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Liver ethoxyresorufin-O-deethylase and brain acetylcholinesterase in two freshwater fish species of South America; the effects of seasonal variability on study design for biomonitoring

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ABSTRACT

Responses at low levels of biological organization to evaluate environmental changes and water quality have been used for many years. South America is no different, and recently biochemical endpoints in fish have been used to assess the impacts of industrial and sewage effluents on wild fish populations. For Chilean native freshwater fish, basic biological data is scarce and data on 7-ethoxyresorufin-O-deethylase (EROD) and Acetylcholinesterase (AChE) activity is practically absent. Moreover, extensive variation in these two biochemical endpoints exists among species and seasons. In this article we evaluate seasonal variation in liver EROD and brain AChE activities in *Trichomycterus areolatus* and *Percilia gillissi*, two widely distributed native freshwater fish species in central Chile. We observed a marked seasonality in hepatic EROD activity in both species, with maximums for *P. gillissi* during winter months and sex differences in February, July, August and December. *T. areolatus* showed no sex differences, and peaks in EROD activity in the middle of summer, winter and late spring. Species differences in EROD activity were observed with activity being 1–2 orders of magnitude higher in *P. gillissi* compared to *T. areolatus*. Scarce seasonal variation and no sex related differences in brain AChE for both species were observed. Multivariate analysis (PCA) indicated that physical water quality parameters had some degree of responsibility for the seasonal responses found. The seasonal variability data of these biochemical endpoints were used to optimize study design for future monitoring programs, planning timing of sampling, increasing statistical power by collecting specific sample sizes required.

1. Introduction

The increasing anthropogenic pressure on inland water systems has generated the need to evaluate and understand the effects of xenobiotics on water quality and health of the biota present. In this regard, an increasing number of investigations have used biochemical responses in fish as functional tools to assess toxicity of xenobiotics in natural populations (Fuentes-Rios et al., 2005, Webb et al., 2005, Chiang et al., 2011a), in situ bioassays (Chuiko, 2000, Whitehead et al., 2005, Orrego et al., 2006) and laboratory experiments (Orrego et al., 2005, Inzunza et al., 2006). Fish are frequently used due to their wide spatial distribution in aquatic environments and their ability to integrate environmental changes (Beyer et al., 1996, Munkittrick et al., 2000).

Mixed function oxygenases (MFOs) and cholinesterase activity are two widely studied biochemical responses in fish (Burnison et al., 1999, Munkittrick et al., 1992, Payne et al., 1996, Van der Oost et al., 2003). In fish, MFOs are concentrated mainly in the liver, since this is the main detoxifying organ, but are also present in the kidney, gastrointestinal tract, gills and other tissues (Varanasi et al., 1989; Stegeman and Hahn, 1994). They are a diverse multigene family of proteins found in many organisms and have an extensive ability to metabolize xenobiotic compounds and endogenous molecules (e.g., sex steroids and fatty acids) (Bernhardt, 1996). One of these families is the Cyp P4501A1 isoenzyme (cytochrome P 450 1A1), which are involved in phase I of biotransformation and further excretion of many metabolites. The induction of CYP450 is currently used to monitor exposure to

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pollutants such as polychlorinated biphenyls (PCBs), dibenzo-p-dioxins, furans and polycyclic aromatic hydrocarbons (PAHs) found as pollutants in all environmental matrices, including air, water, soil, sediment, fauna and flora (Cormier et al., 2000; Leonard and Hellou, 2001). An indirect measure to quantify the activity of CYP1A1 is the dealkylation of 7 ethoxyresorufin (EROD). Its catalytic activity can be measured by monitoring fluorimetric conversion of CYP1A1 substrates. This reaction is well established as a biomarker of exposure in fish (Bucheli and Fent, 1995; Goksøyr and Förlin, 1992; Stegeman and Hahn, 1994; Whyte et al., 2000).

Moreover, cholinesterases are present in all animals, and acetylcholinesterase (AChE) in the nervous system, hydrolyzes the neurotransmitter acetylcholine modulating the nervous signal (Murphy, 1986). Brain AChE inhibition in fish has been suggested as a way to demonstrate exposure to neurotoxic compounds, mainly organophosphates and carbamates (Van der Oost et al., 2003), but other authors have shown a decreased activity in fish exposed to wood leachates (Payne et al., 1996) and downstream of pulp and paper mill effluents (Orrego et al., 2006). Inhibition of these enzymes is essentially non-reversible with most pesticides and has been demonstrated to persist for several weeks. This inhibition often increases with chronic exposure, showing that inhibition of AChE integrates the exposure of aquatic organisms over time (Payne et al., 1996).

As many abiotic (i.e. pH, DO, temperature) and biotic (i.e. sex, reproductive period, maturity) factors modulate these biochemical responses within an organism, it is often difficult to interpret differences between sites and to propose using these types of biological endpoints in environmental risk assessment (Van der Oost et al., 2003, Whyte et al., 2000). A couple of approaches that can be used to improve the interpretation of these data are (1) a better characterization of confounding factors such as water physico-chemical parameters and reproductive status of the fish, which can help explain the observed variability and discriminate induced responses from background noise by applying an appropriate statistical analysis (Sturm et al., 1999); and (2) the proper design of the sampling program, optimizing the conditions for data collection (sampling over a short period of time, reducing the size range of the fish, maximization of statistical power with an adequate sample number; Payne et al., 1996) and particular care taken related to the "windows of sensitivity" for each species (Dissanayake et al., 2011). To address these requirements, extensive knowledge of the endogenous activity of the species and related seasonal variability of the subindividual responses is needed. The interpretation of monitoring data and the exact description of the "*in situ*" effects require some reference values.

Understanding the physiological variability and sensitivity of these biochemical responses between different fish species is essential for the proper design of monitoring programs (Van der Oost et al., 2003). Detailed understanding of the statistical power associated to the collections during different seasons for each species, using different Type I and II errors, is also required (Munkittrick et al. 2009). Thus, this study examines the seasonality of hepatic EROD activity and brain AChE of two native freshwater fish species widely distributed in Chile (*T. areolatus* and *P. gillissi*). Despite their large distribution, there is still limited knowledge of their basic biology. Both species live mainly in rhitron like zones of the river with shallow riffle and rapid habitats with *T. areolatus* highly associated with the substrates, while *P. gillissi* is a midwater dweller. Both species have small bodies (maximum recorded total length was 15 cm for *T. areolatus* and 9 cm for *P. gillissi*; Arratia, 1983; Ruiz and Marchant, 2004) and have a similar diet composed by benthic macroinvertebrates (Duarte et al., 1971; Ruiz, 1994). Spawning season for both species is described from late winter (August) to midsummer (January) (Manríquez et al., 1988; Habit et al., 2005; Chiang et al., 2011b). The influence of biotic and abiotic factors in the variability of these responses is also documented.

2. Methods

2.1. Study area

The Itata river basin drains seven major rivers, the Cholguan, Ñuble, Diguillin, Chillan, Cato, Lonquen and Itata rivers, in an area of about 11,500 km^2. The basin headwaters are in the Andes mountain range and flow from the Andes, through 195 kms of the central valley, through the coastal mountain range, the coastal plains and into the Pacific Ocean (Dussaillant, 2009). Like many other rivers in the oceanic mediterranean climatic zone, the Itata River is located in an area under high human pressure and has high seasonal fluctuations (Figueroa et al., 2007). The Itata River watershed has a mean annual flow of 120 m^3/s in the middle to lower reaches, but during the summer season it can decrease to less than 10 m^3/s with annual floods from May to September which are highly dependent of precipitation (Dussaillant, 2009). The Itata River basin has two overlapped thermal cycles, with daily mean oscillations close to 8 °C, with lower temperatures during August (≤ 5 °C) and maximum during January (≥ 25 °C) (Link et al. 2009).

2.2. Fish sampling

Adult individuals (>40 mm total length) of *T. areolatus* (484 female; 387 male) and *P. gillissi* (330 female; 308 male) were collected monthly, between February 2007 and January 2008 at three reference sites (S1, 36°42′17, 21″S 72°26′47,04″W; S2, 36°41′40,13″S 72°26′47,04″W; S3, 36°38′30,00″S, 72°27′12,64″W). The fish were captured using a backpack electrofishing equipment (Halltech Environmental, Canada) and a 6 mm mesh size seine net in similar habitats, as described in previous studies (Chiang et al., 2011b). Fish (sample size maximum 30 individuals per site and month) were sacrificed by spinal severance, then the liver and brain were weighed ($+0.0001$ g) and immediately placed in liquid nitrogen and later stored at -80 °C prior to analysis.

2.3. Physical–chemical water variables

Surface water samples (triplicate per site) were collected during the fish collections at the same three sites. A total of 48 physical–chemical parameters were analyzed. Some abiotic parameters like pH, conductivity, DO, temperature and water flow were analysed *in situ*. Water chemical analysis of nutrients, metals, microbiological parameters and other environmentally concerned pollutants including 22 pesticides (organochlorinated, carbamates and organophosphates) (see Table 1) was carried out in the laboratory according to standard methodologies (Eaton et al., 2005).

2.4. Liver EROD activity

The cytochrome P450 activity (CYP1A) was assessed by measuring 7-ethoxyresorufin-O-deethylase (EROD) activity in the post-mitochondrial fraction (S9) of the liver samples. This fraction was obtained following the homogenization of the livers in sucrose buffer (0.1 M pH 7.5) and centrifugation at 9000 g for 20 min at 4 °C. Analysis was performed in triplicate and using a Resorufin calibration curve (0–5 μg/ml) for each set of samples. It is expressed as pmol/min^{-1}/mg $protein^{-1}$ according to the methodology of Lubert et al. (1985). If the liver sample weight was <0.0400 g, livers were pooled in order to obtain the minimum sample volume required by the protocol, pooling livers from the same month, species, sex site and size.

2.5. Brain AChE activity

Brain tissue was homogenized in sodium phosphate buffer 0.1 M +1% Triton pH 8 at a ratio of 1:39 and centrifuged at 1000 g for 10 min. The activity of brain AChE was analyzed by a modification of the method of Ellman et al. (1961), as follows: For each sample, 50 μl of homogenate was transferred to a 1.5 ml microfuge tube, containing 900 μl 0.1 M sodium phosphate buffer (pH 8.0, 385 ml dibasic phosphate buffer 0.1 M +15 ml monobasic phosphate buffer 0.1 M) and 50 μl DTNB (10 mM). The solution was thoroughly mixed and incubated at room temperature for 10 min to allow non-enzymatic activity to stabilize. Samples and blank were transferred to plate wells in triplicate (160 μl each). To the blank wells, 40 μl of 0.1 M sodium phosphate buffer (pH 8.0) was added prior to the initiating reaction. Acetylthiocholine iodide (40 μl, 10 mM) was used as substrate and subsequent detection of thiocholine release by a reaction with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) which was monitored after incubation for 10 min at room temperature for a period of 6 min at 12 s intervals,