Aquatic Toxicology 179 (2016) 72–81

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Sensitive biomarker responses of the shrimp *Palaemonetes argentinus* exposed to chlorpyrifos at environmental concentrations: Roles of alpha-tocopherol and metallothioneins

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ARTICLE INFO

Article history:
Received 26 May 2016
Received in revised form 15 July 2016
Accepted 23 August 2016
Available online 24 August 2016

Keywords:
Organophosphate insecticide
Metallothioneins
Alpha tocopherol
Native species
Palaemonetes argentinus
Oxidative stress

ABSTRACT

The aim of this study was to evaluate the toxic effects of chlorpyrifos (CPF) at environmental concentrations on the shrimp *Palaemonetes argentinus*, a South American native species. Organisms were exposed to environmentally relevant concentrations of CPF (from 3.5 to 94.5 ng CPF L^{-1}) at laboratory conditions for 96 h. A wide battery of biochemical responses including bioaccumulation, damage and defense biomarkers were measured in cephalothorax and abdomen of shrimp.

The concentration of CPF was below the detection limit of the method in both body sectors (8 ng CPF g^{-1} ww), probably indicating fast biotransformation of the parental compound. Our results showed that CPF exposure inhibits acetylcholinesterase activity from 3.5 ng CPF L^{-1}, a concentration below the suggested Argentinean guidelines for the protection of aquatic biota. Moreover, oxidative stress was evidenced by increased H_2O_2 content and increased levels of TBARs and carbonyl groups in proteins. The induction of antioxidant enzymes like catalase, glutathione *S*-transferase and glutathione peroxidase seems not be sufficient to prevent oxidative damages. In addition, the mobilization of α-tocopherol from abdomen to cephalothorax was observed and reported for the first time in non-reproductive condition. Likewise, a strong diminution of metallothioneins occurred in cephalothorax from the lowest CPF concentration while induction occurred from the same treatment in abdomen as an oxidative stress response. Finally, significant correlation between Integrated Biomarker Response values and exposure concentrations suggest the usefulness of *P. argentinus* as bioindicator of CPF exposure at concentrations as low as environmental ones.

1. Introduction

After decades of extensive use of organochlorine compounds with high environmental persistence, the use of organopho sphorus (OP) pesticides increased greatly in the last twenty years. Although other groups of pesticides such as pyrethroids are being increasingly used, the OPs are still widely applied because their rapid degradation and low persistence in the environment. Usually, pesticides are found in aquatic habitats at varying concentrations because of direct overspray, drift, atmospheric transport and runoff (Gilliom and Hamilton, 2006). Many of them lack target specificity and have high acute toxicity toward non-target aquatic species with some taxa among crustaceans, insects and fish being the most sensitive (Van Wijngaarden et al., 2005).

Abbreviations: [C10.5], exposure to 10.5 ng CPF L^{-1}; [C3.5], exposure to 3.5 ng CPF L^{-1}; [C31.5], exposure to 31.5 ng CPF L^{-1}; [C94.5], exposure to 94.5 ng CPF L^{-1}; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; cGST, glutathione-*S*-transferase in the cytosolic fraction; ChEs, cholinesterase enzymes; CP, Carbonyl group content in proteins; CPF, chlorpyrifos; GC–ECD, Gas Chromatography coupled with Electron Capture Detector analysis; GR, glutathione reductase; GSSG, glutathione disulphide; HPLC–ESI-qTOF, high performance liquid chromatography coupled to mass spectrometry using a quadrupole time-of-flight analyzer, with an electrospray ionization source; IBR, Integrated Biomarker Response; LC5096h, median lethal concentration after 96 h of exposure; LD, limit of detection; LQ, limit of quantification; mGST, glutathione-S-transferase in the microsomal fraction; MTs, Metallothioneins; TBARs, thiobarbituric acid-reactive substances; ww, wet weight; α-Toco, α-tocopherol.

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http://dx.doi.org/10.1016/j.aquatox.2016.08.014
0166-445X/

Among OP pesticides, Chlorpyrifos (CPF), *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate is a broad spectrum insecticide extensively used for the control of pests. After the herbicide glyphosate, CPF is the second most sold pesticide in Argentina, representing the insecticide more used in the country (www.casafe.org). In this context, runoff from fields to rivers and lakes is expected, with consequent water contamination. Previous studies have reported CPF water contamination, with levels up to 17000 ng CPF L^{-1}, in different regions of Argentina (Mugni et al., 2011; Bonansea et al., 2013).

When the pesticides reach the aquatic ecosystems, their interaction with organisms can occur. Non target effects of these compounds can be evaluated using biomarkers as "early warning" signs, providing data on the potential adverse impacts on aquatic species (Booth et al., 2003). OPs including CPF were initially designed to inhibit cholinesterase enzymes (ChEs), mainly acetylcholinesterase (AChE), an enzyme associated with nerve impulse transmission (Collange et al., 2010). This mode of action is the basis of most studies on CPF effects in invertebrates (Cooper and Bidwell, 2006; Gagnaire et al., 2008), and particularly crustaceans, (Kumar et al., 2010), which have evaluated ChE inhibition. Nevertheless, other enzyme systems, physiological mechanisms or biomolecules can be affected by the insecticide exposure in aquatic organisms and have been also used to assess the effects of OP (Livingstone, 2003 Narra et al., 2013; Griboff et al., 2014).

Oxidative stress has been proposed as one of the other molecular mechanisms involved in pesticide-induced toxicity (Wang et al., 2013). In South America, the use of biomarkers in biomonitoring surveys is increasing, but not always in native species (Carriquiriborde and Bainy, 2012). The sensitivity to a toxic compound varies among species, therefore the study of biomarkers in native bioindicators is relevant (van der Oost et al., 2003). Moreover, many of the studies have exposed the organisms to high or not environmentally relevant concentrations. The question arises, whether the exposure to low concentrations such as those encountered in natural environments affects native species. Therefore, it is important to investigate whether low concentrations of CPF will induce significant response in crustaceans.

The decapod *Paleomonetes argentinus* shows sensitivity to pollution both in laboratory and in field tests (ChiodiBoudet et al., 2015; Galanti et al., 2013; Bertrand et al., 2016). Montagna and Collins (2007) reported a median lethal concentration after 96 h of exposure ($LC50_{96h}$) for this species to be 0.49 ± 0.25 μg CPF L^{-1} and showed negative effects on survival and growth in organisms exposed to this contaminant. Therefore, they proposed that this species might be used as a bioindicator to provide information on environmental quality, but values of several biomarkers have not been previously measured.

The aim of this study was to evaluate the effects of CPF at environmental concentrations on *P. argentinus*, a South American native species, exposed in laboratory conditions for 96 h. For this purpose bioaccumulation, damage and defense biomarkers were measured in cephalothorax and abdomen of shrimps. The results of these biological responses help to clarify the mode of toxic action of this insecticide on non-target species. An integrated biomarker index was also calculated for a more holistic view of the biological responses, to evaluate the association between the responses and the exposure concentrations tested to reinforce the usefulness of *P. argentinus* as bioindicator. The hypothesis was that *P. argentinus* exposed to low concentrations of CPF is able to show changes in biological responses evidencing stress or damages.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical grade supplied by Sigma–Aldrich and Sintorgan (Argentina). All laboratory ware used was left with sulfuric-nitric acid solution overnight and then washed with ultrapure water to avoid contamination. Chlorpyrifos (CPF), analytical standard, diluted in acetone was used as stock solution for calibration of insecticide quantification method.

2.2. Organisms

Adult freshwater shrimps, *Palaemonetes argentines,* were collected from a reference site (Monferrán et al., 2011). Organisms were acclimated for two weeks in 40 L glass aquaria filled with artificial freshwater, maintained at constant laboratory temperature (25 °C ± 1 °C) and under 12 h:12 h photoperiod. Throughout the acclimation period, shrimps were fed daily *ad libitum* with commercial fish food (Vita Fish, Argentina) to which protein was added until the protein content was 54% (Griboff et al., 2014).

2.3. Exposure

Two days before the beginning of exposure, adult organisms (body length > 2.2 cm; Donatti, 1986) of *P. argentinus* (0.204 ± 0.013 g wet weight (ww); 2.876 ± 0.056 cm) were transferred to aquaria filled with artificial freshwater, maintaining the density of two individuals per liter.

The experimental design involved five experimental conditions: control (Ctrol), organisms exposed to 3.5 ([$C_{3.5}$]), 10.5 ([$C_{10.5}$]), 31.5 ([$C_{31.5}$]) and 94.5 ng CPF L^{-1} ([$C_{94.5}$]). CPF exposure was carried out for 96 h at similar temperature and photoperiod as acclimation. From the stock solution of CPF a standard solution was prepared in water and aliquots were taken to provide the above nominal concentrations. The exposure solution with higher concentration of CPF also contained 0.002% of acetone. Therefore, acetone was added at 0.002% to control condition.

Due to unstable nature of CPF in the exposure conditions, all the exposure media were replaced every 24 h with freshly made solutions. CPF concentrations were measured at 0 and 24 h, before replacement. CPF was extracted from exposure media by solid phase extraction as described by Bonansea et al. (2013). The extracts were analyzed by an Agilent 6890 gas chromatograph (Santa Clara, CA, USA) equipped with a microelectron capture detector and a Varian VF–5 ms 30 m × 0.25 mm × 0.25 μm capillary column (Palo Alto, CA, USA) to separate and identify the pesticide residue (Maggioni et al., 2012). The obtained limits of detection (LD) and quantification (LQ) in aquarium water were 1 and 2 ng L^{-1}, respectively. Quantified CPF concentrations (ng L^{-1}) at 0 h were: Control = < LD; $[C_{3.5}] = 3.5 \pm 1.2$ ng L^{-1}; $[C_{10.5}] = 10.9 \pm 1.3$ ng L^{-1}; $[C_{31.5}] = 29.6 \pm 1.0$ ng L^{-1} and $[C_{94.5}] = 94.8 \pm 2.6$ ng L^{-1}. At 24 h: Control = < LD; $[C_{3.5}]$ = < LD; $[C_{10.5}] = 3.3 \pm 1.4$ ng L^{-1}; $[C_{31.5}] = 7.0 \pm 2.1$ ng L^{-1} and $[C_{94.5}] = 17.2 \pm 15.4$ ng L^{-1}. The decay of CPF in the media could be explained by volatilization of the compound from the surface, the degradation by photolysis, hydrolysis, or oxidation, as well as the interaction with the exposed organisms, including bioaccumulation (Solomon et al., 2001).

Chlorpyrifos concentrations tested were selected according to:

1-relevant environmental concentration (CPF = 1.2–17000 ng L^{-1}, Mugni et al., 2011; Bonansea et al., 2013);

2-LC10 $_{96h}$ for this specie (54 ± 39 ng CPF L^{-1}, Montagna and Collins, 2007);

3- the limit recommended by the Canadian Council of Ministers of the Environment (CCME, 2012) and the Argentinean Environmental Water Quality Guidelines (AEWQG, 2003) for the protection of aquatic biota (≤ 2 ng L^{-1} and ≤ 6 ng L^{-1}, respectively).

During exposure shrimps were fed with 3.5 mg of food (Vita fish complemented with proteins) per organism per day. Mortality of *P. argentinus* varied from 3% in Control condition to 12% in [$C_{94.5}$].

For each treatment 90 organisms were exposed in 6 independent aquaria. Six to ten or fifteen organisms were taken randomly from at least six independent aquaria for biomarker of effect or exposure measured, respectively. A total of 450 shrimps were used for the experimental exposure. All measurements were performed in triplicate.

At the end of exposure shrimps were cryoanesthetized and they were washed three times with ultra-pure water, weighed and dissected in cephalothorax and abdomen according to Bertrand et al. (2015). Finally, samples were frozen with liquid nitrogen and kept at −80 °C until analysis.

2.4. Biomarkers analysis

2.4.1. Bioaccumulation

Three cephalothoraxes or abdomens, without exoskeleton, were pooled to get enough quantity for analysis of CPF (five pools of three body sectors each, taken randomly from six exposure tanks conducted for each treatment). Approximately 150–200 mg of pooled tissue sample were homogenized in a mortar with 2 g of anhydrous Na_2SO_4. Then, 3 g of fluorisil (60–100 mesh) were added and samples homogenized again. The mixture of sorbent material and shrimp tissue was packed into a 10 mL cartridge, which was fitted with 2 g of silica for sample clean-up. The elution was carried out with 10 mL of ethyl acetate. Subsequently, the elute was evaporated under nitrogen current until dryness, re-dissolved with 0.5 mL of ethyl acetate and transferred to an auto sampler vial for Gas Chromatography coupled with Electron Capture Detector analysis (GC–ECD). The remaining extracts were afterwards concentrated to dryness, and re-dissolved in 0.3 mL of acetonitrile: ultrapure water (70:30, v/v) to be confirmed by high performance liquid chromatography coupled to mass spectrometry using a quadrupole time-of-flight analyzer, with an electrospray ionization source (HPLC–ESI–qTOF).

Recovery percentages of CPF were previously evaluated from spiked samples at 350 ng CPF g^{-1} ww, obtaining values of 85 ± 9%.

2.4.2. Defense and damage biomarkers

2.4.2.1. Hydrogen peroxides. The hydrogen peroxide (H_2O_2) concentration was determined according to Jana and Choudhuri (1981). Tissue samples were homogenized with 1.5 mL of sodium phosphate solution (50 mM, pH 6.5) and centrifuged at 10000*g* for 2 min and 4 °C. Reaction started when 75 μL of cephalothorax or abdomen extracts were added at 225 μL of titanium sulfate (4.16 mM) dissolved in a H2SO4 (20% v/v). The pertitanic acid generation, after the reaction of H2O2, was measured by spectrophotometry at 415 nm. Results were expressed in mg H2O2 g^{-1}-ww.

2.4.2.2. Lipid peroxidation. Lipid peroxidation was estimated by determining the thiobarbituric acid-reactive substances (TBARs) content in aqueous extracts of cephalothorax and abdomen of *P. argentinus* and according to Heath and Parker (1968). Tissue samples were homogenized with 2.5 mL of ultra-pure water and 2.5 mL of solution with 2-thiobarbituric acid (TBA, 34 mM) and trichloroacetic acid (TCA, 20% v/v) was added to homogenate. Samples were incubated for 30 min at 95 °C, cooled in ice and centrifuged for 10 min (1000 rpm). After that the absorbance of supernatant was measured at 532 nm. The amount of TBARs present was calculated using an extinction coefficient of 155 mM cm^{-1}. Results were expressed in nmol g^{-1} ww.

2.4.2.3. Determination of carbonyl groups in proteins. Carbonyl group content in proteins (CP) was quantified in extracts by the reaction of this functional group with 2,4-dinitrophenylhydrazine (Levine et al., 1990; Dalle-Donne et al., 2003). Tissue samples were homogenized with 1.2 mL of sodium phosphate buffer (10 mM, pH 7.4) and 140 mM NaCl and centrifuged twice (5 min at 11,000*g*, 4 °C) to eliminate all particulate matter that might interfere with the reaction. For nucleic acids precipitation, 60 μL of protamine sulfate solution (1%) were added to 600 μL of obtained tissue extract supernatant. After incubation for 30 min at 4 °C, samples were centrifuged (5 min at 11,000*g*, 4 °C). A solution of 2,4-dinitrophenylhydrazine (DNPH, 10 mM in 2 M HCl) was added to 250 μL of supernatant of each sample to give a final protein concentration of 1–2 mg mL^{-1}. Reagent blanks were obtained by adding only 2 M HCl to the sample alquot. Samples were allowed to stand in the dark at room temperature for 1 h with vortex agitation every 10 min; they were then precipitated with 20% TCA (final concentration) and centrifuged for 5 min. The supernatants were discarded, the protein pellets were washed three times with 1 mL of ethanol: ethyl acetate (1: 1, v/v) to remove any free DNPH. Samples were then resuspended in 6 M guanidine hydrochloride dissolved in sodium phosphate buffer (20 mM, pH 2.3) at 37 °C for 15 min with vortex mixing. The 2,4-dinitrophenylhydrazone generation was measured at 366 nm, using a microplate reader (Bio-Tek, Synergy HT), considering a molar absorption coefficient of 22000 M^{-1} cm^{-1}. Results were expressed in μmol carbonyl groups μg^{-1} proteins.

2.4.2.4. Enzyme extraction and activity measurements. Enzyme extracts of cephalothorax and abdomen of *P. argentinus* were prepared according to Wiegand et al. (2000) . Tissue samples were homogenized in sodium phosphate buffer (NaP, 0.1 M, pH 6.5) containing glycerol (20%), 1,4-dithioerythritol (DTE, 1.4 mM) and ethylene diamine tetra acetic acid (EDTA, 1 mM). After removal of cell debris (10 min at 13,000 *g*, 4 °C), the membrane fraction of the extracts was separated by centrifugation at 105.000*g* for 60 min. The remaining supernatant, defined as the soluble (cytosolic) and the pellet (microsomal) fractions, previously re-suspended in NaP (20 mM, pH 7), containing glycerol (20%) and DTE (1.4 mM), were used for enzyme measurement. Enzymatic activities were determined by spectrophotometry, using a microplate reader (Bio-Tek, Synergy HT).

2.4.2.4.1. Glutathione-S-transferase. The activity of cytosolic and membrane bound glutathione-*S*-transferase (cGST and mGST; EC 2.5.1.18) was measured according to Habig et al., 1974. Enzyme extracts were incubated with 166 μL of NaP (0.1 M, pH 6.5), 7 μL of reduced glutathione (GSH; 58.6 mM) and 7 μL of the substrate 1-chloro-2, 4-dinitrobenzene (CDNB; 29.6 mM). The absorbance was then determined at 340 nm.

2.4.2.4.2. Glutathione reductase. The glutathione reductase activity (GR; EC 1.8.1.7) was assayed according to Tanaka et al. (1994). Cytosolic enzyme extracts were incubated with 160 μL of NaP (0.1 M, pH 7.5), 10 μL of glutathione disulphide (GSSG; 18.6 mM) and 10 μL of Nicotinamide adenine dinucleotide phosphate (NADPH; 1.4 mM). The decrease of NADPH was followed at 340 nm.

2.4.2.4.3. Glutathione peroxidase. The activity of glutathione peroxidase (GPx; EC 1.11.1.9) was determined as reported by Drotar et al. (1985) using hydrogen peroxide (H_2O_2) as substrate. Cytosolic enzyme extracts were incubated with 160 μL of NaP (0.1 M, pH 7.5),

2 μL GSH-reductase (0.4 U), 8 μL of GSH (32.5 mM), 8 μL of NADPH (1.4 mM) and 2 μL of H_2O_2 solution (2.68 mM).

2.4.2.4.4. Catalase. Catalase activity (CAT; EC 1.11.1.6) was measured in shrimp using H_2O_2 as substrate, immediately after enzyme extraction (Beutler, 1982). Cytosolic enzyme extracts were incubated with 200 μL solution of reaction containing TRIS base (50.5 mM), EDTA (0.25 mM) and H_2O_2 (0.01 mM). The absorbance was then determined at 240 nm.

2.4.2.4.5. Superoxide dismutase. Superoxide dismutase activity was determined by using the photochemical p-nitroblue tetrazolium chloride (NBT) reduction method as described by Aiassa et al. (2010) (SOD; EC 1.15.1.1). Reaction started when 30 μL methionine (47.7 mM), 10 μL NBT (0.82 mM in PSB), 30 μL EDTA (0.37 μM) and 30 μL of riboflavin (7.33 μM) were added to enzyme extracts, in the presence of light (20 W). After 30 min, absorbance was determined at 595 nm.

2.4.2.4.6. Cholinesterase. The activities of cholinesterase using acetylcholine and butyrilcholine as substrates were determined as reported by Ellman et al. (1961). Cholinesterases (ChEs) with capacity to hydrolyze acetylcholine and butyrylcholine will be referenced as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively. Enzyme extracts were incubated with 187 μL of NaP (0.1 M, pH 8), 1.3 μL of substrate acetylcholine or butyrylcholine (75 mM), 6 μL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB,10 mM) in NaP (0.1 M, pH 7) with $NaHCO_3$ (17.8 mM).

AChEs activities were measured in cytosolic and microsomal fractions (cAChE and mAChE) while BChE was measured in cytosolic fraction (cBChE). The reaction of thiocholine with DTNB forming a yellow compound was measured at 412 nm.

The activity of all enzymes assessed was calculated in terms of the protein content of the sample extract (Bradford, 1976) and, apart from SOD, are reported in nanokatals per milligram of protein (nkat mg $prot^{-1}$), where 1 nkat is the conversion of 1 nmol of substrate per second. SOD activities were reported in unit (U) per milligram of protein, where one unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT. The protein quantification was performed using bovine serum albumin as standard.

2.4.2.5. Metallothioneins. The amount of metallothioneins (MTs) was determined in cephalothorax and abdomen of *P. argentinus* by differential pulse polarography (DPP), a technique based on SH-compound determination according to the Brdicka reaction as described by Olafson and Olsson (1991). A MDE 150 Stand Polarographic (Radiometer, Copenhagen) Tracelab 50, controlled by the computer software Tracemaster 5 through a Polarographicanalyser POL 150 was used. MTs results were reported in μg MTs g^{-1} ww.

2.4.2.6. Alpha-tocopherol levels. Tocopherol extraction was performed in cephalothorax and abdomen of shrimp as described by Griboff et al. (2014). The α-tocopherol (α-Toco) content was determined by high performance liquid chromatography with fluorescence detection (excitation wavelength 276 nm and emission wavelength 316 nm). α-tocopherol concentrations were reported in μg gr^{-1} ww.

3. Data analysis

3.1. Statistical treatment

Data were expressed as the average ± standard deviation (SD) in tables and median and quartiles were given in boxplot graphs. Generalized Linear Mixed Model was used for the statistical analysis. Models were fitted and then normality and variance homogeneity were tested and variance function was applied if necessary. A posteriori test (LSD Fisher) was used to determine significant differences between the means of control and other treatments ($p < 0.05$). R Studio and Infostat (Version 2013p, Di Rienzo et al., 2013) were used for all statistical analyses.

3.2. Boosted regression trees and integrated biomarker response

The aim of this analysis is to determine those biomarkers with higher capacity than other to explain the variation in the exposure concentrations of CPF. Boosted regression trees (BRT) were fitted according to procedure described by Bertrand et al. (2016). Briefly, the model was constructed with the GBM packages (Ridgeway, 2013) in R (version 0.98.953), using the code described by Elith et al. (2008) including those biomarkers with significant differences with respect to control condition. This method builds a sequence of models of increasing complexity, in order to describe relationships between the response variable (exposure CPF concentration) and the predictor variables (effect biomarkers) getting the variance percentage of the response variable explained by each biomarker (BRT%). The tree complexity was set at 2 in accordance with Elith et al. (2008), where the authors suggest a low tree complexity (2 or 3) for a small data set. The PRESS function was applied to know the capacity of each biomarker selected to predict the CPF exposure concentration.

One general stress index, termed "Integrated Biomarker Response", was afterwards calculated with the selected biomarkers in each body sector of *P. argentinus*. This IBR was performed in accordance to Beliaeff and Burgeot, (2002) with modifications by Devin et al. (2014). Several IBRs were calculated from the same data changing the order of the biomarkers and using the median of all the index values as the final index value. Pearson correlation (CPF exposure levels vs. IBR median) and Kruskall Wallis test to identify IBR difference between treatments were carried out.

4. Results and discussion

4.1. Bioaccumulation

The concentration of CPF was below the detection limit of the method in all the extracts of cephalothorax and abdomen of analyzed shrimps (8 ng CPF g^{-1} ww, data not shown).Our results could indicate that the biotransformation mechanisms in shrimp are adequate for avoiding the accumulation of the parental compound, at least at the exposure concentrations tested. In living organism, the oxidative activation of CPF by cytochrome P450 enzymes to become oxon-analogs, which are much more potent AChE inhibitors than CPF, has been described (Jin-Clark et al., 2002). In crustaceans, this pathway could be possible since mammalian cytochrome P450 2 and 3 family-like enzymes, showed activity for 7-pentoxyresorufin and dibenzylfluorescein substrates, mainly in hepatopancreas (Koenig et al., 2012). Moreover, in various species of crustaceans the transformation of the organophosphate insecticide fenitrothion to fenitrothion-oxon has been observed when the organisms have been exposed to this pesticide (James and Boyle, 1998). The following detoxification pathway involving the formation of more polar compounds is species-dependent. In fish and mammals, the oxon-CPF transformation to dephosphorylated metabolite 3,5,6-trichloro-2-pyridinol (TCP) as the major product of the biotransformation has been reported as one of possible biotransformation pathways (Barron et al., 1993; Bicker et al., 2005). Conversely, in oyster *Crassostrea virginica* the compound *O*,*O*-diethyl-*O*-[3,5-dichloro-6-methylthio-2-pyridyl]phosphorothioate has been described as the single metabolite, as a consequence of the mercapturic acid pathway (Woodburn et al., 2003). Further studies in metabolite analysis should be performed in *P. argentinus*.

4.2. Defense and damage biomarkers

Crustaceans have been reported as sensitive organisms to organophosphate pesticides, especially CPF (Kumar et al., 2010). Several studies report the inhibitory effect of organophosphorus pesticides over ChEs, especially AChEs, in vertebrates and invertebrates (Mehler et al., 2008; Kumar et al., 2010). However, *P. argentinus* exposed to low concentrations were particularly sensitive. Our results showed a significant response in the activity of mAChE in cephalothorax of *P. argentinus* at the lowest exposure concentration (3.5 ng CPF L^{-1}) with a nearly 40% decrease compared to control condition. Maximal inhibition was reached at [$C_{10.5}$] with 48% inhibition and at [$C_{94.5}$] with a 45% inhibition (Fig. 1). The abdomen showed a significant inhibition from [$C_{10.5}$] with an activity decrease of 55% at [$C_{94.5}$]. Those results are in concordance with the mode of action of CPF which consists of impeding the acetylcholine and butyrylcholine neurotransmitter degradation in postsynaptic space by the inhibition of AChE as the result of a chemical reaction between the phosphoryl moiety and a hydroxyl group of serine in the active site of AChE, producing an inactive phosporylated enzyme. In crustaceans, the decrease in AChE activity has been reported in the shrimps *Paratya australiensis* and *Macrobrachium lanchesteri* exposed for 96 h to 10 and 500 ng CPF L^{-1}, respectively (Kumar et al., 2010; Tongbai et al., 2012). Our results would indicate a higher sensibility of *P. argentinus* in comparison to the previously mentioned species. The strong mAChE inhibition from [$C_{3.5}$] onwars might indicate the oxidation of CPF to CPF-oxon with higher affinity for this enzyme, which could be explaining the absence of the parental compound accumulation.

Detoxification and toxic sequestration through cytosolic cholinesterase have been described for numerous kinds of compounds (Johnson and Moore, 2012). In cephalothorax an inhibition of cAChE occurred at [$C_{94.5}$] while no significant changes in cBChE activity were observed (Fig. 1). In contrast, in abdomen cAChE and cBChE were activated at intermediate and high exposure concentrations, respectively (Fig. 1). The increase in cytosolic ChE could be indicating an attempt to detoxify the toxic compound to avoid further effects in microsomal ChE.

Overall, the protection activity of cAChE and cBChE was insufficient to avoid inhibition of mAChE in both body sectors, probably due to the chemical properties of CPF with high affinity to lipophilic structures like membranes (Jokanovic, 2001). AChE inhibition in crustaceans has been directly related with immobility and mortality. Nevertheless, the relation between mortality and AChE inhibition does not exist for all species of invertebrates. In *Gammarus pulex,* when inhibition of AChE reached the 50% of the activity, it was directly related with mortality (Day and Scott, 1990). In *Daphnia magna*, the relationship between AChE inhibition and immobility might depend on the pesticide studied. After CPF exposure, a 50% reduction in AChE activity was associated with detrimental effects on mobility (Printes and Callaghan, 2004).

The exposure of organisms to pesticides like CPF may provoke an imbalance between endogenous and exogenous Reactive Oxygen Species (ROS) and, subsequently, a decrease in the efficiency of antioxidant defences or even oxidative damage (Valavanidis et al., 2006; Wang et al., 2013). However, biological systems have adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cellular components from oxidative damage. The first line of defense consists of antioxidant molecules, such as reduced glutathione, ascorbic acid, carotenoids, retinol and α-tocopherol (vitamin E) (Martínez-Álvarez et al., 2005; Lushchak, 2011). They usually work as free radical scavengers. Another defensive mechanism is comprised of antioxidant enzymes including GPx, GST, SOD, CAT, DT-diaphorase and associated ones providing needed cofactors such as glutathione reductase and glucose-6-phosphate dehydrogenase. Some of these antioxidants, like tocopherol and carotenoids, are obtained by aquatic animals with food, while most are produced metabolically (Lushchak, 2011).

Table 1
Biomarkers measured in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of CPF for 96 h: Peroxides (H_2O_2), Carbonyl Group content in proteins (CP), and Thiobarbituric Acid Reactive Species (TBARS). The data represent mean ± standard deviation (SD). Different letters indicate significant differences ($p < 0.05$) among treatments and body sectors.

	Cephalothorax				Abdomen			
		Mean	SD			Mean	SD	
mg H_2O_2 g^{-1} ww	**Ctrol**	2.26	0.15	***a***	**Ctrol**	2.23	0.32	***a***
	[$C_{3.5}$]	2.32	0.34	***a***	**[$C_{3.5}$]**	2.40	0.53	***a***
	[$C_{10.5}$]	3.19	0.58	***b***	**[$C_{10.5}$]**	2.89	0.12	***b***
	[$C_{31.5}$]	3.18	0.41	***b***	**[$C_{31.5}$]**	2.91	0.14	***b***
	[$C_{94.5}$]	3.4	0.35	***b***	**[$C_{94.5}$]**	3.27	0.27	***b***
		Mean	SD			Mean	SD	
μmol CP g^{-1} proteins	**Ctrol**	2.9	0.5	***a***	**Ctrol**	3.8	1.4	***a***
	[$C_{3.5}$]	3.2	0.7	***a***	**[$C_{3.5}$]**	3.5	0.2	***a***
	[$C_{10.5}$]	8.2	1.1	***b***	**[$C_{10.5}$]**	13.1	2.9	***c***
	[$C_{31.5}$]	9.3	1.8	***b***	**[$C_{31.5}$]**	8.1	4.2	***bc***
	[$C_{94.5}$]	4.3	1.7	***a***	**[$C_{94.5}$]**	6.8	4.1	***b***
		Mean	SD			Mean	SD	
nmol TBARs g^{-1} ww	**Ctrol**	12	3	***c***	**Ctrol**	6	2	***a***
	[$C_{3.5}$]	14	2	***c***	**[$C_{3.5}$]**	6	1	***a***
	[$C_{10.5}$]	14	2	***c***	**[$C_{10.5}$]**	8	1	***a***
	[$C_{31.5}$]	14	3	***c***	**[$C_{31.5}$]**	6	1	***a***
	[$C_{94.5}$]	23	3	***d***	**[$C_{94.5}$]**	10	1	***b***

However, the information about negative effects over the oxidative status of crustaceans caused by CPF at environmental levels is less known. In our study, a significant increase in H_2O_2 levels was observed in organisms exposed to higher CPF levels than [$C_{10.5}$], with maximal H_2O_2 concentration at [$C_{94.5}$] in both body sectors (Table 1). This response was accompanied by oxidative damage to biomolecules indicating that the antioxidant system was overwhelmed. TBARs showed enhanced levels at [$C_{94.5}$] in both body sectors, with concentration significantly higher in cephalothorax compared to abdomen. The concentration of carbonyl groups in proteins increased from [$C_{10.5}$], especially in abdomen (Table 1). Those results could be indicating than proteins suffer oxidative stress at lower exposure concentrations than lipids in *P. argentinus*, at least under the tested conditions. Unexpectedly, in cephalothorax, at [$C_{94.5}$], levels of carbonyl groups dropped in comparison to [$C_{31.5}$], and were comparable to control condition. This diminution in CP could be indicating an increased protein turnover due to oxidative stress. This effect is consistent with the decrease in protein concentration accompanied by the presence of free amino acid due to protein degradation, as described by Narra et al. (2013) in a freshwater crab exposed to CPF.

Vitamin E is considered to be an essential dietary nutrient for crustaceans and α-tocopherol (α-Toco) the most active form in shrimp tissues. The α-Toco is widely accepted to be a lipophilic antioxidant, protecting biological membranes from oxidation by scavenging organic free radicals and producing α-tocopheroxyloxyl radicals (Wang and Quinn, 2000;) besides fulfilling a structural function (Lie et al., 1994). Those functions have been reported in different genuses of shrimps, *Penaeus* (He and Lawrence, 1993), *Litopenaeus* (Du et al., 2006) and *P. argentinus* (Griboff et al., 2014). Cahu et al. (1995) identified the hepatopancreas as the main storage organ. However, the muscle, representing more than 50% of the total body mass, contained a great amount of this biomolecule.

P. argentinus exposed to CPF showed an α-Toco level variation with higher contents in both body sectors at [$C_{10.5}$] when compared with control condition (Fig. 2). From [$C_{10.5}$] to [$C_{94.5}$] these increased α-Toco levels do not show changes in cephalothorax. However, the biomolecule concentration decreased significantly in abdomen in the last two exposure concentrations. On the one

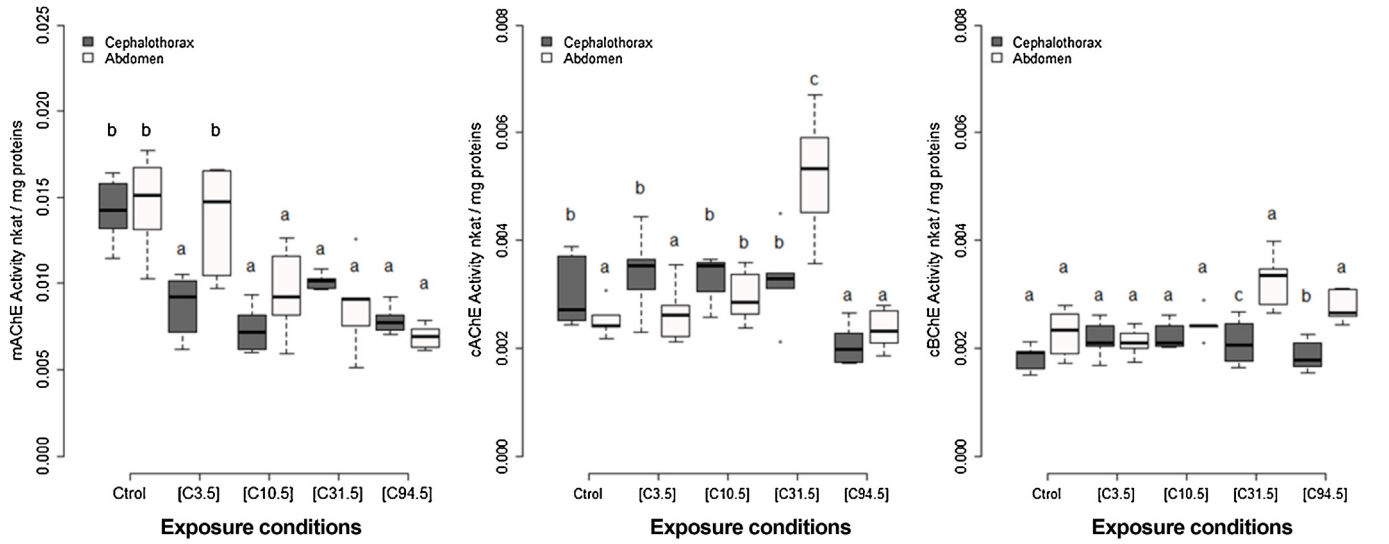

Fig. 1. Microsomal and cytosolic Acetylcholinesterase (mAChE and cAChE) and cytosolic Butyrylcholinesterase (cBChE) activities in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of CPF for 96 h. Different letters indicate significant differences ($p < 0.05$) among treatments and body sectors.

hand,Verma et al. (2007) described the occurrence of lipid peroxidation in rats exposed to CPF and the important role played by α-Toco in reducing it. Griboff et al. (2014) described that supplementation of vitamin E in the diet could enhance the resistance of shrimp to acute exposure to atrazine, decreasing the oxidative stress suffered by the organims. On the other hand, previous studies describe α-Toco transport mechanisms in reproductive periods from muscle to liver in fish (Lie et al., 1994). Cahu et al. (1995) proposed a similar redistribution mechanism in *Penaeus* shrimps, with a transport of this molecule from muscle to hepatopancreas. In our study, despite the increase of α-Toco in cephalothorax of *P. argentinus,* lipid peroxidation and carbonyl group rise ocurred. Thus, probably, the oxidative damage reaches so high levels that they cannot be repaired, triggering alternative mechanisms. Variations in α-Toco levels between the body sectors of *P. argentinus* could be indicating the existence of α-Toco transport from abdomen to cephalothorax, in order to try to prevent oxidation of vital molecules.

Metallothioneins (MTs) are well known as useful biomarkers of metal exposure and they have been used in biomonitoring studies (Luoma and Rainbow, 2008). In *P. argentinus* exposed to zinc, MTs showed a significant induction in cephalothorax but no response has been observed in abdomen (Bertrand et al., 2015). Moreover, the ability of MTs to sequester ROS has been described in vertebrates and aquatic invertebrates (Buico et al., 2008). Buico et al. (2008) decribed a greater antioxidant ability of the MTs in aquatic organisms than in rabbit. The antioxidant function of MTs has been linked to susceptibility of the sulfydryl groups (-SH) of their cysteine residues to be oxidized by ROS. As a consequence of −SH oxidation, the bound metal ion is released, being able to induce further synthesis of MTs, as ROS levels increase (Haq et al., 2003). Finally, MTs are more susceptible for degradation by lysosomes or through cytosolic protease activity, when they have lost the metal ion (Miles et al., 2000).

MTs levels measured in *P. argentinus* exposed to CPF are shown in Fig. 2. Cephalothorax had MT levels significantly higher than abdomen probably due to the presence of hepatopancreas in the first body sector, as has been decribed in Bertrand et al. (2015). In shrimps exposed to CPF, a significant decrease in MTs of cephalothorax ocurred from [$C_{3.5}$] to higher exposure concentrations, while MT concentrations in abdomen increased. The increased H_2O_2 levels found in cephalothorax could be causing the oxidation of MTs, accompanied by metal ion release, increasing their susceptibility for degradation, which causes the decline of the MT concentrations. In abdomen, it seems that the increased H_2O_2 levels did not promote the MT degradation; on the contrary, they may be playing a role in MT induction. The antioxidant role of MTs has been previously described in crustacean genus *Gammarus* according to Correia et al. (2002). In their study a significant

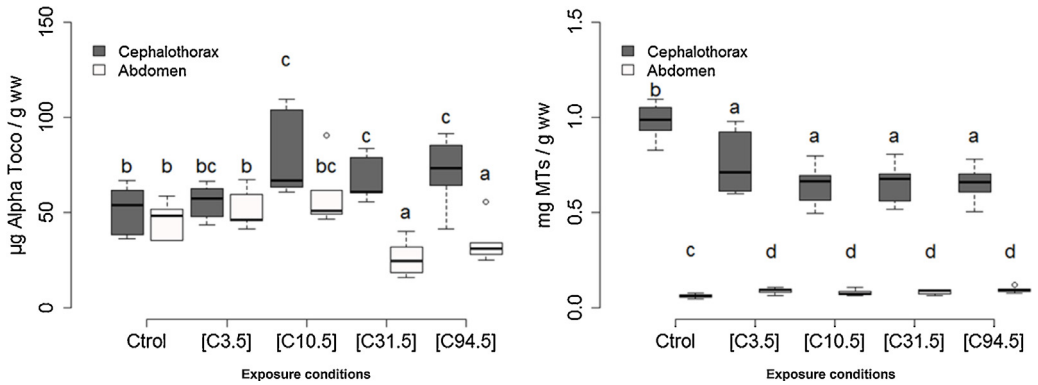

Fig. 2. alpha–tocopherol (α-Toco) and metallothionein (MTs) levels measured in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of CPF for 96 h. Different letters indicate significant differences ($p < 0.05$) among treatments and body sectors.

Table 2
Enzyme activities in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of CPF for 96 h: Catalase (CAT), Glutathione Peroxidase (GPx), cytosolic and microsomal Glutathione *S*-Transferase (cGST and mGST), Superoxide Dismutase (SOD). Enzyme activities are expressed in nanokatal per milligram of proteins except for SOD, which is expressed in units per milligram of protein. The data represent mean ± standard deviation (SD). Different letters indicate significant differences ($p < 0.05$) among treatments and body sectors.

		Cephalothorax			Abdomen			
		Mean	SD			Mean	SD	
Catalase *nkat mg*$^{-1}$ *proteins*	**Ctrol**	9.4	1.8	***a***	**Ctrol**	8.79	1.3	***a***
	[$C_{3.5}$]	11.2	1.6	***a***	**[$C_{3.5}$]**	8.10	1.6	***a***
	[$C_{10.5}$]	8.8	1.6	***a***	**[$C_{10.5}$]**	8.13	0.5	***a***
	[$C_{31.5}$]	11.9	2.1	***b***	**[$C_{31.5}$]**	9.13	1.9	***ab***
	[$C_{94.5}$]	12.1	1.5	***b***	**[$C_{94.5}$]**	8.59	1.8	***ab***
		Mean	SD			Mean	SD	
GPx *nkat mg*$^{-1}$ *proteins*	**Ctrol**	0.179	0.030	***b***	**Ctrol**	0.127	0.031	***a***
	[$C_{3.5}$]	0.310	0.088	***c***	**[$C_{3.5}$]**	0.124	0.034	***a***
	[$C_{10.5}$]	0.308	0.082	***c***	**[$C_{10.5}$]**	0.170	0.021	***b***
	[$C_{31.5}$]	0.254	0.066	***c***	**[$C_{31.5}$]**	0.141	0.019	***a***
	[$C_{94.5}$]	0.260	0.014	***c***	**[$C_{94.5}$]**	0.184	0.023	***b***
		Mean	SD			Mean	SD	
mGST *nkat mg*$^{-1}$ *proteins*	**Ctrol**	0.57	0.02	***f***	**Ctrol**	0.39	0.05	***c***
	[$C_{3.5}$]	0.40	0.03	***e***	**[$C_{3.5}$]**	0.32	0.02	***b***
	[$C_{10.5}$]	0.41	0.02	***e***	**[$C_{10.5}$]**	0.33	0.03	***b***
	[$C_{31.5}$]	0.50	0.03	***d***	**[$C_{31.5}$]**	0.34	0.03	***b***
	[$C_{94.5}$]	0.50	0.04	***d***	**[$C_{94.5}$]**	0.29	0.03	***a***
		Mean	SD			Mean	SD	
cGST *nkat mg*$^{-1}$ *proteins*	**Ctrol**	0.64	0.08	***b***	**Ctrol**	0.34	0.04	***a***
	[$C_{3.5}$]	0.63	0.06	***b***	**[$C_{3.5}$]**	0.37	0.05	***a***
	[$C_{10.5}$]	0.65	0.08	***b***	**[$C_{10.5}$]**	0.43	0.03	***a***
	[$C_{31.5}$]	0.66	0.05	***b***	**[$C_{31.5}$]**	0.43	0.06	***a***
	[$C_{94.5}$]	0.85	0.05	***c***	**[$C_{94.5}$]**	0.43	0.07	***a***
		Mean	SD			Mean	SD	
SOD *U mg*$^{-1}$ *proteins*	**Ctrol**	0.06	0.02	***a***	**Ctrol**	0.08	0.02	***a***
	[$C_{3.5}$]	0.09	0.05	***a***	**[$C_{3.5}$]**	0.09	0.04	***a***
	[$C_{10.5}$]	0.11	0.05	***a***	**[$C_{10.5}$]**	0.10	0.02	***a***
	[$C_{31.5}$]	0.09	0.07	***a***	**[$C_{31.5}$]**	0.11	0.05	***a***
	[$C_{94.5}$]	0.09	0.04	***a***	**[$C_{94.5}$]**	0.09	0.02	***a***

decrease of lipid peroxidation occurred together with MT level rise in tissues.

In *P. argentinus* exposed to CPF, the increase of H_2O_2 levels was accompanied by an enzymatic antioxidant response induction (Table 2). CAT activity increased from [$C_{31.5}$], in cephalothorax, probably indicating an attempt to reduce H_2O_2 to H_2O. Likewise, in cephalothorax, the GPx activity increased from [$C_{3.5}$], while in abdomen the enzyme induction was observed only at [$C_{31.5}$] and [$C_{94.5}$]. GPx is also involved in the reduction of H_2O_2 to H_2O, but uses glutathione as electron donor. SOD activity did not show significant differences among treatments.

The TBARs increase observed at [$C_{94.5}$] in cephalothorax probably induces the enhanced response of cGST, an enzyme with capacity to reduce lipid hydroperoxides (Regoli et al., 2011). In abdomen, a similar trend, although not significant. was observed for cGST activity. According to Griboff et al. (2014), *P. argentinus* exposed to atrazine also presented an induction of the antioxidant enzyme GST. In the same way, induction of CAT, GPx and GST activities in other crustacean exposed to CPF have been previously observed by Narra (2014).

Lipophilic compounds, like CPF ($\log K_{ow} = 4.9$), have been suggested to alter the phospholipid and fatty acid composition, as well as the fluidity of membranes. As a consequence, any change in the lipid composition of membranes will directly affect the activities of membrane-bound enzymes (Antunes-Madeira et al., 1993 Sahoo et al., 1999; Tagliari et al., 2004; Narra, 2014). These effects could be explaining the mGST inhibition observed in both body sectors. Even though the cephalothorax displayed a higher activity than the abdomen, a similar inhibition pattern was observed from the lowest exposure concentration, the enzyme activity was inhibited by 30% at [$C_{94.5}$]. Overall considered, oxidative stress damage observed for proteins and lipids described above demonstrate the insufficient protective role of antioxidant enzyme system.

According to our results, the cephalothorax showed a more sensitive and enhanced oxidative stress response, compared with the abdomen, which could explain the variation in α-Toco levels between body sectors. Probably, and considering the biomarkers here meassured, the requirements of cephalothorax to attempt to reduce oxidative effect over cells and tissues is higher in terms of antoxidant mechanisms. The decrease observed in MT

Table 3
Boosted Regression Tree (BRT) and PRESS values for *P. argentinus*: Percentages (%) of Y variability (exposure concentration) explained by each predictor variable (biomarker response). PRESS values are shown for each biomarker. Bold values indicate selected biomarkers.

	Body Sector	BRT (%)	PRESS
cGST	Cephalothorax	**25.8**	**0.46**
TBARs	Abdomen	**22.4**	**0.46**
mAChE	Abdomen	**20.3**	**0.39**
H_2O_2	Abdomen	**16.1**	**0.39**
α-Toco	Abdomen	**5.3**	**0.40**
cAChE	Abdomen	2.3	0.05
cAChE	Cephalothorax	1.3	0.29
CP	Cephalothorax	1.1	0.08
CAT	Cephalothorax	0.9	0.20
H_2O_2	Cephalothorax	0.8	0.34
mGST	Abdomen	0.7	0.25
GPx	Abdomen	0.6	0.26
cBChE	Abdomen	0.5	0.13
mAChE	Cephalothorax	0.3	0.01
MTs	Cephalothorax	0.3	0.19
MTs	Abdomen	0.2	0.06
CP	Abdomen	0.2	0.06
CAT	Abdomen	0.2	0.07
TBARs	Cephalothorax	0.2	0.46
mGST	Cephalothorax	0.2	0.04
GPx	Cephalothorax	0.2	0.05
α-Toco	Cephalothorax	0.1	0.01

Table 4
Integrated Biomarker Response (IBR) at different exposure concentrations in *P. argentinus* exposed to CPF. Different letter indicate significant differences ($p < 0.05$) among treatments.

IBR						
	Median		Mean	SD	Min	Max
Control	0.06	**a**	0.06	0.07	0.00	0.13
[$C_{3.5}$]	0.31	**b**	0.31	0.04	0.25	0.37
[$C_{10.5}$]	1.44	**c**	1.44	0.50	0.84	2.05
[$C_{31.5}$]	2.64	**d**	2.64	0.71	1.66	3.62
[$C_{94.5}$]	12.56	**e**	12.56	0.03	12.53	12.60

concentration in cephalothorax could be consistent with this hypothesis, even though in *P. argentinus* it would not be enough.

4.3. Boosted regression trees (BRT) and integrated biomarker response (IBR)

Biomarkers from both body sectors with a significant variation with respect to control condition were used to fit a BRT model. Table 3 shows the percentages of variation at different exposure concentrations explained by each predicting variable (biomarkers). According to our results cGST in cephalothorax, TBARs, mAChE, H_2O_2 and α-Toco in abdomen, explained 89.9% of exposure concentration variability. They also showed PRESS values with a good capacity to predict exposure concentrations.

With the biomarkers selected by the BRT method, an Integrated Biomarker Response (IBR) was calculated. The obtained results are shown in Table 4 and Fig. 3, where the area in grey integrates the IBR for each treatment, and it is represented as a star plot. Significantly increased IBR values were observed, and all CPF exposures were significantly different from the control condition. According to these results *P. argentinus* exposed to CPF responded significantly differently with respect to control organisms even at the lowest concentration tested (3.5 ng L^{-1}). This result would be consistent with the sensitivity of crustacean species to OP exposure as before mentioned.

In a similar manner, IBR values and CPF exposure concentrations showed a significant positive correlation ($R^2 = 0.98$, $p < 0.001$). This association suggests the usefulness of *P. argentinus* as bioindicator. Even when these statistics tools are useful to reduce the analysis of big data sets, the toxicological relevance of individual biomarkers should be certainly considered.

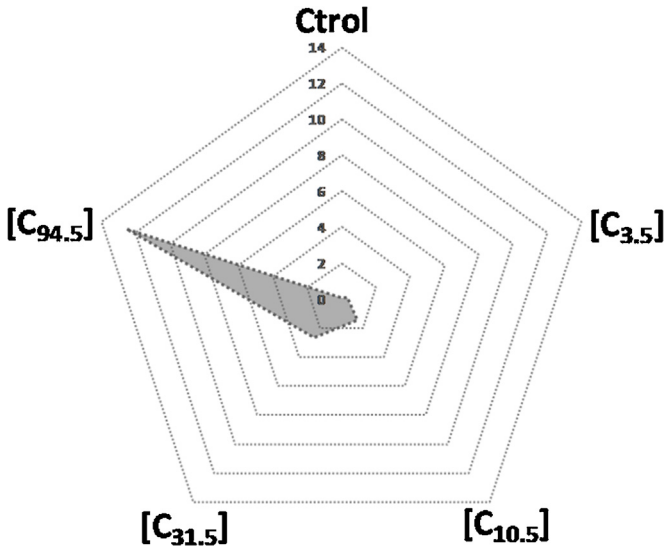

Fig. 3. Integrated Biomarker Response values at different exposure concentrations. Ctrol (not exposed to CPF); [$C_{3.5}$] (3.5 ng CPF L^{-1}); [$C_{10.5}$] (10.5 ng CPF L^{-1}); [$C_{31.5}$](31.5 ng CPF L^{-1}); [$C_{94.5}$](94.5 ng CPF L^{-1}).

5. Conclusions

In the present study, toxic effects of CPF at environmental concentrations on *P. argentinus* were demonstrated using a wide battery of biochemical responses including bioaccumulation, defense and damage biomarkers. Even when CPF was not detected in shrimp tissues, our results showed that this insecticide inhibits AChE activity and causes oxidative stress in *P. argentinus* after an acute exposure. The parental compound is probably transformed to oxon-CPF, with higher toxicity and affinity for AChE than CPF itself, before entering detoxification pathways. The mChE inhibition demonstrated the high sensitivity of the tested species to this OP, since significant inhibition was observed at 3.5 ng CPF L^{-1}, a concentration below the suggested guidelines for the protection of aquatic biota by AEWQG (2003). Oxidative stress was evidenced by increased H_2O_2 content, and biomarkers of antioxidant defense which are overwhelmed, since damages were observed as shown through increased TBARs and carbonyl groups in proteins. The induction of antioxidant enzymes like cGST, CAT and GPx seems not to be sufficient to prevent oxidative damages. Furthermore, the mobilization of α-Toco from abdomen to cephalothorax as well as the decrease in the level of MTs could be possible alternative mechanisms triggered in the cephalothorax to prevent those damages. These last results are interesting if we consider than no previous studies have reported the α-Toco mobilization in a non-reproductive condition and only few reports associate MTs changes with oxidative stress response.

Biological responses reported in the present study in *P.argentinus* from the first exposure concentration were in concordance with IBR values, being significantly higher than in control condition. Finally, significant correlation between IBR values and exposure concentrations could suggest the usefulness of *P. argentinus* as bioindicator of CPF exposure at concentrations as low as environmental ones.

Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Técnica (FONCyT-PICT/2011-1597; 2013-1348), Secretaría de Ciencia y Técnica (SECyT) and CONICET (National Research Council, Argentina). The present work is part of the PhD thesis of L. Bertrand, who gratefully acknowledges fellowship from CONICET. The authors thank Mélanie Bruneau for her technical assistance for MT measurements.

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