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Biomarkers of oxidative damage and antioxidant defense capacity in *Caiman latirostris* blood

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ABSTRACT

Several xenobiotics, and among them pesticides, can produce oxidative stress, providing a mechanistic basis for their observed toxicity. Chronic oxidative stress induces deleterious modifications to DNA, lipids and proteins that are used as effective biomarkers to study pollutant-mediated oxidative stress. No previous report existed on the application of oxidative damage and antioxidant defense biomarkers in *Caiman latirostris* blood, while few studies reported in other crocodilians were done in organs or muscles of dead animals. The aim of this study was to characterize a new set of oxidative stress biomarkers in *C. latirostris* blood, through the modification of conventional techniques: 1) damage to lipids by thiobarbituric acid reactive substances (TBARS), 2) damage to DNA by comet assay modified with the enzymes FPG and Endo III, and 3) antioxidant defenses: catalase, superoxide dismutase and glutathione; in order to apply them in future biomonitoring studies. We successfully adapted standard procedures for CAT, SOD, GSH and TBARS determination in *C. latirostris* blood. Calibration curves for FPG and Endo III showed that the three dilutions tested were appropriate to conduct the modified comet assay for the detection of oxidized bases in *C. latirostris* erythrocytes. One hour of incubation allowed a complete repair of the damage generated. The incorporation of these biomarkers in biomonitoring studies of caiman populations exposed to xenobiotics is highly important considering that this species has recovered from a serious endangered state through the implementation of sustainable use programs in Argentina, and represents nowadays a relevant economic resource for many human communities.

1. Introduction

As a consequence of aerobic life, organisms must deal with the continuous production of reactive oxygen species (ROS: O_2-, H_2O_2, •OH), generated by products of biological molecule metabolism; the rate of ROS generation is closely related to oxygen consumption and is proportional to the amount of mitochondria in the tissue (Costantini and Verhulst, 2009). Besides, ROS are derived from other cellular activities including the autoxidation of various small molecules, the microsomal cytochromes P450 and b5, microsomal flavoprotein reductases, and superoxide leakage from the electron transport chain (Storey, 1996; Costantini and Verhulst, 2009). In addition, animals encounter exogenously generated ROS, which are either natural (e.g. UV radiation, ozone exposure) or of anthropogenic origin (e.g. pollutants; Monaghan et al., 2009).

In order to counterbalance ROS-mediated injury, endogenous antioxidant defense (AOD) systems exist and function by quenching and clearing intracellular ROS activity and accumulation, thus maintaining redox equilibrium. The enzymatic antioxidant defenses exist as a coordinated system and include superoxide dismutase (SOD) which catabolizes superoxide radicals, and catalase (CAT) and glutathione peroxidase which degrade hydrogen peroxide and hydroperoxides, respectively; while non-enzymatic antioxidants include glutathione (GSH), alpha-tocopherol (vitamin E), ascorbic acid, β-carotene, and uric acid, among others (Ziech et al., 2010).

When ROS and AOD are in balance, cells are in a dynamic redox state; typically, if ROS moderately increase, the AOD systems respond

Abbreviations: ROS, Reactive oxygen species; AOD, Antioxidant defense; OS, Oxidative stress; SOD, Superoxide dismutase; CAT, Catalase; GSH, Glutathione; MDA, Malondialdehyde; FPG, Formamidopyrimidine DNA glycosylase; Endo III, Endonuclease III; TBARS, Thiobarbituric acid reactive substances; H_2O_2, Hydrogen peroxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; TCA, Trichloroacetic acid; TBA, 2-Thiobarbituric acid; BHT, Butylated hydroxytoluene; EDTA, Ethylenediaminetetraacetic acid; CA, Comet assay; DI, Damage index; DIR, Damage index of repair.

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and reset the former oxidative balance. However, in excess, ROS can overwhelm the normal antioxidant buffering capacity of the cell, causing oxidative damage, a situation defined as *Oxidative Stress* — OS (Sies, 1993; Somogyi et al., 2007; Costantini and Verhulst, 2009). In some instances, the destructive actions of ROS are actually beneficial to an organism; for example, phagocytic cells generate bursts of ROS to kill engulfed microorganisms or other kinds of cells (Robinson, 2009; Dupré-Crochet et al., 2013); however, ROS must be rapidly eliminated to minimize their destructive nature, as all cellular components are susceptible to attack by ROS. The occurring damage to cellular macromolecules (DNA, lipids and proteins) depends on their susceptibility and will result in irreversible modifications of cellular viability and function which can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage (Pamplona, 2008; Costantini and Verhulst, 2009; Monaghan et al., 2009). Damage to lipids is of great significance, as this can have major consequences for the membrane structure and function in particular; peroxidized membranes become rigid and lose permeability and integrity. Furthermore, oxidative damage to lipids has widespread effects, as lipid peroxidation triggers a complex chain reaction involving a range of reactive intermediates that can then also cause protein and DNA damage (Hulbert et al., 2007), and both malondialdehyde (MDA) and 4-hydroxynonenal constitute the major aldehyde products of lipid peroxidation. DNA damage induced by ROS occurs by oxidative modification of the bases in nucleic acids, and regarding this, a simple modification of the widely used comet assay, incorporating the digestion of DNA with a lesion-specific endonuclease, makes it possible to measure oxidized bases with high sensitivity. Thus, formamidopyrimidine DNA glycosylase (FPG) allows to recognize oxidized purines, while Endonuclease III (Endo III) detects oxidized pyrimidines (Collins, 2009, 2014).

Oxidation of proteins changes their formation and eventually impairs their function. The magnitude of the damage will depend in part on the location of the proteins relative to the site of ROS generation, and their composition and structure (Hulbert et al., 2007). The tripeptide GSH is extremely important in the antioxidant defenses of the cell and has multiple roles: as a substrate for antioxidant enzymes, as an independent scavenger of hydroxyl and singlet oxygen, having a function in the reactivation of some enzymes inhibited under oxidizing conditions, and a role in vitamin E regeneration (Storey, 1996).

A great amount of evidence suggests that several xenobiotics, and among them pesticides, can augment ROS production that, in turn, provides a mechanistic basis for their observed toxicity, so they are referred to as pro-oxidants. These compounds are capable of interrupting normal mitochondrial oxidation by diverting the electron flow away from the electron transport chain and toward ROS generation. This property, together with their ability to act as redox agents, shifts the steady state redox equilibrium, overwhelms the intracellular antioxidant defense mechanisms and consequently results in the generation of an oxidative state. Chronic and accumulative oxidative stress induces deleterious modifications to DNA, lipid peroxidation products, and modifications in endogenous oxygen free radical scavengers, including SOD, CAT and GSH, that are used as effective biomarkers to study pollutant-mediated oxidative stress (Limón-Pacheco and Gonsebatt, 2009; Ziech et al., 2010).

No previous report existed on the application of oxidative damage or antioxidant defense biomarkers in *C. latirostris* blood and only a few reports were found in crocodilians, including *C. yacare* (Furtado Filho et al., 2007; Hermes-Lima et al., 2012) and *Alligator mississippiensis* (Gunderson et al., 2004; Lance et al., 2006), but all of them were conducted in organs or muscles, so that animals were sacrificed or found recently dead to obtain the sample. Taking into account that one of the main probed mechanisms of pesticide toxicity is the production of ROS, and considering our previous findings showing clear evidence of the genotoxic and immunotoxic effects of pesticides and pesticide mixtures on *C. latirostris* (Poletta et al., 2009, 2011; Latorre et al., 2013; López González et al., 2013; Siroski et al., in press), the possibility to include OS biomarkers in biomonitoring studies conducted routinely by our research group on caiman populations exposed to pesticides, represents a noteworthy advance, as we can obtain blood samples without causing any damage to the animals. Therefore, the aim of this study was to characterize a new set of biomarkers of oxidative stress in *C. latirostris* blood through the modification of conventional techniques: 1) damage to lipids by thiobarbituric acid reactive substances (TBARS), 2) damage to DNA by comet assay modified with the enzymes FPG and Endo III, and subsequent repair 3) antioxidant defenses: CAT, SOD and GSH; in order to apply them in future biomonitoring studies on xenobiotics effects.

2. Materials and methods

2.1. Chemicals

Hydrogen peroxide (H_2O_2), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), acridine orange and SOD Kit (19160-1KT) were from Sigma-Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), sodium chloride (NaCl), 1,1,3,3′-tetraethoxypropane, ethylenediaminetetraacetic acid (EDTA), Tris–HCl, and sodium hydroxide (NaOH) were from Cicarelli (Argentina). RPMI-1640 was from Gibco® (Life Technologies Argentina), and normal and low melting point agarose were from Invitrogen® (Life Technologies Argentina).

2.2. Blood samples

This study was evaluated and approved by the 'Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina)' (N° 05-11) and is in accordance with the 'EU Directive 2010/63/EU for animal experiments'.

Blood samples were obtained from the spinal vein of ten juvenile caimans (18 months old, five males and five females) from *Proyecto Yacaré* (Yacare Project; Gob. Santa Fe/MUPCN), with heparinized syringes. Peripheral blood was used immediately for the modified comet assay and repair assay. For the rest of the techniques, blood was centrifuged, and erythrocytes were washed with saline solution twice, then lysed with ice-cold demineralized ultrapure water (Milli-Q plus reagent grade) and stored at −20 °C until analysis. Three different dilutions of erythrocytes (1:10, 1:20, and 1:40) were tested in order to determine the most appropriate way of conducting each technique in this species.

3. Oxidative damage

3.1. Lipid peroxidation in erythrocytes (TBARS)

MDA as a marker of lipid peroxidation in red blood cells was determined by measuring the formation of the color produced during the reaction of TBA with MDA (TBARS Assay), according to a modification of the method of Buege and Aust (1978) (Simoniello et al., 2010) with some variations for this species: lysed erythrocytes dilution (50 μl) was mixed with potassium chloride buffer (0.154 M) plus protease inhibitors (100 μl; Sabatini et al., 2011) and distilled water (100 μl). Then it was thoroughly mixed with four volumes of the solution: 15% w/v TCA, 0.375% w/v TBA, 0.25 mol l^{-1} HCl acid and 4% BHT to inhibit peroxidation stimulated by Fe^{3+}, without affecting the formation of the MDA–TBA chromogen. The mixture was heated in a dry bath at 95 °C for 45 min. After cooling, the flocculent precipitate was removed by centrifugation at 6700 ×*g* for 10 min. The sample absorbance was determined at 535 nm and TBARS concentration was calculated using the extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$. To avoid interferences, iron-free tubes and deionized water were used for the assay. MDA concentration in erythrocytes is expressed as nmol/mg Hb.

3.2. Determination of oxidized bases through the FPG and Endo III-modified comet assay

The alkaline comet assay (CA) was performed as previously described by our group for *C. latirostris* erythrocytes (Poletta et al., 2008), introducing modifications in order to determine oxidized bases. As no previous data exists on the application of the modified CA with FPG or Endo III in *C. latirostris* or any other reptile species, different dilutions of the enzymes were tested and calibration curves were established for them using 25 μM H_2O_2 as an oxidative genotoxic agent. Dilutions tested for FPG were 1:120, 1:240 and 1:480 while for Endo III were 1:30, 1:300 and 1:3000, considering reference data from previous studies conducted by our group (unpublished results). So, for each animal eight slides were prepared and different treatments were applied to each of them as shown in Table 1. Exposure to 25 μM H_2O_2 was done on seven of the slides (C+) by adding 50 μl of the solution and a cover slip during 5 min at room temperature (25 °C), whereas one remained as a negative control (C−). After lysis, slides were washed three times with enzyme buffer and excess liquid was dabbed off with tissue. Then, 50 μl of FPG or Endo III enzyme solution, or enzyme buffer alone as control (Table 1) were placed on the gel, covered with a cover slip, and incubated into a moist box (to prevent desiccation) for 30 min at 37 °C.

Unwinding was carried out in alkaline buffer during 10 min and then electrophoresis was performed in the same buffer during 15 min at 0.90 V/cm. One hundred randomly selected comet images were analyzed, classified into five arbitrary classes, and a single DNA damage index (DI = $n1 + 2\,n2 + 3\,n3 + 4\,n4$) was calculated for each animal (Poletta et al., 2008). An increase in DI after incubation with the enzyme, compared with incubation with buffer alone, indicates the presence of oxidized bases. So oxidative damage to DNA was calculated by subtracting breaks with buffer from breaks with FPG or ENDO III as follows:

FPG/Endo III sites
= DI with FPG/Endo III – DI with enzyme buffer alone (Collins, 2009).

3.3. DNA damage repair assay

In order to test the time necessary for *C. latirostris* erythrocytes to repair DNA damage, we exposed them *in vitro* to 25 μM H_2O_2. Briefly, 50 μl of whole blood was diluted in 925 μl RPMI 1640 medium, and 25 μl of H_2O_2 solution (1 mM) was added to obtain a final concentration of 25 μM, and allowed to damage the DNA for 20 min at 25 °C. The reaction was quenched using 2% DMSO solution in PBS. Each sample was centrifuged at 600 ×*g*, washed again with RPMI, and then resuspended in RPMI supplemented with 10% fetal bovine serum. Samples were incubated at 29 °C for different periods to evaluate the time necessary for DNA damage to repair: 30 min, 1, 2, 4, 8 and 24 h. Immediately after incubation time, we prepared the slides following the standard procedure for comet assay application in *C. latirostris* (Poletta et al., 2008) and damage index of repair (DIR) was calculated as explained for DI.

Table 1
Different treatments applied for FPF and Endo III calibration. C− B: negative control without enzymes; C+ B: positive control without enzymes; C+ FPG 1–3: treatments with different dilutions of FPG; C+ Endo 1–3: treatments with different dilutions of Endo III.

Slides	Treatment applied
C− B	enzyme buffer
C+ B	25 μM H_2O_2 + enzyme buffer
C+ FPG 1	25 μM H_2O_2 + FPG 1:120
C+ FPG 2	25 μM H_2O_2 + FPG 1:240
C+ FPG 3	25 μM H_2O_2 + FPG 1:480
C+ Endo 1	25 μM H_2O_2 + Endo 1:30
C+ Endo 2	25 μM H_2O_2 + Endo 1:300
C+ Endo 3	25 μM H_2O_2 + Endo 1:3000

4. Antioxidant defense systems

4.1. Catalase (CAT) activity in erythrocytes

CAT activity in lysed erythrocytes was measured spectrophotometrically by monitoring the decrease in H_2O_2 concentration over time (Aebi, 1984). The specific activity of each sample was calculated on the basis that one unit of enzyme activity is defined as the activity required to degrade 1 mole of hydrogen peroxide during 60 s/g Hb, considering that the signal continues being linear over that period of time (Simoniello et al., 2010). An aliquot (10 μl) of lysed erythrocytes (1:10; 1:20 or 1:40) was diluted again in Milli Q water (1:10), and then added to 3 ml 50 mM phosphate buffer, pH 7.0, in a quartz cuvette. H_2O_2 was added to a final concentration of 54 mM and absorbance was measured at 240 nm, 25 °C during 60 s in the spectrophotometer. Results are expressed in arbitrary units as the Activity of CAT (KU/gr Hb).

4.2. Superoxide dismutase (SOD) activity in erythrocytes

SOD was determined using the commercial kit 19160-1KT (SIGMA). SOD Assay Kit-WST utilizes a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity, as an inhibition activity, can be quantified by measuring the decrease in the color development at 440 nm. The three erythrocytes dilutions were tested for the assay.

4.3. Glutathione concentration (GSH).

GSH was determined by a modification of the technique of Ellman (1961). Different dilutions of lysed erythrocytes (1:10; 1:20 and 1:40) were mixed with 5% trichloroacetic acid and centrifuged at 6700 ×*g*. Then DTNB was added to the supernatant, absorbance read at 412 nm, and GSH concentration was calculated from a standard curve and expressed as μmol mg^{-1} Hb.

5. Statistical analysis

Statistical analysis was performed using the Software SPSS 15.0 for Windows. Data were tested for normality with Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by Levene's test. Results obtained for CAT, SOD, GSH and TBARS at the different dilutions tested in each case were analyzed by Kruskal–Wallis followed by Mann–Whitney test (nonparametric data). Results from the modified comet assay with FPG and Endo III and from the DNA damage repair assay were done by one-way ANOVA followed by Dunnet test. Data from males and females were compared through T-test or Mann–Whitney test, depending on the variable analyzed.

6. Results

Different modifications made to the technique protocols allowed us to obtain good results for all biomarkers applied in *C. latirostris* blood. Table 2 shows the results of CAT, SOD, TBARS and GSH (mean ± standard deviation) obtained from each dilution of lysed erythrocytes tested. No statistically significant differences were observed among the three dilutions tested in any of the parameters analyzed ($p > 0.05$;

Table 2
Results obtained from CAT, TBARS, GSH and SOD from different dilutions of *Caiman latirostris* lysed erythrocytes tested (1:10; 1:20; 1:40).

Dilutions	CAT (KU/gr Hb)	TBARS (nmol/g Hb)	GSH μmol/mg Hb	SOD (% activity)
1: 10	591.87 ± 132.74	827.45 ± 196.54	1.66 ± 0.02	31.12 ± 7.34
1: 20	457.17 ± 219.06	769.32 ± 204.90	1.88 ± 0.6	27.68 ± 7.64
1: 40	511.53 ± 308.37	990.67 ± 248.66	1.45 ± 0.8	29.49 ± 19.46

Mann–Whitney U-Test). No differences were observed between males and females in any of the parameters analyzed ($p > 0.05$).

In the case of FPG and Endo III, calibration curves showed that the three dilutions tested in each case were appropriate to conduct the modified comet assay for the detection of oxidized bases in *C. latirostris* erythrocytes, as all of them showed an increase in DNA damage in comparison to the enzyme buffer ($p < 0.001$ ANOVA–Dunnet; Figs. 1 and 2). The FPG sites were as follows: C+ FPG1:71 ± 22.48; C+ FPG2: 52 ± 20.14; C+ FPG3: 67 ± 19.6; while Endo III sites were: C+ Endo1: 56 ± 25.0; C+ Endo2: 69 ± 23.94; C+ Endo3: 65 ± 19.20.

Fig. 3 depicts the results obtained from the DNA damage repair assay. As can be seen, H_2O_2 induced a statistically significant increase in DNA damage with respect to the basal DI (BDI; $p = 0.009$, ANOVA–Dunnet). Allowing damage to repair for different periods of time showed that, at 30 min, repair is already taking place, but it is not enough to repair all the damage generated by H_2O_2 and we still observed statistically significant differences with BDI ($p = 0.036$, ANOVA–Dunnet). All the other repair periods tested (1, 2, 4, 8 and 24 h) allowed a complete repair of the damage generated, with DI returning to values similar to the BDI ($p > 0.05$ ANOVA–Dunnet).

7. Discussion

In recent years, ecologists have taken to study antioxidants and oxidative stress in free-ranging organisms and have integrated principles of oxidative stress into several core evolutionary concepts, such as life-history trade-offs (e.g. survival vs. reproduction), senescence and sexual selection (Costantini et al., 2010). The crocodilians comprise many of the largest extant ectothermic species. As such, their success through recent geologic time is of special interest. All the studies previously made on oxidative stress biomarkers in crocodilians were applied in tissues from kidney, muscles, gonads or liver, so animals were slaughtered or samples were obtained from animals that have recently died (Gunderson et al., 2004; Lance et al., 2006; Furtado Filho et al., 2007). In our study, we successfully adapted standard procedures for CAT, GSH, SOD and TBARS to be applied in *C. latirostris* blood, without any suffering to the animals. Even though there were no statistical differences among dilutions tested for any parameter, taking into account the data dispersion we consider that the better dilution for conducting CAT and GSH is 1:10, while in the case of TBARS and SOD both 1:10 and 1:20 can be used. As it is more practical to make only one dilution of the sample before freezing it, we recommended the dilution to be 1:10, which demonstrated to be suitable for all OS determination. Because many tissues are inaccessible to sample, or sampling process could be harmful for the organism, blood is considered a substitute tissue suitable to study the effects of xenobiotic exposure, both *in vitro* and *in vivo* (Carballo and Mudry, 2006). Blood is generally easy to obtain without damage to the individual and is in constant interchange with most of the other tissues and organs of the body. Methods utilizing blood or other body fluids are favored in studies on wild species because they are less invasive and do not require terminal sampling. Considering that these techniques are going to be used as biomarkers in natural populations of caimans environmentally exposed to xenobiotic, especially pesticides, and because we need to obtain samples without any damage to the animals, this alternative is particularly interesting. Besides, all the biomarkers we applied routinely to the evaluation of these compounds are made in blood.

Nowadays, it is possible to measure all four components of oxidative stress (free radical production, antioxidant defenses, oxidative damage and repair mechanisms), but not equally easily. However, many ecological studies have only quantified a single component, usually an indicator of antioxidant capacity, as this is relatively easy, on the erroneous assumption that by itself this can provide information on levels of oxidative stress. On the contrary, the antioxidant capacity is not a simple additive function of the concentration of individual antioxidants in a sample because of the synergistic and antagonistic interactions among antioxidants (Somogyi et al., 2007). Moreover, high levels of ROS do not necessarily result in oxidative stress if this can be balanced by an up regulation of defenses; nor does it follow that individuals having

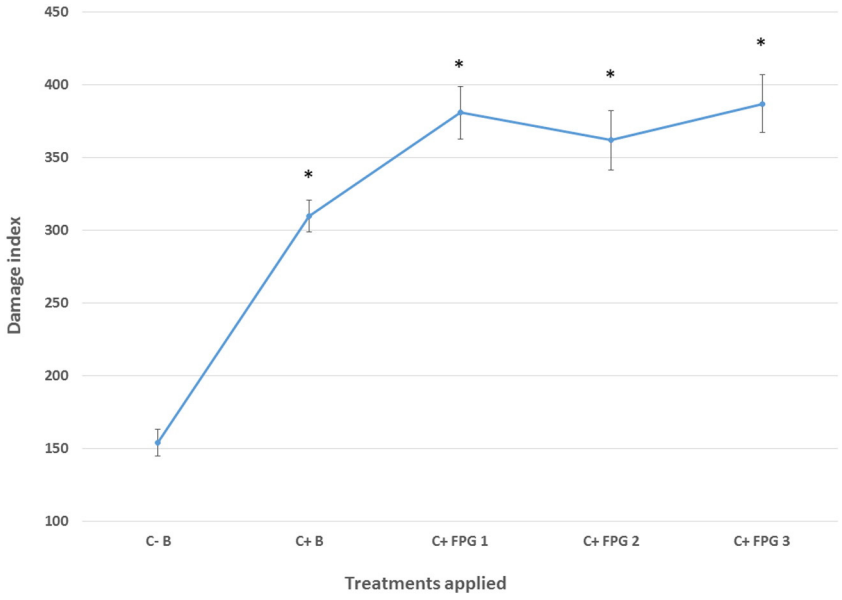

Fig. 1. Calibration curve for DNA damage obtained with formamidopyrimidine DNA glycosylase (FPG) in *Caiman latirostris* erythrocytes. Values are mean DI ± SD from ten specimens. C − B: Negative control plus enzyme buffer; C + B: exposed to H_2O_2 plus enzyme buffer; C + FPG 1: exposed to H_2O_2 plus 1:120 FPG dilution; C + FPG 2: exposed to H_2O_2 plus 1:240 FPG dilution; C + FPG 3: exposed to H_2O_2 plus 1:480 FPG dilution. *Statistically significant with respect to C − B (p ˚ 0.001, ANOVA–Dunnet).

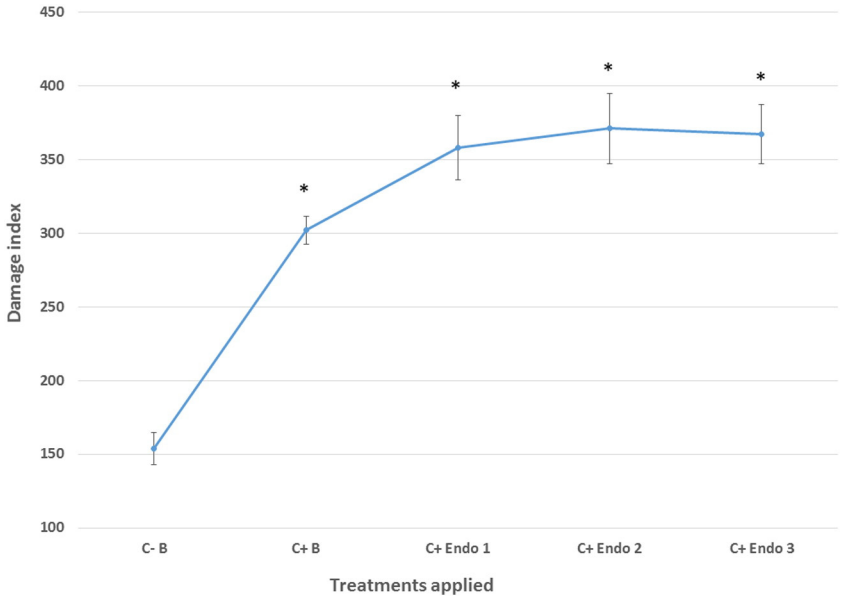

Fig. 2. Calibration curve for DNA damage obtained with Endonuclease III in *Caiman latirostris* erythrocytes. Values are mean DI ± SD from ten specimens. C − B: Negative control plus enzyme buffer; C + B: exposed to H_2O_2 plus enzyme buffer; C + Endo 1: exposed to H_2O_2 plus 1:30 Endo III dilution; C + Endo 2: exposed to H_2O_2 plus 1:300 Endo III dilution; C + Endo 3: exposed to H_2O_2 plus 1:3000 Endo III dilution. *Statistically significant with respect to C − B ($p < 0.001$, ANOVA–Dunnet).

relatively high levels of antioxidants are necessarily in a better redox state than those with lower levels, as this will depend on the level of ROS that these defenses have to deal with (Storey, 1996). Therefore, oxidative stress cannot be inferred simply by measuring just one side of the delicate balance that generally exists between ROS generation and damage limitation by the antioxidant system. In practice, researchers infer oxidative stress from measuring either damage or the presence of increased levels of ROS that suggest quenching is inadequate. In terms of the consequences for the organism, it is the outcome of oxidative stress that matters, i.e. the level of impaired function as a consequence of oxidative damage (Monaghan et al., 2009). In fact, species may greatly differ in their tolerance to stressors generating oxidative stress, so, it is suggested that a marker of antioxidant capacity should always be associated with at least a marker of oxidative damage, when the aim is to make inferences about oxidative stress (Costantini and Velhurst, 2009). In the present study, we adapted a complete battery of biomarkers of oxidative stress, including damage to lipids and DNA, enzymatic (CAT and SOD) and non-enzymatic (GSH) antioxidants and DNA damage repair, for their application in the blood of a native reptile species, which is one of the two living crocodilians in Argentina.

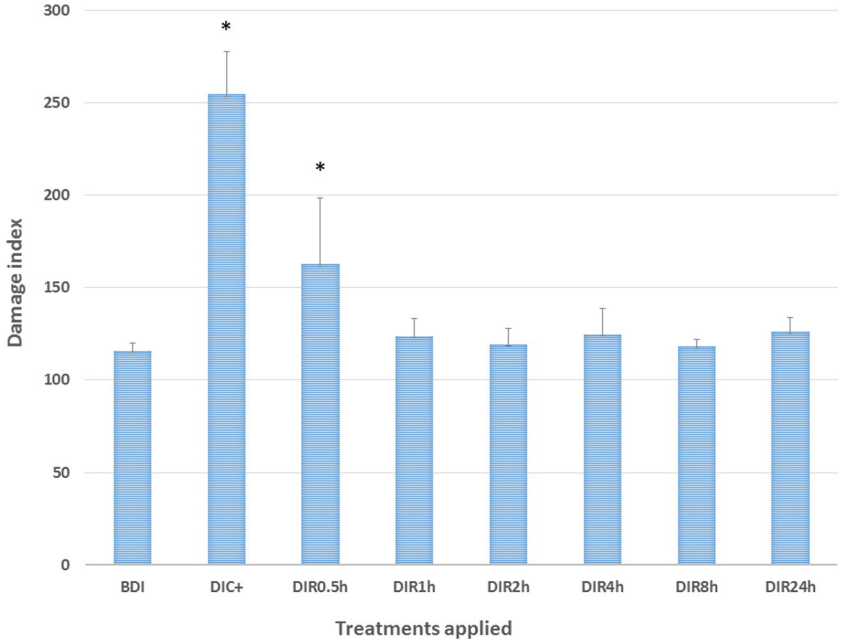

Fig. 3. Results from the DNA damage repair assay on *Caiman latirostris* erythrocytes. Values are mean DI ± SD from ten specimens. BDI: basal damage index; DIC +: damage index after *in vitro* exposure to 25 μM H_2O_2 during 20 min (positive control); DIR 0.5–24 h: damage index obtained after different periods of repair (0.5, 1, 2, 4, 8 and 24 h) of DNA damage produced by H_2O_2. *Statistically significant with respect to BDI ($p < 0.01$, ANOVA–Dunnet).

The consequent loss-of-function and structural integrity of modified bio-molecules through oxidative stress can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage (Pamplona, 2008). Lipids are one of the major targets of oxidative stress. As mentioned earlier, lipid peroxidation gives rise to a number of secondary, highly damaging products, known to further perpetuate ROS production. The two most used as biomarkers of lipid peroxidation are F2-isoprostanes and MDA. Both are important secondary decomposition products of polyunsaturated fatty acid (PUFA). Membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, while peroxidized membranes and lipid peroxidation products represent constant threats to aerobic cells (Storey, 1996; Monaghan et al., 2009). Furtado Filho et al. (2007) reported that lipid peroxidation products determined in *Caiman yacare* by three different assays were generally highest in brain, liver and kidney (in comparison with all other organs: 37–66 nmol/g wet wt.), and lower in white muscles from the tail and hind legs (5–10 nmol/g wet wt.). In eastern Pacific green turtles (*Chelonia mydas agassizii*), TBARS levels determined in different organs were between 45 and 110 nmol/g tissue, approximately. The highest level was found in hepatic tissue, being 47% higher than in the heart, 57.1% higher than in the kidney, and 64.2% above the levels found in the lung. TBARS levels in muscle were intermediate (63.7 ± 7.4 nmol/g), and not significantly different from any other tissues (Valdivia et al., 2007). In *Trachemys scripta elegans* TBARS in muscle was 9.54 ± 0.9 nmol/g, while those in the kidney and the liver were higher, with values of 20.7 ± 1.4 and 71.0 ± 14.1 nmol/g, respectively (Willmore and Storey, 1997). In the present work we determined basal lipid peroxidation by TBARS in *C. latirostris* blood, without differences between males and females. The comparison between our values and all the other studies available in reptile species is not possible, as they were conducted in organ tissues or muscle.

Available evidence suggests that long-lived species, evolved by reducing the relative abundance of those structural components that are highly susceptible to oxidative damage, but without renouncing them, thus conferring to the cellular constituents a higher structural stability and lower susceptibility to oxidative damage. In addition, long-lived species also show low rates of mitochondrial ROS generation and oxidative molecular damage. In this scenario, membrane phospholipids play a causal role in aging and longevity by modulating oxidative stress and molecular integrity (Pamplona, 2008).

GSH is probably the most active antioxidant in biological systems, and is especially effective at neutralizing the destructive hydroxyl radicals against which there is no enzymatic neutralization (Finkel and Holbrook, 2000). Higher levels of GSH can not only function as an "extra" non-enzymatic antioxidant defense, but can also increase the *in situ* activities of glutathione S-transferase (GST), Se-glutathione peroxidase (Se-GPX) and some peroxiredoxins, as GSH is a substrate of these enzymes. GSH was reported in skeletal muscle (0.240 ± 0.029 μmol/g tissue) and liver (1.4 μmol/g wet wt) of wild summer-captured juvenile *C. yacare* (Hermes-Lima et al., 2012), a caiman species that shares a wide area of the geographic distribution with broad-snouted caiman in the north of Argentina, south of Bolivia, Paraguay and Brazil. Similar values were found in muscle of control garter snakes (0.45 ± 0.04 μmol/g wet wt) but those in liver (1.02 ± 0.09 mol/g wet wt; Hermes-Lima and Storey, 1993) were three OM higher.

Activity of CAT in tissues of turtles, crocodiles and lizards varies from 70 to 240 U/mg protein in liver and kidney and from 10 to 47 U/mg protein in skeletal muscle (Hermes-Lima and Storey, 1993; Valdivia et al., 2007; Hermes-Lima et al., 2012).

In the few studies available in the literature about SOD activity in reptiles, all of them are informed in arbitrary units, and are highly variable, from 8 to 48 U/mg protein in liver and from 3 to 24 U/mg protein in the skeletal muscle of snakes and turtles (Hermes-Lima and Storey, 1993; Valdivia et al., 2007; Willmore and Storey, 1997). In the lizard *Lacerta vivipara* values reported were extremely lower in both tissues (0.0055 U/mg protein in muscle and 0.023 U/mg protein in liver; Voituron et al., 2006).

As can be seen, enzymatic and non-enzymatic antioxidant values found in tissues of different reptile species are extremely variable at inter and intra specific levels, including the tissue, the season and especial situation of the sampling. Again, we cannot make any comparison between these and our values, as we applied all biomarkers in blood, precisely to avoid animal sacrifice.

When compared with other non-mammalian vertebrates, reptile organs in general displayed high antioxidant enzyme activities and large cellular pools of GSH. Moreover, activities of antioxidant enzymes and GSH levels are mostly in the range of those found in mammals despite the much lower aerobic metabolic rate of these ectothermic animals (Storey, 1996; Hermes-Lima and Zenteno-Savín, 2002). This may be a consequence of metabolic adjustments to deal with oxidative stress during recovery in animals that repeatedly experience prolonged periods of anoxia or ischemia, as part of their strategy for survival (Storey, 1996). In accordance to this, *C. latirostris* seem to have high antioxidant enzyme activity and concentration of GSH to deal with ROS.

Biomarkers of oxidative DNA damage are of significant importance since ROS can directly attack the deoxyribosyl backbone and/or the deoxyribose residues and thus forming oxidative DNA lesions. ROS can also mediate an indirect attack to DNA primarily by reacting with other cellular components resulting in the generation of secondary reactive intermediates that irreversibly couple to DNA bases, forming DNA adducts. To this end, MDA is suggested to have both mutagenic and carcinogenic effects (Ziech et al., 2010). Recent evidence has implicated MDA as a key molecule in DNA adduct formation by interacting with DNA bases, thus forming adducts all capable of inducing DNA–DNA inter-strand cross-links and/or DNA-protein cross-links. Lipid peroxidation induced formation of DNA-protein cross-links is one of the many mechanisms by which protein oxidative damage is generated. If the adducts formed escape cellular repair mechanisms and persist, they may lead to miscoding and ultimately to mutations (Ziech et al., 2010; Azqueta et al., 2009). In its various forms, the comet assay is now an invaluable tool in genotoxicity testing and human biomonitoring as it can be used to measure not only DNA damage, but also to monitor the cellular repair of strand breaks or oxidized bases. With modifications, the comet assay has become the most popular method for measuring DNA damage of various sorts, including oxidative damage inflicted by ROS. Endo III is specific for oxidized pyrimidines, while FPG acts on oxidized purines, mainly 8-oxo-7,8-dihydroguanine (8-oxoGua). For this technique, both enzyme concentration and incubation time need to be optimized, and this is done by a series of titration experiments with different concentrations of enzyme and different times. A plateau should be reached at a certain concentration, indicating full lesion detection (Collins, 2014). Among the studies that applied the comet assay to assess genotoxicity in wild species, only a few of them use lesion-specific enzymes to detect specifically oxidized bases (Azqueta et al., 2009). It is highly noteworthy that, up to our knowledge, this is the first study to evaluate oxidative damage to DNA through the modified CA in any reptile species.

The final line of defense against the damaging effects of oxidants is the removal or repair of damaged molecules; repair of DNA is very important to cell function, and several complex damage recognition and repair pathways exist. Similarly, damaged proteins and lipids are either repaired, or destroyed and replaced, but these processes are less well understood (Halliwell and Gutteridge, 2007; Hulbert et al., 2007). Repair of damaged DNA, lipids and proteins is essential to cell function and ultimately the fitness of the organism; several methods are available to estimate repair capacity or repair enzyme activity (Monaghan et al., 2009).

Oxidative stress has also been implicated in inducing mutations that debilitate the normal functioning of a variety of repair mechanisms (base excision, transcription-coupled repair, mismatch repair, etc.) and thus allow for the loss-of-function of cell cycle control genes.

Under normal physiological conditions, base excision repair mechanisms are responsible for the repair of modified bases; however, under conditions of oxidative stress generation such repair is not favored and the resulting DNA lesions could potentially serve as biomarkers of detection. In this context, accrued amounts of unrepaired oxidative DNA lesions are directly proportional to mutagenic potential and as repair mechanisms decay with age such lesions tend to accumulate (Collins, 2009; Ziech et al., 2010).

A hypothesis exists on the fact that long-lived organisms have either better repair mechanisms or more efficient repair than short-lived organisms (Bronikowski, 2008). The relative contributions of genetic variation and environmental induction have yet to be resolved. A high intrinsic repair capacity might be expected to lead to a low steady state level of DNA damage; but on the other hand, a high repair rate might be a reflection of exposure to a high level of DNA-damaging agent (Collins, 2014; Monaghan et al., 2009).

Recent evidence has suggested that xenobiotics can augment ROS production that, in turn, provides a mechanistic basis for their observed toxicity. Their capacity to interrupt normal mitochondrial oxidation by diverting electron flow away toward ROS generation together with their ability to act as redox agents, provides for a wide variety of toxicological effects. The cyclic and continuous reduction–oxidation of one xenobiotic molecule leads to the reduction of numerous superoxide anions, which can shift the steady state redox equilibrium, overwhelm the intracellular antioxidant defense mechanisms and consequently result in the generation of an oxidative state (Ziech et al., 2010).

Life stage and metabolic rate are extremely important for oxidative stress indicators, due to the higher rate of ROS production that can accompany greater cellular activity (Hermes Lima et al., 1998; Monaghan et al., 2009). Hermes Lima et al. (2012) and Furtado Filho et al. (2007) found that the fast growth in Paraguayan juvenile caimans (with an increase of approximately 100-fold in body size compared to hatchlings) may be accompanied by an increased metabolic rate – which is normally proportional to body size in reptiles – and by a rise in oxidative stress indicators. Such changes are likely to make juveniles more suited to face environmental conditions that promote a greater generation of ROS, suggesting that a physiologically functional antioxidant protection is achieved in the juvenile stage. Embryos however, can be in a chronic state of oxidative stress, which could be caused by a mild hypoxic environment inside the eggs. Exposure to low oxygen tensions increase mitochondrial ROS formation in some biological systems, leading to oxidative stress, thus, a putative increase in ROS formation plus the low levels of hepatic antioxidant defenses would favor GSH oxidation and a rise in lipid peroxidation in embryos (Hermes Lima et al., 2012), considering them as an especially vulnerable stage under high risk. Differences in oxidative stress markers were also observed in relation to seasonal changes, being more elevated in summer, so it is necessary always to minimize seasonal and diurnal variation.

8. Conclusions

Taking into account our previous studies on the genotoxicity, inmunotoxicity and developmental effects of pesticides and pesticides mixtures on *C. latirostris* embryos and yearlings (Poletta et al., 2009, 2011; Latorre et al., 2013; López González et al., 2013; Siroski et al., in press), the incorporation of multiple response non-destructive biomarkers that includes oxidative damage, antioxidant defenses, DNA oxidative damage and repair mechanisms, is highly important considering that this species has recovered from a serious endangered state through the implementation of sustainable use programs during the last 25 years in Argentina, and represents nowadays a relevant economic resource for many human communities.

Conflict of interest

The authors declare no conflicts of interest.

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References

Aebi, H., 1984. Catalase in vitro. Method. Enzymol. 105, 121–126.
Azqueta, A., Shaposhnikov, S., Collins, A.R., 2009. DNA oxidation: investigating its key role in environmental mutagenesis with the comet assay. Mutat. Res. 674, 101–108.
Beauge, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Method. Enzymol. 52, 302–310.
Bronikowski, A.M., 2008. The evolution of aging phenotypes in snakes: a review and synthesis with new data. Age 30, 169–176.
Carballo, M.A., Mudry, M.D., 2006. Indicadores y marcadores biológicos. In: Mudry, M.D., Carballo, M.A. (Eds.), Genética Toxicológica, Cap. 4. De los Cuatro Vientos Editorial, Buenos Aires, Argentina, pp. 83–108.
Collins, A.R., 2009. Investigating oxidative DNA damage and its repair using the comet assay. Mutat. Res. 681, 24–32.
Collins, A.R., 2014. Measuring oxidative damage to DNA and its repair with the comet assay. Biochim. Biophys. Acta 1840 (2), 794–800.
Costantini, D., Verhulst, S., 2009. Does high antioxidant capacity indicate low oxidative stress? Funct. Ecol. 23, 506–509.
Costantini, D., Rowe, M., Butler, M.W., McGraw, K.J., 2010. The ecology of antioxidants and oxidative stress in animals. From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. Funct. Ecol. 24, 950–959.
Dupré-Crochet, S., Erard, M., Nüβe, O., 2013. ROS production in phagocytes: why, when, and where? J. Leukocyte Biol. 94 (4), 657–670.
Ellman, G.L., Curtney, K.D., Andrews, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetyl cholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of aging. Nature 408, 239–247.
Furtado-Filho, O.V., Polcheira, C., Machado, D.P., Mourão, G., Hermes-Lima, M., 2007. Selected oxidative stress markers in a South American crocodilian species. Comp. Biochem. Physiol. C 146 (1-2), 241–254.
Gunderson, M.P., Oberdörster, E., Guillette Jr., L.J., 2004. Phase I and II liver enzyme activities in juvenile alligators (*Alligator mississippiensis*) collected from three sites in the Kissimmee–Everglades drainage, Florida (USA). Comp. Biochem. Physiol. C 139 (1-3), 39–46.
Halliwell, B.H., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. 4th Ed. Oxford University Press, Oxford.
Hermes-Lima, M., Storey, K.B., 1993. Role of antioxidants in the tolerance of freezing and anoxia by garter snakes. Am. J. Physiol. 265, R646–R652.
Hermes-Lima, M., Zenteno-Savin, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp. Biochem. Physiol. C 133, 537–556.
Hermes-Lima, M., Storey, J.M., Storey, K.B., 1998. Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comp. Biochem. Physiol. B 120, 437–448.
Hermes-Lima, M., Carreiro, C., Moreira, D.C., Polcheira, C., Machado, D.P., Campos, É.G., 2012. Glutathione status and antioxidant enzymes in a crocodilian species from the swamps of the Brazilian Pantanal. Comp. Biochem. Phys. A 163, 189–198.
Hulbert, A.J., Pamplona, R., Buffenstein, R., Buttemer, W.A., 2007. Life and death: metabolic rate, membrane composition, and life span of animals. Physiol. Rev. 87, 1175–1213.
Lance, V.A., Horn, T.R., Elsey, R.M., de Peyster, A., 2006. Chronic incidental lead ingestion in a group of captive-reared alligators (*Alligator mississippiensis*): possible contribution to reproductive failure. Comp. Biochem. Physiol. C 142, 30–35.
Latorre, M.A., López González, E.C., Larriera, A., Poletta, G.L., Siroski, P.A., 2013. Effects of In Vivo Exposure to Roundup® on Immune System of *Caiman latirostris*. J. Immunotoxicol. 10, 349–354.
Limón-Pacheco, J., Gonsebatt, M.E., 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res. 674, 137–147.
López González, E.C., Latorre, M.A., Larriera, A., Siroski, P.A., Poletta, G.L., 2013. Induction of micronuclei in broad snouted caiman (*Caiman latirostris*) hatchlings exposed in vivo to Roundup® (glyphosate) concentrations used in agriculture. Pestic. Biochem. Physiol. 105, 131–134.
Monaghan, P., Metcalfe, N.B., Torres, R., 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol. Lett. 12, 75–92.
Pamplona, R., 2008. Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. Bioch. Biop. Acta 1777, 1249–1262.
Poletta, G.L., Larriera, A., Kleinsorge, E., Mudry, M.D., 2008. *Caiman latirostris* (broad-snouted caiman) as a sentinel organism for genotoxic monitoring: basal values determination of micronucleus and comet assay. Mutat. Res. 650, 202–209.
Poletta, G.L., Larriera, A., Kleinsorge, E., Mudry, M.D., 2009. Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test. Mutat. Res. 672, 95–102.
Poletta, G.L., Kleinsorge, E., Paonessa, A., Mudry, M.D., Larriera, A., Siroski, P.A., 2011. Genetic alterations observed in *Caiman latirostris* exposed in ovo to pesticide formulations and mixtures in an experiment simulating environmental exposure. Ecotoxicol. Environm. Saf. 74, 852–859.

Robinson, J.M., 2009. Phagocytic leukocytes and reactive oxygen species. Histochem. Cell Biol. 131 (4), 465–469.
Sabatini, S.E., Rocchetta, I., Nahabedian, D.E., Luquet, C.M., Eppis, M.R., Bianchi, L., Ríos de Molina, M.C., 2011. Oxidative stress and histological alterations produced by dietary copper in the fresh water bivalve *Diplodon chilensis*. Comp. Biochem. Physiol. C 154, 391–398.
Sies, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215, 213–219.
Simoniello, M.F., Kleinsorge, E.C., Scagnetti, J.A., Mastandrea, C., Grigolato, R.A., Paonessa, A.M., Carballo, M.A., 2010. Biomarkers of cellular reaction to pesticide exposure in a rural population. Biomarkers 15 (1), 52–60.
Siroski, P.A., Poletta, G.L., Latorre, M.A., Merchant, M.E., Ortega, H.H., Mudry, M.D., 2015. Immunotoxicity of Round Up in broad snouted caiman (*Caiman latirostris*). Chemico-Biol. Interact. (in press; august 2015).
Somogyi, A., Rosta, K., Pusztai, P., Tulassay, Z., Nagy, G., 2007. Antioxidant measurements. Physiol. Meas. 28, R41–R55.
Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715–1733.
Valdivia, P.A., Zenteno-Savín, T., Gardner, S.C., Alonso Aguirre, A., 2007. Basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*). Comp. Biochem. Physiol. C 146 (1-2), 111–117.
Voituron, Y., Servais, S., Romestaing, C., Hervé Barré, T.D., 2006. Oxidative DNA damage and antioxidant defenses in the European common lizard (*Lacerta vivipara*) in supercooled and frozen states. Cryobiol. 52, 74–82.
Willmore, W.G., Storey, K.B., 1997. Antioxidant systems and anoxia tolerance in a freshwater turtle, *Trachemys scripta elegans*. Mol. Cell. Biochem. 170, 177–185.
Ziech, D., Franco, R., Georgakilas, A.G., Georgakila, S., Malamou-Mitsi, V., Schoneveld, O., Pappa, A., Panayiotidis, M.I., 2010. The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. Chem. Biol. Inter. 188, 334–339.