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Beta-naphthoflavone-induced*CYP1A* expression in the guppy *Jenynsia multidentata*: Time-dependent response, anesthetic MS-222 effect and fin analysis

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ABSTRACT

Cytochrome P450 1A (CYP1A) expression in fish is used as a biomarker of exposure to organic contaminants, such PAHs, PCBs and dioxins, in the aquatic environment. South American guppy fish *Jenynsia multidentata* were exposed to the prototypical aryl hydrocarbon receptor (AHR) agonist beta-naphthoflavone (BNF; 1 μM) and the fins were biopsied to characterize different aspects of *CYP1A* induction. RTq-PCR was used to quantify *CYP1A* mRNA levels in fish tissues. *CYP1A* induction in the gill, liver and anal fin (gonopodium) occurred within the first hour of waterborne exposure to BNF and persisted throughout 2, 4, 8, 24, 48 and 96 h compared to controls (DMSO vehicle; $p < 0.05$). The organ-specific temporal pattern of induction was marked by mRNA levels consistently augment as duration of exposure increases and tend to a sustained induction from 24 h to 96 h for gill and liver (~15-fold and ~50-fold over control, respectively). In gonopodium, there was a maximum *CYP1A* mRNA level at 4 h (~34-fold over control). Basal *CYP1A* mRNA levels and its induction following BNF exposure were not affected by administration of a chemical anesthetic (fish immersion in 100 mg l^{-1} MS-222 for 2–5 min) in the gill, liver, gonopodium, dorsal or tail fin ($p < 0.05$). In an *ex vivo* assay, in which small pieces of biopsied fins were exposed to BNF for 4 h, high *CYP1A* induction was observed in the tail and gonopodium (~49-fold and ~69-fold, respectively) but not in the dorsal fin compared to controls. To our knowledge, this is the first study to show that a 1 h waterborne exposure to an AHR agonist is sufficient to cause *CYP1A* induction in fish organs and fins. The present study added new information to the field regarding the use of MS-222 as an anesthetic on fish and the analysis of biopsied fins as an alternative non-lethal*ex vivo* assay for evaluating the *CYP1A* biomarker in fish. This observation could be useful for planning fish toxicological bioassays and biomonitoring studies on the aquatic environments in South America.

1. Introduction

Aquatic ecosystems are significantly impacted by organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins (*e.g.*, TCDD), which are potentially carcinogenic, teratogenic and mutagenic compounds (Aas et al., 2001). The induction of cytochrome P450 1A (CYP1A) in fish has been extensively used as a biomarker in monitoring and ecotoxicological studies (Whyte et al., 2000) and is often measured using enzymatic assays (*e.g.*, ethoxyresorufin-O-deethylase; EROD) (Billiard et al., 2004) and protein quantification (Bucheli and Fent, 1995; VanVeld et al., 1997). CYP1A induction occurs *via* aryl hydrocarbon receptor (AHR) activation in vertebrates such as mammals, birds and fish (Hahn, 2002). The *CYP1A* mRNA levels in fish have been evaluated using reverse transcription followed by real time PCR (RTq-PCR) for biomarker analysis (Pina et al., 2007).

The CYP1A mRNA transcript level and protein are primarily abundant in liver but can also be found in extra-hepatic organs, where it is strongly induced by organic contaminants such as PAHs, PCBs and dioxin (Jonsson et al., 2007; Ortiz-Delgado et al., 2008; Zanette et al., 2009). In addition, fish fins may be targets for

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CYP1A promoter activation following organic chemical exposure, as demonstrated using GFP transgenic fish models (Ng and Gong, 2013; Kim et al., 2013) and histological immunolocalization (Zodrow et al., 2004). The possible advantages of *CYP1A* biomarker evaluation in fish fins remain poorly explored. The peak of *CYP1A* induction by contaminants occurs in a time-dependent manner in the different organs of fish (Kim et al., 2008). Thus, it is important to understand this pattern to choose target organs and optimal exposure times to use in ecotoxicological studies and to understand the toxicokinetics of compounds.

Biomarker analysis in organs classically used in aquatic toxicological studies, such as the gills and liver, requires destructive (lethal) sampling, which could possibly endanger fish populations (Ress et al., 2005). To minimize this effect, nonlethal sampling methods, in which tissue removal occurs without a major damage to the animals, are potential alternatives that could be used in different animal species (Schmitt and Brumbaugh, 2007). CYP1A activation in external epithelial organs responds rapidly following waterborne exposures of fish or *ex vivo* exposure of gills (Kim et al., 2008; Jonsson et al., 2002). Based on that, the use of fins for analysis to develop a non-lethal biopsy method may be proposed.

The use of the chemical anesthetic tricaine methanesulfonate (MS-222) emerged around the 1960s and has been extensively used in fish experimentation (see Popovic et al. (2012) for a review). Reports on the possible influence of MS-222 on cytochrome P450 (CYP) responses are conflicting (Popovic et al., 2012). Evaluation of the MS-222 influence on the *CYP1A* biomarker response and its influence on other biological responses is required for the ethical use of fish as a surveillance species.

The Cyprinodontiforme fish that are widespread in aquatic environments, such as the North American killifish *Fundulus heteroclitus* and the South American guppies *Poecilia vivipara* and *Jenynsia multidentata*, are potential species for use in environmental studies (Dorrington et al., 2012; Elskus et al., 1999; Ferreira et al., 2012). The guppy *J. multidentata* (Cyprinodontiformes, Anablepidae) inhabits the region from Rio Negro, Argentina to Rio de Janeiro, Brazil and possesses unique characteristics, such as ovoviviparity and sexual dimorphism. In these fish, there is a modification of the male anal fin into a thin and elongated copulatory organ denominated gonopodium with regeneration capacity (Offen et al., 2008; Turner, 1947). Growth and gene expression profiles in the gonopodium have been used as important endpoints to study endocrine disruptor effects caused by contaminants in mosquitofish *Gambusia sp.* (Brockmeier et al., 2013). As far as we know, there are no studies that have investigated CYP1A induction in the gonopodium or the potential for a biopsy of this modified fin to be used in a non-lethal toxicological assay.

In the present study, the time-dependent and organ-specific*CYP1A* response to beta-naphthoflavone (BNF) exposure was evaluated in *J. multidenta*. The influence of the MS-222 anesthetic procedure on these responses was also investigated using *in vivo* and *ex vivo* (biopsied fin exposure) experiments. This study also contributes to the comprehension of *CYP1A* responses in fins, to develop non-lethal biopsy methodology. The results present evidence for the use of *J. multidentata* as an alternative model organism for ecotoxicology studies in the South American environment.

2. Materials and methods

2.1. Animal collection

Male *J. multidentata* fish (3–5 cm length; 0.5–2.5 g whole body weight; $n=150$) were collected in a watercourse from an uninhabited area 15 km away from the district of Balneário Cassino (Rio Grande, RS, Brazil; 32°17′48.36′′S and 52°16′01.96′′O) in August 2013 and November 2013 and were used in the experiments described in Sections 2.2, 2.3 and 2.4. Males were identified by the presence of the gonopodium morphological dimorphism. Fish were acclimated for seven days in the laboratory before beginning the experiments. The fish were maintained in dechlorinated water at 24 °C, salinity 5, on a 12 h light/12 h dark photoperiod with constant aeration and fed twice per day with *Alcon BASIC®MEP 200 Complex*. The procedures were approved by the Animal Care and Use Committee at the Universidade Federal do Rio Grande (FURG).

2.2. Time-dependent CYP1A induction following BNF exposure

Male fish ($n=84$) were divided into 14 experimental groups with $n=6$ fish in each group (1 fish per liter) and maintained in similar conditions to those mentioned in Section 2.1. BNF (Sigma-Aldrich, Germany) was dissolved in pure DMSO and was added to seven experimental groups to make a final concentration of 1 μM BNF and 0.002% DMSO in the aquarium. BNF and DMSO concentrations were chosen based on previous experiments in zebrafish (Jonsson et al., 2007) and pufferfish (Kim et al., 2008). DMSO was also added to seven experimental groups in an equal volume to make 0.002% DMSO control groups. BNF and DMSO were replaced in the exposed and control aquarium, respectively, every 24 h. Fish from the BNF and control groups were euthanized at 1, 2, 4, 8, 24, 48 and 96 h after the first addition of BNF or DMSO by putting the fish in ice followed by cervical transection. The gill, liver and gonopodium were dissected and immediately preserved in RNAlater® (Ambion) according to the manufacturer's instructions.

Total RNA was isolated with Trizol reagent (Invitrogen) and reversed transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Specific *CYP1A* and *β-actin* primers pairs (forward 5′-CATGGGCAGTGATGTACCTTGTGG-3′ and reverse 5′-GGAGTTCGATCCAGACCAATTTGC-3′ for *CYP1A* and forward 5′-AAAGCCAACAGGGAGAAGATGAC-3′ and reverse 5′-GCCTGGATGGCAACGTACA-3′ for *β-actin*; IDT Integrated DNA Technologies) were designed based on the GenBank nucleotide sequences EF362746 and EF362747, respectively. The real-time PCR conditions for the *CYP1A* and *β-actin* primers were previously tested and established (Ferreira et al., 2012).

Analyses were performed in duplicate using the GoTaq qPCR Master Mix kit (Promega) and a 7300 Real-Time PCR System (Applied Biosystems) set for the following program: 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The $E^{-\Delta ct}$ method was used to calculate the relative transcriptional level in experimental groups using *β*-actin as a reference gene as described by Schmittgen and Livak (2008). The threshold used for Ct inference was fixed at 0.2 ΔRn (fluorescence normalized by internal ROX dye) for all runs. Because no differences in transcriptional levels were observed between the control groups at the different experimental times evaluated, all values from control groups were pooled ($n=42$) and the mean value was used as a calibrator to evaluate the *CYP1A* induction to BNF at the different exposure times. The *CYP1A* fold induction to BNF was represented as a ratio of BNF exposed to control. Data from all groups were logarithmically transformed to adhere to the ANOVA assumptions of normality and homoscedasticity, and the differences between the groups were determined by one-way ANOVA followed by the Tukey-HSD post-hoc test for unequal number of samples ($p<0.05$). The software Prism 5 for windows (Version 5.00, GraphPad software Inc.) was used for all statistic analysis used in this manuscript.