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Biomarkers of Organic Contamination in the South-American Fishes *Poecilia vivipara* and *Jenynsia multidentata*

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Abstract

South American Cyprinodontiform fishes are potential candidates to be used as model species in environmental toxicology. We sought for molecular and biochemical biomarkers of pollution in *Poecilia vivipara* (Poeciliidae) and *Jenynsia multidentata* (Anablepidae). Partial nucleotide sequences for the *cytochrome P450 1A* (*cyp1A*), a classical biomarker of exposure to organic contaminants in fish, were identified in *P. vivipara* and *J. multidentata* (~ 650 nucleotides) using degenerated primers and PCR. These sequences shared ~ 90 % identity in the predicted amino acid sequence with the corresponding Cyp1A region of *Fundulus heteroclitus*. RT-qPCR analysis confirmed that *cyp1A* transcription was strongly induced in the liver and gills of *J. multidentata* (~185-fold and ~20-fold, respectively) and *P. vivipara* (122-fold and 739-fold, respectively), after 24-hrs exposure to 1 μ M of the synthetic *cyp1A* inducer β -naphthoflavone (BNF). After 24 hs of injection with 1 μ g.g⁻¹ of the environmental carcinogenic contaminant benzo[a]pyrene (BaP), a decreased total antioxidant capacity against peroxyl radicals was observed both in liver of *J. multidentata* and gills of *P. vivipara*. BaP injection in both fishes did not cause changes in lipid peroxides (TBARS) levels, suggesting an absence of an oxidative stress situation caused by BaP injection in this study. The newly identified *cyp1As* would serve as general biomarkers of exposure to organic contaminant in future studies using *P. vivipara* and *J. multidentata*. The results also points out to the important species-specific differences in the biomarker responses in those South American cyprinodontiform fishes, which would suggests distinct resistance/susceptibility to polycyclic aromatic hydrocarbons.

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Keywords

fish; pollution; PAH; Cyp1A; *cytochrome P450*, oxidative stress; *Jenynsia multidentata*, *Poecilia vivipara*

1. Introduction

Following the example of the Atlantic killifish *Fundulus heteroclitus* (Cyprinodontiform, Fundilidae), extensively studied in the North America (Burnett et al., 2007), Cyprinodontiform fishes that inhabit the South American Coast, e.g. *Jenynsia multidentata* (Anablepidae) (from Argentina to Brazil) and *Poecilia vivipara* (Poeciliidae) (from Argentina to Venezuela) (Froese and Pauly, 2001), have been suggested as “novel” model species in environmental toxicology (Amado et al., 2009; Ame et al., 2009; Cazenave et al., 2008; Mattos et al., 2009). The uses of those model cyprinodontiform fishes in toxicology, relies in the study of the fate of contaminants in the aquatic environment (Burnet et al., 2007, Matson et al., 2008) and also in the understanding of the genetic/evolutionary aspects involved in the adaptation to survive in extreme polluted conditions (Williams and Oleksiak, 2008, Wirgin et al., 2011) that helps to elucidate the fundamental biochemical and molecular mechanisms in toxicology (Hahn et al., 2004).

The understanding of the fate and the toxicity mechanisms caused by organic contaminants requires the study of cytochrome P450s and the biochemical aspects of the oxidative stress. Cytochrome P450 (Cyp) enzymes catalyze oxidative metabolism of thousands of drugs, environmental pollutants, and endogenous compounds. Environmental pollutants includes many substrates of mammalian and fish Cyp1s (e.g., Schober et al., 2006) such as the halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAH), herbicides, and pesticides (Nebert and Russell, 2002). While metabolism often results in detoxification, the action of Cyp1 enzymes also can generate toxic metabolites, and induced production of reactive oxygen species (ROS), contributing to increased risks of cancer, birth defects, and other toxic effects (Nebert and Karp, 2008).

Mammalian Cyp1A1 and fish Cyp1As could be strongly induced in the mRNA, protein and catalytically activity levels, by environmental contaminants such PAHs, planar polychlorinated biphenyl (PCB), dibenzo-p-dioxin (PCDD), and dibenzofuran (PCDF) congeners, and some natural products, via activation of the aryl hydrocarbon receptor (AHR) (Hahn, 2002). These features have led to the widespread use of Cyp1A as a marker of environmental exposure to AHR agonists in humans and wildlife (e.g., Stegeman, 1986; Fujita et al., 2001; Lambert et al., 2006). Although some substances (e.g. the synthetic β -naphthoflavone, BNF) are potent AHR agonists and Cyp1A inducers without causing major toxicity, other compounds, such as benzo[a]pyrene, BaP and PCB126) are potent AHR agonists, Cyp1A inducers and highly toxic.

It has been reported that both reactive oxygen species (ROS) derived from BaP biotransformation by Cyps (through redox cycling of hydro- and semi-quinone intermediates) and its metabolites as BaP-semiquinone radicals are responsible by oxidative damage to lipids and proteins or to modulate the antioxidant defense system (Kim et al.,

2000; Lin et al., 2007). In fact, lipid peroxides are oxidative damage products frequently found after BaP exposure in many animal models (Pan et al., 2006; Alsop et al., 2007).

Our objectives in this study were: (1) to identify Cyp1A sequences for both *J. multidentata* and *P. vivipara*; (2) to evaluate the *cyp1A* transcriptional induction after exposure to BNF, a model Cyp1A inducer (via AHR activation), in the aim to give support for the use of this biomarker of organic contaminant in environmental studies; and (3) to evaluate the oxidative related effects (in terms of total antioxidant capacity and lipid peroxidation) in those fishes injected with BaP, an environmental contaminant that is an Cyp1A inducer and toxic carcinogenic compound. This study will help to understand, whether or not, inter-specific differences exist between the South American Cyprinodontiform fishes *P. vivipara* and *J. multidentata*, and if there is an acquired resistance or increased susceptibility to organic contaminant exposure, as it occurs and have been extensively reported in the N. American *F. heteroclitus* fish populations.

2. Material and Methods

2.1 Fish collection and maintenance

Male *P. vivipara* and *J. multidentata* were caught at Cassino Beach (Rio Grande, Brazil) in March 2011, using minnow traps. Fish were acclimated at 20 °C in 100 L aquarium at UV treated water, adjusted to salinity 15 ppt with dechlorinated water, for one month and were fed twice a day with Alcon BASIC® MEP 200 Complex during the acclimation period. The procedures used in these experiments were approved by the Animal Care and Use Committee (CEUA) at the Universidade Federal do Rio Grande (FURG).

2.2 Identification of *cyp1A* sequences in *J. multidentata* and *P. vivipara*

Liver was dissected from one untreated male *J. multidentata* and one male *P. vivipara*, randomly selected. Total RNA was isolated using Qiazol reagent (Qiagen). The RNA quality was determined in a standard agarose gel electrophoresis, running 5 µL of sample plus 2 µL of loading buffer. The RNA quantity and quality was also determined spectrophotometrically (Nanodrop ND 1000; NanoDrop Technologies, Wilmington, DE). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), a mix of anchored oligo(dT) primer (MWG Biotech, Inc.) with random hexamer primer (provided in the reverse transcriptase kit) and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen).

Degenerate primers were designed using highly conserved regions of previously known *cyp1A* sequences from *F. heteroclitus* (Cyprinodontiform), Japanese medaka *Oryzias latipes* (Belontiiform) and Stickleback *Gasterosteus aculeatus* (Gasterosteiformes) (Genbank Accession numbers AF026800, NM_001105087 and HQ202281, respectively) and avoiding conserved regions present in other *cyp1* subfamilies (e.g. *cyp1B1*, *cyp1C1*, *cyp1C2* and *cyp1D1*). The list of primers used is presented in the table 1. PCR reactions were carried out for Cyp1A and β -actin in liver cDNA of *P. vivipara* and *J. multidentata*. PCR products were resolved on a 1% agarose gel and then isolated and purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Nucleotide

sequences were obtained using an ABI Prism 3130xl platform (Applied Biosystems), translated to the predicted amino acid sequences and aligned with other Cyp1 family members using ClustalW (Thompson et al., 1994). Calculation of identities among *P. vivipara*, *J. multidentata* and *F. heteroclitus* nucleotide and amino acid predicted sequences were performed using the Sequence Identity Matrix tool, Bioedit Sequence Alignment Editor Software (Hall, 1999).

2.3 BNF exposure experiment and quantification of cyp1A transcripts

Twenty male *P. vivipara*, with 3-5 cm length and 0.5-2.5 g whole body weight were randomly distributed in two 10 L glass aquaria (10 fish per aquaria). One of those aquaria was further used as control group, and another was used as the BNF exposed group. Fish stayed without food in those aquaria with aerated water, temperature 20 °C and salinity 15, for 24-h before starting the exposure experiment. After this period, 200 µL of a 0.05 M BNF stock solution (dissolved in DMSO) was introduced in one of the aquaria to make a final concentration of 1 µM BNF. BNF was purchased from Sigma-Aldrich Co.(MO, USA). An equivalent volume of DMSO was also introduced in the control group aquaria. The BNF concentration used here (1 µM) has been shown to be high enough to induce ethoxyresorufin O-deethylase (EROD) activity in gills of various fish species (Brunstrom et al., 2002, Jonsson et al., 2003). The same experimental procedures described above using *P. vivipara*, was also done with *J. multidentata* fish (3-5 cm length, 0.5-2.5 g whole body weight, n=20) in order to setup a control group (n=10 fish) and a BNF exposed group (n=10 fish).

Twenty four hours after the exposure experiment all *P. vivipara* and *J. multidentata* fishes (n=40 in total) were killed by cervical transection and liver and gill of individual fish were dissected and immediately placed in RNA later (Ambion). The samples were held 24-h at 4 °C, and then stored at -20 °C according to the RNAlater manufacturer's instructions.

Total RNA was extracted from gills and liver and cDNA was synthesized (n=5 per experimental group), using the methods described in the item 2.2 Gene-specific primers for the *P. vivipara* and *J. multidentata* *cyp1A* were designed with Primer3 (Rozen and Skaletsky, 2000). Specific *β-actin* primers for *J. multidentata* and *P. vivipara* were designed based on GenBank (Accession EF362747) and a recently identified sequence (unpublished data), respectively. Primers were obtained from IDT Integrated DNA Technologies (Primer sequences are shown in Table 1). Real-time PCR (qPCR) was performed in duplicate using GoTaq qPCR Master Mix (Promega, Madison, WI) according to manufacturer's instructions and a 7500 Real-Time PCR System (Applied Biosystems) using the program: 50 °C for 2 min, 95 °C for 2 min and 40 cycles 95 °C for 15s and 60 °C for 30s. Melting curve analysis was performed to all the PCR products at the end of each qPCR run to ensure that a single product was amplified. The E^{ct} method using *β-actin* as house-keeping gene was used to evaluate the *cyp1A* relative expression levels and the fold-induction in response to BNF treatment comparing to the respective control group. Gill's and liver's cDNAs from five fish per experimental group were used in the qPCR analysis.

2.4 BaP injection experiment and biochemical analysis

Ten previously acclimated male *J. multidentata* and ten male *P. vivipara* (3-5 cm length, 0.5-2.5 g whole body weight), were individually weighed and intraperitoneally injected with benzo[a]pyrene (BaP) (previously dissolved in DMSO) to make the dose of 1 µg BaP per g of fish (1 mg.kg⁻¹). Previous studies showed that an equivalent injected dose of BaP is high enough to induce ethoxyresorufin O-deethylase (EROD) activity, to alter oxidative stress parameters (Regoli et al., 2009) and to induce *cyp1A* in the transcriptional level (Bugiak et al., 2009) in fish liver. Ten *J. multidentata* and ten *P. vivipara* fishes were also injected with an equivalent volume of DMSO alone (control groups). Twenty four h after the BaP or DMSO injections in the both fish species (four experimental groups in total) the gills and liver were excised from all the forty fishes used in the experiment (n=10 fish per experimental group) and immediately stored at – 80 °C for biochemical analysis.

Total antioxidant competence against peroxyl radicals was determined through reactive oxygen species (ROS) determination in sample tissues treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2, 2'-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) (Winston et al., 1998). ROS concentration was measured along 30 min with the fluorogenic compound 2',7'-dichlorofluorescein diacetate (H2DCF-DA) at a final concentration of 40 mM, according to the methodology of Amado et al. (2009).

Results were expressed as relative area difference, using the following expression: Relative area = (FU 30 min_{ABAP} – FU 30 min_{without ABAP})/FU 30 min_{without ABAP}

According to this expression, low relative area difference means high antioxidant capacity against peroxyl radicals.

Oxidative damage was measured through lipid peroxidation using the TBARS method (Oakes and Van Der Kraak, 2003). This methodology involves the reaction of malondialdehyde (MDA), a degradation product of lipid peroxidation, with thiobarbituric acid (TBA) under conditions of high temperature and acidity, producing a chromogen which was quantified by fluorometry (excitation: 520 nm, emission: 580 nm). Briefly, after homogenization, aliquots were incubated at 95 °C during 30 min with 35 µM of butylated hydroxytoluene (BHT), 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid and 0.8% TBA. After cooling to room temperature it was added n-butanol and centrifuged at 3,000 × g for 10 minutes at 15°C. Tetramethoxypropane (ACROS Organics) was used as standard. Fluorescence was read at room temperature using a plate reader fluorimeter (Victor 2, Perkin Elmer).

2.5 Statistics

All results in the bar graphs were expressed as mean ± standard deviation. For the 1 µM BNF exposure experiment, homogeneity of variance for the *cyp1A* data was tested through Bartlett's test. Data were logarithmically transformed if the test rejected the assumption of variances homogeneity. Differences between the transcript levels of BNF and control (DMSO), for each fish species and organ, were analyzed using Student's t-Test. For the BaP injection experiment, significant differences in the total antioxidant competence and TBARS

were assessed by two-factor analysis of variance (ANOVA), being the factors BaP exposure (Control and 1 mg.kg⁻¹ fish BaP injected) and fish species (*P. vivipara* and *J. multidentata*). Post hoc comparisons were performed using the Newman-Keuls test or orthogonal contrasts. In all cases, type I error probability (α) was fixed in 5%. ANOVA assumptions (normality and variance homogeneity) were previously checked (Zar, 1984).

3. Results and discussion

Although poorly studied so far, *J. multidentata* and *P. vivipara*, as well as other cyprinodontiform fishes from S. America, possess one or more of the following features that support its use as model species in toxicology: (1) cosmopolitan (see figure 1) and adaptation to live in a wide range of stressing conditions (e.g., pollution, salinity, oxygen and temperature stresses); (2) different from the model species killifish *F. heteroclitus*, most are ovoviviparous, being interesting alternative models to understand mother embryo toxicological interaction during the embryonic development; (3) a huge diversity of species exists, and possibly a diversity of mechanisms of resistance/sensitivity to chemical stress; (4) some species are rare, thus possibly endangered. In the present study we added important information regarding biomarkers of organic contaminants using *J. multidentata* and *P. vivipara* as model species in environmental toxicology.

3.1 Identifying new cyp1A transcripts in *J. multidentata* and *P. vivipara*

Using degenerate primers based on teleost Cyp1A, we were able to amplify partial Cyp1A sequences (about 650 bp) from *J. multidentata* and *P. vivipara*. Aligning the amino acid predicted sequences from these PCR products with *F. heteroclitus* Cyp1A, Cyp1B1, Cyp1C1, Cyp1C2 and Cyp1D1 (obtained from GenBank, accession numbers AF026800, FJ786959, DQ133570, FJ786960 and FJ786961, respectively) resulted in the preliminary classification of the *J. multidentata* and *P. vivipara* sequences as *cyp1As* (Figure 2 and Table 2). The partial sequences obtained presents four of the six known Cyp1A substrate recognition sites (SRS2, SRS3, SRS4 and SRS5), and are located between amino acid positions 210 and 420 based on the full-length *F. heteroclitus* sequence (Figure 2). Comparisons of *F. heteroclitus* Cyp1A with the other Cyp1A sequences showed a marked identity between the cyprinodontiform Cyp1As in SRSs 2, 4 and 5, and some differences in the SRS3, as it could be expected based on previous studies that compared Cyp1A alignments among fishes before (Goldstone et al., 2009). The partial *J. multidentata* and *P. vivipara* Cyp1A sequences display 90 % and 90 % pair-wise identity to Cyp1A of *F. heteroclitus*, respectively (Table 2), and much lower identity to other *F. heteroclitus* Cyp1s (Cyp1B1, Cyp1C1, Cyp1C2 and Cyp1D1) that ranged from 38 % to 49 % identity (Table 2). Notably, the Cyp classification based on the amino acid percent identities suggested by Nelson et al. (1996), accepting identities higher than 55 % for the same subfamily, is in agreement with our preliminary annotation of these partial sequences. This preliminary analysis of SRSs and the high identities among the referred sequences (~90-95 %) could suggest that substrate overlapping could exist for Cyp1A in those S. American cyprinodontiform and the N. American *F. heteroclitus*.

3.2. BNF induces the expression of the *J. multidentata* and *P. vivipara* cyp1A genes

No mortality was observed during the *J. multidentata* and *P. vivipara* exposure to BNF or the carrier DMSO (control). The 24-hs exposure to 1 μ M of BNF strongly induced the expression of *cyp1A* in gill and liver of *J. multidentata* and *P. vivipara* comparing to the respective control groups ($p < 0.0001$ in all cases) (Figure 3). BNF is a commonly studied AHR agonist and a potent Cyp1A inducer (Jonsson et al., 2007). The present results suggest that the newly identified *cyp1As*, have potential to be additional sensitive biomarkers of exposure to AHR agonist contaminants in *J. multidentata* and *P. vivipara* likely it is well-known in other fishes.

The induction of Cyp enzymes in fish liver was first suggested as an indicator of aquatic contamination in the 1970s (e.g. Payne, 1976). Since then, many studies have shown that Cyp1As in vertebrate liver (often measured by activity assay, e.g. EROD, and protein detection by Western blot) are strongly induced by some organic contaminants that represent risk for human and wildlife (e.g., PAHs, coplanar PCBs, polychlorinated dibenzofurans, and dibenzodioxins) (Bucheli and Fent, 1995). The analysis of Cyp1A transcriptional levels by qPCR has been compared with traditional methods (e.g. EROD) and also has showed to be a sensible and robust biomarker for organic contaminants (Piña et al., 2007). Based on the presented results, we could suggest that the transcript quantification of the newly identified *cyp1As* by RT-qPCR, could be employed as potential biomarkers for organic contaminant exposure using *J. multidentata* and *P. vivipara* S. American cyprinodontiform fishes.

Although strongly induced by BNF, the quantified levels of *cyp1A* induction in exposed groups vary when different organs/species were considered. The most substantial changes in *cyp1A* expression in response to BNF were in *P. vivipara* gills (~739-fold induction) followed by *J. multidentata* liver (~185-fold), *P. vivipara* liver (~122-fold) and *J. multidentata* gill (~20-fold). This result suggests that different patterns for Cyp1A induction could occur when comparing those organs and cyprinodontiform species, after exposure to AHR-agonists, and possibly will reflect differences in the detoxification/bioactivational processes that are linked to Cyp1A activity. It is also possible that the observed differences rely in the characteristic interval of time that each organ/species show the “peak” of induction. It is well documented that in a time-exposure window, few hours can influence strongly the levels of induction at the gene transcription levels for Cyp1A in putterfish organs, after 6-, 12-, 24-, 48- and 96-hs exposure to 1 μ M BNF (Kim et al., 2008).

3.3. Biochemical biomarkers in *J. multidentata* and *P. vivipara* after exposure to the carcinogenic PAH BaP

As showed in Figure 4a, total antioxidant capacity against peroxy radical was decreased (higher relative area difference) in liver of *J. multidentata* liver and gills of *P. vivipara* after BaP exposure ($p < 0.05$; Figure 4b). The reduction in antioxidant competence, however, did not result in oxidative stress, since no changes on lipid peroxidation (TBAR) were observed after BaP treatment in tissues of both species ($p > 0.05$; Figure 5). Several evidences indicate that BaP alters the cellular redox state, triggering apoptosis (Soulhaug et al., 2005). Mussels *Mytilus galloprovincialis* exposed to BaP showed a peak of activity in the antioxidant enzyme catalase after 48 h exposure (Banni et al., 2010). In present study the selected BaP

dose and exposure period induced an intermediary response considering the previous evidences that showed an induction of the antioxidant defense (Lin and Yang, 2007; Banni et al., 2010) or the generation of oxidative stress (Costa et al., 2010). Our results indicated that BaP exposure caused loss of antioxidants, with specific differences, since liver was the responsive organ in *J. multidentata* and gills for *P. vivipara* 24-hs after injection. However the antioxidants consumption in liver and gills of the two species seemed to cope the pro-oxidant effect of BaP, since no evidences of oxidative damage was observed.

4. Conclusions

The high identity of Cyp1A sequences observed among *P. vivipara*, *J. multidentata* and *F. heteroclitus* associated with the Cyp1A transcriptional responses observed in fish exposed to BNF and treated with BaP support that proposition to use these South American Cyprinodontiform fishes as model species in environmental toxicology. Interestingly, depending on the species and tissue analyzed, we observed different responses in the oxidative stress associated parameters which suggest distinct resistance/susceptibility to PAHs. Population studies can be subsequently carried out in order to identify possibly polymorphisms in detoxification associated genes that enable the fish to adapt to highly contaminated sites, similarly to what has been described in *F. heteroclitus*.

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References

- Amado LL, Garcia ML, Ramos PB, Freitas RF, Zafalon B, Ferreira JLR, Yunes JS, Monserrat JM. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. *Sci Tot Environ*. 2009; 407:2115–2123.
- Ame MV, Baroni MV, Galanti LN, Bocco JL, Wunderlin DA. Effects of microcystin-LR on the expression of P-glycoprotein in *Jenynsia multidentata*. *Chemosphere*. 2009; 74:1179–1186. [PubMed: 19124144]
- Alsop D, Brown S, Van Der Kraak G. The effects of copper and benzo[a]pyrene on retinoids and reproduction in zebrafish. *Aquat Toxicol*. 2007; 82(4):281–95. [PubMed: 17433458]
- Banni M, Negri A, Dagnino A, Jebali J, Ameer S, Bousetta H. Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. *Ecotoxicol Environ Saf*. 2010; 73:842–848. [PubMed: 20071027]
- Brunstrom B, Jonsson EM, Brandt I. Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. *Environ Sci Technol*. 2002; 36:3340–3344. [PubMed: 12188363]
- Bucheli TD, Fent K. Induction of Cytochrome-P450 as a Biomarker for Environmental Contamination in Aquatic Ecosystems. *Critical Reviews in Environ Sci Technol*. 1995; 25:201–268.
- Bugiak B, Weber LP. Hepatic and vascular mRNA expression in adult zebrafish (*Danio rerio*) following exposure to benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Aquat Toxicol*. 2009; 95:299–306. [PubMed: 19403181]
- Burnett KG, Bain LJ, Baldwin WS, Callard GV, Cohen S, Di Giulio RT, Evans DH, Gomez-Chiarri M, Hahn ME, Hoover CA, Karchner SI, Katoh F, MacLachy DL, Marshall WS, Meyer JN, Nacci DE,

- Oleksiak MF, Rees BB, Singer TD, Stegeman JJ, Towle DW, Van Veld PA, Vogelbein WK, Whitehead A, Winn RN, Crawford DL. Fundulus as the premier teleost model in environmental biology: Opportunities for new insights using genomics. *Comp Biochem Physiol, Part D: Genomics Proteomics*. 2007; 2:257–286. [PubMed: 18071578]
- Cazenave J, Nores ML, Miceli M, Diaz MP, Wunderlin DA, Bistoni MA. Changes in the swimming activity and the glutathione S-transferase activity of *Jenynsia multidentata* fed with microcystin-RR. *Water Res*. 2008; 42:1299–1307. [PubMed: 17959219]
- Costa C, Catania S, De Pasquale R, Stancanelli R, Scribano R, Melchini A. Exposure of human skin to benzo[a]pyrene: Role of CYP1A1 and aryl hydrocarbon receptor in oxidative stress generation. *Toxicology*. 2010; 271:83–86. [PubMed: 20307623]
- Froese, R., Pauly, D. FishBase. World Wide Web electronic publication; 2011. www.fishbase.org, version (06/2011)
- Fujita S, Chiba I, Ishizuka M, Hoshi H, Iwata H, Sakakibara A, Tanabe S, Kazusaka A, Masuda M, Masuda Y, Nakagawa H. P450 in wild animals as a biomarker of environmental impact. *Biomarkers*. 2001; 6:19–25. [PubMed: 23886053]
- Goldstone JV, Jonsson ME, Behrendt L, Woodin BR, Jenny MJ, Nelson DR, Stegeman JJ. Cytochrome P450 1D1: A novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. *Arch Biochem Biophys*. 2009; 482:7–16. [PubMed: 19103147]
- Hahn ME, Karchner SI, Franks DG, Merson RR. Aryl hydrocarbon receptor polymorphisms and dioxin resistance in Atlantic killifish (*Fundulus heteroclitus*). *Pharmacogenetics*. 2004; 14:131–143. [PubMed: 15077014]
- Hahn ME. Aryl hydrocarbon receptors: diversity and evolution. *Chem-Biol Interact*. 2002; 141:131–160. [PubMed: 12213389]
- Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser*. 1999; 41:95–98.
- Jonsson M, Abrahamson A, Brunstrom B, Brandt I, Ingebrigtsen K, Jorgensen EH. EROD activity in gill filaments of anadromous and marine fish as a biomarker of dioxin-like pollutants. *Comp Biochem Physiol, Part C: Toxicol Pharmacol*. 2003; 136:235–243.
- Jonsson ME, Jenny MJ, Woodin BR, Hahn ME, Stegeman JJ. Role of AHR2 in the expression of novel cytochrome p450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci*. 2007; 100:180–193. [PubMed: 17686920]
- Kim HS, Kwack SJ, Lee BM. Lipid peroxidation, antioxidant enzymes, and benzo[a]pyrene-quinones in the blood of rats treated with benzo[a]pyrene. *Chem-Biol Interact*. 2000; 127(2):139–50. [PubMed: 10936229]
- Kim JH, Raisuddin S, Ki JS, Lee JS, Han KN. Molecular cloning and beta-naphthoflavone-induced expression of a cytochrome P450 1A (CYP1A) gene from an anadromous river pufferfish, *Takifugu obscurus*. *Mar Pollut Bull*. 2008; 57:433–440. [PubMed: 18304588]
- Lambert GH, Needham LL, Turner W, Lai TJ, Patterson DG, Guo YL. Induced CYP1A2 activity as a phenotypic biomarker in humans highly exposed to certain PCBs/PCDFs. *Environ Sci Technol*. 2006; 40:6176–6180. [PubMed: 17051818]
- Lin T, Yang MS. Benzo[a]pyrene-induced elevation of GSH level protects against oxidative stress and enhances xenobiotic detoxification in human HepG2 cells. *Toxicology*. 2007; 235(1-2):1–10. [PubMed: 17416446]
- Matson CW, Clark BW, Jenny MJ, Fleming CR, Hahn ME, Di Giulio RT. Development of the morpholino gene knockdown technique in *Fundulus heteroclitus*: A tool for studying molecular mechanisms in an established environmental model. *Aquatic Toxicol*. 2008; 87:289–295.
- Mattos JJ, Siebert MN, Luchmann KH, Granucci N, Dorrington T, Stoco PH, Grisard EC, Bainy ACD. Differential gene expression in *Poecilia vivipara* exposed to diesel oil water accommodated fraction. *Mar Environ Res*. 2010; 69:S31–S33. [PubMed: 19963263]
- Nebert DW, Russell DW. Clinical importance of the cytochromes P450. *Lancet*. 2002; 360:1155–1162. [PubMed: 12387968]

- Nebert DW, Karp CL. Endogenous Functions of the Aryl Hydrocarbon Receptor (AHR): Intersection of Cytochrome P450 1 (CYP1)-metabolized Eicosanoids and AHR Biology. *J Biol Chem*. 2008; 283:36061–36065. [PubMed: 18713746]
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW. P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*. 1996; 6:1–42. [PubMed: 8845856]
- Oakes KD, Van Der Kraak GJ. Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. *Aquatic Toxicol*. 2003; 63:447–463.
- Pan LQ, Ren J, Liu J. Responses of antioxidant systems and LPO level to benzo(a)pyrene and benzo(k)fluoranthene in the haemolymph of the scallop *Chlamys farreri*. *Environ Pollut (Barking, Essex: 1987)*. 2006; 141(3):443–51.
- Payne JF. Field Evaluation of Benzopyrene Hydroxylase Induction as a Monitor for Marine Petroleum Pollution. *Science*. 1976; 191:945–946. [PubMed: 1251203]
- Pina B, Casado M, Quiros L. Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *TrAC, Trends Anal Chem*. 2007; 26:1145–1154.
- Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. *Environ Toxicol Chem*. 2003; 22:2120–2129. [PubMed: 12959540]
- Rozen, S., Skaletsky, HJ. Primer3 on the WWW for general users and for biologist programmers. In: K, S., M, S., editors. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press; Totowa, NJ: 2000. p. 365–386.
- Schober W, Luch A, Soballa VJ, Raab G, Stegeman JJ, Doehmer J, Jacob J, Seidel A. On the species-specific biotransformation of dibenzo[a,l]pyrene. *Chem-Biol Interact*. 2006; 161:37–48. [PubMed: 16581046]
- Solhaug A, Øvrebø S, Møllerup S, Låg M, Schwarze PE, Nesnow S, Holme JA. Role of cell signaling in B[a]P-induced apoptosis: characterization of unspecific effects of cell signaling inhibitors and apoptotic effects of B[a]P metabolites. *Chem Biol Interact*. 2005; 151:101–119. [PubMed: 15698582]
- Stegeman JJ, Kloepper-Sams PJ, Farrington JW. Monooxygenase induction and chlorobiphenyls in the deep sea fish *Coryphaenoides armatus*. *Science*. 1986; 231:1287–1289. [PubMed: 17839567]
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994; 22:4673–680. [PubMed: 7984417]
- Williams LM, Oleksiak MF. Signatures of selection in natural populations adapted to chronic pollution. *BMC Evol Biol*. 2008; 8:282. [PubMed: 18847479]
- Winston GW, Regoli F, Dugas AJ Jr, Fong JH, Blanchard KA. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Rad Biol Med*. 1998; 24:480–493. [PubMed: 9438561]
- Wirgin I, Roy NK, Loftus M, Chambers RC, Franks DG, Hahn ME. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River. *Science*. 2011; 331:1322–1325. [PubMed: 21330491]
- Zar, JH. *Biostatistical Analysis*. 2nd. Prentice Hall; New Jersey: 1984.

Figure 1. Distribution of *Fundulus heteroclitus* (+), *Poecilia vivipara* (•) and *Jenynsia multidentata* (Δ) Cyprinodontiform fishes in the North and South American Atlantic coast and the location of the sampling site at Praia do Cassino, Rio Grande, RS, Brazil (↗) (adapted from Froese and Pauly, 2011).

Figure 2. Alignment of the *F. heteroclitus* (FUNHE), *P. vivipara* (POEVI) and *J. multidentata* (JENMU) amino acid sequences. The identified substrate recognition sites (SRS2-5) are showed in boxes. POEVI and FUNHE residues that are identical to the FUNHE sequence were indicated by a dot. Numbers indicates the position of *F. heteroclitus* amino acids in the full-length sequence. The dashes (-) are missing aminoacids in the sequences.

Figure 3.

Relative *cyp1A* expression in liver and gill of *P. vivipara* and *J. multidentata* exposed to 1 μ M β -naphthoflavone (BNF) (gray bars) and control (white bars). β -actin was used as a housekeeping gene. * indicate difference between treatments and control groups ($p < 0,0001$).

Figure 4.

Total antioxidant capacity against peroxy radicals in liver (a) and gill (b) from *P. vivipara* and *J. multidentata*. Data are expressed as mean $\pm$ 1 SE (n = 3-8). Similar letters indicate absence ($p > 0.05$) of statistical differences treatments following Newman-Keuls test. Asterisk (*) means significant differences ($p < 0.05$) in the indicated treatments after orthogonal contrasts.

Figure 5. Levels of thiobarbituric reactive substances (TBARS) in liver (a) and gill (b) from *P. vivipara* and *J. multidentata*. Data are expressed as mean $\pm$ 1 SE (n = 3-10). Similar letters indicate absence ($p > 0.05$) of statistical differences treatments following Newman-Keuls test.

Table 1

Forward (F) and reverse (R) degenerated primers employed in the *cyp1A* identification and specific primers employed in the *cyp1A* and β -Actin qPCR analysis in *P. vivipara* (Pv) and *J. multidentata* (Jm) fishes.

Primer name	Primer sequence 5'-3'	Location
Cyp1A degenerated F	TGTGGCATGTGCTTTGGMCGACG	610–632
Cyp1A degenerated R	GGGTCRTGGTTTATYTGCCACTGG	1245–1268
Pv_Cyp1A_qPCR_F	AATCCTGCAGCATTCATCCCTGCT	709–732
Pv_Cyp1A_qPCR_R	TGTCCTTGTCAAAGGTGGCGTAGT	821–844
Jm_Cyp1A_qPCR_F	CATGGGCAGTGATGTACCTTGTGG	995–1018
Jm_Cyp1A_qPCR_R	GGAGTTCGATCCAGACCAATTTGC	1062–1085
Pv_β-Actin_qPCR_F	ACCATCACCGGAGTCCATGACGA	<i>a</i>
Pv_β-Actin_qPCR_R	ATGTACGTTGCCATCCAGGCCGT	<i>a</i>
Jm_β-Actin_qPCR_F	AAAGCCAACAGGGAGAAGATGAC	<i>a</i>
Jm_β-Actin_qPCR_R	GCCTGGATGGCAACGTACA	<i>a</i>

^aNo full sequence available

Identities between nucleotide sequences (presented in the right-top) and amino acid sequences (left-bottom) among *J. multidentata* (JENMU), *P. vivipara* (POEVI) and *F. heteroclitus* (FUNHE) *cyp1* partial sequences. The full *F. heteroclitus* *Cyp1A*, *Cyp1B1*, *Cyp1C1*, *Cyp1C2* and *Cyp1D1* sequences were obtained from GenBank (accession numbers AF026800, FJ786959, DQ133570, FJ786960 and FJ786961, respectively).

Table 2

	JENMU_1A	POEVI_1A	FUNHE_1A	FUNHE_1B1	FUNHE_1C1	FUNHE_1C2	FUNHE_1D1
JENMU_1A							
POEVI_1A	0.96	0.95	0.91	0.48	0.47	0.49	0.58
FUNHE_1A	0.90	0.89	0.90	0.47	0.47	0.48	0.58
FUNHE_1B1	0.40	0.39	0.39	0.47	0.46	0.48	0.58
FUNHE_1C1	0.42	0.42	0.41	0.52	0.61	0.58	0.51
FUNHE_1C2	0.38	0.38	0.39	0.51	0.66	0.68	0.49
FUNHE_1D1	0.48	0.49	0.48	0.36	0.36	0.37	0.49