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Oxidative stress response induced by atrazine in *Palaemonetes argentinus*: The protective effect of vitamin E

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ABSTRACT

The widespread contamination and persistence of the herbicide atrazine residues in the environment resulted in the exposure of non-target organisms.

The present study was undertaken to investigate the effect of atrazine in the response of oxidative stress biomarkers in the freshwater shrimp *Palaemonetes argentinus* and the protective effect of vitamin-E against atrazine-induced toxicity. Therefore, two batches of *P. argentinus* were fed for 21 days with a commercial food enriched in proteins (D1) or with D2, composed of D1 enriched with vitamin-E (6.8 and 16.0 mg% of vitamin-E, respectively). Subsequently, half of the individuals of each group were exposed to atrazine (0.4 mg L^{-1}) for 24 h and the others remained as controls.

Atrazine promoted oxidative stress response in *P. argentinus* fed with D1 as indicated by enhanced H_2O_2 content and induction of superoxide dismutase, glutathione-S-transferases and glutathione reductase. This antioxidant activity would prevent the increment of thiobarbituric acid reactive substances in the shrimp tissues. *P. argentinus* fed with D2 reversed the response of the biomarkers measured. However, the activation of antioxidants response had an energetic cost, which was revealed by a decrease in lipids storage in shrimps.

These results show the modulatory effect of vit-E on oxidative stress and its potential use as an effective antioxidant to be applied in chemoprotection strategies during aquaculture.

1. Introduction

Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine, ATZ) belongs to the triazine herbicide family which is predominantly applied to the areas cultivating graminaceous crops (e.g. corn, sorghum, and sugar cane) for controlling broadleaf weeds. It is one of the most effective and inexpensive herbicides in the world and, as a consequence, is more frequently used than any other herbicide. ATZ is soluble in water, but its slow degradation generates leachates and contamination of surface and groundwater. The concentration of ATZ varies from 0.2 to 1000 μg L^{-1} in freshwaters directly adjacent to treated fields. The highest reported concentrations are associated with the first rainfall after application (Graymore et al., 2001). As ATZ administration covers most areas of developing countries in recent years, it has become one of the most serious environmental problems (Knauert et al., 2010; Lu et al., 2013).

Once in the aquatic environment, ATZ may cause stress within aquatic communities. With some degree of controversy among the scientific community, different studies have demonstrated the toxic effects of ATZ in aquatic animals (Solomon et al., 2008; Paulino et al., 2012). The role of ATZ as endocrine disruptor has been proved in teleost fish, amphibians and reptiles (Hayes et al., 2011). With this function ATZ alters male reproductive tissues when animals are exposed during development, among other effects (Hayes et al., 2011).

Abbreviations: ATZ, atrazine; CAT, catalases; CDNB, 1-chloro-2,4-dinitrobenzene; D1, diet 1; D1+ATZ, control shrimps fed with diet 1 and exposed to atrazine; D1C, control shrimps fed with diet 1; D2, diet 2; D2+ATZ, control shrimps fed with diet 2 and exposed to atrazine; D2C, control shrimps fed with diet 2; GPx, glutathione-dependent peroxidises; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferases; GSTm, glutathione-S-transferases measured in microsomal fraction; GSTs, glutathione-S-transferases measured in cytosolic fraction; ROS, reactive oxygen species; SOD, superoxide dismutases; SPE–SPME–GC–MS, solid phase extraction–solid phase microextraction–gas chromatography coupled to Mass Spectrometry; TBA, thiobarbaturic acid; TBARs, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; Vit-E, vitamin E

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Moreover, ATZ is classified as highly toxic to slightly toxic to aquatic invertebrates. There is a wide range of EC50/LC50 values for freshwater invertebrates with values ranging from 720 to $>33{,}000\ \mu g\ L^{-1}$ (USEPA, 2007).

Oxidative stress has become an important subject in aquatic toxicology (Livingstone, 2001, 2003), and ATZ may be directly involved in this process.

Reactive oxygen species (ROS) are an undesirable part of aerobic life. Their steady-state concentration is a balance between production and elimination providing certain steady-state ROS level (Lushchak, 2011). The exposure of organisms to some xenobiotics, especially toxic chemical pollutants, may produce an imbalance between endogenous and exogenous ROS and subsequently, a decrease of antioxidant defenses or even oxidative damage (Valavanidis et al., 2006). Biological systems have developed during their evolution 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 (GSH), ascorbic acid (vitamin C), carotenoids (including β-carotene), retinol (vitamin A) and α-tocopherol (vitamin E – vit-E) (Martinez-Alvarez et al., 2005; Lushchak, 2011). They usually function as free radical scavengers. Another defensive mechanism comprises antioxidant enzymes including glutathione-dependent peroxidases (GPx), glutathione-S-transferases (GST), superoxide dismutases (SOD), catalases (CAT), DT-diaphorase and associated ones providing needed cofactors as glutathione reductase (GR) 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).

There is an increasing evidence that vit-E has vital antioxidant functions in tissues of aquatic animals (Conklin, 1997). Vit-E enhances the oxidative stability of organisms owing to its ability to protect polyunsaturated fatty acids from peroxidation and to scavenge free radicals (Evstigneeva et al., 1998). Since aquatic animals have high levels of unsaturated fatty acids to maintain cell membrane fluidity, especially at low temperatures, it is assumed that vit-E should play an important role (Blazer, 1992). Moreover, non-antioxidant and non-pro-oxidant molecular mechanisms of tocopherols have been also described. α-Tocopherol specific inhibitory effects have been seen on protein kinase C, on the growth of certain cells and in regulation of expression of certain genes (CD36 and collagenase) (Lushchak and Semchuk, 2012 and authors referenced therein).

In the common carp (*Cyprinus carpio* L.), ATZ was linked to the induction of oxidative stress by interfering with different endpoints (diminution of GPx and SOD activities) as well as increase in the malonaldehyde content (MDA) associated to ROS production in liver and gill tissues (Xing et al., 2012).

Decapods are a component of the aquatic community due to their density and their role in the energy transfer (Spivak, 1997; Collins et al., 2006). *Paleomonetes argentinus* is a species of ecologic interest because of its wide distribution in different countries of South America (Morrone and Lopreto, 1995). Some publications have reported the sensitivity of *P. argentinus* to pollution in laboratory tests (Collins and Cappello, 2006; Galanti et al., 2013), and proposed that this species might be used as a bioindicator crustacean to provide information on environmental quality (Montagna and Collins, 2007). The potential employ of *P. argentinus* for biomonitoring purposes, together with its use as live bait for fishing, a recreational activity with high economic impact in some regions of Argentina, define the species as potentially interesting also for aquaculture.

Therefore, this study aimed to assess the effect of ATZ in the response of oxidative stress biomarkers in *P. argentinus* considering also the influence on ATZ toxicity of a well-known antioxidant such as vit-E. Additionally, the metabolic energy consumed by the shrimps during the ATZ exposure was quantified by changes in contents of fat, carbohydrates, and proteins between exposed and control organisms.

2. Materials and methods

2.1. Acclimation period

Adult freshwater shrimps, *P. argentinus*, were collected from a low polluted site (La Calera, Suquía river, Córdoba, Argentina, Monferrán et al., 2011) and immediately transported to the laboratory after collection. Organisms were acclimated in glass aquaria filled with artificial freshwater (ultra-pure water containing $0.100\ g\ L^{-1}$ sea salt, $0.200\ g\ L^{-1}$ $CaCl_2$, and $0.103\ g\ L^{-1}$ $NaHCO_3$, pH=7.6), maintained at constant laboratory temperature ($25\pm1\ ^{\circ}C$) and under a 12 h:12 h light: dark photoperiod for 30 days. All along this period, the organisms were fed daily ad libitum with commercial food for fish (Vita Fish, Argentina). During acclimation period and further treatments, two shrimps within a 1 L of exposure media were considered (Giri and Collins, 2003).

2.2. Dietary plans and diet pre-atrazine exposure

After acclimation period, 120 shrimps were randomly divided in two groups and differentially fed with diet 1 (D1) or diet 2 (D2) twice a day (0.1 g/aquarium/day) along 21 days. The time of feeding was selected in order to allow the shrimps to complete one molting cycle (Díaz et al., 2001). Temperature and photoperiod were maintained like in the acclimation period.

The first diet (D1) was formulated with commercial fish food (VitaFish, Argentina) enriched with proteins through the addition of lyophilized shrimp, adjusting the protein content to 54 percent according to Díaz et al. (2001). The second diet (D2) was formulated with D1 but now added with 11.4 percent of peanut oil. The chemical composition of both diets was determined according to standard methods (AOAC, 1995). Total proteins were estimated from nitrogen content by the Kjeldahl method; fat content was determined by ether extraction using Soxhlet apparatus; moisture was measured by the Karl Fischer method; ashes were determined by incineration of the sample (525–550 °C) while available carbohydrates were calculated by difference (FAO/WHO, 2003). Vit-E content was also measured as described in Section 2.4. All assays were performed in duplicate.

2.3. Atrazine exposure

After 21 days of feeding shrimps with D1 (diet without vit-E addition) or D2 (vit-E enriched diet), the intermoult organisms were split into four groups: D1C; D1+ATZ; D2C and D2+ATZ. D1+ATZ and D2+ATZ groups were exposed to $0.4\ mg\ L^{-1}$ of ATZ (98 percent purity, Sigma Aldrich, Germany) during 24 h. D1C and D2C correspond to animals differentially fed with D1 or D2 but maintained in artificial freshwater without the addition of ATZ. Each group was composed of fifteen shrimps. Each exposure was carried out twice, meaning 30 specimens by condition.

The sublethal concentrations (50 percent 48-h LC1) were chosen according to lethal 48-h toxicity test previously conducted (LC1: $0.8\pm1.5\ mg\ L^{-1}$; LC50: $8.9\pm2.3\ mg\ L^{-1}$, data not shown) and environmental concentrations reported. An adult grass shrimp 96-h atrazine LC50 of $9\ mg\ L^{-1}$ has been previously published by Ward and Ballantine (1985). The 50 percent LC1 criterion for sublethal toxicity test has been previously reported in fish studies (Bacchetta et al., 2011).

The measurement of ATZ in the exposition medium was performed by solid phase extraction–solid phase microextraction–gas chromatography coupled to Mass Spectrometry (SPE–SPME–GC–MS) according to Bonansea et al. (2013). The ATZ concentration was determined at the beginning ($D1+ATZ=0.38\pm0.03\ mg\ L^{-1}$; $D2+ATZ=0.35\pm0.06\ mg\ L^{-1}$) and at the end of the exposure ($D1+ATZ=0.37\pm0.01\ mg\ L^{-1}$; $D2+ATZ=0.37\pm0.02\ mg\ L^{-1}$). Atrazine concentrations in D1C and D2C were below the detection limits of the method ($1.1\ ng\ L^{-1}$). At the end of the exposure, organisms were cryoanesthetized, washed three times with ultra-pure water, measured (rostrum–uropod length), weighted, snap-frozen in liquid nitrogen and maintained at $-80\ ^{\circ}C$ for further samples determination.

2.4. Vitamin E

Vit-E, in its natural state, has eight different isomers, four tocopherols and four tocotrienols (Almeida et al., 2011). Thus, tocopherols and tocotrienols extraction was performed in samples of peanut oil, formulated diets and shrimps as described by Fraser et al. (2000) with minor modifications. In brief, after the addition of 1.5 mL MeOH, samples (1.5 mL of peanut oil, 0.25 g of formulated diet or one shrimp) were vortexed for 1 min. Then, 1 mL of chloroform was added and

sonicated for 5 min prior to the addition of 2.5 mL Tris–HCl (50 mM, pH 7.5 containing 1 mM NaCl). The extracts were mixed and centrifugated for 5 min at 1000*g*. The supernatant was separated and the methanol phase (remaining pellet) re-extracted with chloroform (2 mL). Chloroform extracts were combined and adjusted to a final volume of 4 mL. Two milliliters were dried under nitrogen gas and re-suspended in 0.2 mL of 99.5:0.5 hexane:isopropanol. The tocopherol and tocotrienols content was determined using a Hewlett-Packard series 1100 HPLC system equipped with a fluorescence detector (Agilent Technologies series 1200) and a normal-phase column Metasil Si (250 mm × 4.6 mm, 5 μm, Varian; Metachem, USA) for the separation, maintained at room temperature using an isocratic solvent system (mobile phase) consisting of 99.5:0.5 hexane:isopropanol with a flow rate of 1 mL min^{-1}. Fluorescence detection was recorded at excitation wavelength 276 nm and emission wavelength 316 nm. Identification and quantification of compounds was performed by comparison with the retention times and peak areas of standard substances. The content of tocopherols and tocotrienols in *P. argentinus* was measured individually in six organisms at each experimental condition. All measurements were performed in triplicate.

2.5. Oxidative stress biomarkers

2.5.1. Peroxides

The peroxides content was measured within 24 h after exposure by spectrophotometry according to Jana and Choudhuri (1981). Samples were homogenized with 50 mM sodium phosphate buffer (pH = 6.5) and centrifuged at 10,000*g*, 2 min at 4 °C. Afterwards, 75 μL supernatant was mixed with 225 μL 0.1 percent titanium sulfate dissolved in a H_2SO_4 solution (20 percent v/v), measuring the absorbance due to pertitanic acid (H_2TiO_4) formation at 415 nm in microplates. The amount of H_2O_2 in shrimp tissues was determined using a calibration plot, constructed from solutions containing known amount of H_2O_2, and its concentration is expressed as mg H_2O_2/g of fresh tissue. The peroxides content in *P. argentinus* was measured individually in six organisms at each experimental condition. All measurements were performed in triplicate.

2.5.2. Antioxidant enzymes

Enzyme extracts were prepared from six organisms at each experimental condition according to Wiegand et al. (2000) with few modifications. Thus, one shrimp was homogenized using a glass homogenizer in 0.1 M potassium phosphate buffer, pH 6.5, at 4 °C. Then samples were centrifuged at 4 °C for 10 min at 13,000*g*. Supernatants were removed and centrifuged at 4 °C for 1 h at 105,000*g*. Supernatants were conserved to cytosolic enzyme activity determination. Obtained pellet was resuspended in 20 mM potassium phosphate buffer, pH 7, and was used to microsomal enzyme activity determination. Enzyme extracts were frozen and maintained at −80 °C until the use.

The protein content in each fraction was determined according to Bradford (1976). GST activity, in cytosolic (GSTs) and microsomal (GSTm) fractions, was measured using as substrate 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of glutathione (GSH) as described by Habig et al. (1974). GR activity, in cytosolic fraction, was measured as described by Carlberg and Mannervik (1985). GST and GR activities were expressed in nanokatals per milligram of protein (nkat mg $prot^{-1}$).

SOD activity was determined in cytosolic fraction using a commercial kit (Ransod, Randox, United Kingdom) which employs xanthine and xanthine oxidase to produce superoxide radicals which react with chloride of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium (INT), to form a red colored formazan measured at 505 nm (Suttle, 1986). Then, the SOD activity was measured by the degree of inhibition of this reaction. One unit of SOD is that which caused a 50 percent inhibition of the rate of reduction of INT under the conditions of the assay and was expressed per milligram of protein (SOD unit mg $prot^{-1}$).

Each enzymatic assay was carried out by triplicate.

2.5.3. Lipid peroxidation

The thiobarbaturic acid (TBA) method described by Heath and Parker (1968) was used to evaluate the peroxidation of lipids in *P. argentinus*. Briefly, one shrimp was homogenized with 2.5 mL of ultra-pure water. Thereafter, 2.5 mL of TBA/trichloroacetic acid (TCA) solution (0.5 percent TBA and 20 percent TCA) were added to homogenate and incubated for 30 min at 95 °C. The reaction was stopped with an ice bath and samples were centrifuged at 1000*g* for 10 min. Absorbance of each supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The reaction was performed in triplicate. The rate of lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactive substance (TBARs) formed per milligrams of fresh tissue ($n = 6$).

2.6. Energy reserves analyses

2.6.1. Lipids and carbohydrates

The measurements of lipid and carbohydrates were performed in the same sample according to Van Handel (1965). Samples were homogenized in a solution of methanol:water saturated with Na_2SO_4 (6:4). Homogenates were extracted twice using chloroform:methanol (1:1). Chloroformed supernatant was used to lipid determination. Pellets were extracted with a solution KOH (30 percent) and ethanol to carbohydrates determination. Total lipids were determined by the sulfophosphovanillin reaction, a colorimetric determination at 540 nm (Frings and Dunn, 1970), while glycogen concentration was determined spectrophotometrically at 620 nm by the anthrone method (Scott and Melvin, 1953). Total lipids and glycogen contents in *P. argentinus* were measured individually in six organisms at each experimental condition. All measurements were performed in triplicate.

2.6.2. Proteins

The protein content was determined in an extract obtained by homogenization of an individual in 0.1 M potassium phosphate buffer, pH 6.5 and measured according to Bradford (1976). The remaining extract was immediately processed for enzyme extraction as indicated in Section 2.5.2. Calibration curve was obtained using bovine serum albumin as a standard. Protein content in *P. argentinus* was measured individually in six organisms at each experimental condition. All measurements were performed in triplicate.

2.7. Statistics

All values are expressed as mean ± standard deviation. Normal distribution for data was analyzed by Shapiro Willks test, while Levene test was used to test the homogeneity of variance. ANOVA test was used to compare different treatments to analyze normal variables, followed by Tukey test. When the data showed abnormal distribution, they were subjected to a non-parametric statistical analysis (Kruskal–Wallis) followed by Dunn test. The InfoStat/P software (Di Rienzo et al., 2011) was employed in all the cases. Significance was accepted for $p < 0.05$.

3. Results and discussions

3.1. Experimental diets

According to Díaz et al. (2001) 54 percent of protein content in the diet of *P. argentinus* promotes an overall healthy condition of this species. For that reason, to the present study, the protein content of shrimp diet was adjusted to this amount (D1).

Afterwards, in order to test the protective effect of vit-E, peanut oil was added to the formulated food as source of this vitamin (D2). The resulting centesimal composition of formulated diets was for D1: 8 percent lipids, 54 percent protein, 16 percent carbohydrates, 15 percent ash and 7 percent moisture; and for D2: 17 percent lipids, 54 percent protein, 11 percent carbohydrates, 12 percent ash, and 6 percent moisture. The calculated metabolizable energy for D1 and D2 was 14.72 and 16.06 kJ g^{-1}, respectively (Lee et al., 2013). The tocopherols and tocotrienols contents in peanut oil and formulated diets are shown in Table 1. Vit-E concentration in the D2 was more than two times higher than in the D1 (16.0 and 6.8 mg%, respectively) with α-tocopherol and γ-tocopherol being the main differences between D1 and D2 vit-E composition. β-Tocotrienol and γ-tocotrienol were below detection limits in all samples. Lee and Shiau (2004) reported that the 8.5–8.9 mg vit-E percent diet is required for maximal growth and non-specific immune responses of juvenile grass shrimp *Penaeus monodon*. Barim (2009) reported that 10 mg% supplemental vit-E during gonadal development reduced the degree of tissue malondialdehyde (MDA) in Turkish crayfish *Astacus leptodactylus*.

Table 1
Tocopherols and tocotrienols content in peanut oil and formulated diets (D1 and D2). Mean ± S.D., $n = 3$. Different letters indicate mean significant differences between samples ($p < 0.05$).

Vitamin-E	Peanut oil (mg%)	Diet 1 (mg%)	Diet 2 (mg%)
α-Tocopherol	16.1 ± 0.2^{c}	6.2 ± 0.3^{a}	13.7 ± 0.7^{b}
α-Tocotrienol	0.2 ± 0.1^{a}	0.09 ± 0.01^{a}	0.07 ± 0.03^{a}
β-Tocopherol	0.36 ± 0.02^{b}	0.07 ± 0.01^{a}	0.1 ± 0.1^{a}
γ-Tocopherol	13.5 ± 0.7^{b}	0.22 ± 0.01^{a}	2.0 ± 2.0^{a}
δ-Tocopherol	0.42 ± 0.02^{b}	$0.2 \pm 0.2^{a,b}$	0.10 ± 0.06^{a}
δ-Tocotrienol	0.05 ± 0.03^{a}	0.01 ± 0.01^{a}	0.01 ± 0.01^{a}
Total	30.6 ± 0.8^{c}	6.7 ± 0.6^{a}	16.4 ± 1.2^{b}

In this study, the protein-enriched diet (D1) would provide between 68 percent and 80 percent of the daily requirement, while vit-E enriched diet (D2) exceeds this requirement by 75–85 percent. In this way two significantly different situations were able to be compared.

Success of aquaculture depends on healthy cultured stock. Artificial feed cannot meet all the elements required for the growth of aquatic organisms. Thus, supplemented artificial feed is an alternative to maintain a disease free healthy stock. Fish oil has been frequently used as a source of lipids in aquaculture feedstuff. However, the increasing cost of this oil, which limits its use, has promoted the investigation of some alternatives for many years (Tacon and Metian, 2008). According to our study, peanut oil seems an interesting alterative due to high vit-E content comparable to other plant oils already studied (Lee et al., 2013).

3.2. Effect of atrazine on P. argentinus fed with D1 and D2

3.2.1. P. argentinus survival, growth performance, and vitamin E content

The mortality registered before and after ATZ exposure varied between 2 and 3 percent, regardless the treatment. Consequently, mortality observed was assumed not to be related to the diet or pesticide exposure.

No significant differences were observed in length or weight of the shrimps among post-acclimation period, before and after exposure to ATZ for each diet or between diets at those times (Table 2). Therefore, even though diets have different metabolizable energies, it does not seem to affect the weight or size of the organisms over the time the diets were supplied. However, when tocopherols and tocotrienols were measured in *P. argentinus* the levels of vit-E in the tissues of organisms fed with the enriched diet (D2) were 1.8-fold higher than in the ones fed with D1 (Table 3). This difference could be attributed to α-tocopherol, which was the only isomer that also showed significant difference between tissues of *P. argentinus* fed with D1 and D2 and is in accordance with similar studies conducted in red hybrid tilapia *Oreochromis* sp. (Lee et al., 2013). These results indicate that the organisms fed with the enriched diet incorporated greater amount of this vitamin than that fed with D1, placing this group in a potential differential antioxidant status to deal with toxicant challenge.

3.2.2. Biomarkers of oxidative stress in P. argentinus

Pesticides may provoke oxidative stress leading to the generation of free radicals and cause lipid peroxidation as molecular mechanisms involved in pesticide-induced toxicity (Wang et al., 2013). Recent studies indicated that the toxic manifestations induced by ATZ might be associated with the enhanced production of ROS, which might provide an explanation for the multiple types of toxic responses (Wang et al., 2011). However, the information about ATZ negative effects in invertebrates is less known.

On the other hand, numerous reports are available in the literature showing protective effect of antioxidants against the pesticide-induced toxicity (Singhn et al., 2011 and authors referenced therein).

In the present investigation we studied whether ATZ promotes oxidative stress in *P. argentinus* and if vit-E has the potential to attenuate this ATZ-induced oxidative stress by different endpoints.

Fig. 1A shows no significant difference in peroxide content in control organisms of *P. argentinus* fed with D1 and D2. Nevertheless, when they were exposed to ATZ, the amount of peroxides generated by the organisms fed with D1 was 1.7-fold increased, indicating that the herbicide promotes the generation of ROS. On the other hand, this effect was not observed in organisms fed with the vit-E enriched diet. Vit-E is a potent peroxy radical scavenger that prevents the propagation of free radicals in membranes and

Table 2
Length and weight of *P. argentinus* after acclimation period, after been fed with D1 or D2 (pre-exposure to ATZ), and after exposure to ATZ. Mean ± S.D. No significant differences were observed between diets or among treatments ($p < 0.05$).

Treatment	Length (cm)		Weight (g)	
	D1	D2	D1	D2
Post-acclimation	2.8 ± 0.4	2.7 ± 0.3	0.14 ± 0.05	0.13 ± 0.05
Pre-exposure	2.5 ± 0.2	2.5 ± 0.2	0.12 ± 0.04	0.12 ± 0.03
Post-exposure	2.7 ± 0.4	2.6 ± 0.3	0.12 ± 0.05	0.10 ± 0.03

Table 3
Tocopherols and tocotrienols content in *P. argentinus* fed with D1 and D2 in mg per 100 g of fresh tissue. Mean ± S.D., $n=6$. Different letters indicate mean significant differences between samples ($p < 0.05$).

Vitamin E	*P. argentinus* fed with D1 (mg%)	*P. argentinus* fed with D2 (mg%)
α-Tocopherol	19 ± 7^a	38 ± 7^b
α-Tocotrienol	0.86 ± 0.01^a	1.0 ± 0.2^a
β-Tocopherol	0.25 ± 0.01^a	0.8 ± 0.7^a
γ-Tocopherol	3 ± 3^a	5.2 ± 0.2^a
δ-Tocopherol	1 ± 2^a	0.3 ± 0.3^a
δ-Tocotrienol	0.21 ± 0.01^a	0.3 ± 0.2^a
Total	25 ± 7^a	45 ± 5^b

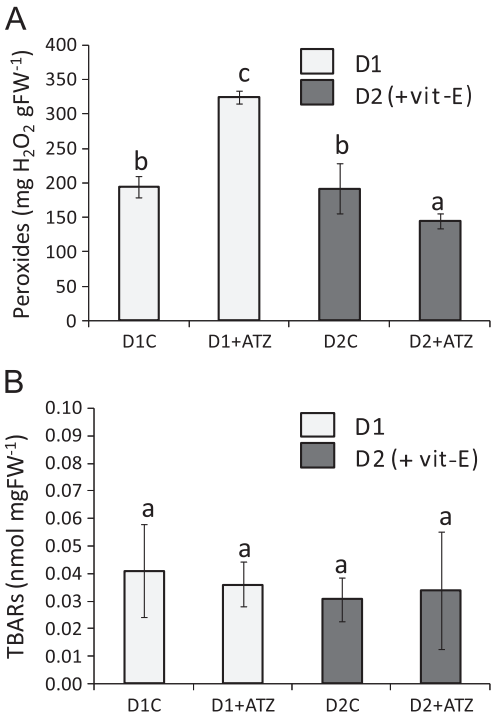

Fig. 1. (A) Peroxides and (B) TBARs content in *Palaemonetes argentinus*. D1C: control shrimps fed with diet 1; D2C: control shrimps fed with diet 2; D1+ATZ: control shrimps fed with diet 1 and exposed to atrazine; D2+ATZ: control shrimps fed with diet 2 and exposed to atrazine. Means not sharing the same letter (a, b, or c) are significantly different at $p < 0.05$.

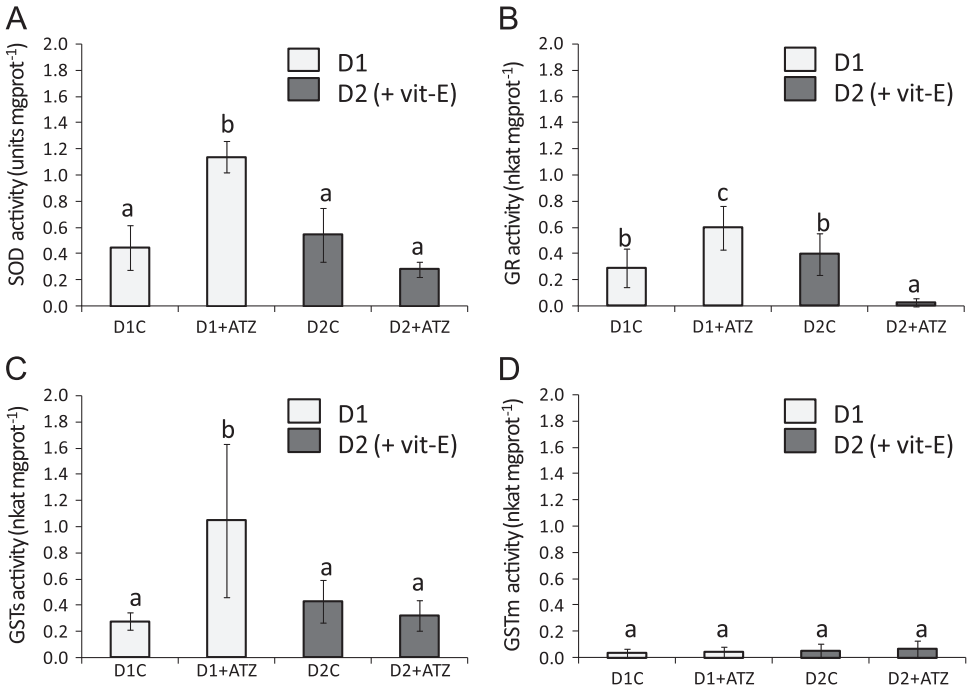

Fig. 2. (A) Superoxide dismutase, (B) glutathione reductase, (C) cytosolic glutathione-S-transferase, and (D) microsomal glutathione-S-transferase activities measured in *Palaemonetes argentinus*. D1C: control shrimps fed with diet 1; D2C: control shrimps fed with diet 2; D1+ATZ: control shrimps fed with diet 1 and exposed to atrazine; D2+ATZ: control shrimps fed with diet 2 and exposed to atrazine. Means not sharing the same letter (a, b, or c) are significantly different at $p < 0.05$.

lipoproteins. This prevention could result in lesser peroxide tissue content, since they are intermediates of lipid peroxidation (Liu et al., 2007).

A similar tendency was observed for most of the antioxidant enzymatic activities monitored (Fig. 2). The activities of SOD, GSTs and GR showed 2.5, 3.9 and 2.1-fold increase, respectively, in *P. argentinus* fed with the first diet plan after the exposure to ATZ (D1+ATZ). In contrast, the organisms fed with the second dietary plan and exposed to ATZ (D2C and D2+ATZ, respectively) did not show significant changes in SOD and GSTs activities. This result may indicate that the action of these defence enzymes was not required by the organisms against the herbicide, being enough the action of vit-E as main antioxidant.

SOD activity decreases oxidative stress by dismutation of O_2^- and provides the first line of defense against oxygen derived free radicals (McCord and Fridovich, 1969). The increase in SOD activity after ATZ administration appears to be an adaptive response to increased generation of ROS. It has been reported in the literature that exposure of animals to xenobiotics increases SOD activity in various tissues (Datta et al., 1992; John et al., 2001). The increase in the activity of SOD in our study reflects compensatory mechanism to increased oxidative stress. Vit-E, as an antioxidant, reduces the oxidative stress and hence normalizes SOD activity to some extent. Similar results have been observed in livers of male Wistar rats fed with enriched vit-E diets and exposed to ATZ (Singhn et al., 2011).

GSTs are a multi-gene family of enzymes involved in the detoxification of electrophilic compounds during phase II metabolism by conjugation with GSH. However, the α-class GSTs also can reduce peroxides of free fatty acids and phospholipids, as well as cholesterol hydroperoxides efficiently (Lushchak, 2012). The methodology used in the present study to determine GST activity includes the antioxidant action carried out by GST α-class. Thus, changes in GST activity could mean both biotransformation and antioxidant function. According to Elia et al. (2002) the detoxification reactions of ATZ in plants and mammals can be divided into a phase I reaction, with a cytochrome P450-mediated N-dealkylation, and a phase II reaction with GST catalyzed conjugation with GSH. However, in the present study, a significant activity increase was only observed for GSTs measured at D1+ATZ. This activity was attenuated to control values for D2+ATZ organisms. Thus, it is highly probable that the changes observed in GSTs activity could be more related to the antioxidant function of these enzymes than to biotransformation. The basal GSTs activity could be enough to metabolize the compound. In contrast, no significant changes were detected in GSTm activity in any of the tested conditions. Because vitamin E is lipophilic, it partitions preferentially into fat deposits, oil storage organs and in cell membranes. Of all the subcellular membrane fractions, the greatest concentrations of α-tocopherol were found in the Golgi membranes and lysosomes where it is believed that vitamin E functions as an antioxidant. The principle role of α-tocopherol is to scavenge the lipid peroxyl radical before it is able to attack the target lipid substrate producing α-tocopheroxyloxyl radicals (Wang and Quinn, 2000 and other authors referenced therein). This local antioxidant action could make unnecessarily an increased GSTm activity.

GR activity followed a similar trend that the other antioxidant enzymes, but showing a significant decrease in its activity after the exposure to ATZ in the batch of shrimps fed with D2 (Fig. 2B). It is worth mentioning here that GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced GSH using electrons from NADPH (Reed, 1986). Thus, the activity of this enzyme is directly associated with the ratio GSH/GSSG. The higher availability of vit-E as non-enzymatic antioxidant could mean a lesser oxidation of GSH and consequently, a lower need of GR activity. On the other hand, the quenching and scavenging of ROS and lipid peroxy radicals by tocopherols can result in the formation of various tocopherol oxidation compounds. These in turn, can be recycled by a multi-step pathway, where ascorbate and GSH are involved. The nature of the reductive step still remains unclear (Dixon et al., 2011), but shows that the full understanding of the redox status of glutathione in the cell is complex.

It is also possible that other components of peanut oil could contribute with the observed response and that should be the point of further studies.

Finally, there were no significant differences observed on TBARs when measured in *P. argentinus* control and exposed to ATZ, fed either with D1 or D2 (Fig. 1B). As TBARs are a by-product of lipid peroxidation, the results indicate that there was no significant progress with this reaction in the studied organisms (with or without enriched vit-E food), possibly due to the right functioning of the antioxidant system.

Similar responses to ATZ exposure have been observed in the liver of zebrafish with SOD, CAT and GPx up-regulation as well as depletion in GSH (Jin et al., 2010). The authors also reported an increase in TBARs content after ATZ exposure; however, this damage was observed after 14 days of being in contact with the herbicide.

According to Kanazawa (1985) vit-E may play a significant role in shrimp nutrition as an antioxidant. Moreover, some information is available on antioxidant role of vit-E in marine prawns (He et al., 1992; He and Lawrence, 1993). Previous studies conducted with decapoda *Chasmagnathus granulates* exposed to the cyanotoxin Microcystin-LR showed the antioxidant characteristic of vit-E (Pinho et al., 2005). After the cyanotoxin exposure, CAT activity was reduced in posterior gills of crabs supplemented with vit-E. A lower increment in GST activity was observed in organisms pre-treated with vit-E and then exposed to microcystin with respect to those exposed to the toxin but not pre-treated with the vitamin.

Moreover, in *Litopenaeus vannamei*, a significantly increased SOD, CAT, GPX and Na+/K+-ATPase activities were observed in shrimp fed diets supplemented with vit-E compared to shrimp fed the unsupplemented control diet. The results demonstrated that vit-E might have a potentially useful role as an effective antioxidant by regulating osmotic balance and resistance to salinity changes in shrimp (Liu et al., 2007).

The results obtained in the present study would indicate that a higher content of vit-E in the organism prevents the generation of peroxides with following oxidative damages which denotes its antioxidant role.

3.2.3. Energy reserves in P. argentinus

The activities of antioxidant and biotransformation enzymes observed in this study result in benefits for the shrimps, mainly due to the prevention to oxidative damage. However, the activation of this defence metabolism implies the use of additional energy to support this system. Therefore, the next step of the research is focused on the components of the organism, which would be the most suitable source for this additional energy required for defence.

The lipids content of *P. argentinus* was 1.4-fold higher when the shrimps were fed with D2 instead of D1. Considering the different lipid contents of both diets, it can be said that this result is expected. However, by exposing the organisms to ATZ a decrease (not significant for D1 but significant for D2) in the lipid content was observed (Fig. 3A). The glycogen content of *P. argentinus* when fed with D2 was 1.9-fold higher than those fed with D1. Even though the carbohydrate content was higher in the D1, this difference was not reflected in the energy storage of the organisms, indicating that the D2 induces a better nutritional condition in the organism regardless of its individual components. Subsequently, when the organisms were exposed to ATZ, there was a non-statistically significant loss of glycogen content in *P. argentinus* fed with both diets, though more marked for organisms fed with D2 (Fig. 3B).

The protein content of the shrimps fed with diets D1 and D2 was adjusted to 54 percent considering that this percentage promotes an overall healthy condition of this species (Díaz et al., 2001). Therefore both batches ingested the same quantity and quality of proteins and this is reflected in a similar protein content

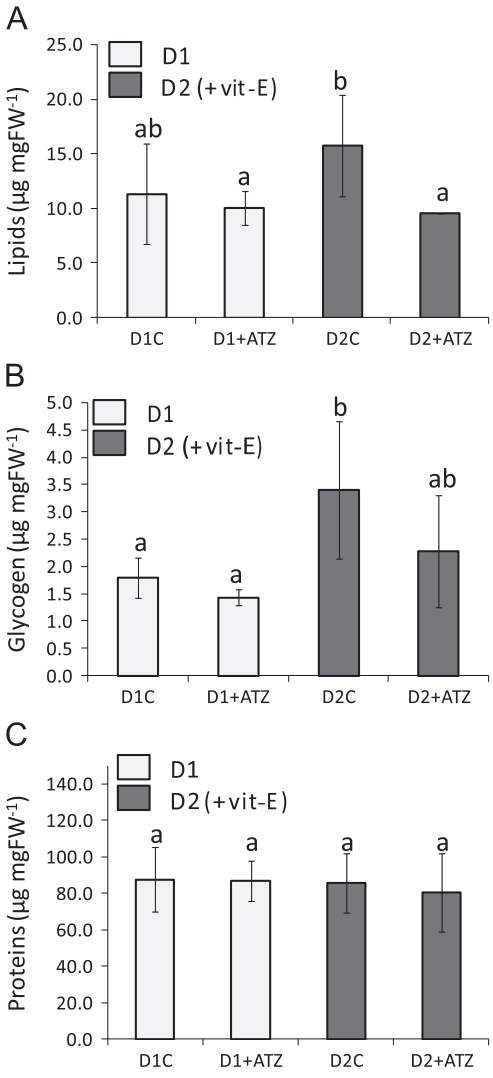

Fig. 3. (A) Lipids, (B) glycogen and (C) protein content in *Palaemonetes argentinus*. D1C: control shrimps fed with diet 1; D2C: control shrimps fed with diet 2; D1+ATZ: control shrimps fed with diet 1 and exposed to atrazine; D2+ATZ: control shrimps fed with diet 2 and exposed to atrazine. Means not sharing the same letter (a or b) are significantly different at $p < 0.05$.

of *P. argentinus* (Fig. 3C). After being exposed to ATZ no significant differences were observed in any of the two lots.

In shrimps no absolute dietary requirement for carbohydrate has been established (Tacon, 1987). To a large extent this has been due to the ability of shrimp to synthesize carbohydrates from non-carbohydrate substrates by gluconeogenesis and to the capability to satisfy their dietary energy requirements through protein and lipid catabolism alone if so required (Tacon, 1987). Thus, the higher lipid content of D2 could be the cause of higher glycogen content in shrimps fed with this dietary plan.

In the present study, the results allow suggesting that an extra consumption of reserved energy, stored as lipids, could be caused by the entrance of the toxic into the organisms, probably associated with the activation of biotransformation and antioxidants processes as it has been previously described in other species (Smolders et al., 2003; Cazenave et al., 2006).

4. Conclusions

Our findings demonstrate ATZ promotes oxidative stress in *P. argentinus* after an acute exposure as indicated by enhanced

H_2O_2 content and induction of some antioxidant enzymes like SOD, GSTs and GR. This antioxidant activity seems to be enough to prevent TBARs increment in the shrimp tissues. Nevertheless, the activation of biotransformation and antioxidants response has an energetic cost, which was compensated to the expense of the lipids storage in shrimps.

Supplementation of vit-E in diet could enhance the resistance of shrimp to acute exposure to ATZ as indicated by the reversion in the response of the biomarkers of oxidative stress measured.

Moreover, the results indicate that vit-E might have a potentially useful role as an effective antioxidant to be applied in chemoprotection strategies during aquaculture.

Conflict of interest

None.

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