

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 μ M and 13 μ M 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.

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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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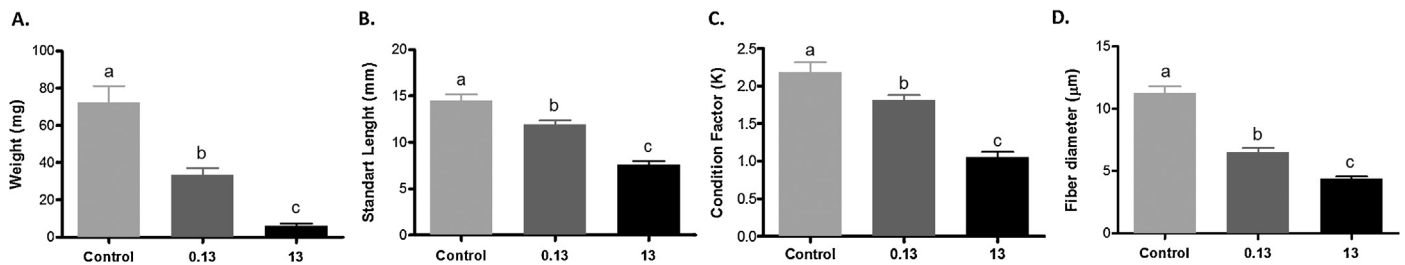


Fig. 1. Weight (A), standard length (B), condition factor (C) and fiber diameter (D) of transgenic zebrafish exposed to 0.13 µM and 13 µM of methyl parathion over 40 days. Different letters indicate significantly different means ($p < 0.05$). Data are expressed as mean $\pm$ SEM.

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 µm²/100 µm². 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 IMAGEJ (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 $\pm$ 8.8; 0.13 µM: 33.4 mg $\pm$ 3.7; 13 µM: 5.9 mg $\pm$ 1.4). Similarly, length (Fig. 1B) decreased significantly when exposed to MP (control: 14.5 mm $\pm$ 0.6; 0.13 µM: 11.9 mm $\pm$ 0.4; 13 µM: 7.6 mm $\pm$ 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

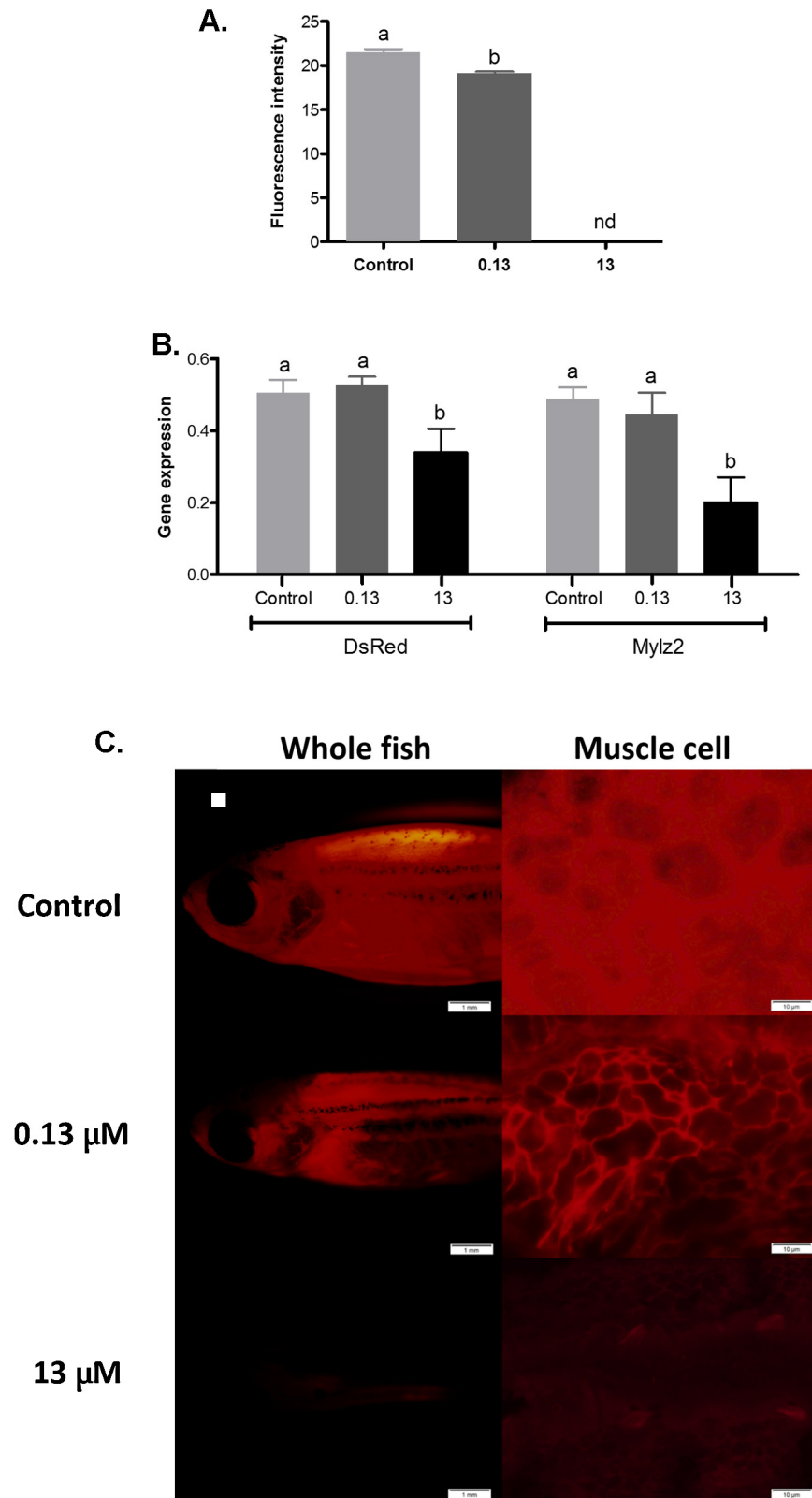


Fig. 2. Expression of genes (A) and red fluorescence protein (DsRED) expression quantified (B) and visualized using an epifluorescence microscope (C) in transgenic zebrafish. Fish were treated with acetone 0.01%, (control); 0.13 μM ; and 13 μM of methyl parathion. Gene expression was normalized by the expression of elongation factor 1 alpha (ef1a) and beta-actin (β -actin). Data are median $\pm$ SEM ($n = 5$). Different letters indicate significantly different means ($p < 0.05$). DsRED = red fluorescent protein; Myl2 = myosin light chain.

consume muscle proteins. Proteins are immediate and primary energy providers for fish exposed to stressful conditions.

The fluorescence intensity of DsRED (Fig. 2A and C) was evaluated for verifying if the expression of this protein, which is modulated by the myosin light chain promoter, could be related to growth. Similarly to growth data, fluorescence intensity decreased with MP concentration (control: 21.5 ± 0.39 ; $0.13 \mu\text{M}^{-1}$: 19.1 ± 0.21 ; $13 \mu\text{M}$: non detected). Several transgenic fish models have been developed associating promoters involved in important biological processes with fluorescent reporter proteins (Fan et al., 2011; Kusik et al., 2008; Perz-Edwards et al., 2001; Petersen et al., 2013; Seok et al., 2007, 2006). Fluorescent reporter proteins such as GFP, YFP and RFP are tools that can effectively detect and quantify genetic and molecular events (Ghim et al., 2010). These proteins are advantageous mainly because: (i) they do not require additional substrate; (ii) quantification is non-invasive and can be evaluated *in vivo* and (iii) analysis can range from one cell to an entire organism (Chalfie et al., 1994; Tsien, 1998). On the other hand, extremely long half-lives can lead to accumulation of these proteins in the cell, making studies of transitory gene expression changes challenging (Ghim et al., 2010; Mateus and Avery, 2000). Another disadvantage of fluorescent proteins is the expression background, where interference of endogenous and reporter molecules can alter the signal-to-noise ratio. Therefore, it is important to reduce background, mainly when a dynamic evaluation of the expression signal is necessary (Ghim et al., 2010). For solving this issue, different varieties of fluorescent proteins, such as GFP (Andersen et al., 1998), YFP (Hentschel et al., 2013) and DsRED (Li et al., 1998), have been developed.

Finally, we quantified mRNA in order to verify if the fluorescence emission was related to the transcriptional rate of exogenous genes inserted in the MYO-RED model. Gene expression data (Fig. 2B) showed that DsRED and Myl2 genes were significantly down-regulated only at the higher MP concentration (33% and 59%, respectively). These results suggest that, at the lower MP concentration, the decrease in fluorescence intensity is directly associated with protein degradation, since transcriptional levels were unaltered. Conversely, the reduced fluorescence of the higher MP concentration is caused not only by protein degradation, but also by a decrease in gene transcription, which limits protein turnover capacity in muscle tissue. Considering that transcriptional control is a complex process that demands adequate metabolic status (Kadonaga, 2004), we infer that high MP concentrations promoted an additional catabolic effect by influencing both on transcriptional and post-transcriptional response in the evaluated model.

In conclusion, our results indicate that the production of transgenic models expressing destabilized fluorescent proteins under transcriptional control of muscle growth-related promoters can be considered a valuable tool for assessing the effect of pollutants on growth. The fluorescence observed in these models depend on the balance between gene expression and protein degradation. Thus, any decrease in fluorescence may indicate a reduction in transcription and/or increase in catabolism of muscle protein to compensate for the additional energy demand caused by any environmental stress.

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