Pesticide residue analyses and biomarker responses of native Costa Rican fish of the Poeciliidae and Cichlidae families to assess environmental impacts of pesticides in Palo Verde National Park

F. Mena[1]*, M. Fernández San Juan[2], B. Campos[2], J. Sánchez-Ávila[2], M. Faria[2,3], M. Pinnock[1],
E. de la Cruz, S. Lacorte, A.M.V.M.Soares and C. Barata[2]

[1]Central American Institute for Studies on Toxic Substances/Instituto Regional de Estudios en Sustancias Tóxicas (IRET).
Universidad Nacional, Campus Omar Dengo, 3000, Heredia, Costa Rica
[2]Department of Environmental Chemistry, IDÆA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain[1]
[3]CESAM & Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal
*Corresponding Author E-mail: fmena@una.ac.cr

Abstract

Pesticide chemical residues in water samples and biomarker responses in transplanted fish were used to monitor environmental hazards of pesticides in Palo Verde National Park (Costa Rica). The Costarican fish, *Parachromis dovii* (Ciclhidae) and *Poecilia gillii* (Poecillidae), were selected as sentinel species. Contaminant analyses detected up to 15 different pesticide residues in water with hexachlobenzene (2261 ng l^{-1}), phorate (473 ng l^{-1}), epoxiconazole (314) and bromacil (117 ng l^{-1}) being the compounds found in higher concentrations. Biomarker responses evidenced impacts on cholinesterase activities in transplanted fish at Barbudal site probably due to the presence of organophosphate insecticides such as phorate. High enzyme activities of glutathione S-transferase and catalase and elevated levels of lipid peroxides were also observed at a site impacted by rice fields (Cabuyo); those effects could be associated with the presence of hexachloro benzene and triazole fungicides. In general, *P. dovii* biomarkers were affected to a greater extent than those of *P. gillii* in fish transplanted to sites associated with agriculture, which suggests the former species is a good candidate for future surveys.

Key words

Biomarker, Costa Rica, Fish, Pesticides, Tropical, Water

Introduction

In most countries of the EU, Ecological Risk Assessment procedures of contaminants are well established (i.e. Directive 93/67/EEC, Regulation 1488/94, Directive 98/9/EC, Directive 91/414/EEC). However, same is not the case in Latin American countries where despite environmental legislations, risk assessments are not required to address regional or local problems. For example, in Costa Rica, environmental risks of pesticides are usually limited to establish maximum dosage of use without taking into account their effects or environmental behaviour (de la Cruz and Castillo, 2002; de la Cruz *et al.,* 1998,

2004). This is unsatisfactory since pesticides have a complex environmental fate under tropical conditions and hence it is not possible to predict their environmental concentration levels from usage doses only.

Tropical ecosystems also hold high biodiversity communities which likely make them susceptible to be impacted by pesticides. For the above mentioned reasons, there is a need to improve pesticide analytical and biological methods to detect their effects. The integrated use of chemical analyses, biochemical and cellular responses to pollutants is considered to be one of the best procedures for detecting impacts of

contaminants in aquatic systems (Porte *et al.,* 2001; Walker and Livingstone, 1992).

Recently, the development of multi-analyte mass spectrometry methods combined with liquid and gas chromatographic techniques have allowed detection of low environmental concentrations of a broad range of pesticides in water samples contaminated with complex pesticide mixtures (Sánchez-Avila *et al.,* 2011; Suárez-Serrano *et al.,* 2010). Thus, these methods offer opportunity to determine precisely the occurrence of pesticides in surface waters. The use of biomarkers is a suitable strategy for studying sublethal effects of pollutants, providing an early indication of possible adverse effects in the organisms (Van der Oost *et al.,* 2003). Fish are often selected as sentinel species in monitoring and impact assessment studies due to their known physiological responses to pollutants, their trophic importance in the aquatic food web, their commercial importance and their sensitivity to pollutants (Van der Oost *et al.,* 2003).

Aquatic communities impacted by agriculture are usually affected by complex mixtures of pesticides and other pollutants, many of them with unknown mechanisms of action to fish, thus, a battery of biomarkers is often used. In environmental assessment, some biomarkers are frequently applied. Among these, liver biotransformation enzymes that aid organisms to detoxify and eliminate many hydrophobic pollutants by making them more water soluble. Glutathione S-transferase (GST) is a phase II enzyme that aids conjugating pollutants or/and their metabolites with glutathione, favouring their further excretion (Livingstone, 2001; Ketterer *et al.,* 1988). High GST activities are usually associated with the presence of organic pollutants or pro-oxidant conditions (Livingstone, 2001). Detoxification enzymatic processes have a metabolic cost and often produce reactive oxygen species that are detoxified by a battery of antioxidant defences. One of such defence is enzyme catalase that converts hydrogen peroxide into water (Aebi, 1974). Overproduction of reactive oxygen species may produce oxidative damage in proteins, lipids and DNA. One of such products, lipid peroxides are used as a marker of oxidative stress (Halliwell and Gutteridge, 1999). Certain pollutants such as organophosphate or carbamate pesticides act by inhibiting cholinesterase activity and hence disrupting the transmission of nerve impulse causing death by asphyxia (Boone and Chambers, 1997).

The present study aimed to use multi-residue mass spectrometry analytical methods and a battery of biomarkers to assess possible detrimental biological effects of pesticides in two local Costa Rican fish species. Up to 40 pesticide residues including organo phosphorus, acaricides, carbamates, fungicides and herbicides were determined in water using solid phase extraction and gas or liquid chromatography - mass spectrometry (GC-MS, LC-MS) methods (Sánchez-Avila *et al.,* 2011; Hilde brandt *et al.,* 2007). A set of four biomarkers including the phase II

metabolizing GST enzyme, antioxidant enzyme, cholinesterase enzyme and lipid peroxidation levels were determined in fish transplanted across clean and contaminated sites surrounding Palo Verde National Park, Pacific coast of Costa Rica. This study was part of a project intended to characterize the environmental risk of pesticides used in the production of rice and sugar cane in the aquatic communities of Palo Verde.

Materials and Methods

Experimental animals : Two fish species *Parachromis dovii* (Ciclhidae) and *Poecilia gillii* (Poecillidae) were used for the experiments. Both species are native with a broad distribution in Costa Rica (Bussing, 2008; Mendiola, 2008). Immature juveniles of *P. dovii* (mean standard length 2.91.1±0.13 cm; mean body weight 0.705±0.09 g) were acquired from the Aquaculture Laboratory at the Universidad Nacional, Heredia, Costa Rica. Adult specimens of *P. gillii* (mean standard length 4.33.1±0.15 cm; mean body weight 1.79±0.19 g) for transplants were captured in a reference site included in field experiments (site B).

Environmental area and fish sampling : The Palo Verde National Park (10° 21' N; 85° 21 W) is located at the Tempisque River low watershed and has an extension of 200 km^2 (www.sinac.go.cr/acat_paloverde.php). The land bordering Palo Verde is characterized by the alternation of wetlands, livestock production and intensive agriculture (rice, sugar cane, melon), associated with an intensive use of pesticides. Water for agriculture is provided by a system of channels that interconnect cultured areas with protected wetlands, including the ones located inside the Palo Verde National Park. The studied locations (Fig. 1) included putative agriculture-contaminated sites in Río Cabuyo at a point surrounded by rice plantations (CS) and the former basin of Río Tempisque, which now is a main channel that collects water from smaller channels that carry water from rice and sugar plantations (CVT). Putative eference sites were Río Cabuyo, at a point inside the wildlife reserve Lomas de Barbudal (CL) and Río Barbudal (B), which rises in the same reserve and severely diminishes its water flow until almost dry during the late summer months. Two sampling campaigns were conducted: in August and December 2009 water samples were collected to determine pesticide residue levels in the rainy and dry seasons, respectively. Due to adjustments in fish caging protocol, fish exposures were carried out in November 2010 and April 2011 (rainy, dry seasons),fish were exposed in cages to study bio marker responses.

Surface water samples were collected using 1 l glass bottles previously washed with acetone and acid; samples were stored at 4°C until processed within the day. Fish were transported to the field in an aerated container and then deployed in each site in groups of ten fish per cage. Exposure was carried out in rectangular PVC cages (25 cm x 30 cm x 15 cm) covered with a mesh of 0.4 cm pore. In each deployment a non-exposed
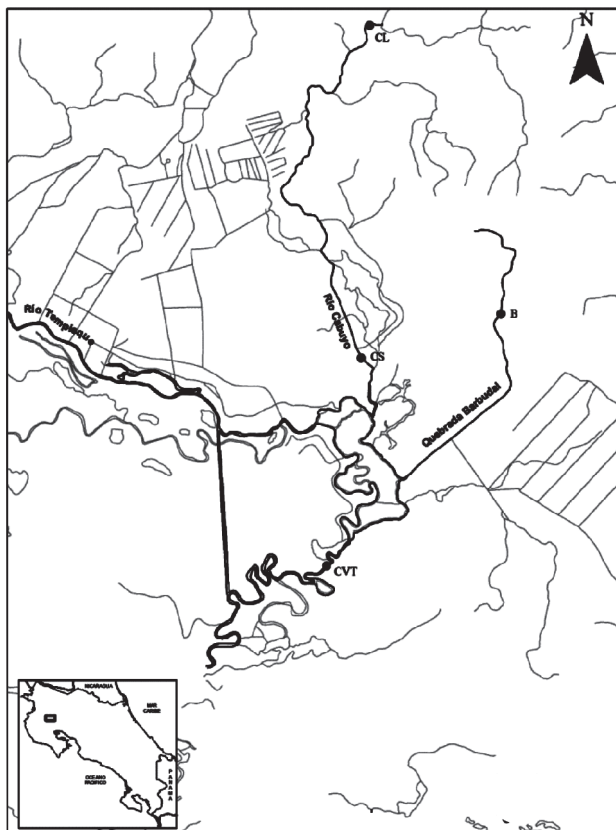
Fig 1. Map of Palo Verde National park with the indications of the sampling sites: Putative polluted sites (CS and CVT); putative reference sites (CL and B). See text for detailed description of sites

control group of randomly selected fish (same n than exposed per site) was kept in an aerated plastic container until the end of exposure. After two days of exposures, caged and control fish were sacrificed in ice and their livers and dorsal muscle dissected, frozen in liquid N_2 and preserved at -80°C until biomarker determination.

Pesticide analyses : Pesticides of different chemical families including insecticides, acaricides, fungicides and herbicides were analyzed. Tables 1 and 2 show the target analytes. Pesticides were analyzed either by gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Water samples were filtered through 0.45 μm glass fiber filter. Target pesticides were pre-concentrated by Solid Phase Extraction (SPE) using Isolute ENV+ (200 mg Biotage). Samples were spiked with the surrogate standard ethion at a concentration of 500 ng ml⁻¹. SPE cartridges were previously conditioned using 5 ml of ethyl acetate, 10 ml of methanol and 10 ml of ultra pure water. One litre of sample aliquot was percolated through the cartridges. The cartridges were finally rinsed with 5 ml of ultrapure water and dried under vacuum until dryness. For GC-MS analysis, elution was performed with 5 ml x 2 ethyl acetate. In a parallel

extraction step for LC-MS analysis, elution was performed with 5 ml x 2 of methanol. The extracts were evaporated at 25 ºC under nitrogen current until dryness. Extracts were reconstituted with 500 μl of a mixture of 0.1% of formic acid in acetonitrile/water (30:70). Sample blanks were rigorously performed to eliminate any external source of contamination.

GC-EI-MS analysis : Extracts were analyzed on a GC-MS Trace GC coupled to a Trace MS (Thermo Finnigan) in electron ionization mode (EI+) at 70 eV. A fused-silica capillary column (30 m, 0.25 m mi.d., 0.25 μ m film thickness), containing 5% phenyl and 95% methyl polysiloxane (HP 5MS) was used. Helium was the carrier gas at a flow rate of 1.2 ml min⁻¹. A 2-μl extract was injected in split less mode (split less time of 0.80 min). The oven was set at 60°C (1 min), increased to 130°C at 30°C min⁻¹, to 250°C at 5°C min⁻¹ and to 300°C at 10°C min⁻¹ (5 min). The total run time was 38 min. The injector and interface temperatures were set at 290°C and 280°C, respectively. Acquisition was performed in time-scheduled selected ion monitoring (SIM) using retention time windows. Table 1 shows the retention time of the target compounds and the monitored ions used for their quantification and confirmation. Detection limits (LOD) calculated using a signal-to-noise ratio of 5 (the ratio between the peak intensity and the noise) were between 1.9 ng l⁻¹ for ametrina and 36 ng l⁻¹ for α-endosulfan. Ethion was used as surrogate standard for GC-MS analysis and was recovered at 79 ± 8%.

LC-MS/MS analysis : Analyses were performed using an ACQUITY UPLCTM (Ultra Performance Liquid Chromatography) System from Waters (Milford, MA, USA) interfaced to a triple quadruple mass spectrometer AQUITY TQD (Waters, USA) equipped with an electrospray ionization (ESI) interface. Analyses were performed in both positive and negative ion modes. The chromatographic column was a LiChroCART HPLC RP-18e column (125 mm x 2 mm x 5 μm particle size, Merck, Germany). 10 μl of extracts were analyzed. The mobile phase consisted in acetonitrile (solvent A) and a solution of 0.1% of formic acid in ultrapure water (solvent B). Mobile phase was delivered at a flow rate of 0.4 ml/min. Gradient elution program started from 30% of solvent A, held for 0.5 min and increased to 70% A in 0.5 min; then increased to 100% A in 9 min and held for 1 min. Initial conditions were gathered in 1 min and finally the system was stabilized for 5 min. The total run time was 17 min. Ions were acquired in Multiple Reaction Monitoring (MRM). The optimal MRM conditions used for each compound are presented in Table 3. Quantification was performed by the external standard method. A 5 points calibration curve was constructed at 0.01, 0.05, 0.1, 0.5, and 1.0 ng ml⁻¹ with good linearity over this concentration range (R2> 0.99). Recoveries were calculated using ultrapure water spiked with 50 ng l⁻¹ of the target pesticides. Limits of detection (LODs) were calculated by a signal-to-noise ratio of 5, using the spiked ultrapure water samples (Table 2). Good recovery yields were obtained for all pesticides (between 77 and 105%). Blank samples were below MDLs despite the chromatographic methodology used. The optimized conditions permitted to determine low ng l⁻¹ level.

Table 1 : Compound identification number. list of compounds studied ordered by retention time (Rt. in minutes) in the GC-MS conditions used. Retention time and characteristic ions and the Methodological Detection Limits (MDL) of target compounds analyzed in GC-MS.

ID	Compound	Rt (min)	Quantification Ion (m z^{-1})	Confirmatory Ions (m z^{-1})	MDL (ng l^{-1})
1	Carbofuran	7.12	164	147. 121	35.0
2	DEET	11.42	119	91.19	3.6
3	Ethoprophos	12.4	158	200.242	11.0
4	Hxachlorobenzene (HCB)	14.06	286	249.142	16.1
5	Terbufos	15.18	231	153.288	2.9
6	Phorate	15.65	121	260.97	3.0
7	Diazinon	15.68	304	137.152	4.2
8	Chlorothalonil	16.13	266	264.268	19.0
9	Parathion-methyl	17.4	109	125.163	9.0
10	Ametryne	17.73	227	212.185	1.9
11	Bromacil	18.65	205	231.26	3.1
12	Chlorpyrifos	19.21	314	199	3.5
13	Triadimefon	19.33	128	208.168	21.0
14	Thiabendazole	20.56	174	57.181	5.0
15	Alpha endosulfan	21.6	195	241.277	36.0
16	Fenamiphos	22.18	154	303.195	11.0
17	Imazalil	22.36	215	173.217	11.0
18	Beta endoulfan	23.74	195	241.159	27.0
19	Ethion	24.4	231	97.125	2.0
20	Propiconazole	25.35	173	259.261	13.84
21	Carbofuran phenol	25.59	273	273	1940.0
22	Epoxiconazole	26.61	192	194	4.5
23	Bitertanol	30.29	170	112.141	3.9
24	Cypermethrin	31.71	181	152.163	18.0
25	Difenoconazole	33.36	165	323.267	18.0

Table 2 : Experimental LC-ESI-MS/MS conditions and the Methodological Detection Limits (MDL)

Pesticide	Ionization type	Precursor ion (m/z)	Cone (eV)	Product ion(m/z)	Col. E. (eV)	Product ion(m/z)	Col. E. (eV)	RT (min)	MDL (ng l^{-1})
Formetanate	Positive	222.9	25	166.1	15			1.31	0.025
hydrochloride		221.9	25	165.1	20			1.31	
Spinosin A	Positive	733.2	40	142.1	35	98.1	60	2.82	0.072
Spinosin D	Positive	746.2	40	142.1	30	98.0	60	2.91	0.124
Metalaxyl	Positive	279.9	20	220.2	15	160.1	20	3.16	8.00
Triadimenol	Positive	295.9	15	111.0	50	70.0	10	3.45	1.31
Terbutryn	Positive	242.9	25	187.1	20	96.0	35	3.47	14.0
Fludioxonil	Negative	246.9	25	181.1	20	180.1	30	3.77	19.40
Myclobutanil	Positive	288.9	30	125.0	35	70.0	20	3.88	1.78
Alachlor	Positive	269.9	15	238.2	10	162.2	20	4.50	0.753
Cyprodinil	Positive	226.9	40	108.0	25	93.0	35	4.51	1.70
Chlorfenvinphos	Positive	360.7	20	155.1	15	98.9	30	5.27	0.853
Pyraclostobin	Positive	387.8	20	194.1	15	163.1	25	5.33	21.3
Pyrimiphos-Me	Positive	306.8	35	165.2	25	109.0	30	5.74	1.10
Tebufenpyrad	Positive	333.9	45	147.1	25	117.0	35	6.27	3.51
Hexythiazox	Positive	352.8	20	228.1	15	116.0	50	7.37	0.663

Biochemical estimation : Livers and muscle tissue were homogenized in 1:5 w/v cold 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ containing 100 m MKCl, and 1.0 m Methylene di aminetetra-acetic acid (EDTA). Liver homogenates used for lipid peroxidation analyses also included 0.2 mM butylhydroxytoluene (BHT) to prevent lipid oxidation. The rest of homogenate was centrifuged at 12000 x g for 20 min and the supernatant used for enzyme activities. Protein content was measured in all samples by the Bradford method Bradford (1976) using bovine -globulin as standard.

Glutathione S-transferase conjugation activity was measured in liver homogenates using 1-chloro-2, 4-

Table 3 : Pesticide residue levels determined by GC-MS and LC-MS in water samples (ng l^{-1}) collected from the studied sites. n.d: below detection limit

Compounds	Sites and dates						
	B	CL	CS	B	CL	CS	CVT
GC-MS	August	August	August	December	December	December	December
Ametryne	n.d	13.0	n.d	n.d	n.d	10.4	21.9
Hexachlobenzene	n.d	16.6	2199	n.d	n.d	n.d	17.9
Bromacil	46.5	33.1	12.57	8.01	4.92	5.36	6.72
Ethoprophos	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Epoxiconazole	n.d	n.d	187.8	n.d	n.d	n.d	124
Diazinon	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Phorate	n.d	n.d	n.d	461	12.4	n.d	n.d
Chlorothalonil	n.d	n.d	n.d	n.d	n.d	n.d	n.d
LC-MS							
Alachlor	n.d	3.16	n.d	n.d	n.d	0.83	1.27
Chlorfenvinphos	5.79	31.8	5	n.d	6.49	4.05	3.43
Cyprodinil	12.2	7.11	n.d	4.71	n.d	2.88	6.05
Myclobutanil	n.d	2.96	3.93	n.d	n.d	n.d	2.35
Pyrimiphos-Me	n.d	n.d	3.97	1.24	1.9	1.7	2.77
Spinosin	n.d	4.3	n.d	n.d	1.98	n.d	n.d
Terbutryn	n.d	37.7	23.6	n.d	n.d	n.d	n.d

Table 4 : ANOVA results of the effects of sex, season and site on ChE, GST and CAT activities and LPO levels in *P. gillii* and *P. dovii* transplanted at the studied locations in November and April. For clarity only degrees of freedom (df), Fisher's coefficient (F) and probability levels are depicted. For clarity sex effects were not considered for ChE, GST, CAT and LPO

P. gillii	ChE			GST			CAT			LPO		
	df	F	P	df	F	P	df	F	P	df	F	P
Season	1,55	15.90	<0.01	1,57	0.20	0.66	1,57	3.94	0.05	1,58	24.20	<0.01
Site	4,55	5.99	<0.01	4,57	1.85	0.13	4,57	1.81	0.14	4,58	7.14	<0.01
Season * site	4,55	1.65	0.17	4,57	7.83	<0.01	4,57	2.98	0.03	4,58	1.09	0.37
P. dovii												
Season	1,66	43.42	<0.01	1,68	0.59	0.44	1,63	38.76	<0.01	1,64	0.08	0.78
Site	4,66	1.04	0.39	4,68	21.12	<0.01	4,63	11.70	<0.01	4,64	8.12	<0.01
Season * site	4,66	2.23	0.08	4,68	21.45	<0.01	4,63	10.79	<0.01	4,64	1.17	0.33

dinitrobenzene (CDNB) following the method of Habiget al. (1974). The reaction mixture contained 100 mM phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM GSH. The formation of S-(2, 4-dinitrophenyl)-glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm during 3 min (= 9.6 mM-1cm-1) and expressed as nmol min^{-1} mg^{-1} protein. CAT activity was measured in liver homogenates by the decrease in absorbance at 240 nm due to H_2O_2 consumption (= 40 m^{-1} cm^{-1}) according to Aebi *et al.* (1974), and expressed as µmol min^{-1} mg^{-1} protein. The reaction volume and reaction time were 0.5 ml and 20 sec respectively and contained 50 mM phosphate buffer, pH 6.5, 50 mM H_2O_2Ni *et al.* (1990). Lipid peroxidation was measured by the thiobarbituric reactive species (TBARS) assay, which measures the production of malonaldehyde (MDA) that reacts with thiobarbituric acid Oakes and Van Der Kraak, (2003). Measurement of TBARS was carried out following the method of Ohkawa *et al.* (1979), using a Genesys 20, Thermo spectro fluori

meter. Briefly, 1 ml of 12% trichloroacetic acid, 0.8 ml of 60 mM Tris–HCl pH 7.4 with diethylene triamine pentaacetic acid (DTPA) 0.1 mM and 1 ml of 0.73% thiobarbituric acid were added to 0.2 ml of liver homogenate. After an incubation bath during 60 min at 100 °C, the solution was centrifuged at 2000 x g during 5 min and LPO levels were determined at 535 nm and expressed as nmol TBARS per mg of protein. ChE in muscle was determined by a modification of the Ellman method, Ellman (1961) in the presence of 1 mM acetylthiocholine and 0.1 mM 5.5' dithiobis-2-dinitrobenzoic acid (DTNB), and the increase of absorbance was measured at 415 nm and expressed as nmol min^{-1} mg^{-1} protein.

Statistical analysis : Site, season and/or sex effects on the studies responses were determined using ANOVA tests. Prior to analyses biochemical determinations were log transformed to improve ANOVA assumptions of normality and variance homocedasticity (Zar, 1984). The spatial and temporal

distribution of pesticide residue levels measured in the different water samples was compared using principal component analyses. Non detected analytes were substituted by half the detection limit to avoid empty cells. Statistical tests were performed using SPSS 17 (SPSS Inc, Chicago, III) package.

Results and Discussion

Pesticide residue levels are depicted in Table 3 and their distribution across the studied sites and periods determined by principal component analyses is shown in Fig 2. The PCA analysis defined five variables that explained 99% of data variance. The first two PC explained up to 62% of variance and separated four of the seven water samples analyzed. The water sample collected in August from CS had highest level of HCB, epoxiconazole and pyrimiphos and those collected from site B in December had highest level of phorate, respectively. The rest of water samples had low level of pesticides.

Biochemical markers depicted in Fig 3 were not significantly (P<0.05) affected by sex in *P. gillii* (data not shown).Thus, sex was not further considered in the ANOVA from Table 4. In both species, significant (P<0.05) site effects within

and across the study periods (Site by season interaction effects) were observed in all the studied responses (Table 4). Transplanted fish of *P. dovii* at site CS in April showed higher activities of GST, CAT and higher levels of TBARs than those from the other sites (Fig 3). In contrast, in transplanted *P. gillii* there was not a consistent response pattern across sites and seasons (Table 4, Fig 3). Muscle ChE activity was inhibited at site B in April relative to control fish (C in Fig. 3), liver GST activity inhibited at all sites in April but in November it was higher at site CS; CAT activity was inhibited in April CL, CS and CVT sites. Unexpectedly, lipid peroxidation level was higher in control fish than in those transplanted across most sites (Fig 3).

Pesticide residues have a complex environmental fate in tropical aquatic ecosystems (Carvalho *et al.,* 2002; Daam and Van Den Brink, 2011). The relative low environmental persistence of most pesticides combined with elevated temperatures makes difficult to predict their environmental occurrence from usage data (Laabs *et al.,* 2007). The study area is affected primarily by herbicides, insecticides and fungicides used for rice, sugar cane and melon production (de la Cruz and Castillo, 2002; de la Cruz *et al.,* 2004). Off the 40 pesticide analyzed, the most used are carbofuran, diazinon, chlorthalonil, ametryne, chlorpyrifos, endo
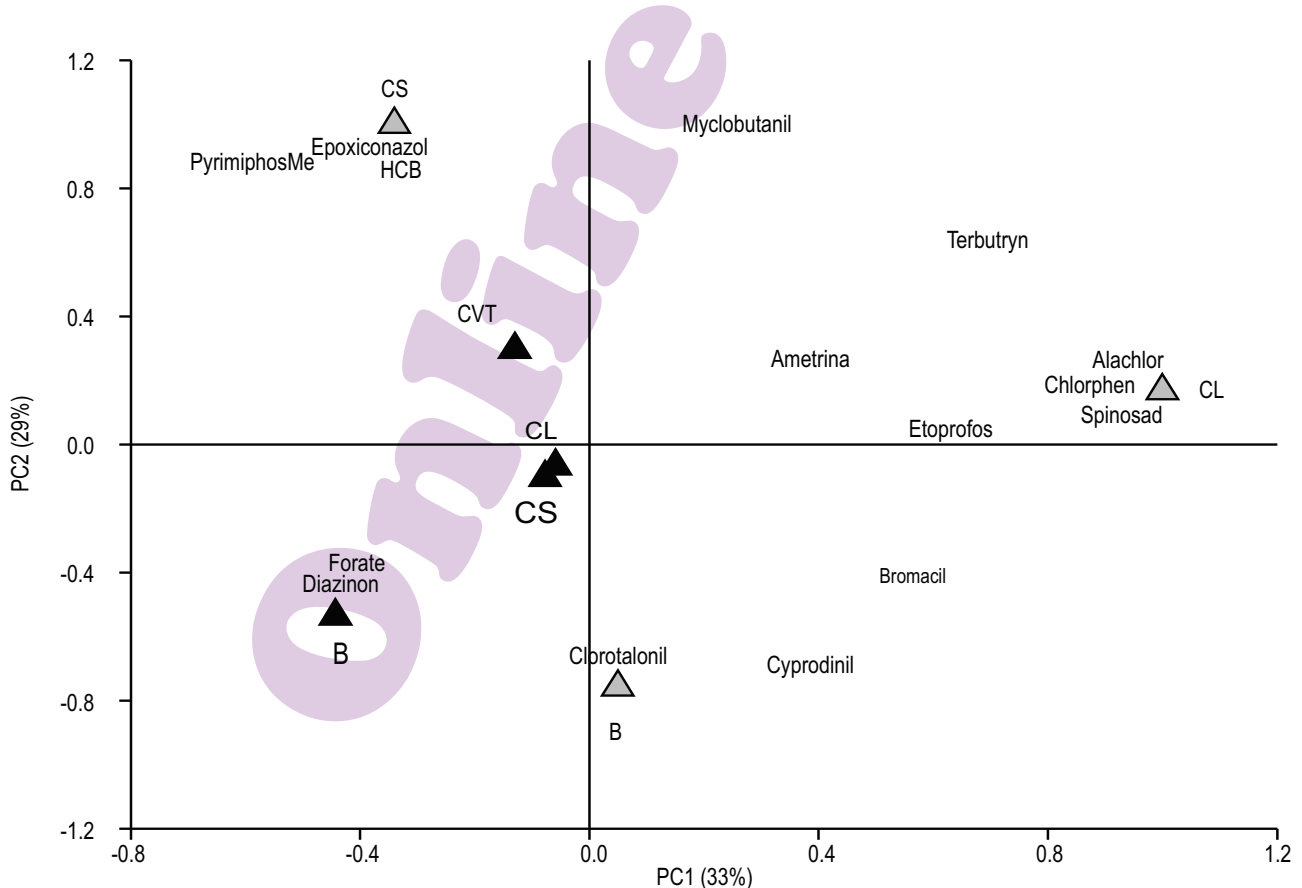

Fig. 2 : Bi-plot of pesticide loadings and site scores of the first two principal components for measured pesticide residues. Grey and black triangles correspond to August and December water samples, respectively
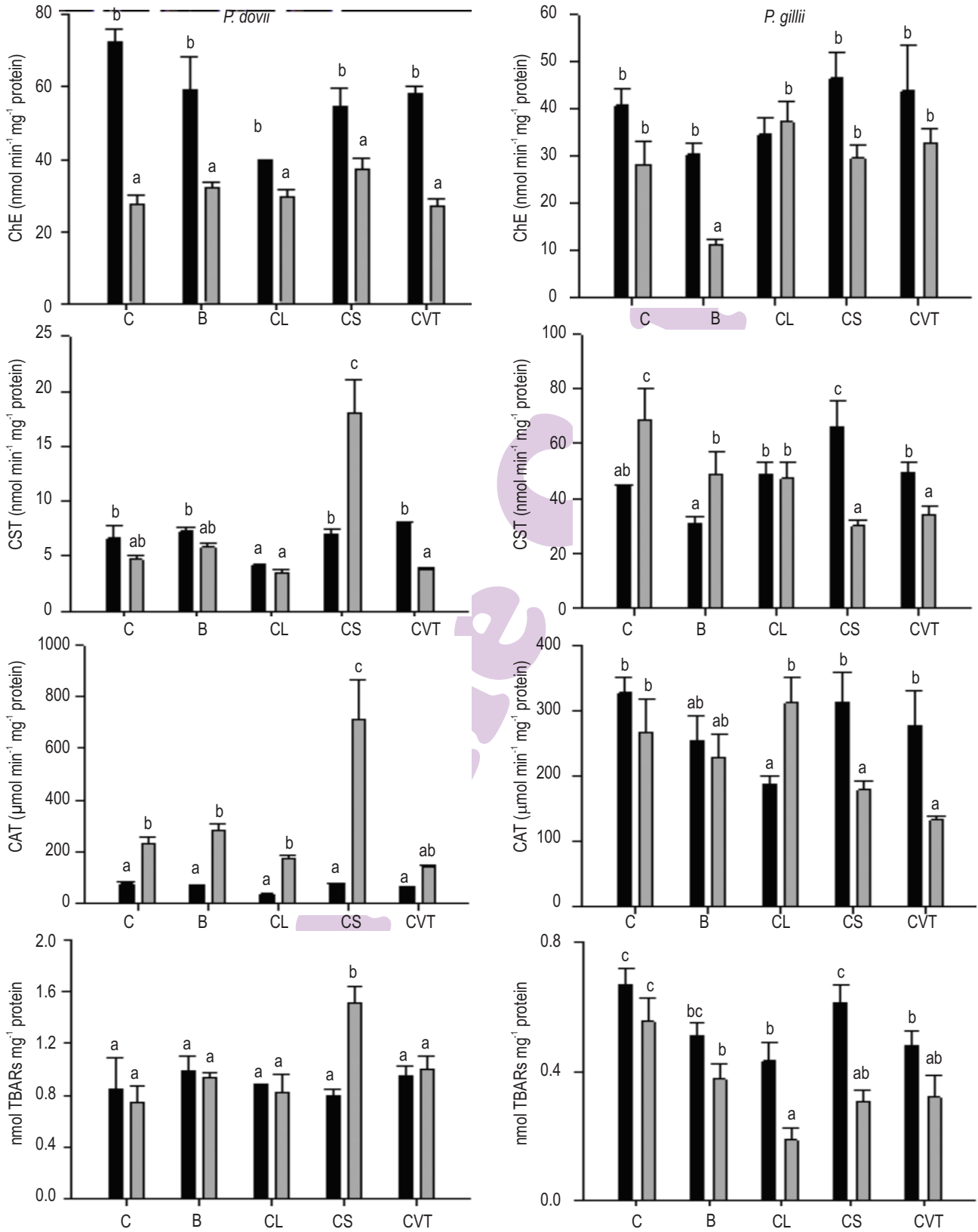
Fig. 3 : Enzymatic activities of ChE, GST, CAT activities and level of LPO in *P. dovii* and *P. gillii* fish caged for two days four studied sites in November (black bars) and April (grey bars) . Control fish (C) responses are also included. Different letters indicate statistically significant differences among groups following ANOVAs. Values represented by base are mean of replicates $\pm$ SE (n = 5 and 10)

sulfan, cypermethrin and terbutryn (de la Cruz *et al.,* 1998, 2004) Within the analyzed water samples, only 15 pesticides were detected (Table 3) from which only four (diazinon, ametryne, cypermethrin and terbutryn) were known to be used. The pesticides found in higher concentrations in water samples, such as hexachlobenzene (2261 ng l^{-1}), phorate (473 ng l^{-1}), epo xiconazole (314) and bromacil (117 ng l^{-1}) were not known to be used in the studied area.

These results corroborate our previous arguments that in tropical aquatic systems it is very difficult to predict pesticide level in water from utilization data. Furthermore, in a previous study conducted in rice fields of Delta del Ebro (Spain) during and after pesticide application, Suarez *et al.* (2010) reported that the studied pesticides only were detected within two days after their application irrespective of their water solubility, lipophility and environmental persistence. Thus, it is feasible to conclude that the occurrence of studied pesticides in collected water samples were due to their recent use in surrounded agricultural fields. Indeed, hexachlobenzene, despite its low water solubility (<0.005 mg l^{-1}) and low persistence in water (half life< 12 h) (www.epa.gov/safewater/hfacts.), was detected at the highest concentrations (2261 ng l^{-1}), indicating that it was applied within the days of sampling.

Selected biochemical markers included ChE activity, which is the specific target of organophosphorous and carbamate pesticides. Low ChE activity was only showed by specimens of *P. gillii* transplanted at Barbudal site in April (dry season). In a chemical survey conducted a year before we found trace residues of phorate, in Barbubal during dry season (December, Table 3). Thus, it is possible that in April, presence of organophosphorous pesticides may have affected measured ChE activity in this location. The other measured biomarkers included the antioxidant and phase II metabolizing enzymes CAT and GST that detoxifying reactive oxygen species and secondary metabolites (Livingstone, 2001) that may also include peroxidated products (Ketterer *et al.,* 1988), thus they are physiologically linked with lipid peroxidation levels, which is a marker of oxidative stress mediated tissue damage (Halliwell and Gutteridge, 1999). Accordingly, the relationship of these three markers with pro-oxidant factors such as organic pollutants is expected to occur (Halliwell and Gutteridge, 1999; Di Giulio, *et al.,* 1995). Our results confirm the previous arguments since high activities of GST and CAT measured in liver of *P. dovii* transplanted at the impacted Cabuyo site (CS) during dry season in April were also related to high level of lipid peroxides. Meanwhile, in *P. gillii*, oxidative stress-related markers responses kept at the level, or even lower then control fish, suggests that *P. gillii* was a less sensitive species. Regarding the responsiveness of biomarkers in fish exposed to pollutants during a short period, Sayeed *et al.* (2003) reported responses of oxidative stress-related biomarkers (LPO, CAT, GST) in fish exposed to pyretroids during 48 hr.

In a survey conducted in 2009, it was found that pesticide residue level at CS was 15 fold higher than reported at other sites. In this survey, level of hexachlorobenzene in water was 2.2 µg l^{-1} and of other six pesticides ranged from 3 ng l^{-1} for mychlobutanyl and pyrimiphos methyl to 187 ng l^{-1} for epoxiconazol. Song *et al.* (2006) reported the effect of hexachlorobenzene on antioxidant status of liver and brain of common carp (*Cyprinus carpio*) found enhanced level of antioxidant enzyme activities and lipid peroxidation. Triazole fungicides are also known to alter antioxidant enzymatic activities and promote oxidative stress in rainbow trout (Li *et al.,* 2010). Therefore, it is reasonable to consider that hexachlobenzene and triazole residues in water present at Cabuyo site may have caused the observed effects in transplanted *P. dovii*.

Finally, it is interesting to consider that studied fish species behaved differently at study areas. Both species belonged to different families with known differences in sensitivity to pollutants. Poecilid fish are known to have genera such as *Poecillia* that are adapted to harsh conditions and as such may be quite tolerant to pollution (Chiyokubo *et al.,* 1998; Shikano and Fujio, 1997; Magurran and Phillip (2001). Cichlids are a diverse family of fish that include tolerant and sensitive species to pollution (Katunzi *et al.,* 2010; Mosepele *et al.,* 2011; Ribbink, 1987). In particular, *P. dovii* is considered a relatively sensitive fish (Tabash and Guadamuz, 2000), thus it is not surprising that showed a greater response to pollution than *P. gillii*. Our data provides evidence that biomarkers are responsive in Costa Rican native fish species exposed at sites impacted by agricultural influence. Biomarker responses were observed in both species exposed, being *P. dovii* responses more congruent with putative impacted sites than those of *P. gillii*.

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