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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,

Table 1
Seasonal physical–chemical parameters in the three sites evaluated in the Itata River. Mean (standard error).

Parameter	Unity	Feb07	Mar07	Apr07	May07	Jun07	Jul07	Aug07	Oct07	Nov07	Dec07	Jan08
Alkalinity	$mgCaCO_3/L$	40.00(2.00)	45.33(0.33)	39.67(0.67)	38.00(1.00)	39.67(1.33)	25.67(0.67)	27.83(0.83)	26.17(1.33)	28.67(0.67)	44.00(1.00)	49.83(0.83)
Colour	Pt/Co	15.33(2.67)	17.67(0.67)	31.33(6.33)	24.67(2.67)	18(1.00)	11.33(0.33)	10.67(0.33)	19.33(0.67)	14.33(0.33)	18.67(0.67)	17.00(1.00)
Conductivity	μS/cm	72.43(1.67)	104.33(1.84)	48.3(1.67)	64.63(0.47)	66.67(1.09)	38.13(1.31)	65.67(1.02)	48.7(8.07)	72.83(3.72)	103.23(1.82)	105.60(1.65)
BOD5	mgO_2/L	1.97(.23)	1.63(0.13)	1.83(0.23)	1.93(0.03)	1.83(0.13)	1.90(0.20)	1.50(0.10)	1.3(0.00)	1.13(0.13)	1.47(0.07)	2.30(0.10)
COD	mg/L	8.33(6.33)	13.67(0.67)	8.67(1.67)	5.33(0.33)	7.00(0.00)	2.33(0.33)	2.00(0.00)	3.00(1.00)	3.00(0.00)	7.33(3.33)	16.33(1.33)
Dissolved oxygen	mgO_2/L	9.37(0.44)	9.20(0.21)	9.47(0.13)	11.50(0.21)	11.97(0.15)	12.07(0.24)	12.1(0.10)	10.3(0.15)	10.3(0.12)	10.03(0.24)	9.27(0.07)
pH	–	8.1(0.15)	8.3(0.03)	8.03(0.03)	8.07(0.19)	7.67(0.09)	7.17(0.03)	7.77(0.07)	7.73(0.09)	8.53(0.04)	8.16(0.19)	8.63(0.02)
Total suspended solids	mg/L	9.27(2.07)	3.53(0.07)	10.93(0.33)	7.07(0.33)	10.13(2.57)	5.33(0.57)	3.33(0.27)	6.17(1.07)	7.70(0.30)	7.23(0.63)	5.00(1.30)
Temperature	°C	24(0.0)	24(0.0)	16.4(0.7)	12.53(0.33)	9(0.00)	7(0.00)	8.47(0.13)	15.67(0.33)	19.00(0.00)	21.83(0.13)	27.3(0.10)
Ammonium	mg/L	0.04(0.01)	0.02(0.00)	0.02(0.00)	0.02(0.00)	0.02(0.00)	0.07(0.03)	0.03(0.01)	0.02(0.00)	0.02(0.00)	0.02(0.00)	0.02(0.00)
Total phosphorous	mg/L	0.09(0.02)	0.08(0.03)	0.11(0.01)	0.11(0.00)	0.11(0.01)	0.08(0.01)	0.06(0.01)	0.07(0.01)	0.07(0.01)	0.08(0.02)	0.10(0.04)
Nitrate	mg/L	0.09(0.02)	0.05(0.00)	0.63(0.17)	0.56(0.25)	0.42(0.28)	0.45(0.09)	0.73(0.10)	0.19(0.02)	0.22(0.03)	0.25(0.08)	0.06(0.01)
Nitrite	mg/L	0.010(0.003)	0.006(0.001)	0.019(0.007)	0.018(0004)	0.016(0.008)	0.007(0.002)	0.013(0.006)	0.017(0.005)	0.018(0.003)	0.018(0.005)	0.017(0.016)
Total nitrogen	mg/L	0.26(0.07)	0.23(0.01)	0.32(0.02)	0.32(0.00)	0.28(0.05)	0.27(0.07)	0.26(0.03)	0.27(0.02)	0.17(0.05)	0.21(0.03)	0.25(0.01)
Orthophosphate	mg/L	0.08(0.06)	0.14(0.07)	0.09(0.04)	0.04(0.02)	0.08(0.04)	0.06(0.02)	0.05(0.01)	0.07(0.03)	0.13(0.03)	0.11(0.06)	0.13(0.10)
Sulfate	mg/L	3.6(0.6)	2.7(0.2)	3(0.5)	6(0.0)	5.67(0.033)	4.33(0.33)	4(0.00)	2.67(0.17)	3.00(0.00)	5.33(0.67)	5.50(0.50)
Silica	$mgSiO_2/L$	17.35(1.85)	19.68(1.33)	18.90(0.97)	20.13(1.27)	19.17(1.26)	19.47(0.59)	19.61(0.50)	15.49(0.71)	15.34(1.39)	14.23(1.23)	19.51(1.97)
Fatty acids	mg/L	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.01(0.00)	0.013(0.003)	0.013(0.003)	0.010(0.000)	0.020(0.006)
Total hydrocarbons	mg/L	0.11(0.03)	0.61(0.13)	0.10(0.003)	0.21(0.06)	0.23(0.06)	0.27(0.08)	0.09(0.01)	0.18(0.02)	0.14(0.03)	0.08(0.00)	0.327(0.003)
Phenol index	μg/L	5(0.9)	2.1(0.2)	2.4(0.2)	2.3(0.3)	2.93(0.23)	2.93(0.43)	1.00(0.00)	1.97(0.07)	1.60(0.60)	1.67(0.67)	1.00(0.00)
AOX	mg/L	0.006(0.000)	0.005(0.002)	0.022(0.003)	0.022(0.006)	0.007(0.001)	0.007(0.003)	0.033(0.009)	0.008(0.000)	0.014(0.003)	0.012(0.000)	0.012(0.009)
Total aluminum	mg/L	0.289(0.053)	0.12(0.023)	0.197(0.037)	0.243(0.063)	0.143(0.037)	0.300(0.060)	0.27(0.06)	0.340(0.110)	0.147(0.054)	0.371(0.028)	0.273(0.070)
iron	mg/L	0.02(0.004)	0.03(0.009)	0.049(0.001)	0.041(0.005)	0.067(0.014)	0.028(0.002)	0.025(0.004)	0.049(0.004)	0.055(0.006)	0.042(0.002)	0.030(0.002)
Zinc	mg/L	0.001(0.0)	0.001(0.000)	0.017(0.005)	0.001(0.000)	0.001(0.000)	0.004(0.003)	0.008(0.003)	0.011(0.002)	0.004(0.001)	0.002(0.000)	0.006(0.003)
Fecal coliform	NMP/100 ml	27(0.0)	40.33(9.67)	166.67(36.67)	153.33(73.33)	280(110.00)	130(50.00)	26.67(3.33)	216.667(83.33)	56.667(13.33)	226.67(73.33)	50.00(0.00)
Flow	m^3/s	17.83(0.433)	19.27(3.73)	n.d	n.d	n.d	n.d	155.57(7.87)	n.d	n.d	n.d	7.8(0.80)
Pesticides[a]	mg/L	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL

[a] Pesticides: γ-hexachlorocyclohexane, α-hexachlorocyclohexane, β-hexachlorocyclohexane, δ-hexachlorocyclohexane, hexachlorobenzene, heptachlor, heptachlor epoxide, aldrin, dieldrin, endosulfan I and II, endrin, pp-DDE, pp-DDD, pp-DDT, op-DDT, metoxychlor, trifuralin, chlorothalonil, parathion, captan.

using a microplate spectrophotometer (Bausch and Lomb DNM-9602G) at 412 nm. The activity was expressed as IU mg of protein^{-1}.

2.6. Statistical analysis

Statistical analysis between the three reference sites showed no site differences for physical–chemical water quality parameters or for biochemical data from both fish species, so data were pooled within the month for the three sites for the following analysis. Due to the seasonal nature of the study, the analysis evaluated responses between months with sexes separate for each species. The responses between sexes and months were evaluated using analysis of variance (ANOVA, $p<0.05$) for biochemical endpoints (EROD, AChE) (SYSTAT ©11.0). To characterize the seasonal changes of the sites, a principal component analysis (PCA) was performed using a multivariate matrix of the set of abiotic parameters, using the software PRIMER V.6 developed in Plymouth Marine Laboratory (Clarke and Gorley, 2005).

3. Results

3.1. Physico-chemical water variables

While a marked seasonality is shown in some of the water quality parameters, no significant differences between the sites within the sampling period were present during the study period (Table 1). Within the high number of physical–chemical parameters analyzed in the water column, the PCA (Fig. 1) showed seasonal variation in water quality that could be separated between dry, rainy and snowmelt seasons.

The most evident seasonal differences are shown by Factor 1 and Factor 2, where total hydrocarbons and fatty acids are clearly influenced by water temperature, pH and alkalinity during the summer, while BOD_5, silica, total nitrogen, total iron and faecal coliforms are influenced by rainfall and runoff during the autumn and winter seasons. Factor 1 and Factor 2 explained just 50.5% of the variation, and separated the variation on ammonium, zinc and aluminium during the snowmelt season (Fig. 1, Table 2). This seasonal fluctuation in the water quality was strongly marked, with temperature, pH, alkalinity, total hydrocarbons and fatty acids grouped by the dry season. All the other physical and chemical parameters grouped less to the three autumn–winter and spring seasons and explained a cumulative total of 63.2% of the variation (Fig. 1, Table 2).

3.2. Liver EROD and brain AChE activities

No significant differences were observed between sites (EROD, ANOVA, $p=0.996$; AChE, ANOVA $p=0.390$) or sex (EROD ANOVA $p=0.053$; AChE $p=0.887$) for *Trichomycterus areolatus* so data were pooled to assess seasonal variation in these parameters. The EROD activity in *Percilia gillissi* differed between males and females during certain months; so for this analysis, sexes were not pooled.

Males and females of *T. areolatus* showed marked seasonality in hepatic EROD activity, with multiple peaks during late spring, midsummer and mid to late winter periods. Both sexes exhibit small EROD activity in the period from April to June (mid-autumn to early winter), but lowest activities were recorded in October and January (ANOVA, $p<0.001$), (0.118 ± 0.032 ρmol/min/mg protein, $0.278+0.179$ ρmol/min/mg protein, respectively). Maximal EROD activities (recorded in February, July–August and November–December) ranged from $3.525+0.264$ to $7.647+0.830$ ρmol/min/mg protein (Fig. 2A). Brain AChE for *T. areolatus* did not exhibit major variation between months, with maximum of $0.345+0.016$ IU/mg protein during June and a minimum of $0.184+0.004$ IU/mg protein during October. The annual observed mean for AChE was 0.281 IU/mg protein. AChE activity was significantly lower than all other months during October (ANOVA, $p<0.05$) (Fig.3).

P. gillissi adults showed no significant differences between sites during the year long collection period for both endpoints, EROD activity (ANOVA, $p=0.946$) and AChE activity (ANOVA, $p=0.273$). There were some significant differences between males and females for EROD ($p=0.035$) in February and December, but not for AChE activity ($p=0.936$). Despite the differences between sexes, EROD activity showed similar seasonal patterns for males and females, except for the December collections in which EROD activity was below the detection limit for males (Fig. 2B). Thus, both sexes showed the lowest activities during October and January (0.063 ± 0.023 a 0.544 ± 0.132 ρmol/min/mg protein), with an increase between November and December. There was a gradual increase in EROD activity from the summer to late winter (August) with a maximum EROD activity of 35.087 ± 6.492 ρmol/min/mg protein in males and 26.201 ± 4.463 ρmol/min/mg protein in females during August. Seasonally, there were significant differences in AChE activity for *P. gillissi* adults (ANOVA, $p<0.05$), demonstrating minimum activity between

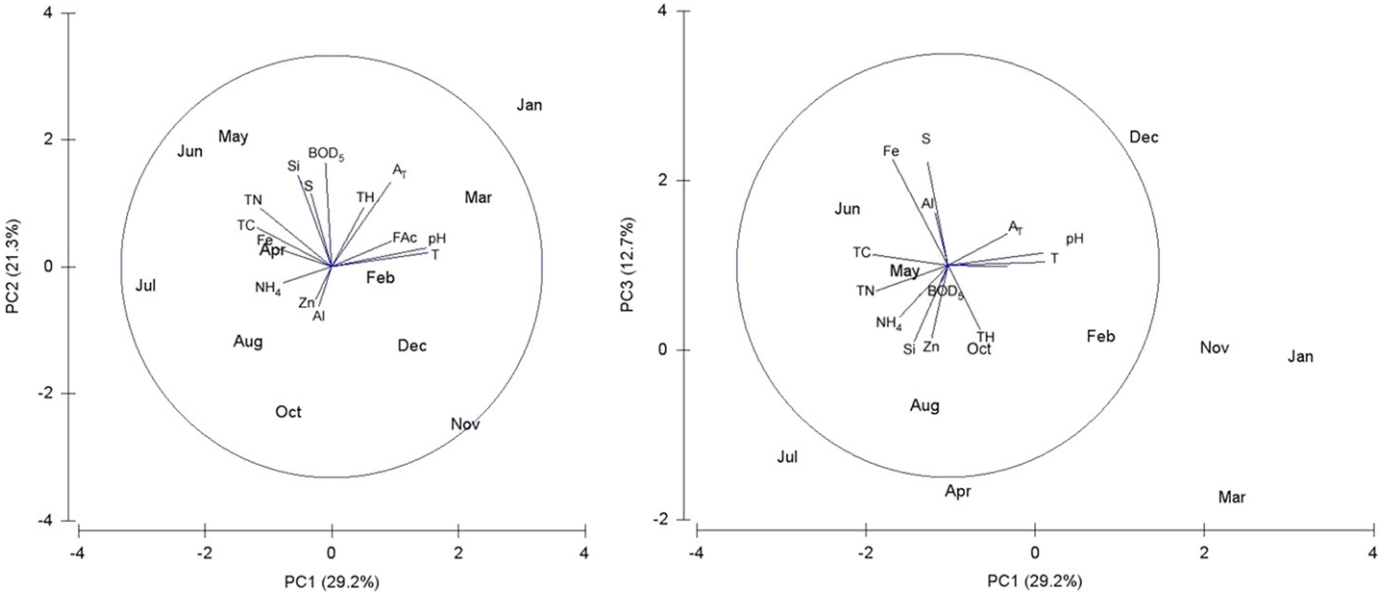

Fig. 1. Principal Component Analysis (PCA) of water physicochemical parameters between February 2007 and January 2008. A_T: Alkalinity; Al: aluminium; TH: Total Hydrocarbons; Fac: Fatty Acids; pH; *T*: temperature; Zn: Zinc; Fe: iron; TC, total coliforms; TN; total nitrogen; Si: Silica; S: Sulphur; BOD_5: Biochemical oxygen demand; NH_4: ammonia.

Table 2
Correlations of variables and principal components (PC1, PC2, PC3) for water physicochemical parameters in the Itata River during February 2007–January 2008.

Variable % total variance	**PC1** 29.2	**PC2** 21.3	**PC3** 12.7
A_T	0.279	0.399	0.148
BOD_5	−0.029	**0.693**	−0.074
pH	**0.548**	0.088	0.059
T	**0.658**	0.066	0.016
NH_4	−0.23	−0.076	−0.246
TN	−0.339	0.277	−0.12
S	−0.097	0.343	**0.690**
Si	−0.16	**0.534**	−0.359
Fac	0.283	0.12	−0.006
TH	0.153	0.282	−0.302
Al	−0.061	−0.19	0.249
Fe	−0.261	0.087	**0.698**
Zn	−0.077	−0.157	−0.346
TC	−0.355	0.185	0.051

A_T: alkalinity; Al: aluminium; TH: total hydrocarbons; Fac: fatty acids; pH; *T*: temperature; Zn: Zinc; Fe: iron; TC, total coliforms; TN; total nitrogen; Si: Silica; S: sulphur; BOD_5: biochemical oxygen demand; NH_4: ammonia. Bold numbers indicate significant correlations.

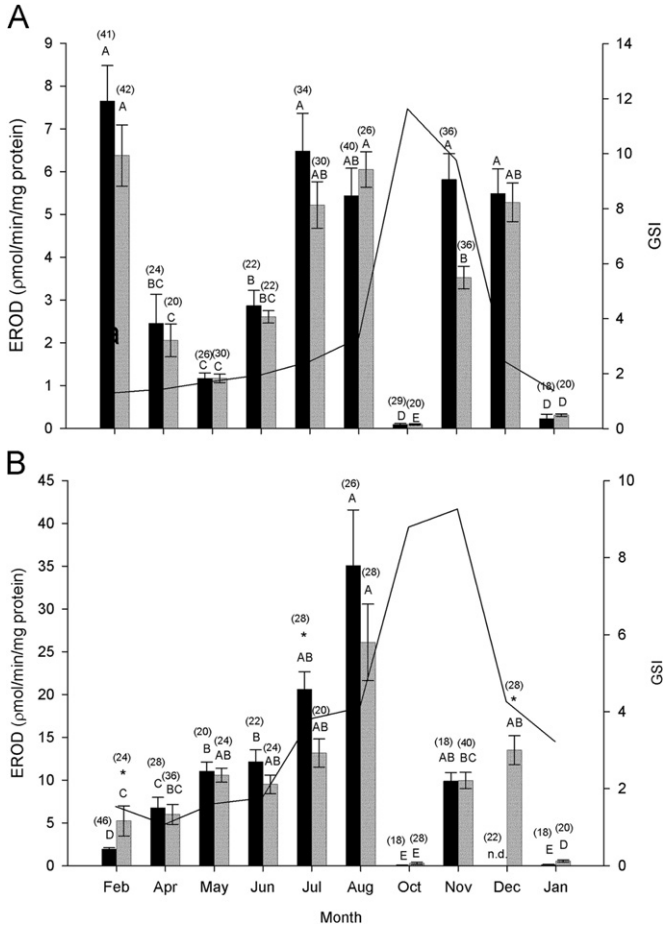

Fig. 2. Seasonal variation on liver EROD activity in **A**: *T. areolatus and* **B**: *P. gillissi.* Black bars: male; grey bars: female. Number above bars indicate sample size for each month and sex. Line represents mean female GSI (modified from Chiang et al., 2011b) Equal letters indicate no statistical differences for the same sex. * indicates significant differences between sexes for that month. One way-ANOVA, $p < 0.05$.

October and November (0.132 ± 0.004 and 0.138 ± 0.007 IU/mg protein), rising between December and April and peaking in May (0.307 ± 0.03 IU/mg protein), before returning to mean values between June and August (Fig. 3).

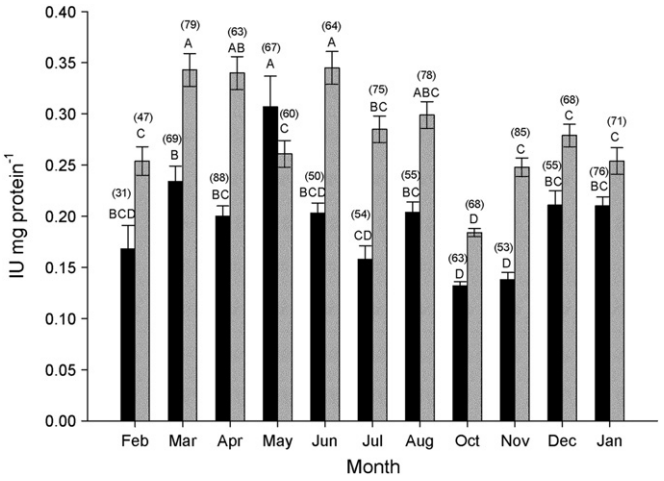

Fig. 3. Seasonal variation on brain AChE in *T. areolatus* (grey bar) *and P. gillissi* (black bar). Equal letters indicate no statistical differences between months for each species. One way-ANOVA, $p < 0.05$.

4. Study design

The traditional statistical design sets up Type I error (α) at 0.05 and Type II error (β) at 0.20, with a statistical power of 80%. Using this approach with equal α/β values (0.1 and 0.05), we were able to calculate sample size requirements for each species and enzyme activity, depending on the effect size we wanted to test, in the two seasons that had the lowest and the highest coefficients of variance (COV).

AChE activity showed lower COV's for both species in each season tested (16.9%–34.4%) and sample sizes of ≤ 20 individuals were calculated to detect 25% and 50% differences between sites for March when COV's were highest. October was the season with the lowest COV for AChE, and sample size requirements were below 10 individuals per site, for both species (Fig. 4A and B). Sample sizes were similar using $\alpha=0.05/\beta=0.2$ and $\alpha=0.1/\beta=0.1$, but using $\alpha=0.05/\beta=0.05$ in almost every case, sample size requirements nearly doubled (Fig. 4A and B).

COV for EROD activity was very high for both species during the whole study period. April and October showed the lower and higher COV's respectively, with values for *P. gillissi* (73.3–145.5%) about double of that for *T. areolatus* (37–79.7%). April sample sizes (55% effect sizes) ranged from 30 to 49 individuals, with higher values using error values of $\alpha=0.05/\beta=0.2$ for *P. gillissi*, while for *T. areolatus* the sample sizes were lower and ranged from 11 to 17, with equal values for $\alpha=0.05;0.1/\ \beta=0.2;0.1$, and higher for $\alpha=0.05/\beta=0.05$. Using the sample sizes that we used in our study (<30 individuals), we observed that effect sizes had to be 95% for *P. gillissi* and 60% for *T. areolatus*. Statistical design using $\alpha=0.05/\beta=0.05$ almost doubles the sample size requirements, while the sample size requirements using $\alpha=0.05;0.1/\beta=0.2;0.1$ were similar and lower for both species (Fig. 5A and B).

5. Discussion

For the species studied here, basic biological data was scarce (Manríquez et al., 1988; Chiang et al., 2011b) and the seasonal variability of two biochemical markers of pollution did not exist. While there was a marked seasonality in hepatic EROD activity in both species, there were only differences between sexes for *P. gillissi*. Such differences have been reported for other species and were linked to the stage of gonadal maturation (DeZwart et al., 1995,

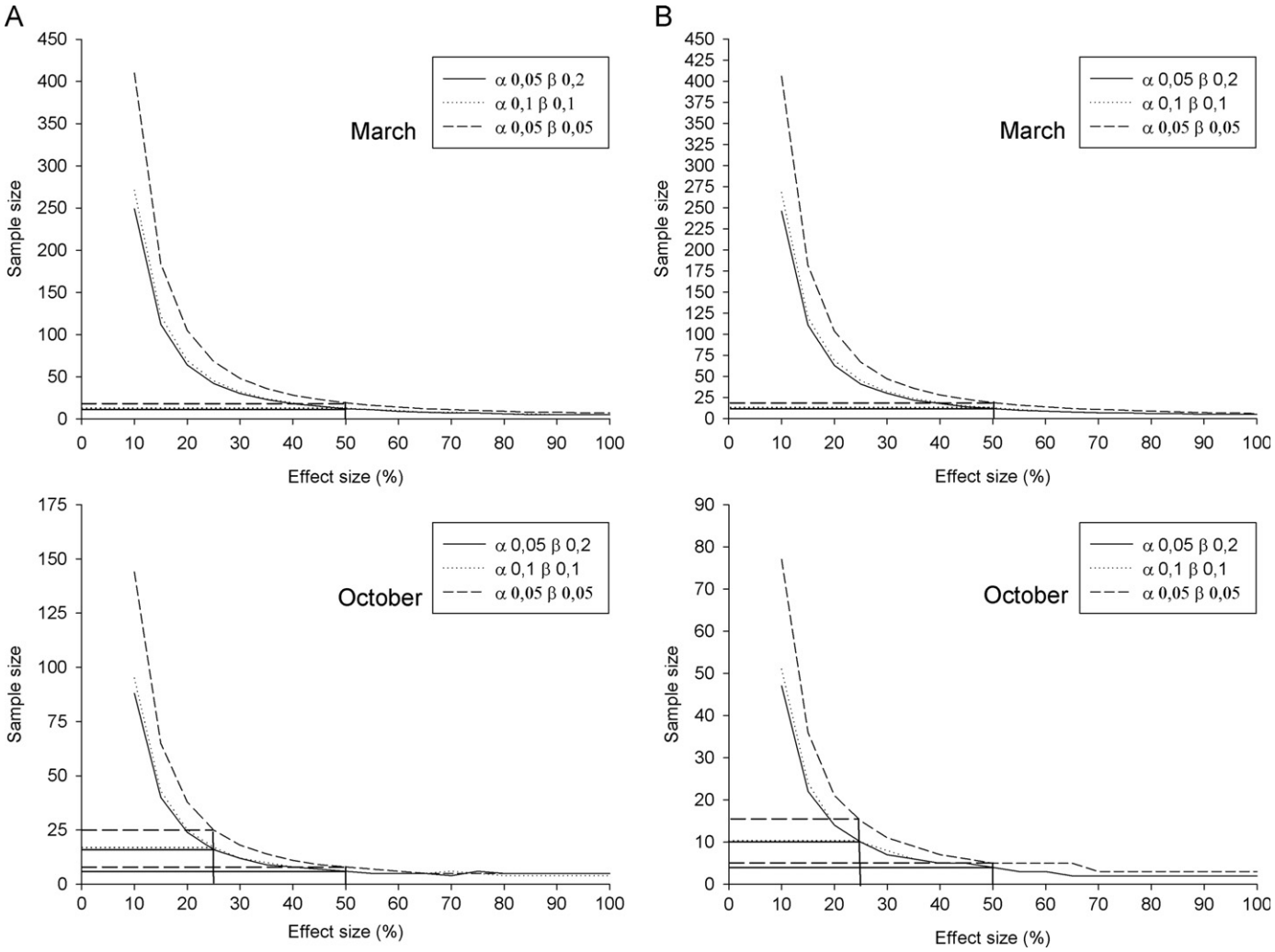

Fig. 4. Influence of statistical design on sample size at multiple effects sizes on brain AChE activities between wild populations of *P. gillissi* (A) and *T. areolatus* (B).

Levine et al., 1995, Sánchez et al., 2008). Although the absence of gender differences in hepatic EROD activity in *T. areolatus* is not uncommon (Collier et al., 1995, Förlin et al., 1995), comparing the induction of EROD activity between species without establishing baselines would be highly inappropriate.

On the other hand, EROD activity in this fish species had marked differences between seasons, especially during the spawning season. In fish with strong sex differences, the decreased EROD activity could be due to the negative effect of endogenous estradiol in the catalytic activity of the CYP450 complex (Arukwe and Goksøyr, 1997) or due to the dilution of proteins during vitellogenesis in female fish (Andersson et al., 2007). In general EROD activity and CYP1A protein levels of females gradually decline towards the onset of ovulation and then rise again during the postspawning period (Elskus et al. 1989, Arukwe and Goksøyr, 1997). This was precisely the case for our fish species under study here. Namely, *P. gillissi* and *T. areolatus* female GSI peaks during October and November (Manríquez et al., 1988; Chiang et al., 2011b). As we have stated, EROD activity could be related to reproductive status of the adult fish, and in previous studies (Chiang, 2010; Chiang et al., 2011b) we found a marked spawning season of both species that starts in late winter, has a maximum development of the gonad during midspring and in larger fish is prolonged to early summer. Both species are multiple spawners and histological data showed batches of mature oocytes and a cycle of sex steroids production that peaks before the gonad in late winter and a second peak of sex steroid production in late spring–early summer, presumably to extend the oocyte production and the spawning season. These peaks in sex steroid production could be responsible for the marked seasonality observed in EROD activity, explaining that very fast decline of EROD activity occurs during this peak of gonadal development and again in January for *P. gillissi* with a gradual increase towards winter.

Brain AChE activity in both species demonstrated a low seasonal variability, with no differences between sexes throughout the current study. This is consistent with other similar studies where no seasonal patterns were observed (Sánchez et al., 2007, 2008, Sturm et al., 1999), with healthy reference fish in the environment showing only 17% differences in activity (Williams and Sova, 1966).

Although, the marked seasonality in EROD activity and scarce seasonality observed in AChE, might be influenced by the reproductive–metabolic stage of the fish; we observed that the correlations of seasonal variation within physical–chemical water parameters might give us some insights. Seasonal variability in EROD activity may be strongly linked to the physiological condition of the fish, as demonstrated by Levine et al. (1995) for *Dorosoma cepedianum*. For this species, peaks in EROD activity positively correlated to condition factor and inversely correlated to LSI and water temperature. Brain AChE activity in *P. gillissi* in our study was correlated with silica levels, which may indicate

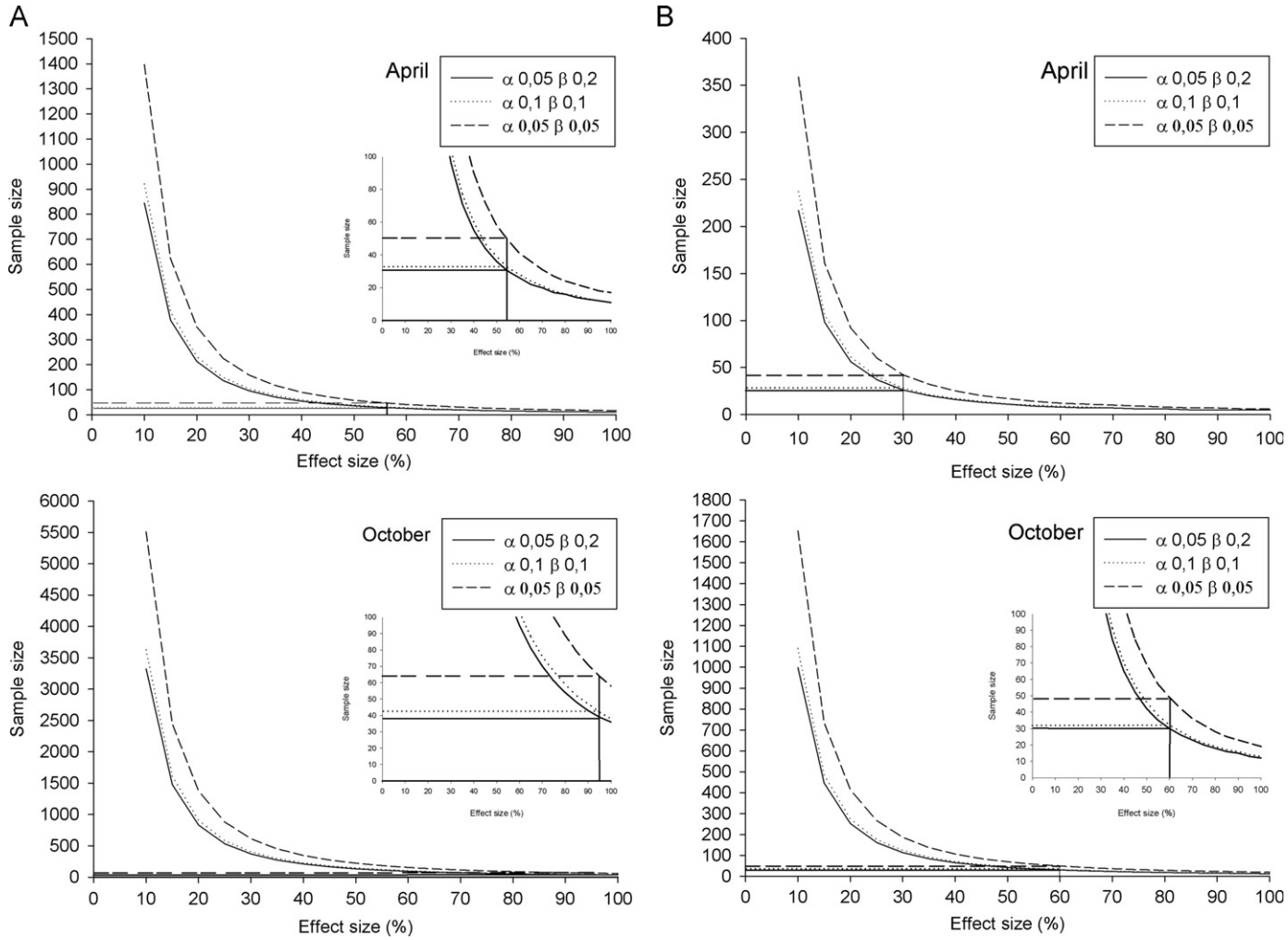

Fig. 5. Sample size required to assess liver EROD activities differences on wild fish populations of *P. gillissi* (A) and *T. areolatus* (B), using different Type I (α) and Type II (β) errors.

the drag of allochthonous material to the basin (silica), along with compounds capable of decreasing cholinesterase activity such as pesticides (< DL), with bioaccumulation possibly having a modulating effect on AChE activity (Payne et al., 1996). Inhibition has also been observed following low exposures and even after weeks of exposure to OP (Fulton and Key, 2001). In addition in poikilotherms, the regulation of enzyme-based reactions is dependent on temperature compensation mechanisms, due to the influence of environmental temperature (Sleiderink and Boon, 1996). This is clear in *P. gillissi*, where temperature is one of the two main variables that account for the variability in all the seasonal biological responses of these species. We showed a small, but clear increase in AChE activity starting in early summer and peaking to early autumn, where temperatures decrease and rainy season contributes to runoff of allochtonous material to the river and AChE activities decreased, especially in *P. gillissi*. Indeed, the differences in EROD activity between both species are very strong, with values in *P. gillissi* 1–2 orders of magnitude higher than that observed in *T. areolatus*. This might indicate a lower threshold for this species, with a temperature based compensation mechanism, increasing the enzymatic activities to regulate lower reaction rates at lower temperatures (Ankley et al., 1985).

Despite authors having shown that certain fish species are capable of surviving brain AChE inhibition of up to 95% (Fulton and Key, 2001), we have to set similar thresholds for each species. Other authors have shown in laboratory tests that $\geq$20% inhibition of AChE indicates a mild exposure to pesticides whereas lethal effects occurred mainly at inhibitions $\geq$70% (Fulton and Key, 2001; Golombieski et al., 2008). Moribund fish exposed to insecticides in the environment have shown AChE activity levels depressed by 50% in comparison to reference fish (Williams and Sova, 1966).

Among the most important reasons for the variability observed, there might be individual differences in physiology, nutritional, reproductive and health status. To minimize this variability and make environmental assessments possible, the comparisons should be restricted to groups of fish with the same general characteristics as we have shown (i.e. same species, sex, reproductive status). Using this information we can properly design a sampling program, maximizing statistical power using an adequate sample number (Beliaff and Burgeot, 1997, Payne et al., 1996, Munkittrick et al. 2009).

EROD coefficients of variability (CV) were very high and sample sizes similar for other endpoints ($\approx$30 individuals, Chiang et al., 2011b) allowed us to detect effect sizes >30%. When EROD activity was induced, it has been shown to be at least 2 fold greater than in fish collected from reference sites (Orrego et al. 2009, Chiang et al., 2011a).

Therefore, using a traditional sampling design with α=0.05 and β=0.20, or using an equivalent level of error (α=0.1, β=0.1) we could calculate similar minimum sample sizes for both species at different times of the year for each biochemical endpoint. This

way, we could increase the probability of detecting significant induction in EROD and inhibition in AChE activity between fish populations from different sites. Knowing the COV for each species within a certain season where variability is the lowest we would use March and October for EROD and April and October for AChE (min–max COV, respectively).

6. Conclusions

In these baseline studies, we observed that the activities of both enzyme complexes have different modulations, with liver EROD activity linked to endogenous changes of individuals and physical parameters in the river. Both species present a marked seasonality, which was particularly evident in *Percilia gillissi*. Both species showed a minimum EROD activity during October inversely correlated to the period of maximum gonadal development. Brain AChE activity was relatively constant throughout the year decreasing during the rainy season that could be correlated to the contribution of allochthonous material and low water temperatures. This study showed not only the seasonal variation in EROD and AChE activities in two native freshwater fish, but the importance of assessing these seasonal changes in biota to optimize study designs for developing a monitoring plan using biochemical indicators of exposure and effect. Despite the possible interannual variability of the biological responses, this approach showed to be the most appropriate to reduce sample size of species in conservation concern, along with reducing Type I and II errors without sacrificing our statistical power of the study. The present study represents a baseline data for improving biomonitoring studies using this species and responses for Chilean freshwater environmental assessment

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