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Physiological response of alligator gar juveniles (*Atractosteus spatula*) exposed to sub-lethal doses of pollutants

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Abstract Alligator gar populations have declined because of overfishing, habitat loss and pollution. Over time, the exposure to different pollutants have affected these fishes as a consequence of their high trophic level, bottom-dwelling habits and long life span. In order to evaluate the physiological effects of pollutants on alligator gar, juveniles (6, 12 and 24 months) were exposed to sub-lethal doses of diazinon, β-naphthoflavone (BNF) and 17 β-estradiol (E2) by intraperitoneal injection. After 2 days of exposure, liver samples were taken to determine the activities of acetylcholinesterase, butyrylcholinesterase and carboxylesterase; alkaline and acid phosphatases (ALP and ACP); ethoxyresorufin o-deethylase (EROD); glutathione s-transferase (GST); superoxide dismutase (SOD), and vitellogenin (VTG) concentration. Two additional bioassays consisting on the exposure of compounds through water or food were performed and after 4 and 28 days, respectively, biomarkers were determined. All esterases were inhibited in organisms exposed to diazinon as well as in 6-months gar exposed to E2 and BNF. In contrast, ALP activity increased in gar exposed to diazinon and E2, while ACP activity did not show any variations. No EROD activity was registered after exposure to the different pollutants, despite being one of the most sensitive and common detoxification biomarkers used for fishes. GST activity reduction was detected when gar were exposed to E2 and BNF, while SOD activity increased after exposure to diazinon and E2. Finally, VTG levels were higher in animals exposed to E2 compared to other treatments. Overall, these results suggest that alligator gar juveniles have a low biotransformation metabolism and show that they are especially sensitive to those pollutants affecting the nervous system.

Keywords Alligator gar · Biotransformation enzymes · Cholinesterases · Vitellogenin · EDCs

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Introduction

Alligator gar (*Atractosteus spatula*) belongs to the seven extant species of an ancient group of non-teleost bony fishes of the family Lepisosteidae. At present, lepisosteids are distributed in North America, Central America and Cuba. As other ancient fish, alligator gar have undergone few morphological and physiological changes in 180 million years and possess a non duplicated genome, as occurred in modern bony fish,

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making important the study of their evolutionary physiology (Amores et al. 2011). Furthermore, gars are appreciated as sport and edible fish. Unfortunately, many lepisosteid populations have declined due to overfishing, habitat loss and alteration (road and dams construction, channel straightening and pollution) (Mendoza et al. 2008). Among these causes, pollution is a rather unexplored factor that may have a great influence on gar's health, considering their biological characteristics, namely their high trophic position, long life span and bottom-dwelling habits (O'Connell et al. 2007). Additionally, other aspects that may contribute to their exposure to pollutants are their ability to withstand high concentrations of ammonium and nitrites and their tolerance to water anoxic conditions as a result of their ability to breathe atmospheric air (Mendoza et al. 2008; Solé et al. 2009). These attributes may also imply a different sensitivity to the toxicity of specific pollutants compared to other fish species (Goldstone and Stegeman 2006). Moreover, during larval and juvenile stages, these fishes show one of the fastest growth rates among fish (Mendoza et al. 2008), an aspect that may forward the processes of biotransformation and bioaccumulation of contaminants. Gars have a very long life cycle in which reproductive maturation starts between ages 4 and 6 years and can exceed 50 years of life reaching larger sizes close to 3 m in length, an aspect that may increase the potential for mobilization of contaminants accumulated during periods of starvation or reproduction associated with lipidic reserves (Van der Oost et al. 2003).

At the present, there is a paucity of data on the potential effects of pollutants on the physiology of primitive fish, and the available information is mainly limited to the determination of the tissue concentrations of residual pollutants. Overall, these studies have shown that lepisosteids displayed higher concentrations of pollutants than other fish in the same habitat. In this regard, several authors have reported the highest concentration of polychlorinated biphenyls and chlorinated pesticides in *Lepisosteus oculatus* and *A. tropicus* compared to other fish inhabiting the same water bodies (Carvalho et al. 2009). In the same way, an elevated concentration of metals have been registered in *L. platyrhincus* and *L. osseus* (Rumbold et al. 2008), as well as very high concentrations of polycyclic aromatic hydrocarbons (PAHs) in *L. oculatus* (Luna et al. 2005). The physiological effects reported for these pollutants include liver lesions characterized by the accumulation of melanomacrophage centers, pancreatitis and perivascular cuffing (Hartley et al. 1996), esterases inhibition (Huang et al. 1997) and the reduction in 17 β-estradiol levels (Orlando et al. 2002).

To explain the effects of pollutants, molecular and biochemical biomarkers have been widely used because of their potential to identify the mode of action of xenobiotics. In particular, phase I detoxification enzymes associated with the P450 cytochrome are the most accepted biomarkers for dioxin-like compounds, including PAHs exposure (Solé et al. 2009), while phase II detoxification enzymes such as transferases, associated with organic xenobiotics and heavy metals, are also commonly used (Sen and Kirikbakan 2004). Esterases are widely used as indicators of organophosphate pesticides, carbamates and other neurotoxics (Thompson 1999). Complementary to this set of biomarkers, the detection of vitellogenin (VTG) in males and juveniles is also a valued tool for the identification of endocrine disrupting compounds (EDCs), particularly of those mimicking estrogenic effects (Van der Oost et al. 2003).

Considering this context, the present research was aimed at evaluating the physiological response of alligator gar juveniles exposed to sub-lethal doses of specific pollutants using enzymatic biomarkers acknowledged by international governmental bodies and the scientific community (Solé et al. 2009).

Methods

Chemicals

Within the vast range of existing pollutants, only those to which the gars are more likely to be exposed were selected: a pesticide, an aromatic hydrocarbon and an estrogenic endocrine disruptor.

The commercial formulation (equivalent to 236 g a.i. diluted in one liter) of Diazinon®, an organophosphate pesticide compound (*O*,*O*-diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate, INN—dimpylate), was used (El-Saeid 2010). The cytochrome P4501A inducer, β-naphthoflavone (BNF) (N3633 Sigma) was used as a representative of aromatic polycyclic hydrocarbons (Karimzadeh et al. 2006). And 17β-estradiol (E2) (E8875 Sigma) was used as a standard estrogenic endocrine disruptor (Duong et al. 2010).

Alligator gar juveniles

Alligator gar juveniles used in this study were obtained from the reproduction of broodfish maintained at the Ecophysiology Laboratory of the Biological Sciences Faculty of the Universidad Autónoma de Nuevo León, Mexico, according to methods previously described (Mendoza et al. 2008).

General conditions for the bioassays

Prior to the bioassays, the animals were acclimated during 7 days in 500-L fiberglass tanks (0.70 × 2.30 × 0.30 m) equipped with a water recirculating system provided with a 1/8 hp pump and a fiber filter containing activated charcoal. Water temperature was maintained at 28 °C with the use of thermostats. Oxygen was provided throughout the acclimation period by a compressor to maintain dissolved oxygen levels above 6 mg/L. A 12:12 light/dark photoperiod was maintained throughout this period, and light intensity at the water surface was 2000–3000 lx. Water parameters for the experimental period were kept in the same way.

Bioassays were performed with juveniles of 6 months (37.4116 ± 7.2793 g), 12 months (170.8333 ± 24.5798 g) and 24 months (309.1666 ± 41.6060 g). The juveniles selected for the bioassays were fed the same artificial feed (52 % protein) used for culture maintenance, and the feeding was stopped 24 h prior to the exposure to the different pollutants.

Bioassay 1: pollutants exposure by injection

The different pollutants (diazinon, BNF, E2) were dissolved in 0.5 mL menhaden fish oil, free of contaminants designed for laboratory use (Sigma F8020) and injected intraperitoneally (50 µg/g) to gar juveniles of 6, 12 and 24 months, as a direct exposure method. Control animals were injected with the same volume of menhaden oil. A 500-L fiberglass tank (0.70 × 2.30 × 0.30 m) was divided by means of an aquaculture mesh in four compartments to host each treatment. Four 6-month juvenile gars were assigned to each compartment, corresponding to an exposure treatment. This experimental procedure was repeated with juveniles of 12 and 24 months. Post-injection, the fish were maintained in these compartments and after 48 h they were killed by hypothermia for obtaining liver samples, which were frozen with liquid nitrogen and stored at −70° C for further processing. The experimental protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Biological Sciences (Approval number FCB-CB-701).

Bioassay 2: pollutants exposure through food

In order to simulate natural conditions of exposure, artificial feed was replaced by live tilapia (*Oreochromis niloticus*) fry. Tilapia fry with an average weight of 2.34 ± 0.48 g were individually injected with 0.1 mL menhaden oil containing 120 µg of the different pollutants (diazinon, BNF, E2). Two tilapia fry were supplied on a daily basis to each experimental 12-month juvenile gar, and it was visually assessed that these were consumed. The control group of gars was fed tilapia free of pollutants, only injected with menhaden oil. The bioassay was performed under the same conditions as described above. Juvenile gars were killed after 28 days to obtain liver samples.

Bioassay 3: pollutants exposure through the water

The addition of contaminants to the water was made following the methodology of Jönsson et al. (2002). Briefly, after the acclimation period, four 12-month juvenile gars per exposure treatment were transferred to a static system consisting of polyethylene bags placed in 48-L culture tanks filled with 40 L of water continuously aerated with air-lifts. Pollutants (diazinon, BNF, E2) were first diluted in 1 mL of dimethyl sulfoxide (DMSO; Sigma D8779), and subsequently, this dilution was poured in the corresponding culture tank water to reach a final concentration of 10^{-6} M. To rule out the effect of the diluent, a rearing tank to which 1 mL of DMSO was applied was used as a control. The fish were exposed during 96 h after which they were killed as previously described, to obtain liver samples.

Sample preparation

Liver samples were homogenized at 4 °C using a Wheaton-glass homogenizer (Glas-Col™) at 333 rpm during 4 min in a tris–HCl 50 mM (pH 7.1) buffer to a proportion of 1:10 (W/V). The homogenized material was centrifuged at 15,300 g at 4 °C for 30 min. The supernatant was separated and stored in 0.1 mL

aliquots at −70 °C to be later used as enzymatic extract. Total soluble protein concentrations in the extracts were determined by the Bradford method using bovine albumin serum as a standard (Bradford 1976).

Biomarkers

Esterases activities were determined according to the methodology described by Huang et al. (1997). The reaction mixture for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) consisted of 280 µL of 5,5′-dithiobis 2-nitrobenzoic acid in buffer PBS 0.1 M (pH 7.8) and 10 µL of enzymatic extract. The reaction was initiated adding 10 µL of acetylthiocholine chloride (0.015 M) for the AChE activity or butyrylthiocholine chloride (0.015 M) for the BChE activity. For carboxylesterases (CaE), 200 µL of buffer Tris–HCl 50 mM (pH 7.1), 10 µL of enzymatic extract and 100 µL of *p*-nitrophenyl acetate (2 mM)-like substrate were added to initiate the reaction. Absorbance was immediately registered at 405 nm in intervals of 120 s up to 10 min in a microplate reader (TECAN, Sunrise^{TM}). Alkaline and acid phosphatase (ALP and ACP) activities were measured using *p*-nitrophenyl phosphate as substrate (Mazorra et al. 2002). The reaction was performed using 200 µL of buffer diethanolamine (1.0 M) with 50 mM MgCl2 (pH 9.8) for ALP or buffer 0.1 M sodium acetate-HCl (pH 4.8) for ACP, then 10 µL of the enzymatic extract and 10 µL of the substrate were added at a final concentration of 0.4 mM. Absorbance was immediately registered at 405 nm in the same conditions as the esterases. For each sample, three analytical replications were conducted. In the case of the control, the sample was replaced with buffer. The linearity of the reaction was verified and the enzymatic activity for esterases and phosphatases was expressed as the increase in absorbance per minute by milligram of protein in the extracts (ΔAbs/min/mg protein).

The GST activity was analyzed according to Wilce and Parker (1994). The reaction mixture was carried out in 1.0 mL quartz cells and consisted in 970 µL of Dulbecco' s buffer (pH 7.2), 10 µL of reduced L-glutathione (200 mM) and 10 µL of the enzymatic extract. The reaction was initiated by adding 10 µL of 1-chloro-2,4-dinitrobenzene (CDNB) 100 mM. Absorbance was immediately registered at 340 nm every 30 s during a period of 5 min in SPECTRONIC spectrophotometer, Genesys 2. GST activity was expressed as µmol/mL/min/mg protein using for CDNB a molar extinction coefficient of 9.6 Mmol/cm. To determine superoxide dismutase (SOD), activity a kit was used (CAYMAN 706002). The reaction mixture was carried out in microplates, and consisted of 200 µL of radical detector (tetrazolium salt), in buffer Tris–HCl 50 mM (pH 8.0) with diethylenetriaminepentaacetic acid (0.1 mM) and hypoxanthine (0.1 mM), and 10 µL of enzymatic extract or a SOD standard. The reaction was initiated by adding 20 µL of xanthine oxidase in 50 mM Tris–HCl buffer (pH 8.0), and absorbance was immediately registered at 450 nm. In order to quantify the activity, a SOD standard curve was used and the concentration in the samples was expressed in U/mL.

To determine the EROD activity, the livers were excised, weighed and homogenized with an additional equal volume of ice-cold 140 mM KCl solution. Microsomes were prepared by the calcium chloride precipitation method (Hamilton et al. 1999). Microsomal extracts from adult tilapia injected with β-naphthoflavone under the same conditions were used as positive controls. Total soluble protein concentrations of the microsomal extracts were determined by the Bradford method (Bradford 1976). EROD activity was measured by the spectrophotometric method described by Kennedy (1994). The reaction mixture consisted of 480 µL of ethoxyresorufin (2 µM) in a Tris–HCl 0.1 M (pH 8.0) buffer with NaCl 0.1 M and 10 µL of microsomal extract. The reaction was started by the addition of 10 µL of NADPH 29.9 mM (N1630 Sigma) in a Tris–HCl 50 mM buffer (pH 7.4), with dithiothreitol 1 mM, EDTA 1 mM and glycerol 20 %. The absorbance (572 nm) was recorded every 30 s during 10 min in a Genesys 2, SPECTRONIC spectrophotometer. EROD activity was expressed as nmol of resorufin formed min/mL. Each assay was conducted in duplicate using individual liver microsomes from at least three individual fish for each group.

Liver VTG concentration was assessed by a previously reported competitive enzyme-linked immunosorbent assay (ELISA) (Mendoza et al. 2012). Nalgene microtiter plates were coated with 100 ng of alligator gar VTG in 200 µL of coating buffer (Na2CO3 0.05 M, pH 9.6) and incubated overnight at 4 °C, then 200 µL of blocking solution (PBS-Tween 0.05 %, defatted milk 5 %) were added and incubated 2 h at 37 °C. Competition was carried out

by adding 200 μL of assay buffer (PBS-Tween 1:1000, defatted milk 5 %) containing a concurrently incubated (1 h, 37 °C) mixture of 8.5 ng of homologous VTG-antibody and samples or reference VTG (1.5–300 ng). Secondary antibody (IgG-peroxidase conjugate) diluted in 200 μL of PBS-Tween (1:1000) was added and incubated during 1 h at 37 °C. Peroxidase activity was assayed by adding 100 μL of ortho-phenylene–diamine 1 M in a citrate buffer 0.1 M (pH 4.5), at 37 °C for 20 min in the dark. After incubation, 50 μL HCl 1 N were added to stop the reaction, and absorbance was read at 492 nm.

Statistical analysis

Data normality was assessed using Kolmogorov–Smirnov test. A two-way ANOVA analysis was performed to evaluate the effect of age and pollutants for the first bioassay. The Tukey multiple range test was used to identify differences among ages, and t-Dunnett test was conducted to detect differences between pollutants and the control ($p < 0.05$). Exposure of pollutants through food or water was analyzed by a one-way ANOVA and the t-Dunnett was used to determine differences between pollutant treatments and the control ($p < 0.05$). Statistical analyses were performed using the SPSS Statistics 22.0 software.

Results

The statistical analysis revealed significant differences in AChE, BChE and SOD activities as these increased between 6 and 12 months (Table 1). In contrast, the difference in CaE occurred between 12 and 24 months. Differences in ACP did not show a pattern associated with age. No significant differences in the activities of GST and SOD were observed at any of the three ages, while differences in ALP were only significant at a low probability level ($p = 0.043$).

The activity of ACP of liver samples of juvenile gars of different ages belonging to the control groups was similar to that determined in experimental individuals after exposure to the three different pollutants. Among the pollutants tested, diazinon stood out for showing the greatest effect on the enzymatic activities of the different biomarkers. Only levels of VTG were not significantly affected by diazinon. E2, by contrast, produced greater levels of VTG and important variations in the activities of GST and BChE, while BNF exhibited the most marked effect on the detoxification enzymes CaE and GST.

Significant lower activities of esterases (AChE, BChE, CaE) and GST, compared to those observed in the control groups, were observed in 6-month-old gars exposed to diazinon, BNF and E2 (Table 2). While in 12- and 24-month-old gars, the esterases activities were only significantly lower in those animals exposed to diazinon. A decrease in GST activity was also observed in 12-month-old gars when exposed to diazinon and BNF. This decline in GST activity was observed again in 24-month-old gars exposed to BNF and E2. BChE activity was also reduced in 24-month-old gars exposed to E2.

An opposite response to that observed for esterases was registered for the ALP activity, which rose in response to contaminants. The activity levels were significantly higher for gars of all ages exposed to diazinon and for 12- and 24-month gars exposed to E2. Similarly, an increase in CaE activity was registered in 12 months animals exposed to E2.

No significant variation was detected in the activity of ACP irrespective of the age of juveniles exposed to different pollutants. Taking into account the low activity levels and the absence of significant variations detected for this enzyme its evaluation for the pathway of exposure was discarded.

EROD activity could not be determined in gar microsomal liver samples, as no changes in absorbance (572 nm) could be recorded, indicating the absence of resorufin formation. To discard any methodological complications, EROD activity was assessed using microsomal liver samples from tilapia exposed to BNF as a positive control.

The SOD activity increased in response to the exposure of all contaminants, but the increase was only significant in 6-month-old gars exposed to diazinon and 12-month-old gars exposed to diazinon and E2.

An important increase in VTG concentration (up to ten times) was observed in animals of all ages exposed to E2, in contrast with animals exposed to the rest of the contaminants, which showed VTG levels similar to the control.

In regard to the exposure pathway, a significant reduction in esterases (AChE, BChE and CaE) activities was observed in those animals exposed to

Table 1 Results of the two-way ANOVA to determine the effect of age (6, 12, 24 months) and pollutants (diazinon; β-naphthoflavone: BNF; 17 β-estradiol: E2) on different biomarkers in juvenile alligator gar (*Atractosteus spatula*)

Biomarker	Age (months)			Pollutants			
	6	12	24	Control	DOP	BNF	E2
AChE							
Model			$F = 23.148$; $p = 0.000$; gl = 11				
Factor	$F = 11.448$; $p = 0.000$; gl = 2			$F = 57.944$; $p = 0.000$; gl = 3			
Group	1.031 ± 0.065^{a}	1.610 ± 0.113^{b}	1.335 ± 0.065^{b}	1.785 ± 0.098	0.301 ± 0.098***	1.575 ± 0.098**	1.641 ± 0.098**
BChE							
Model			$F = 49.66$; $p = 0.000$; gl = 11				
Factor	$F = 92.271$; $p = 0.000$; gl = 2			$F = 80.387$; $p = 0.000$; gl = 3			
Group	0.549 ± 0.038^{a}	1.131 ± 0.065^{b}	1.249 ± 0.038^{b}	1.183 ± 0.056	0.334 ± 0.056***	1.381 ± 0.056*	1.008 ± 0.056***
CaE							
Model			$F = 35.197$; $p = 0.000$; gl = 11				
Factor	$F = 83.82$; $p = 0.000$; gl = 2			$F = 24.21$; $p = 0.000$; gl = 3			
Group	3.078 ± 0.135^{a}	3.043 ± 0.121^{a}	4.468 ± 0.135^{b}	3.988 ± 0.107	2.180 ± 0.148***	3.550 ± 0.148***	3.861 ± 0.148
ALP							
Model			$F = 6.469$; $p = 0.000$; gl = 11				
Factor	$F = 3.273$; $p = 0.043$; gl = 2			$F = 16.256$; $p = 0.000$; gl = 3			
Group	$8.215 \pm 0.330^{a,b}$	7.105 ± 0.408^{a}	8.380 ± 0.330^{b}	7.237 ± 0.415	10.079 ± 0.415***	6.124 ± 0.415	8.161 ± 0.415
ACP							
Model			$F = 5.313$; $p = 0.000$; gl = 11				
Factor	$F = 21.25$; $p = 0.000$; gl = 2			$F = 2.074$; $p = 0.110$; gl = 3			
Group	0.404 ± 0.017^{c}	0.261 ± 0.014^{a}	0.331 ± 0.014^{b}	0.316 ± 0.017	0.340 ± 0.017	0.331 ± 0.017	0.370 ± 0.017
GST							
Model			$F = 8.45$; $p = 0.000$; gl = 11				
Factor	$F = 1.468$; $p = 0.240$; gl = 2			$F = 12.55$; $p = 0.000$; gl = 3			
Group	245.15 ± 12.54^{a}	264.69 ± 12.54^{a}	258.35 ± 10.24^{a}	306.18 ± 12.95	234.33 ± 12.95***	230.3 ± 12.95***	241.9 ± 12.95***
SOD							
Model			$F = 6.303$; $p = 0.000$; gl = 11				
Factor	$F = 13.103$; $p = 0.000$; gl = 2			$F = 8.561$; $p = 0.000$; gl = 3			
Group	121.67 ± 7.18^{a}	167.71 ± 10.15^{b}	169.80 ± 7.18^{b}	138.78 ± 9.57	191.08 ± 9.57**	122.05 ± 9.57	160.32 ± 9.57
VTG							
Model			$F = 20.09$; $p = 0.000$; gl = 11				
Factor	$F = 3.182$; $p = 0.050$; gl = 2			$F = 69.575$; $p = 0.000$; gl = 3			
Group	401.93 ± 38.08^{a}	271.15 ± 46.64^{a}	287.71 ± 38.08 a	152.65 ± 47.5	116.27 ± 47.5	125.87 ± 47.5	886.3 ± 47.5***

Groups show the mean ± SE; mean values corresponding to age with different superscripts are significantly different (Tukey: $p < 0.05$). Values corresponding to pollutants marked with an asterisk are significantly different compared to the control (Dunnett: * $p < 0.05$; ** $p < 0.00$; *** $p < 0.000$)

Table 2 Mean values ± SD of different biomarkers determined in liver samples of alligator gar (*Atractosteus spatula*) juveniles of different age (6, 12, 24 months) exposed by injection to pollutants (diazinon; β-naphthoflavone: BNF; 17 β-estradiol: E2)

	6 months				12 months	
	Control	DOP	BNF	E2	Control	DOP
AChE	1.99 ± 0.48	0.27 ± 0.06***	1.23 ± 0.24***	0.62 ± 0.41***	1.67 ± 0.14	0.32 ± 0.12
BChE	0.85 ± 0.21	0.36 ± 0.07***	0.59 ± 0.19*	0.39 ± 0.15***	1.25 ± 0.10	0.3 ± 0.11
CaE	4.38 ± 0.84	2.10 ± 0.14***	3.06 ± 0.24***	2.75 ± 0.31***	2.75 ± 0.62	2.0 ± 0.35
ALP	7.69 ± 3.33	10.12 ± 1.08	7.99 ± 1.49	7.04 ± 1.22	6.17 ± 1.18	11.0 ± 2.1
ACP	0.402 ± 0.05	0.401 ± 0.03	0.408 ± 0.05	0.404 ± 0.01	0.201 ± 0.005	0.23 ± 0.07
EROD	nr	nr	nr	nr	nr	nr
GST	333.4 ± 25.3	168.8 ± 12.1***	236.3 ± 47.5*	241.9 ± 81.7*	282.1 ± 0.2	220 ± 31.7*
SOD	88.4 ± 38.8	170.4 ± 9.9**	115.1 ± 29.2	112.7 ± 43.7	139.0 ± 5.5	180 ± 30.4
VTG	202.56 ± 52.05	161.77 ± 75.60	166.66 ± 91.81	1076.72 ± 409.8***	161.60 ± 80.99	143 ± 50.7

	12 months		24 months			
	BNF	E2	Control	DOP	BNF	E2
AChE	2.01 ± 0.11	2.45 ± 0.34*	1.68 ± 0.43	0.34 ± 0.06***	1.47 ± 0.55	1.84 ± 0.61
BChE	1.47 ± 0.51	1.49 ± 0.29	1.44 ± 0.28	0.33 ± 0.07***	2.08 ± 0.18***	1.12 ± 0.37
CaE	2.75 ± 0.51	4.21 ± 0.78*	4.82 ± 0.57	3.26 ± 0.27***	5.05 ± 0.36	4.72 ± 0.73
ALP	4.26 ± 1.42	8.92 ± 2.39*	7.83 ± 2.23	11.05 ± 3.15*	6.11 ± 0.41	8.51 ± 2.02
ACP	0.201 ± 0.01	0.341 ± 0.013*	0.343 ± 0.09	0.318 ± 0.09	0.298 ± 0.15	0.364 ± 0.06
EROD	nr	nr	nr	nr	nr	nr
GST	222.6 ± 7.7**	264.0 ± 2.2	302.9 ± 19.2	271.4 ± 23.1	228.1 ± 29.4***	230.8 ± 22.7***
SOD	114.6 ± 10.7	199.2 ± 13.3*	188.8 ± 31.6	185.6 ± 55.4	136.3 ± 10.7	168.3 ± 37.2
VTG	136.8 ± 42.0	699.39 ± 269.04***	93.79 ± 19.03	100.23 ± 36.36	74.14 ± 33.72	882.87 ± 353.7***

Values marked with an asterisk are significantly different compared to the control (Dunnett: * $p < 0.05$; ** $p < 0.00$; *** $p < 0.000$)

diazinon supplied trough either water or food (Table 3). In the same sense, those animals exposed to E2 through the water showed a lower BChE activity. When the animals were fed the tilapia fry containing diazinon and BNF a significant reduction in the GST activity was registered, in contrast to the exposure of these contaminants trough the water that did not result in a significant reduction in the activity.

A reverse response to that observed for esterases was registered in the case of ALP activity, as this increased regardless of the pathway of diazinon exposure (water or food). No significant changes in the SOD activity were observed when the animals were exposed to the contaminants either trough water or food. A 10- to 18-fold increase in the levels of VTG was observed when animals were exposed to E2 by the food or water, respectively, compared to the control.

Discussion

Because of the scarcity of toxicological reports for alligator gar, the pollutants as well as the doses and exposure periods were implemented considering similar experiments performed in different species (Karimzadeh et al. 2006; Jönsson et al. 2002). When the animals were exposed to pollutants through food, the amount of tilapia was calculated so that the dose was similar to that injected to gar juveniles of the first bioassay. While the exposure time, 28 days, is commonly used in experiments to evaluate artificial feed or nutrients (Mendoza et al. 2008). Even if the differences in pathways and exposure times do not allow a direct comparison of the results the response of the biomarkers was found to be consistent.

The reduction in the activities of esterases (AChE, BChE, CaE) in gars of all ages and by different

Table 3 Mean values ± SD of different biomarkers determined in liver samples of alligator gar (*Atractosteus spatula*) 12-months juveniles exposed to pollutants (diazinon; β-naphthoflavone: BNF; 17 β-estradiol: E2) trough water or food

Biomarker	Water pathway			
	Control	DOP	BNF	E2
AChE	1.031 ± 0.28	0.331 ± 0.127***	1.314 ± 0.19	1.061 ± 0.23
BChE	1.214 ± 0.18	0.375 ± 0.171***	1.111 ± 0.33	0.785 ± 0.29*
CaE	2.873 ± 0.4	1.719 ± 0.513**	3.399 ± 0.61	3.054 ± 0.52
ALP	3.46 ± 0.9	10.06 ± 1.17***	5.910 ± 2.20*	5.21 ± 2.1
GST	124.91 ± 18.13	95.78 ± 10.57	110.93 ± 20.14	99.50 ± 17.29
SOD	202.57 ± 31.5	205 ± 20.12	194.43 ± 46.9	183.88 ± 45.7
VTG	97.2 ± 61	105 ± 37	78.8 ± 32	1802.8 ± 445***
Biomarker	Food pathway			
	Control	DOP	BNF	E2
AChE	1.252 ± 0.28	0.361 ± 0.17***	1.326 ± 0.35	1.257 ± 0.23
BChE	1.183 ± 0.42	0.279 ± 0.18**	1.063 ± 0.25	0.932 ± 0.27
CaE	3.32 ± 0.41	1.748 ± 0.36**	3.22 ± 0.33	3.43 ± 0.790
ALP	5.58 ± 1.2	19.91 ± 7.70**	6.25 ± 2.23	6.26 ± 1.50
GST	203.94 ± 18.33	79.89 ± 11.45*	140.01 ± 10.96	164.11 ± 20.13
SOD	254.59 ± 51.3	317.63 ± 44.9*	280 ± 47.90	304.73 ± 34.7
VTG	368 ± 42	225.6 ± 130.6	322.1 ± 170.1	3810.9 ± 997***

Values marked with an asterisk are significantly different compared to the control (Dunnett: * $p < 0.05$; ** $p < 0.00$; *** $p < 0.000$)

exposure pathways to diazinon suggests possible neurotoxic effects triggered by this pollutant. The inhibition of these type β esterases by organophosphates and carbamates in fish has been widely documented (Thompson 1999). Diazinon is a synthetic broad-spectrum pesticide, used on a massive scale due to its low cost and easy application that given its chemical structure and stability in alkaline pH remains longer than other phosphorus compounds (El-Saeid 2010). In consequence, there are numerous reports of its negative effects on non-target aquatic organisms (Rakhodaei et al. 2012). The results of the present research are in agreement with the observed reduction in esterases activity in tissues of gar collected in contaminated water bodies by chlorinated hydrocarbons and heavy metals (Huang et al. 1997). It is important to note that 6-month-old gars exposed to E2 and BNF also produced esterases inhibition. This is not surprising considering that chemicals other than carbamates and organophosphates such as PAHs, metals, detergents and other complex mixtures that are environmental contaminants can also affect the activity of esterases (Nunes 2011). In this regard, in vitro exposure to PAHs affected esterases activities in the scallop *Adamussium colbecki* (Bonacci et al. 2009). Cadmium exposure reduced cholinesterase activities of brain and muscle tissues of *Oreochromis niloticus* and *Rhamdia quelen* (Silva and Pathiratne 2008; Pretto et al. 2009). And, in the same way, two surfactants (SDS and a domestic detergent) inhibit in vitro and in vivo AChE activity of *Daphnia magna* (Guilhermino et al. 2000). In the case of E2, besides the known reproductive alterations produced by this estrogen, it has been associated with adverse neuronal effects on a wide range of behaviors including activity, aggression, dominance and other social behaviors (Zala and Penn 2004).

On the other hand, it was observed that individuals of 6 months showed a lower AChE, BChE and SOD activity than gars aged 12 and 24 months, while CaE activities increase significantly in gars of 24 months. In relation to this, intraspecific differences on the activity of esterase enzymes have been reported associated with age, body size and eating habits (Thompson 1999). These results suggest that the sensitivity of gar to pollutants changes with the age. Thus, the lower activity of esterases and SOD observed in organisms of 6 months may imply that at this age gars have not fully developed yet the physiological mechanisms of protection against xenobiotic.

There was no difference in ACP activity between control organisms and those exposed to contaminants, whereas ALP activity increased significantly in all

organisms exposed to diazinon and in animals aged 12 and 24 months exposed to E2. In this regard, it has been reported that slight increases in ALP activity occur in response to minor damage to liver cells, while high increases in ALP activity may indicate liver necrosis and tissue damage (Firat et al. 2011). Moreover, the increase in ALP activity has been associated with the detoxification mechanisms of phosphorus compounds, particularly in insects that become resistant to these pesticides. Therefore, increases in ALP activity observed particularly in organisms exposed to diazinon, in addition to possible liver damage, may be associated with mechanisms of xenobiotics biotransformation. This contention is supported by the reported increase in ALP activity in snakeheads (*Channa punctatus*) exposed to diazinon (Sastry and Malik 1981), and in tilapia (*Oreochromis niloticus*) and trout (*Oncorhynchus mykiss*) exposed to pyrethroids (Firat et al. 2011; Velisek et al. 2009).

Biotransformation enzymes of CYP1A phase I, and EROD activity in particular, are now regarded as some of the most sensitive and useful biomarkers in both laboratory and field for many fish species (Van der Oost et al. 2003). Therefore, the absence of EROD activity in all gar liver samples examined is an unexpected result, particularly in organisms exposed to BNF, which is considered a potent inducer of the CYP1A system. However, this would not be the first report of the absence of EROD activity in fish. Previously, the nonexistence of EROD activity was reported in three fish species of the Loricariidae family, both in wild and captive organisms induced with BNF and dimethylbenz [a] anthracene (Parente et al. 2009). Absence of EROD activity has also been reported in primitive fish such as the sea lamprey *Petromyzon marinus* (Hahn et al. 1998), and the American paddlefish (*Polyodon spathula*) exposed to BNF (Gundersen et al. 2000). The variability of EROD activity levels among species and populations (Parente et al. 2009; Gundersen et al. 2000) may contribute to explain the absence of activity in gar under the experimental conditions established in this research. On the other hand, a very low response of the CYP1A system has been registered in some species when individuals are exposed to inducing agents. This has been interpreted as an adaptive measure of species or populations that are chronically exposed to pollutants in highly contaminated habitats (Couillard et al. 2005). In the present research, this kind of insensitivity of gar could be discarded, as the experimental animals were captive born individuals. Furthermore, the possible influence of sexual hormones on EROD activity, observed in other fish (Gundersen et al. 2000), can be ruled out, as the individuals were sexually immature.

CYP1A phylogenetic studies indicate that the evolution of this system is characterized mainly through gene duplication events or alternative splicing and that these genes likely have gone through an adaptive evolution within aquatic animals (Goldstone and Stegeman 2006). This could contribute to explain the absence of EROD activity in paddlefish and gar, which are ancient species that exist before the genome duplication process underwent by bony fish (Amores et al. 2011). Notwithstanding, other ancient fish such as sturgeons have shown EROD activity when exposed to BNF (Karimzadeh et al. 2006). Therefore, more research is needed on the CYP1A detoxification system in gars and its mechanisms of induction.

In the present study, a reduction in GST activity attributable to xenobiotic was observed and this lower activity was more evident in those organisms exposed to E2 and BNF. Nevertheless, the majority of studies indicate that GST activity increases in response to the presence of xenobiotic (Sen and Kirikbakan 2004). This is because most of the compounds that enter the body undergo a transformation by the enzymes system of the phase I (CYP) allowing a later conjugation with enzymes of phase II, like the glutathione radical (GSH) by GST activity, which converts hydrophobic compounds to hydrophilic ones, to be more easily excreted (Sen and Kirikbakan 2004). In this regard, considering the lack of EROD activity observed in gars, it could be assumed that the products of phase I, which would be the potential substrates for GST, were not being adequately generated. This dependence has been well documented, and an EROD/GST rate (termed biotransformation index: BTI) has been used as a biomarker to assess the phase I/phase II metabolism (Van der Oost et al. 2003). In the case of gars, a reduced biotransformation metabolism may involve a greater likelihood of bioaccumulation of contaminants in the body tissues, which could explain the high residual levels of contaminants reported in lepisosteids (Carvalho et al. 2009). Moreover, Yousef (2004) reported the inhibition of GST activity in rabbits exposed to aluminum, but also detected a strong increase in free radicals. This would mean an increased oxidative stress as a consequence of non-

metabolized xenobiotic, which may affect the GST radical considering that GSH is also involved in the mechanism of defense against free radicals by glutathione peroxidase and glutathione reductase (Sen and Kirikbakan 2004). Therefore, the results concerning the GST could be more related to a response of the defense system against oxidative stress, rather than to the detoxification system. This is also supported by the results of those treatments in which a reduction in GST activity and an increase in the SOD activity were simultaneously observed.

In this regard, a significant increase in SOD activity was observed in gars of 6 and 12 months exposed to diazinon and E2. Several studies have reported that SOD activity may increase in response to xenobiotic, including hydrocarbons and organophosphates (Xu et al. 2012). However, the activity of SOD also depends on other factors, such as the intensity and duration of stress (Xu et al. 2012). This could help to elucidate the variations in the responsiveness of SOD activity between gars of different ages and ways of exposure. It should also be considered that the activity of the enzymes involved in the control of oxidative stress depends on the joint response of these and the content of non-enzymatic antioxidants such as vitamins E and C. Within this context, it can be pointed out that lepisosteids have the ability to synthesize vitamin C (Dabrowski and Moreau 2005); therefore, it would be important to assess in more detail the overall defense mechanism against oxidative stress in lepisosteids and its importance in the presence of xenobiotic.

Currently, the estrogenic effects of EDCs are well known, and a widely accepted measure of estrogenicity is the determination of VTG (Solé et al. 2000). The results of the present research clearly show that the VTG concentration was higher in those organisms exposed to E2 in all age groups and exposure pathways. In the case of injected animals, higher vitellogenin levels could be noticed in 24-months individuals compared to 6- and 12-month-old juveniles, which may reflect an increasing capacity of the liver of the former ones to produce vitellogenin (Table 2). When E2 was administered through food or water, the increase in the concentration of VTG was particularly marked compared to the levels registered when animals were injected with E2 (Fig. 1), reaching levels up to 4000 ng VTG/mg protein. VTG was absent in control organisms,

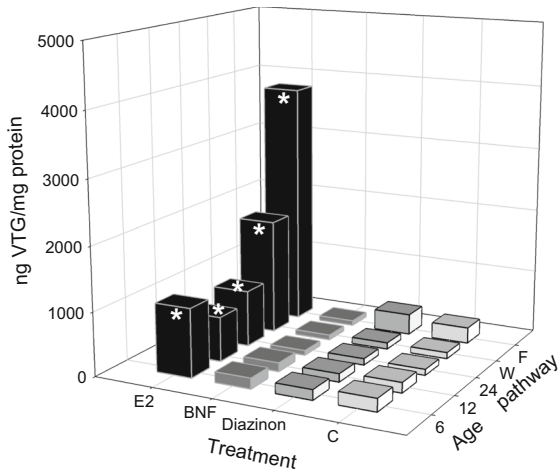

Fig. 1 Hepatic vitellogenin (VTG) concentration (ng VTG/mg protein) of alligator gar (*Atractosteus spatula*) juveniles of different age (6, 12, 24 months) exposed by intraperitoneal injection, through the water (W) or food (F) to diazinon, β-naphthoflavone (BNF) and 17 β-estradiol (E2). Statistical values are presented in Tables 2 and 3. *Asterisks over the bars* represent significant differences compared to their respective control (C) (Dunnett: $*p < 0.05$)

because all animals were young and immature, as gars reach sexual maturity around 5 years (Mendoza et al. 2012). Natural hormones, including estrogens, are commonly released into the environment via sewage effluents and from sources such as animal feedlots (Falconer et al. 2006). Among the diversity of estrogenic compounds identified in the environment, E2, estrone (natural hormones) and 17α-ethinylestradiol (EE2; synthetic estrogen used in birth control pills) are compounds considered responsible for the major estrogenic effects in wild organisms (Duong et al. 2010). Steroids are mostly excreted in a less active conjugated form; however, their deconjugation by microorganisms originates a more potent parent compound (Solé et al. 2000). Although it is known that E2 and EE2 are the main estrogens stimulating hepatic VTG synthesis in teleosts, the molecular mechanism of estrogen action in phylogenetically ancient fish, remains poorly studied. However, it was recently reported that estrogen receptors in lepisosteids are similar to those of teleosts and its activation depends primarily on E2, but are also sensitive to other EDCs such as DDT/DDE (Katsu et al. 2008). This indicates that the reproductive physiology of gars could be altered by estrogen-like EDCs, which could possibly be partially responsible for the current decline of wild

populations of alligator gar, especially taking into account different reports of the presence of these contaminants in the muscle of lepisosteids (Carvalho et al. 2009; Luna et al. 2005). At the same time, considering the lipophilic and persistent nature of most estrogens and their metabolites, these may bioaccumulate and biomagnify to tissue concentrations that could be lethal, carcinogenic or teratogenic (Orlando et al. 2002). For these reasons, EDCs have been identified as possibly responsible for the population declines observed in top predators (Fossi et al. 2006).

Finally, from the results of this research, it can be concluded that different contaminants may be producing negative effects on the nervous system of gars particularly in their early stages as indicated by the reduction in the activity of esterases. In turn, this may lead to a reduction in its predatory capacity and increase the possibility to be preyed upon, with the resulting reduction in their populations and low recruitment of new breeders (Almeida et al. 2010). On the other hand, the observed increase in activity of esterases and SOD with age indicates that the physiological mechanisms of protection against xenobiotic upsurge during the juvenile stage. The absence of EROD activity and the reduction in GST activity may suggest that gars have a low biotransformation metabolism, although there may exist another enzymatic activity with the same detoxification role. This would indicate a tendency toward the bioaccumulation of contaminants, a fact supported by the high residual pollutant concentrations reported in these fish; however, more research is required to clarify this assumption. This may also imply that gars are more susceptible to alterations by oxidative stress, particularly during times of starvation or reproduction, when lipid reserves and associated contaminants are mobilized. Further studies are needed to elucidate their system against oxidative damage to contaminants with better-known effects, such as heavy metals.

The biochemical changes observed in gars exposed to different pollutants may be useful not only to deduce the presence of xenobiotic in wild organisms, but also to understand physiological changes that might lead to changes in their biological performance and the potential alteration of their populations. Moreover, the use of an adequate set of biomarkers can enhance the likelihood of identifying areas/species that are threatened by chemicals, especially in cases where differences in sensitivity may be found among the species.

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Conflict of interest We, the authors state that we do not have any conflict of interest to declare.

References

Almeida JR, Oliveira C, Gravato C, Guilhermino L (2010) Linking behavioural alterations with biomarkers responses in the European seabass *Dicentrarchus labrax* L. exposed to the organophosphate pesticide fenitrothion. Ecotoxicology 19:1369–1381. doi:10.1007/s10646-010-0523-y

Amores A, Catchen J, Ferrara A, Fontenot Q, Postlethwait JH (2011) Genome evolution and meiotic maps by massively parallel DNA sequencing: spotted gar, an outgroup for the teleost genome duplication. Genetics 188:799–808. doi:10.1534/genetics.111.127324/-/DC1

Bonacci S, Corsi I, Focardi S (2009) Cholinesterases in the Antarctic scallop Adamussium col-becki: characterization and sensitivity to pollutants. Ecotoxicol Environ Saf 72:1481–1488

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carvalho FP, Villeneuve JP, Cattini C, Rendón J, Mota de Oliveira J (2009) Ecological risk assessment of PCBs and other organic contaminant residues in Laguna de Terminos, Mexico. Ecotoxicology 18:403–416. doi:10.1007/s10646-008-0295-9

Couillard CM, Lebeuf M, Ikonomou MG, Poirier GG, Cretney WJ (2005) Low hepatic ethoxyresorufin-0-deethylase activity correlates with high organochlorine concentrations in atlantic tomcod from the Canadian east coast. Environ Toxicol Chem 24(10):2459–2469

Dabrowski K, Moreau R (2005) Biosynthesis of ascorbic acid by extant actinopterygians. J Fish Biol 57(3):733–745

Duong CN, Ra JS, Schlenk D, Kim SD, Choi HK, Kim SD (2010) Sorption of estrogens onto different fractions of sediment and its effect on vitellogenin expression in male japanese medaka. Arch Environ Contam Toxicol 59:147–156. doi:10.1007/s00244-009-9429-1

El-Saeid MH (2010) Monitoring of pesticide residues in Riyadh cultured farm fish. World Appl Sci J 9:1075–1080

Falconer IR, Chapman HF, Moore MR, Ranmuthugala G (2006) Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. Environ Toxicol 21:181–191. doi:10.1002/tox.20172

Firat O, Cogun HY, Yuzereroglu TA, Gök G, Fırat O, Kargin F, Kotemen Y (2011) A comparative study on the effects of a pesticide (cypermethrin) and two metals (copper, lead) to

serum biochemistry of Nile tilapia, Oreochromis niloticus. Fish Physiol Biochem 37:657–666. doi:10.1007/s10695-011-9466-3

Fossi MC, Casini S, Marsili L (2006) Endocrine disruptors in mediterranean top marine predators. Environ Sci Pollut 13(3):204–207. doi:10.1065/espr2006.01.018

Goldstone HM, Stegeman JJ (2006) A revised evolutionary history of the CYP1A subfamily: gene duplication, gene conversion, and positive selection. J Mol Evol 62(6):708–717. doi:10.1007/s00239-005-0134-z

Guilhermino L, Lacerda MN, Nogueira A, Soares AMVM (2000) *In vitro* and in vivo inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. Sci Total Environ 247:137–141

Gundersen DT, Miller R, Mischler A, Elpers K, Mims SD, Millar JG, Blazer V (2000) Biomarker response and health of polychlorinated biphenyl- and chlordane-contaminated paddlefish from the Ohio river basin, USA. Environ Toxicol Chem 19(9):2275–2285

Hahn ME, Woodin BR, Stegeman JJ, Tillitt DE (1998) Aryl hydrocarbon receptor function in early vertebrates: inducibility of cytochrome P450 1A in agnathan and elasmobranch fish. Comp Biochem Physiol Part C 120:67–75

Hamilton RL, Moorehouse A, Lear SR, Wong JS, Erickson SK (1999) A rapid calcium precipitation method of recovering large amounts of highly pure hepatocyte rough endoplasmic reticulum. J Lipid Res 40:1140–1147

Hartley WR, Thiyagarajah A, Treinies AM (1996) Liver lesions in the gar fish (Lepisosteidae) as biomarkers of exposure. Mar Environ Res 42(1–4):217–221

Huang TL, Obih PO, Jaiswal R, Hartley WR, Thiyagarajah A (1997) Evaluation of liver and brain esterases in the spotted gar fish (*Lepisosteus oculatus*) as biomarkers of effect in the lower Mississippi river basin. Bull Environ Contam Toxicol 58:688–695

Jönsson EM, Brandt I, Brunström B (2002) Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. Environ Sci Technol 36(15):3340–3344

Karimzadeh K, Mostafaei A, Sari AE, Pourkazemi M, Zahmatkesh A (2006) Induction and purification of cytochrome P4501A1 from ß-naphthoflavon-treated beluga, *Huso huso*. J Appl Ichthyol 22(1):221–225

Katsu Y, Kohno S, Hyodo S, Ijiri S, Adachi S, Hara A, Guillette LJ, Iguchi T (2008) Molecular cloning, characterization, and evolutionary analysis of estrogen receptors from phylogenetically ancient fish. Endocrinology 149(12):6300–6310

Kennedy CJ (1994) Xenobiotics: designing an in vitro system to study enzymes and metabolism. In: Hochachka, Mommsen (eds) Biochemistry and molecular biology of fishes, vol 3, pp 426–427

Luna R, Watanabe KH, Wilding AJ (2005) Spatial bioaccumulation modeling in a network of bayous. Environ Model Softw 21(12):1674–1683

Mazorra MT, Rubio JA, Blasco J (2002) Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. Comp Biochem Physiol B: Biochem Mol Biol 131(2):241–249

Mendoza R, Aguilera C, Ferrara A (2008) Gar biology and culture: status and prospects. Aquac Res 39:748–763. doi:10.1111/j.1365-2109.2008.01927.x

Mendoza R, Santillán O, Revol A, Aguilera C, Cruz J (2012) Alligator gar (*Atractosteus spatula*, Lacépède 1803) vitellogenin: purification, characterization and establishment of an enzyme-linked immunosorbent assay. Aquac Res 43:649–661. doi:10.1111/j.1365-2109.2011.02870.x

Nunes B (2011) The use of cholinesterases in ecotoxicology. Rev Environ Contam Toxicol 212:29–59

O'Connell MT, Shepherd TD, O'Connell AMU, Myers RA (2007) Long-term declines in two apex predators, bull sharks (*Carcharhinus leucas*) and alligator gar (*Atractosteus spatula*), in Lake Pontchartrain, an oligohaline estuary in southeastern Louisiana. Estuaries Coasts 30(4):567–574

Orlando EF, Binczik GA, Kroll KJ, Guillette LJ (2002) The contaminant—associated stress response and its relationship to plasma stress and sex steroid concentrations in the Florida gar, *Lepisosteus platyrhincus*. Environ Sci 9(4):329–342

Parente TEM, De-Oliveira ACAX, Beghini DG, Chapeaurouge DA, Perales J, Paumgartten FJR (2009) Lack of constitutive and inducible ethoxyresorufin-O-deethylase activity in the liver of suckermouth armored catfish (*Hypostomus affinis* and *Hypostomus auroguttatus*, Loricariidae). Comp Biochem Physiol Part C 150:252–260. doi:10.1016/j.cbpc.2009.05.006

Pretto A, Loro VL, Morsch VM, Moraes BS, Menezes C, Clasen B, Hoehne L, Dressler V (2009) Acetylcholinesterase activity, lipid peroxidation, and bioaccumulation in silver catfish (*Rhamdia quelen*) exposed to cadmium. Arch Environ Contam Toxicol 58(4):1008–1014. doi:10.1007/s00244-009-9419-3

Rakhodaei M, Alishahi M, Baboli MJ (2012) Determining the lethal concentration of diazion pesticide (LC 96 H) on *Barbus grypus*. World J Fish Mar Sci 4(4):390–395

Rumbold DG, Lange TR, Axelrad DM, Atkeson TD (2008) Ecological risk of methylmercury in Everglades National Park, Florida, USA. Ecotoxicology 17:632–641. doi:10.1007/s10646-008-0234-9

Sastry KV, Malik PV (1981) Acute and chronic effects of diazinon on some enzymes in certain tissues of a freshwater teleost fish, *Channa punctatus* (Bloch). J Environ Biol 2(3):19–28

Sen A, Kirikbakan A (2004) Biochemical characterization and distribution of glutathione s-transferases in leaping mullet (*Liza saliens*). Biochem (Moscow) 69(9):993–1000

Silva KTU, Pathiratne A (2008) In vitro and in vivo effects of cadmium on cholinesterases in Nile tilapia fingerlings: implications for biomonitoring aquatic pollution. Ecotoxicology 17:725–731. doi:10.1007/s10646-008-0221-1

Solé M, Castillo M, Lopez de Alda MJ, Porte C, Barceló D (2000) Estrogenicity determination in carp, *Cyprinus carpio*: a laboratory and field approach. ANALUSIS 28(9):783–788

Solé M, Rodríguez S, Papiol V, Maynou F, Cartes JE (2009) Xenobiotic metabolism markers in marine fish with different trophic strategies and their relationship to ecological variables. Comp Biochem Physiol Part C 149:83–89

Thompson HM (1999) Esterases as markers of exposure to organophosphates and carbamates. Ecotoxicology 8:369–384

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Velisek J, Svobodova Z, Piackova V (2009) Effects of acute exposure to bifenthrin on some haematological, biochemical and histopathological parameters of rainbow trout (*Oncorhynchus mykiss*). Vet Med 54(3):131–137

Wilce MCJ, Parker MW (1994) Structure and function of glutathione-s-transferases. Biochem Biophys Acta 1205:1–18

Xu W, Liu W, Lu K, Jiang Y, Li G (2012) Effect of trichlorfon on oxidative stress and hepatocyte apoptosis of *Carassius auratus gibelio in vivo*. Fish Physiol Biochem 38(3):769–775. doi:10.1007/s10695-011-9559-z

Yousef MI (2004) Aluminum-induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: protective role of ascorbic acid. Toxicology 199(1):47–57

Zala SM, Penn DJ (2004) Abnormal behaviors induced by chemical pollution: a review of the evidence and new challenges. Anim Behav 68:649–664. doi:10.1016/j.anbehav.2004.01.005