP) fish were exposed to 5.2 mg L^{-1} of methyl-parathion (concentration based on previous results by Bellavere and Gorbi (1984) and on the most commonly used concentrations in aquaculture stated Luvizotto-Santos *et al.* (2009) in the third group (S) fish were exposed to a stressor (60 sec of chasing with a net) and in the last group (MP+S) zebrafish were exposed to both the stressor and the contaminant. The stressor was applied after 96 h of exposure, and fish sampled at hour 97, cryoanaesthetized and euthanized (Wilson *et al.*, 2009) for whole-body cortisol determination (n = 10).

The concentration of MP in water was monitored daily from the moment of the inoculation to until 96 h of exposure period. MP was analyzed by High-Pressure Liquid Chromatography (HPLC) using the method described specifically for MP by Sabharwal and Belsare (1986) and for other compounds general by Zanella *et al.* (2003).

Analytical methods: The extraction and measurement of cortisol was fully described by Barcellos *et al.* (2007). Briefly, fish were captured and immediately frozen in liquid nitrogen and stored at -80 °C until the cortisol extraction. Each zebrafish was weighed and a pool of three fish were minced and placed into a disposable stomacher bag with 2 mL of Phosphate Buffered Saline (PBS, pH 7.4) for 6 min. The contents were transferred to a 10-mL screw top disposable test tube and 5 mL of laboratory grade ethyl ether was added. The tube was vortexed for 1 min and centrifuged for 10 min at 3000 rpm. The tube was then immediately frozen at liquid nitrogen and the unfrozen portion (ethyl ether containing cortisol) was decanted. The ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol. The extract was stored at -20 °C until the ELISA was conducted on the samples suspended with 1 mL of PBS buffer. In order to prevent a possible stress response induced by manipulation, the time elapsed between capture and killing was less than 10 sec. Whole body cortisol was measured in duplicate samples of tissue extract with a commercially available

EIAgen™ CORTISOL test (BioChem Immuno Systems). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions in PBS (pH 7.4) of the tissue extracts. The standard curve constructed with the human standards ran parallel to that obtained using serial dilutions of zebrafish tissue extracts. In the linear regression test, high positive correlation (R_2 = 0.9818) was found between the curves. The intra-assay coefficient of variation was 3.33-3.65%.

Statistical analysis: Data were expressed as Mean±S.E.M. and analyzed with Graph Pad InStat 3.00 statistical package (GraphPad Software, San Diego, California USA), by analysis of variance (ANOVA) followed by Tukey´s multiple range tests. Statistical significance was accepted at $p<0.05$.

RESULTS

The main result of the present work was the attenuation of whole-body cortisol levels in fish from MP+S group with values of 21.1±2.33 ng g^{-1} tissue, statically lower than stressed zebrafish in clear water (34.67±6.25 ng g^{-1} tissue). The mean whole-body cortisol levels are indicated in Fig. 1. No mortality was observed in all groups.

Immediately after inoculation the concentration of MP measured in the water (5.044 mg L^{-1}) was extremely close to the nominal concentration. After 96 h, MP was detected at a concentration 45% lower than inoculated (2.808 mg L^{-1}, Table 1).

Table 1: Daily measured Methyl-parathion concentrations (mg) in water

Measurement moment	Measured concentration (mg L^{-1})
Immediately after inoculation	5.044
24 h after inoculation	4.368
48 h after inoculation	3.692
72 h after inoculation	3.016
96 h after inoculation	2.808

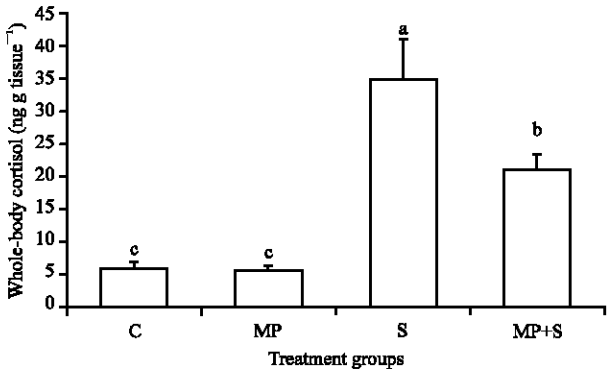

Fig. 1: Mean whole-body cortisol (ng g^{-1} ±SEM) levels of zebrafish exposed to stress (S), Methyl-Parathion (MP) or stress plus acute exposition to methyl-parathion (MP+S). Different letters above the standard error bars indicate a significant difference between treatment groups (ANOVA followed by Tukey's multiple range test, $p<0.01$; n = 10)

DISCUSSION

The main result of the present work was the attenuation of whole-body cortisol levels in fish from MP+S group. A weakened cortisol response to stress is a well-documented phenomenon that occurs in several fish species following prolonged (Hontela *et al*., 1992, 1995, 1997) and acute exposure to xenobiotics (Gravel and Vijayan, 2007; Cericato *et al*., 2008; Hori *et al*., 2008). In general, whole-body cortisol levels measured in the present study were similar to levels previously determined in this species (Ramsay *et al*., 2006; Barcellos *et al*., 2007, 2010).

HPI axis disruption provoked by MP exposure was also verified in the teleost *Rhamdia quelen* (Cericato *et al*., 2008), but the mechanisms involved were not studied. HPI axis disruption is a well know phenomenon in fish (Aluru *et al*., 2004; Gravel and Vijayan, 2006; Gravel and Vijayan, 2007). According to our knowledge, the results presented herein are the first reporting impaired whole-body cortisol levels in zebrafish exposed to MP.

The possibility that MP impairs the HPI axis in zebrafish by acting directly on the interrenal tissue cannot be discarded since we previously found a nonresponsive interrenal tissue in *R. quelen* when challenged with adrenocorticotropic hormone (ACTH) (Cericato *et al*., 2009). Also, we cannot discard the possibility that this disrupting effect may occur elsewhere within the HPI axis. Despite the exact mechanism by which MP blocked the cortisol response, biologically, the fish lost their capacity to mount an adequate response to cope with a standard stressor and maintain homeostasis. This attenuation may reduce the ability of the organism to promote metabolic and ionic adjustments necessary for the stress response. As outlined by Brodeur *et al*. (1997), fish that are incapable of mounting a normal cortisol response are likely to have a reduced ability to respond to the continuous challenges imposed on their homeostatic systems, either by aquaculture practices or by environmental changes.

Regarding the substance tested, the organophosphorous methyl-parathion (Folidol600®, 600 g L^{-1} of MP) is widely used as an insecticide in food storage and agriculture, as well as in fish farms to eliminate predatory aquatic insects. Thus, the current uses and practices easily allow MP to reach non-target organisms like fish (Cericato *et al*., 2008). When we put our results into an environmental context, we perceive that they are very relevant since the concentration used in fish farms to eliminate predatory aquatic insects ranges from 4 to 6 mg L^{-1} (Luvizotto-Santos *et al*., 2009), very close to the concentration used in the present work.

MP was degraded reducing the real concentration to about 55% of nominal inoculated concentration after 96 h of exposure, as verified by Sabharwal and Belsare (1986). Thus, fish were really exposed to sub lethal concentrations for 96 h.

The results presented herein show that MP produced impairment in the cortisol response to stress in zebrafish at a commonly used concentration. Thus, whole-body cortisol measurement might serve as sensitive diagnostic tools for acute exposure of fish to methyl-parathion.

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