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Short communication

Fluorescent transgenic zebrafish as a biosensor for growth-related effects of methyl parathion

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ABSTRACT

Transgenic fish models are potential alternative subjects in toxicological studies, since they can provide *in vivo* information on the deleterious effects of different substances. Here, we used a transgenic zebrafish (*Danio rerio*) lineage, which expresses a destabilized fluorescent protein (DsRED) driven by the myosin light chain promoter (Mylz2), in order to propose a new research tool for environmental biomonitoring. For validating the MYO-RED lineage, we exposed fish to the organophosphorated pesticide methyl parathion (MP). The effect of MP on fish growth was assessed by evaluating weight, length, condition factor and muscle fiber diameter. All factors suffered reduction at both tested concentrations (0.13 μM and 13 μM of MP). Similarly, fluorescence intensity decreased in a concentration-dependent manner, suggesting muscle protein catabolism. However, DsRED gene expression lowered only at the higher MP concentration. Results indicate that the MYO-RED transgenic zebrafish is an interesting model for detecting the growth-related effects of pollutants. Destabilized proteins such as reporter genes are apparently sensitive biomarkers, since effects were observed even at the lower, environmentally acceptable concentration. Therefore, this transgenic fish is a promising candidate model for sensitive, fast, and easy environmental monitoring.

Transgenic fish models are important tools for thoroughly understanding the role of specific genes in biological systems and pathways. These models can be applied in toxicological evaluations since they provide *in vivo* information on the deleterious potential of different substances. In toxicology research, transgenic fish lineages are developed mainly through the induction of genes associated with fluorescent reporter proteins (Chen et al., 2010; Seok et al., 2007). Fluorescent protein expression allows real-time monitoring of developmental processes, as well as a quick evaluation of how chemical substances affect these processes (Chen et al., 2010; Dai et al., 2014; Fan et al., 2011; Hano et al., 2009). Fast reductions in gene transcription can be of interest when they occur in response to regulatory physiological events (Kadonaga, 2004). However, detecting these reductions is unviable in most models due to the low degradation rates of fluorescent proteins. In this context, the use of destabilized fluorescent proteins is an interesting alternative, allowing a dynamic and *in vivo* evaluation of gene expression (Kitsera et al., 2007). The main characteristic of destabilized fluorescent proteins is that in the C-terminal region of mouse ornithine decarboxylase (MODC) contains a PEST sequence that targets the protein for degradation, resulting in rapid protein turnover (Li et al., 1998; Rechsteiner, 1990).

Classic toxicological studies have focused mainly on the effect of environmental pollutants on mortality rates. In an ecological context however, physiological parameters such as development, reproduction and growth are becoming increasingly representative. Growth rate is an important parameter since different pollutants can affect an individual's growth, leading to behavioral alterations that could result, for instance, in a higher risk of predation (Scott and Sloman, 2004). Considering the importance of evaluating growth rates in situations of environmental stress, Gabillard et al. (2010) combined evaluation of growth and molecular responses. These authors developed a transgenic trout model containing green fluorescent protein (GFP) driven by the myosin light chain promoter (Mylz2), and verified through *in vitro*

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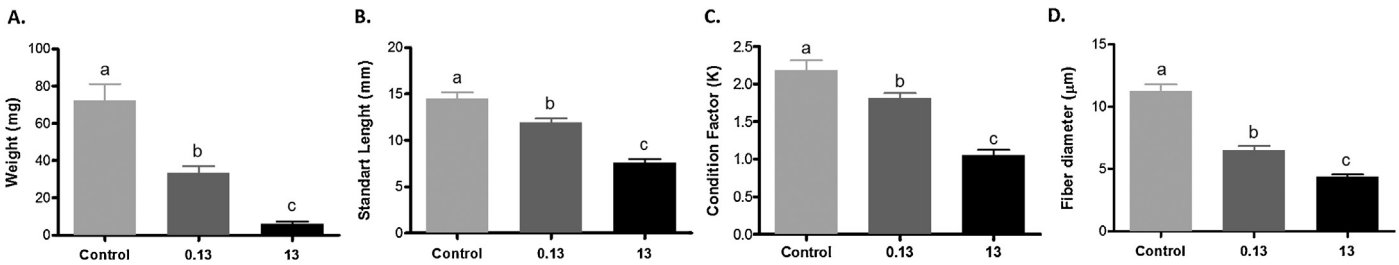

Fig. 1. Weight (A), standard length (B), condition factor (C) and fiber diameter (D) of transgenic zebrafish exposed to 0.13 μM and 13 μM of methyl parathion over 40 days. Different letters indicate significantly different means ($p < 0.05$). Data are expressed as mean ± SEM.

fluorescence that temperature variation can affect the differentiation of myogenic cells.

Our research group recently developed a transgenic zebrafish model (MYO-GHR) containing two genetic constructions. One construction is aimed at increasing signalization related to the growth process, with the growth hormone gene receptor (GHR) driven by the myosin light chain promoter. The second construction was co-injected to mark transgenesis, with the same myosin promoter driving expression of a destabilized red fluorescent protein (RED) (Figueiredo et al., 2012). The MYO-GHR lineage presents a high degree of fluorescence and does not require excitation for *in vivo* visualization. Due to this characteristic, during routine laboratory maintenance we were able to observe that stressful situations led to a decrease in fluorescence. Considering the ecological relevance of evaluating growth parameters and combining different biomarker responses in one study model, we used the MYO-GHR transgenic zebrafish lineage, which expresses a destabilized fluorescent protein, to propose a new research tool for environmental biomonitoring.

In this work we used transgenic zebrafish (*Danio rerio*) from the MYO-GHR lineage developed by Figueiredo et al. (2012). Hemizygotic animals with 30 days post fertilization (dpf) were obtained from crosses between non-transgenic females and hemizygous transgenic males, and bred in a water recirculation system, as described by Westerfield (2007). Fish were kept for 40 days in nine 2 L glass aquariums, at a constant temperature of 24 °C and a 12 h light:12 h dark photoperiod, and fed *ad libitum* twice daily. The experimental design was randomized, with two treatments and a vehicle control (acetone 0.01%) performed in triplicate. The evaluated methyl parathion concentrations were 0.13 μM, value permitted by the Brazilian legislation for Class III freshwater (CONAMA, 2005), and 13 μM. This pollutant was diluted in acetone, maintaining a 0.01% proportion. After exposure, fish were euthanized with tricaine methanesulfonate (500 mg/mL), measured, and weighed. Growth was analyzed in standard length (mm), weight (mg) and condition factor [$K = 100(W/L^3)$]. Animal care and experimentation were performed in compliance with the Federal University of Rio Grande's Ethics Committee (FURG, Brazil).

Following the growth experiment, fish were submitted to histological and gene expression analysis. For fiber diameter and fluorescence intensity analyses, six fish from each treatment were euthanized and microphotographed using an SZX 16 stereomicroscope with a DP72 camera (Olympus, Japan). Fish were then soaked in Tissue Freezing Medium (Leica, Germany) and cut sagittally using a CM 1850 cryostat (Leica, Germany) with 7 μm width. Sections were microphotographed using a DP72 camera with a 40X objective (SAPO) coupled with a BX52 microscope with filters for observation of blue-stained nuclei (U-MWU2 filter: excitation 330–385 nm, emission 420 nm), and DsRED fluorescence (U-MWG2 filter: excitation 510–550 nm, emission 590 nm) (Olympus, Japan). Differences in fluorescence intensity were analyzed through differential absorption method (Ornberg et al., 1999). The intensity of fluorescence micrographs (exposure time 250 ms) of RFP (red fluorescent protein) was analyzed with emission of 590 nm, selecting the red fluorescent marking and deleting the rest (Image > Adjust > Color Threshold > Selected). The selected and total area (μm) was then measured, and were expressed in $\mu m^2/100\ \mu m^2$. Muscle fiber diameter was measured as the smallest transversal diameter of 20 different muscle fibers in each animal. All measures were taken from the micrographs using the open source software IMAGE J (US National Institute of Health, available at http://rsb.info.nih.gov/ij/).

For gene expression analysis, RNA was extracted from five whole fish of each treatment using TRIzol (Invitrogen, Brazil). Extracted RNA was treated with DNAse I, Amplification Grade (Invitrogen, Brazil), and used as a target for cDNA synthesis through a High Capacity Reverse Transcription kit (Applied Biosystems, Brazil). Gene expression was analyzed through quantitative Real Time PCR, in a 7500 Real Time PCR System (Applied Biosystems, Brazil) with Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Brazil), using specific primers based on Figueiredo et al. (2012). Target gene expression was normalized by elongation factor 1 alpha (ef1a) and beta-actin (β-actin) gene expression, which did not vary significantly among experimental groups (data not shown). Growth performance, fluorescence intensity and fiber diameter between groups were analyzed with one-way ANOVA, followed by Tukey's *post hoc* test (0.05% significance). Significant differences in gene expression data among treatments were evaluated through the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The results of this experiment showed no significant effects of methyl parathion (MP) on fish survival. However, growth data evidenced a significant weight decrease (Fig. 1A) in fish exposed to MP (control: 72.4 mg ± 8.8; 0.13 μM: 33.4 mg ± 3.7; 13 μM: 5.9 mg ± 1.4). Similarly, length (Fig. 1B) decreased significantly when exposed to MP (control: 14.5 mm ± 0.6; 0.13 μM: 11.9 mm ± 0.4; 13 μM: 7.6 mm ± 0.4). These results indicate that MP affects zebrafish growth, even at the concentration permitted by Brazilian legislation (0.13 μM). Exposure to this pollutant class has been shown to induce muscular dystrophy in frog embryos (Bonfanti et al., 2004). Furthermore, Cook et al. (2005) reported that sublethal concentrations of malathion induced malformation (reductions in body length and eye diameter) in zebrafish embryos. A P450 monooxygenase enzyme metabolically transforms MP (Jokanović, 2001), producing metabolites such as methyl paraoxon and 4-nitrophenol. Methyl paraoxon inhibits acetylcholinesterase (AChE) activity, provoking accumulation of the acetylcholine neurotransmitter in the synaptic clefts, which can lead to tremors and even death (Chuiko, 2000; Shaonan et al., 2004). In parallel to the deleterious effect of excess acetylcholine on synapses, there is a high energetic cost that directly affects all physiological processes. The significant decrease in fish condition factors (Fig. 1C) and muscle fiber diameters (Fig. 1D) at the tested pollutant concentrations suggests that in order to supply this energetic demand, animals