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Development of quantitative RNA biomarkers for detecting dioxin-like and estrogenic pollutants in Costa Rican native fish species

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Abstract

Monitoring the environmental impact on native species is crucial for the correct management of tropical ecosystems. The Costa Rican fish *Parachromis dovii* (Cichlidae) and *Poecilia gillii* (Poecillidae) were used as sentinel species for freshwater bodies under considerable pressure by intensive agriculture Cichlidae development. Suitable qRT-PCR probes for the quantification of hepatic mRNA levels of two stress-related genes –vitellogen in (estrogenic effects) and cytochrome P4501A (CYP1A, dioxin-like compounds) – for both species were designed and validated in experimental treatments with model effectors (17β-estradiol and β-naphtoflavone, respectively), demonstrating their usefulness as markers of exposure to these two kinds of pollutants. Analysis of fish transplanted across pesticide contaminated sites near Palo Verde National Park, Pacific Coast of Costa Rica did show significant changes on hepatic Cyp1A in both species. In *P. dovii,* Cyp1A levels were enhanced in Barbubal and in the impacted Cabuyo sites in the rainy season whereas in *P. gillii* fish Cyp1A transcripts were down-regulated differently across rainy and dry seasons. Vitellogen in mRNA levels in *P. gillii* varied between males and females with males showing always low values which indicated no estrogenic effects. Within females, vitellogenin levels varied over 100,000 fold depending on their maturation stage, further demonstrating the ability of the method to monitor changes (natural or induced) in the reproductive system of the fish.

Key words

Biomarker, Endocrine disruption, EROD, Fish, Pesticides, Tropical

Introduction

Ecological Risk Assessment should consider not only pollutant loads, but also an estimation of the ecological effects of this exposure. Whereas this principle has been incorporated in legislation of the European Union (i.e. Directive 93/67/EEC, Regulation 1488/94, Directive 98/9/EC, Directive 91/414/EEC) and other legislative bodies, its implementation is still lacking for many tropical areas, including Central American countries. For example, environmental risks of pesticides in Costa Rica are usually limited to establish maximum concentration levels of usage without taking into account their effects (de la Cruz *et al.,* 1998; de la Cruz and Castillo, 2002; de la Cruz *et al.,* 2004). Therefore, there is a need to develop biological methods to monitor toxic effects, like application of biomarkers for pollutant exposure in selected sentinel species (Van der Oost *et al.,* 2003). Hepatic 7-Ethoxyresorufin O-Deethylase (EROD) activity and blood or liver levels of vitellogenin in males are often used as specific markers of exposure to dioxine like and estrogenic compounds in fish, respectively (Van der Oost *et al.,* 2003) . However, the use of biochemical methods (enzymatic activity or Western blots) require large amounts of tissue (e.g, about 1 g of liver), the use of flash-freezing methods for sampling, like immersion in liquid N_2, and preservation of the samples at -80 °C. These requisites are difficult to meet when sampling small fish species collected in tropical remote areas. A plausible alternative,

however, is to develop molecular markers that require less sample and easier storing conditions. For example, quantitative analyses of mRNA levels of CYP1A and vitellogenin have often been used as molecular markers of EROD activity and vitellogenin protein levels, respectively, in field studies conducted in remote areas such as alpine lakes or the Tunisian coast (Jarque *et al.*, 2010; Kessabi *et al.,* 2010). These analyses require minute amounts of sample tissues (less than 50 mg) that can be preserved at 4 °C in RNA later for days and stored for months at -20ºC (Jarque *et al.*, 2010).

In this study we developed and characterize probes to analyze liver mRNA levels of Cyp1A and of Vitellogenin in two local Central American fish species, *Parachromis dovii* (Cichlidae) and *Poecilia gillii* (Poecillidae), considered as good candidates for sentinel species for freshwater bodies in Costa Rica. The study combined laboratory induction experiments with the model Cyp1A and vitellogenin inducers β-naphtoflavone and 17β-estradiol treatments, respectively, and field samples collected from fish transplanted in clean and contaminated sites surrounding the Palo Verde National Park, Pacific coast of Costa Rica. This study is part of a project aimed 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 : *Parachromis dovii* (Cichlidae) and *Poecilia gillii* (Poecillidae) are two Central American native fish species with a broad distribution in Costa Rica (Bussing, 202). 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 of the Universidad Nacional at Heredia, Costa Rica. Juvenile specimens of *P. gillii* (mean standard length 2.56.1±0.06 cm; mean body weight 0.35±0.04 g) were obtained from culture in the laboratory of ecotoxicological studies at IRET. Adult specimens of *P. gillii* were captured in a clean site included in field experiments (site B). Fish captured in the field were acclimated to culture conditions for 1 month prior to any experiment. Fish were kept in the laboratory at 24 °C in filtered, dechlorinated, UV treated water with a photoperiod of 12 hr light: 12 hr dark and fed on flakes twice a day. Juvenile specimens of *P. gillii* were used in laboratory exposure to β-naphtoflavone; only adult males were used in exposure to 17β-estradiol and adult individuals of both sexes were used in field transplants.

Environmental area and fish sampling : The Palo Verde National Park (10°21'N; 85°21W), 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 alternative presence of wetlands and intensive agriculture (rice, sugar cane, melon and livestock production), associated with an intensive use of pesticides (Ramírez *et al.,* 2009). 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 (Falles *et al.,* 2011). Fish were transplanted at four sites (Fig. 1). Putative agriculture-contaminated sites were Río Cabuyo at a point surrounded by rice cultured lands (CS) and the former basin of Río Tempisque, which collects water from smaller draining channels from rice and sugar cane cultured lands (CVT). Putative reference 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 dryness during the late summer months. Sites were selected and considered more or less impacted regarding their association with cultured lands, nevertheless, the ecological water quality of the studied sites has been considered good for sites CL and CS and bad for B and CVT, as determined by macroinvertebrate biodiversity (Costa Rican Bio Monitoring Working Party Scores) (Mora, 2011; Stein *et al.,* 2008).

Fish were transported to the field in aerated containers and 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 control group of ten randomly selected fish was kept in an aerated container until the end of exposure. Deployments

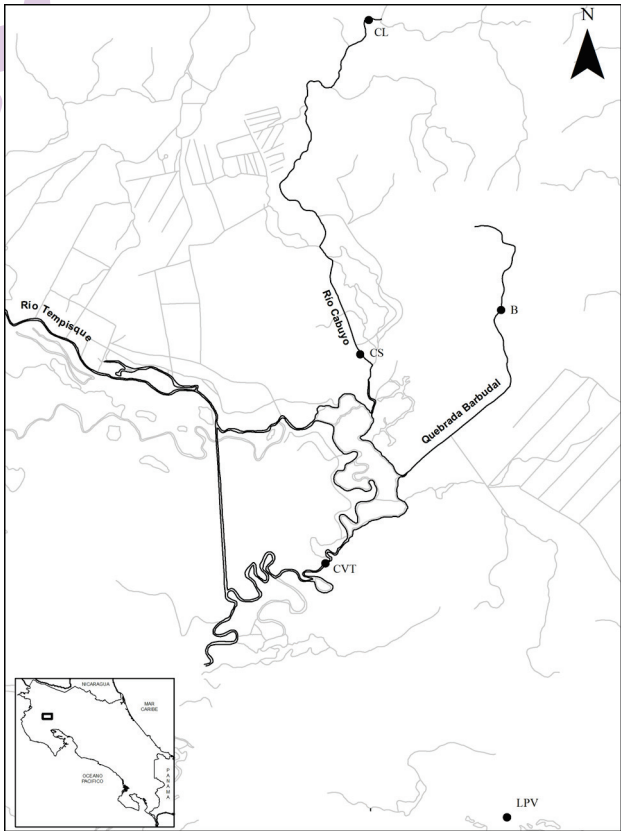

Fig. 1 : Map of Palo Verde National Park with the indications of the sampling sites

Table 1 : Gene primers

Species	Gene	Acronym	Primer Forward (5'-3')	Primer Reverse (5'-3')
P. gillii	B-actin	BACT	AGATCATTGCCCCACCAGAG	ACTCATCGTACTCCTGCTTGCTG
	CytochromeP450	CYP	TCGCCATTCTTCATTCCTGC	TGGTTGATCTGCCACTGATTG
	Vitellogenin b	VT	CAACAACCTGCCATATCAGCA	GCCTCTCATCCAGTCCACAAC
P. dovii	B-actin	BACT	GTCATGGACTCCGGTGATGG	TGGTGAAGGACTAGCCACGC
	CytochromeP450	CYP	CCACCATGATGAGAGCTGG	GAAGCGACCATTGAGGCTCAC
	Vitellogenin b	VT	CACCCAGCGGTTCTCTAATCA	TTTCTGGCATGAGCAACAGG

were conducted in dry and wet season (November 2010 and April 2011, respectively) for *P. gillii* and only in November for *P. dovii*. After a 2-day exposure, caged and control fish were sacrificed in ice and dissected. Whole livers were preserved in 1 ml of RNA later (Sigma Aldrich, St. Louis, MO), transported on ice to the laboratory and stored at -80°C until analysis.

Induction experiments : Laboratory exposures were conducted in 5 l glass tanks filled with filtered, dechlorinated, UV treated and aerated tap water. Fish were exposed in groups of seven to β-naphtoflavone (juveniles of both species) and 17β-estradiol (juveniles of *P. dovii*; mature males of *P. gillii*) treatments. Contaminants were dissolved in ethanol (1 ml l^{-1}), which was also added to control treatments. After exposures for two days, fish were anaesthetized on ice and their livers dissected and preserved in RNA later.

Sequence analysis and primer design : DNA sequences from ß-actin, Cytochrome P4501A (CYP) and Vitellogenin B (VtgB) were obtained using SRS tool from the European Bioinformatics Institute (http://www.ebi.ac.uk/). Conserved sequence regions were derived from the alignment of Cyprinodontiformes for *P. gillii* and from Cichlids for *P. dovii*. Gene specific primer sets depicted in Table 1 were designed from consensus using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA.) Amplifications were performed in a Mini Cycler (MJ Research, Hemel Hempstead, UK), using the following conditions: an initial step of 95ºC for 5 min, 40 cycles at 95ºC for 15 sec, 55ºC for 1 min and 72ºC for 1 min, and a final step of 72ºC for 10 min. The resulting PCR products (amplicons) were cloned into the vector pTZ57R/T from the Ins TAclone TM PCR clone kit (Fermentas, life sciences) and propagated using XL-1 Blue competent cells. Sequencing of DNA was performed on 3730 DNA Analyzer (Applied Biosystems), and the results compared to the corresponding references in GeneBank by the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

RNA extraction and qRT-PCR quantification : Total RNA was isolated from liver using Trizol reagent® protocol (Invitrogen). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-8000 Spectrophotometer (Nano Drop Technologies, Delaware, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). RNA was treated with DNAse I to remove genomic DNA contamination. Quantities from 1 µg to 100 ng of DNAse I-treated RNA were retrotranscribed to cDNA using First Strand cDNA Synthesis Kit Roche® (Germany) and stored at -20 ºC. Aliquots of 25 ng of original RNA preparations were used to quantify specific transcripts in LightCycler® 480 Real Time PCR System, using SYBR®Green Mix (Roche, Germany) and the pair of primers listed in Table 1. Amplified sequences were re-confirmed by cloning and sequencing as above.

Relative mRNA abundances of the different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). Cp values for target genes (Tg) were compared to the corresponding values for the reference gene (ref, ß-actin) to obtain the ΔCp values (ΔCp= Cpref – CpTg). PCR efficiency values for reference and target genes were calculated as described by Pfaffl (2001), and assumed to be close to 100% from these calculations. To facilitate reading of tables and graphs, mRNA abundance values are represented as mRNA copies per 1000 copies of the reference gene mRNA (‰ of reference gene, 1000x2ΔCp).

Statistical analysis : Statistical tests were performed using SPSS 17 (SPSS Inc, Chicago, III) package. All statistical calculations were performed using ΔCp values, as this parameter followed normal distribution (Kolmogorov-Smirnov). Statistical comparisons of mean values were made using analysis of variance (ANOVA).

Results and Discussion

Cloning genes from *P. dovii* and *P. gillii* : Amplicon sequences from putative ß-actin, Vtg and CYP genes from *P. dovii* and *P. gillii* liver confirmed the close sequence homology of the obtained amplicons with the corresponding genes in different fish species (Table 2), thus confirming the initial adscription of the amplified fragments. Obtained sequences from *P. gillii* shared high homology with homologous genes from several Cyprinodontiformes species; in the case of the highly conserved ß-actin sequence, 100% homology was found even for two Perciformes species. In the case of *P. dovii*, the highest homologies corresponded to other cichlids, both for the Old and the New World (Table 2).

Experimental laboratory exposures : Exposure to 17β-estradiol and β-naphtoflavone increased (P<0.05) Vtg and CYP mRNA levels in liver, respectively (Fig 2). Basal levels of mRNA for Vtg were undetectable by qPCR in immature *P. dovii*, but they increased until 100 copies per 1000 copies of β actin in fish exposed to 100 µg l^{-1} of estradiol (Fig 2 A). Levels of Vtg mRNA in *P. gillii* males were low, but detectable, and increased more than 200 fold upon exposure to 100 µg l^{-1} of estradiol (Fig 2C). In both cases, the results indicate a robust response to estrogens for both species.

Basal mRNA level of Cyp1a was substantially higher than the corresponding Vtg mRNA level in *P. dovii* (Fig 2A,B). Exposure to saturating concentrations of ß-naphthoflavone increased these levels 10 to 100 times in both species (Fig 2B,D), also demonstrating the responsiveness of the probed genes to

Table 2 : List of the sequence homology for *P. gillii* and *P. dovii*.It also shows the accession number and the maximum coverage and maximum identification values for the obtained amplicons

Specie	Gene	Similar seq.	Accesion number	Family, Order	Max. Cov.	Max. Indet.
P. gillii						
	ß-actin	*Girellapuncata* ß-actin	JN226151	*Kyphosidae, Perciformes*	100%	100%
		Pargus major ß-actin	JN226150	*Sparidae, Perciformes*	100%	100%
		Aphaniusfasciatus ß-actin	HM017072	*Cyprinodontidae, Cyprinodontiformes*	100%	100%
		Poeciliareticulata ß-actin	EU143772	*Poecilliidae, Cyprinodontiformes*	100%	100%
	CYP1A	*Aphaniusfascinatus* CYP1A	HM017071	*Cyprinodontidae, Cyprinodontiformes*	100%	99%
		Jenynsiamultidentata CYP1A	EF362746	*Anablepidae,Cyprinodontiformes*	100%	96%
		Fundulusheteroclitus CYP1A	AF026800	*Fundulidae, Cyprinodontiformes*	97%	91%
		Cyprinodonvariegatus CYP1A	EF535032	*Cyprinodontidae, Cyprinodontiformes*	93%	93%
	VtgB	*Gambusiaaffinis* VgB	AB181836	*Poecilliidae, Cyprinodontiformes*	100%	92%
		Poeciliareticulata VgB	EF456700	*Poecilliidae, Cyprinodontiformes*	99%	97%
		Fundulusheteroclitus VgB	U70826	*Fundulidae, Cyprinodontiformes*	93%	81%
		Cyprinodonvariegatus VgB	AF239721	*Cyprinodontidae, Cyprinodontiformes*	93%	80%
P. dovii	ß-actin	*Astronotusocellatus ß-actin*	EU553593	*Cichlidae, Perciformes*	99%	83%
		*Oreochromisniloticus*ß-actin	EU887951	*Cichlidae, Perciformes*	99%	82%
		Tilapia massombica ß-actin	AB037865	*Cichlidae, Perciformes*	99%	82%
		Oreochromismassombica ß-actin	Y18689	*Cichlidae, Perciformes*	91%	82%
	CYP1A	*Astronotusocellatus* CYP1A	EU553595	*Cichlidae, Perciformes*	84%	87%
		Oreochromisniloticus CYP1A	FJ664151	*Cichlidae, Perciformes*	84%	83%
		Pleuronectesyokohamae CYP1A	AB120566	*Pleuronectidae, Pleuronectiformes*	84%	82%
		Sparusaurata CYP1A	AF011223	*Sparidae, Perciformes*	77%	83%
	VtgB	*Cichlasomafacetum* Vtg	DQ369701	*Cichlidae, Perciformes*	100%	91%
		Pargus major Vtgb	AB181839	*Sparidae, Perciformes*	99%	89%
		Oreochromisniloticus Vtg	XM_003452575	*Cichlidae, Perciformes*	97%	89%
		Oreochromismassombica Vtg	FJ756399	*Cichlidae, Perciformes*	97%	89%

Table 3 : ANOVA results of the effects of sex, season and site on mRNA levels of Vtg and CYP1A in *P. gillii* fish cages at the studied locations. For clarity only degrees of freedom (df), Fisher's coefficient (F) and probability levels (P) are depicted

	Vtg			CYP1A		
	df	F	P	df	F	P
Sex	1,37	10.7	<0.01	1,41	0.1	0.83
season	1,37	4.2	0.05	1,41	0.9	0.36
site	4,37	1.7	0.17	4,41	4.2	0.01
Sex * season	1,37	4.1	0.05	1,41	0.2	0.7
Sex * site	4,37	0.9	0.45	4,41	0.4	0.82
season * site	4,37	1.1	0.37	4,41	4.6	<0.01
Sex * season * site	4,37	1.1	0.38	4,41	0.6	0.67

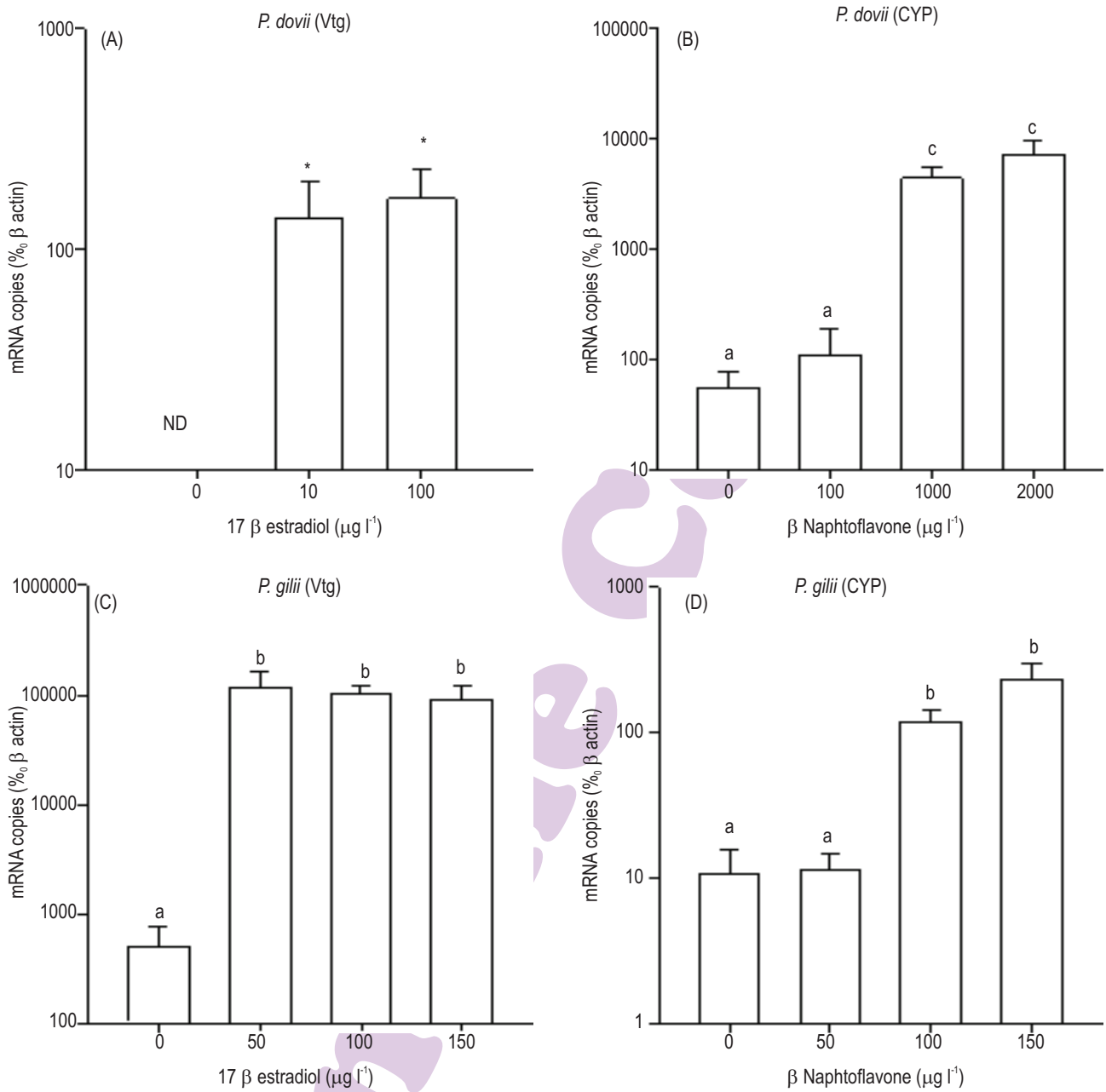

Fig. 2 : Abundance of mRNA (Mean ± SE, 5-10) of Vtg and Cyp1A in immature *P dovii* and male *P gillii* fish exposed to 17β-estradiol and β-naphtoflavone. Y axis is in log10 scale. Different letters in graphs B and C indicate significant differences among groups following ANOVA and pos-hoc Tukey's tests; * in graph A indicate significant ($P<0.05$) differences relative to the putative qPCR detection limit. ND: Bellow detection

Cyp1a inducers.

Field exposures : Levels of Vtg mRNA were not detectable by qRT-PCR in caged *P. dovii* in any of the sites, suggesting no estrogenic activity attributable to putative pollutants present in the corresponding areas. Conversely, *P. gillii* Vtg mRNA levels were essentially determined by sex (males vs. females). Fig. 3 shows Vtg mRNA levels for all analyzed individuals, separated by sex, season and size. The graph shows that Vtg mRNA levels were relatively low and uniform for all males, whereas females showed a much heterogeneous distribution, with differences of more than 105 fold between extreme values. No significant differences were observed between seasons or sizes (Fig. 3, Table 3). The observed distribution may well reflect the natural maturation cycle of *P. gillii* females (Fig. 3), corresponding high levels to ovogenetic females and lower ones to females either immature or in a resting period. The uniform, low Vtg mRNA levels in males confirm the assumption that no estrogenic pollutants were present at the analyzed sites; the presence of three individuals with relatively higher Vtg mRNA levels (around 1% of ß-actin mRNA, Fig. 3)

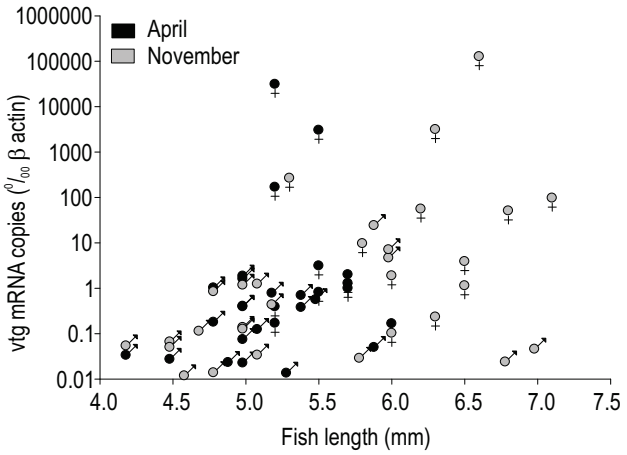

Fig. 3 : Body length versus abundance of mRNA of Vtg in males and females of *P. gillii* transplanted across the studied sites in November and April

might indicate an incipient feminization activity, but the current data does not allow for definitive conclusions.

CYP1A mRNA levels varied significantly among sites and periods but not between females and males (Fig. 4, Table 3). For *P. dovii*, maximal levels were observed in B and CVT sites (Fig. 4A), whereas *P. gillii* showed a decrease, rather than an induction, of CYP mRNA levels in presumably agricultural-impacted sites (Fig. 4B, C). The CYP1A RNA levels varied among sites between the two campaigns for *P. gillii* (Compare Fig. 4B, C). These results might indicate the presence of some pollutants affecting the expression of hepatic metabolic enzymes and affecting differently the two species.

In this study, we cloned and sequence putative mRNA partial sequences encoding for Vtg and CYP1A1 in two local fish species from Costa Rica. Our partial sequences had high homology with those from closely related fish species, and showed a differential expression upon exposure to the model inducers 17 β-estradiol and β-naphtoflavone. These data were considered as evidence that the amplified fragment indeed correspond to the intended genes in *P. gillii* and *P. dovii*. The relatively low levels of Vtg mRNA in juveniles of *P.dovii* were likely to be related to their undifferentiated immature stage. Levels of Vtg mRNA in males and females of *P. gillii* transplanted across the studied sites reflected a typical breeding population distribution with males having always low residual levels of mRNA and females a large variation due to the occurrence of individuals in different reproductive cycle stages (Orlando *et al.,* 2007; Palace *et al.,* 2009). In both species, there was no induction of Vtg mRNA levels in transplanted fish, which means that among the pesticides present in the study sites there was no estrogenic compounds or the concentration of the estrogenic ones, such as endosulfan, were too low. Conversely, site effects on CYP1A mRNA levels were observed for both species, although the results differed substantially between them. Whereas the observed

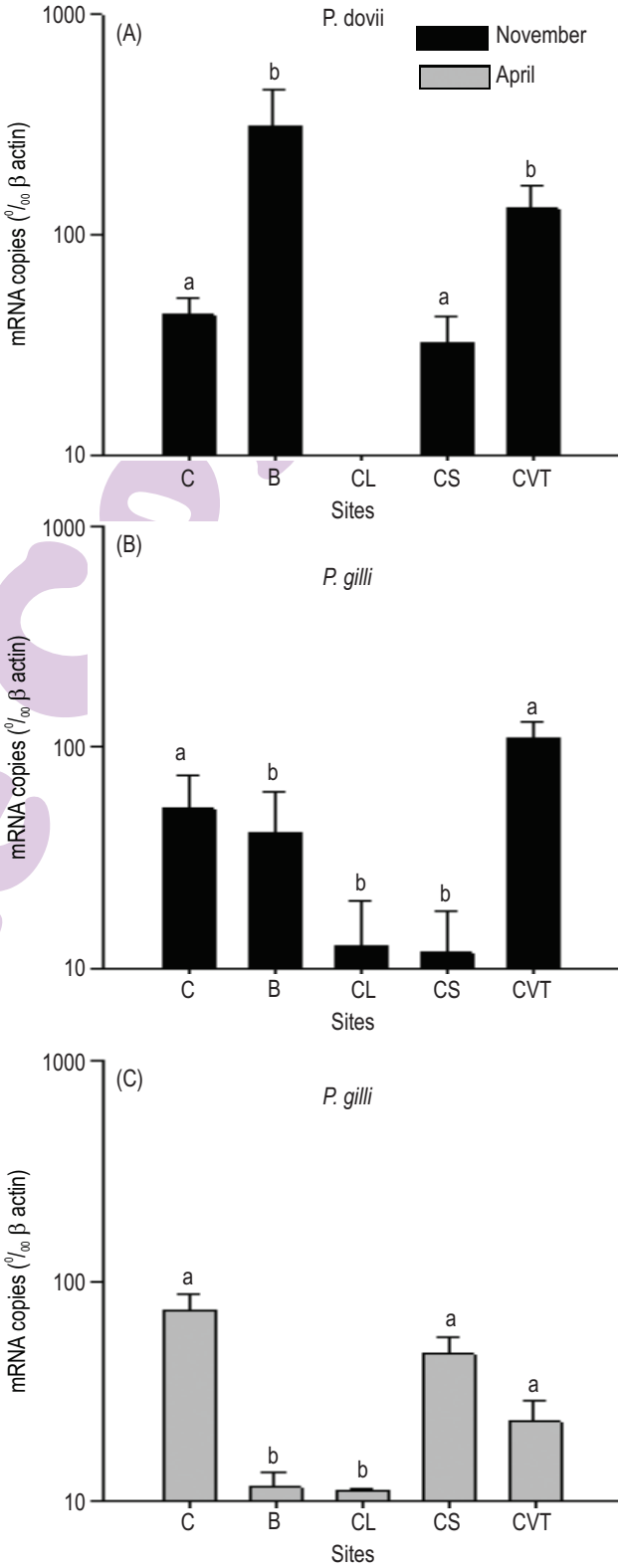

Fig . 4 : Abundance of mRNA (Mean ± SE, 5-10) of Cyp1A in immature *P dovii* and male *P. gillii* fish caged for 2 days into the four studied sites in November and April. Control fish (C) responses are also included. Y axis is in log10 scale. Different letters indicate significant differences among groups following ANOVAs and pos-hoc Tukey's tests.

pattern in *P. dovii* specimens was consistent with a typical CYP1A induction in sites with recognized bad water quality, transplanted *P. gillii* showed a decrease, rather than an increase of CYP mRNA levels in assumed agriculture-polluted sites relative to control. Although there is no satisfactory explanation for this discrepancy, field studies have evidence that hexachlorobenzene at high doses may inhibit the enzymatic activity associated to CYP (Lavado *et al.,* 2004; 2006). Assuming that this effect may be species-specific, the results suggest that some compounds with capacity to alter the xenobiotic metabolism exist in some of the selected sites, although they may not be canonical dioxin-like compounds. In a previous study conducted in Costa Rica during 2009 (Campos *et al.,* 2010) analyzed up to 33 different pesticides residues in surface water and report levels of pesticides quite low for most compounds except those of hexachlorobenzene, epoxichlorazole, phorate and bromacil that had peak values of 2261, 473, 314 and 117 ng l^{-1}, respectively (Lavado *et al.,* 2006). To our knowledge none of the above mentioned compounds had estrogenic neither dioxin like effect. Therefore, the responses obtained in this study are consistent with the results from previous studies.

The data suggests that the method of quantitative RNA biomarkers are feasible to use in two Costa Rican indigenous fish species to evaluate effects of dioxin-like and estrogenic compound in surface water. Increase in vitellogenin and Cyp1A expression after exposure to inducers was clearly assessable with this method in both species. In field, no signs pollution-induced estrogenicity were observed, while a more consistent pattern of CYP1A induction response was observed in *P. dovii*, which suggests this species could be a good model for field monitoring.

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