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House sparrow biomarkers as lead pollution bioindicators. Evaluation of dose and exposition length on hematological and oxidative stress parameters

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ABSTRACT

House sparrows (*Passer domesticus*) have been proposed as a key ecological indicator of urban pollution. Remarkably, we lack knowledge about the physiological effects of lead on this bird species. Therefore, this study was aimed to evaluate the effect of Pb on several physiological parameters in house sparrows exposed to environmental Pb concentrations. In a first experiment, birds were exposed to Pb sub-lethal doses (from 1.3 to 14.0 μg of Pb/g animal/day) during 5 days, which resulted in a dose response increase of blood Pb levels and decrease of blood ALAD activity. However, at the higher doses tested (> 7 μg of Pb/g animal/day) the blood ALAD activity inhibition (~82%) remained constant. Hematocrit and hemoglobin were significantly reduced only at the highest-doses, and the stress indicator, heterophils to lymphocyte (H/L) ratio, did not show apparent changes.

In a second experiment, house sparrows were exposed to Pb in drinking water (12.3 ppm) during either 15 or 30 days. Pb concentration used in this study was enough to produce blood lead levels equivalents to those found recently in house sparrows inhabiting urban areas, reduced blood ALAD activity and inversion of the H/L ratio. Decreasing blood ALAD activities were correlated with increasing blood Pb levels. In addition, Pb exposure produced modification in the levels of hepatic antioxidant enzymes, increased GST activity and decreased CAT activity, without lipid peroxidation.

In conclusion, our results suggest that blood ALAD activity is a reliable and sensitive biomarker for environmental Pb exposure in house sparrows, additionally chronic exposure produce physiological stress (H/L inversion) and small changes in antioxidant enzyme activity. Finally, this specie could be considered a bioindicator for monitoring the urban Pb contamination.

1. Introduction

Lead (Pb) is a toxic element that occurs naturally and is found in small amounts in the earth's crust, but industrial activities and urbanization led to its redistribution in the environment (Levin et al., 2008). Even though, in recent times the Pb released to environments have been significantly reduced, they may remain contaminated for very long time periods because of high past emissions and the persistence of this heavy metal. Industrial and urban soils are frequently enriched in Pb (Kabata-Pendias, 2010) with levels of micrograms per gram in urban areas, while background concentrations in unpolluted soils do not exceed the tenths of micrograms per gram (dry soil) (Kabata-Pendias, 2010). In line with this observation, wildlife that live in industrialized, urbanized and intensive agricultural areas has been reported having augmented trace metal concentrations. For example, urban populations of house sparrows, starlings, and pigeons displayed higher heavy metals concentration than rural populations (Bichet et al., 2013; Kekkonen et al., 2012; Millaku et al., 2015; Nam and Lee, 2006; Swaileh and Sansur, 2006).

Bird biomarkers are useful bioindicators of pollution and very

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frequently used as such (Burger, 1995). Some of the reasons behind this choice are the broad diversity of bird species found in most biomes and the different trophic levels they may occupy according to their position within food webs, which can provide useful information about bioavailability, magnification and bio-transference of pollutants (Cid et al., 2009; Swaileh and Sansur, 2006). Birds accumulate high levels of pollutants in their tissues and are particularly susceptible to display physiological effects. Recently, certain urban birds species were suggested as bioindicators for urban heavy metals contamination (Bichet et al., 2013; Nam and Lee, 2006; Swaileh and Sansur, 2006).

The analysis of bioaccumulation of contaminants in the biotic components of ecosystems is an important and useful tool for understanding persistence, movement and allocation of these compounds. However, ecosystem and species conservation and management require the evaluation of the health risks of organisms as well, which in turn primarily compels to assess the effects of contaminants on organism physiology (Herrera-Dueñas et al., 2014). The adverse effects of Pb have been well documented in birds and other animals (Eisler, 1988). The fastest toxic effect reported after Pb exposure is an inhibition of the δ-aminolevulinic acid dehydratase (ALAD) (Finley et al., 1976), an enzyme involved in heme biosynthetic pathway. Pb exposition also can produce a decrease of blood hemoglobin, hematocrit and increased blood porphyrin levels (Eisler, 1988). Changes in ALAD enzyme activity combined with hematocrit and hemoglobin levels have been extensively used as a proxy for Pb exposure. Nevertheless, ALAD inhibition varies between species, and is influenced by the level and time of Pb exposition (Eisler, 1988). Thus, in spite of the broad and routine use of ALAD assays in numerous wildlife species, its use, especially in comparative studies, must be cautiously interpreted.

Heavy metals are considered as environmental stressors that could produce physiological stress in animals. In this sense, several studies demonstrated that stress increases the heterophils/lymphocyte (H/L) ratio, supporting the use of this hematological parameter as proxy of stress status (Davis et al., 2008). For this reason, H/L index is becoming widely reported as a complementary method for understanding the physiological stress of heavy metals exposure in birds (Grasman and Scanlon, 1995; Plautz et al., 2011). Additionally, the accumulation of δ-aminolevulinic acid (ALA) in cells produced by the inhibition of ALAD activity along with the Pb activity itself, induce the generation of reactive oxygen species (ROS) and produce cellular oxidative stress (Martinez-Haro et al., 2011). Congruously, the assessment of oxidative stress parameters is increasingly used as biomarkers of heavy metal exposure in free-living birds (Berglund et al., 2007; Espin et al., 2014; Martinez-Haro et al., 2011). However, the differences between bird species still quite poorly known (Koivula and Eeva, 2010). To our knowledge, there are not studies on the effects of each heavy metal, particularly Pb, on oxidative stress biomarkers on house sparrows and passerine birds under controlled experimental conditions.

House sparrow (*Passer domesticus*) has been proposed as a suitable bioindicator to evaluate and compare Pb pollution within and between urban zones, because it is sedentary, strongly related to urban environments and has a worldwide distribution (Swaileh and Sansur, 2006). This species have been used to evaluate bioaccumulation of heavy metals by measuring the concentrations of these metals in different biological samples (i.e., bone, liver, kidney, brain, feathers, eggs, etc.) (Bichet et al., 2013; Kekkonen et al., 2012; Millaku et al., 2015; Swaileh and Sansur, 2006). However, strikingly, information on the Pb effects on most functional traits of house sparrows is not available. Therefore, the main objective of this work was to evaluate the effect of different doses and different time of Pb exposure, on the blood lead levels, the activity of ALAD, the hematocrit, the concentration of hemoglobin, the heterophil/lymphocyte (H/L) index and oxidative stress parameters [e.g., glutathione-S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities, and thiobarbituric acid–reactive substance (TBARS)] in house sparrows.

2. Materials and methods

2.1. Animal care and housing

Adult house sparrows were live-trapped (mist net) near the Universidad Nacional de San Luis Campus (San Luis, Argentina). The birds were housed individually in cages (40 × 25 × 25 cm) in a room maintained at constant environmental conditions, temperature 23 ± 1 °C, relative humidity 40 ± 10%, photoperiod of 14:10 h light-dark cycles, and provided with water and food ad libitum (seeds supplied with vitamins and minerals). Animals were acclimated to laboratory conditions at least for eight weeks before using them in experiments.

All animal experiments and procedures were approved by the Animal Care and Use Committee (CICUA UNSL permit number B86/11) of the Universidad Nacional de San Luis, Argentina and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council U.S.).

2.2. Experimental design

2.2.1. Experiment 1: Exposure to different doses of Pb

After the acclimation period, thirty house sparrows weighing 22–27 g were selected and randomly divided in six groups of five animals each. One of these groups was randomly chosen as control group and the other five, as experimental groups. The body masses of animals were not statistically different at the beginning and end of the experiment (data not shown). Additionally, values for hematocrit, hemoglobin, ALAD enzyme activity, and H/L index before treatments were similar between groups (data not shown).

Each individual of each of the five experimental groups received a single daily dose of Pb acetate solution for five days; dose for each group was 1.3, 3.5, 5.5, 7.0, 14.0 μg of Pb/g animal/day, respectively. Pb acetate has been widely used to evaluate Pb toxicity, because it is a form of lead that is highly bioavailable (Eisler, 1988). All the administered doses were lower than those considered as sublethal for other bird species (Eisler, 1988). Control animals received an equivalent sodium acetate solution. A gavage of 270μl Pb or sodium acetate solution was administered in less than 30 s by carefully inserting a blunt-edge cannula deeply through the esophagus of the bird without anesthesia. All gavages were performed between 9:00 and 10:00 a.m. Twenty-four hours after the last administration, animals were weighed, and blood (< 10% total blood volume) was sampled by brachial vein puncture and collected in heparinized capillary tubes.

2.2.2. Experiment 2: Different time Pb exposure

In this experiment, house sparrows were Pb-exposed via drinking water supply for 15 or 30 days. Thirty-three acclimated house sparrows weighing between 22 and 26 g were randomly divided in three groups of eleven individuals each, six males and five females in each group. Negative control (without Pb) and experimental groups were setup to compare the effects of Pb exposure for 15 days (EP15) or 30 days (EP30). Drinking water of experimental groups was prepared with Pb acetate to obtain a concentration of Pb of 12.3 ppm, this concentration produced similar blood Pb levels as those reported recently in urban sparrows (see below). Drinking water of all groups (with and without Pb) and food was daily and freshly supplied ad libitum. Water intake was daily measured every morning between 08:00 and 09:00 a.m., and animals were weighed at the beginning and end of the experiment. After treatments, between 08:00 and 09:00 A.M., birds were blood sampled [< 10% of total blood volume] by brachial wing vein puncture with heparinized capillary tubes, eighteen blood samples were stored in acid-cleaned vials for Pb determination. Then, animals were anesthetized with isoflurane, the abdominal cavity was opened and liver was removed. Eighteen livers were immediately frozen in liquid nitrogen for oxidative stress determinations and fifteen livers were

stored for later histological studies. Samples were analysis. All carcasses were preserved for latter studies in a − 20 °C freezer.

2.3. Analytical procedures

The analytical procedures were as follows:

2.3.1. Hematocrit and Hemoglobin

Blood sample capillary tubes were centrifuged during 5 min at 12,000 rpm in a microhematocrit centrifuge (Cavour model VT-1224) and hematocrits were measured. Hemoglobin was assayed by the cyanmethemoglobin method using a commercial kit from Wiener lab (Wiener Laboratorios SAIC, Rosario, Argentina).

2.3.2. Heterophil/Lymphocyte Index

H/L index was determined by counting one hundred of leucocytes in each blood smear stained with May-Grünwald-Giemsa. The H/L ratio for each bird was calculated using the average of two smear counts.

2.3.3. ALAD assays

Blood ALAD activity was measured as the rate of enzymatic conversion of ALA to its product porphobilinogen (PBG) using heparinized blood according to Gómez-Ramírez et al. (2011) and Scheuhammer (1987a). The absorbance of the color of the product (PBG), that the enzyme forms with the dimethylaminobenzaldehyde, was measured at 555 nm at room temperature on a Beckman DU 64 spectrophotometer. ALAD activity was assayed immediately after blood extraction and it was expressed as μmol PBG/h/l red blood cells (RBC).

Before assays, we determined pH optima and kinetics of ALAD from blood of twelve acclimated house sparrows that had not been experimentally exposed to Pb. Assays were run with pHs ranging from 5.8 to 7.4, at intervals of 0.4 pH units. The pH optimum of ALAD ranged between 6.2 and 7, therefore the mean value, 6.6 was used. The substrate (ALA) curves were performed with different concentrations of substrate (120; 60; 30; 15; 7.5; 3.75 mM of ALA) at the optimum pH obtained. In the range of concentration used, ALAD exhibited saturable kinetics that was adequately described by the equation: relative activity = (Vmax * concentration)/ (Km + concentration). Based on the results obtained from these trials, all ALAD enzyme assays were run at pH optima (6.6) and used a substrate concentration (60 mM) that elicit Vmax.

2.3.4. Determination of Pb in blood samples

Blood samples were stored in vials previously cleaned with 1:10 (v/v) nitric acid solution (analytical grade) and rinsed with distilled water.

The whole blood samples of experiment 1 were digested through a wet protocol as follows: 0.1 g of sample was added with 150 μL of formic acid, left in an ultrasonic bath for 30′; and after that added with 150 μL of H_2O_2, and kept at 90 °C for one hour. The resulting clear solution was diluted up to 10 mL with 1% v/v nitric acid. Lead determination was carried out using an inductively coupled plasma mass spectrometer (ICP-MS) using the ^{207}Pb isotope, from Perkin-Elmer SCIEX, ELAN DRC-e (Thornhill, Canada).

For experiment 2, the whole blood samples were diluted in a matrix modifier solution (0.5% Triton X-100% and 0.2% Ammonium Dihydrogen phosphate solutions in 100 mL 0.2% concentrated Nitric Acid (Merck®, Supra-pure), homogenized, centrifuged and the supernatant were extracted. The Pb determination was performed using graphite furnace atomic absorption spectrometry (GFAAS, Varian - SpectrAA-800) with Zeeman-effect background correction. Validation was carried out on a certified standard reference material (Seronorm™ Trace Elements Whole Blood L-2). Analytical results of the certified samples were in agreement with the certified values (recoveries ranged between 97% and 102%), confirming the reliability of the method used. Results were expressed as μg/dl. All regents were of analytical grade.

2.3.5. Oxidative stress evaluation

2.3.5.1. Preparation of samples and quantitation of protein. Livers were homogenized in 30 mM phosphate buffer (pH 7.4) containing 120 mM KCl, 1% (w/v) Triton X-100 and 0.1 μM EDTA. The homogenates were centrifuged at 10,000 g for 20 min, and the supernatant extracted and further centrifuged at 105,000 g for 60 min. Once again, the supernatant was extracted, aliquoted and stored at a − 80 °C freezer until use to analyze the activity of antioxidant enzymes. All preparative steps were carried out at 0–4 °C.

Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

2.3.5.2. Determination of the activities of the antioxidant enzymes. *Glutathione Reductase* (GR) activity was measured in liver cell cytosol fraction. The reaction solution contained 0.08 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid], 0.63 mM NADPH and the sample. The addition of 3.25 mM GSSG (glutathione disulfide or oxidized glutathione) initiated the reaction. All the reagents were dissolved in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. GSH generated from the excess of GSSG reduced DTNB. The reduction of DTNB was monitored at 412 nm at room temperature. The rate of the increase in absorbance is directly proportional to the amount of GR in the sample. Enzyme activity was calculated using the extinction coefficient for DTNB ($14.15\ M^{-1}\ cm^{-1}$).

Glutathione-S-transferase activity was measured in liver cell cytosol fractions. The reaction mixture contained 0.1 M phosphate buffer (pH 7.5), 1 mM GSH (reduced glutathione), and 2 mM CDNB (1-chloro-2,4-dinitrobenzene) dissolved in DMSO (dimethyl sulfoxide) and the sample. Reaction was started by adding sample, and absorbance at 344 nm at room temperature was monitored. Enzyme activity was calculated using the extinction coefficient of the conjugated product ($9.6\ mM^{-1}\ cm^{-1}$).

Catalase (CAT) activity was measured in liver cell cytosol fractions. A reagent mixture containing 50 mM K-phosphate buffer (pH 6.5) and 3 mM H_2O_2 diluted with 50 mM phosphate buffer (pH 7) was used. Reaction was started by adding sample and the absorbance at 240 nm was monitored at room temperature. The decrease in absorbance was recorded and activity calculated using a molar extinction coefficient of $40\ M^{-1}\ cm^{-1}$.

2.3.5.3. Thiobarbituric acid–reactive substance (TBARS) Determination. Livers homogenates were used for TBARS assay, the levels of lipid peroxidation products, mainly malondialdehyde (MDA), were determined spectrophotometrically. Livers were homogenized with phosphate buffer 30 mM (pH 7.4) containing 120 mM KCl, 1% (p/v) Triton X-100 and 0.1 μM EDTA, centrifuged at 11,000 rpm 30 min. The proteins were precipitated with 20% trichloroacetic acid and 4% BHT (butylated hydroxytoluene), and placed in ice bath for 30 min. The supernatant containing MDA was incubated with a 0.7% thiobarbituric acid solution for 60 min at 100 °C. An acid hydrolysis product of 1,1,3,3-tetramethoxy propane (TMP) was used as standard. The reaction absorbance was read at 532 nm.

2.4. Data analysis and statistics

Results are given as means ± 1 S.E.M. (n = number of individuals per treatment). One or Two-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to examine the effect of Pb exposure on measured parameters (biochemical, enzyme activity, body mass and water consume). The hematological parameters of experiment 2 were analyzed using a two-way ANOVA, with the experimental groups and sex as factors. Pearson's correlation coefficients (r) and regression equations were calculated to estimate the relationship between the blood Pb levels and the measured biomarkers and the association among blood ALAD activity and oxidative stress parameters. Normality of data was checked by Shapiro–Wilk test, homoscedasticity by

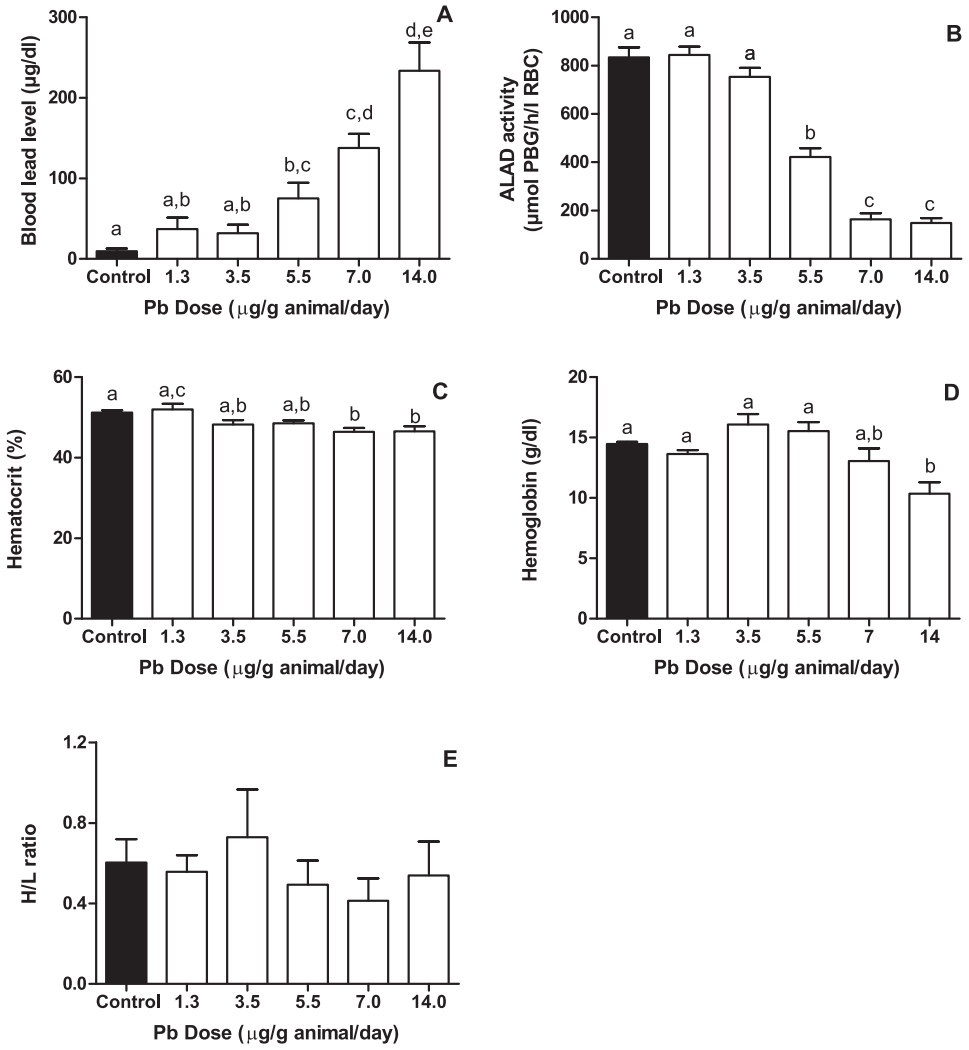

Fig. 1. Effects of different Pb doses in house sparrow on (A) blood Pb levels, (B) red blood cell ALAD activity, (C) hematocrit, (D) hemoglobin, and (E) H/L ratio. Values are expressed as mean ± SE, n = 5 animals per experimental group. Different letters indicate significant differences between groups (Tukey's test, $p < 0.05$).

Bartlett's test, and test for autocorrelation by using the Durbin-Watson statistic. Prior to comparisons, data (H/L ratio of both experiments and blood Pb levels of experiment 1) were transformed when necessary to meet these assumptions. Differences were considered statistically significant at $\alpha < 0.05$. The tests were computed using R version 3.3.1.

3. Results

3.1. Effects of different doses of Pb on house sparrow (Experiment 1)

The blood Pb levels increased with an increase of the Pb dose (Fig. 1A). Contrary the ALAD activity decreased gradually with an increase of the Pb dose by gavage between 3.5 and 7.0 µg Pb g^{-1} animal day^{-1}. Daily doses of 7.0 and 14.0 (µg Pb/g animal/day) produced greater inhibition, 82% less activity than control group ($F_{5,24} = 96.65$, $P < 0.01$; Fig. 1B). Hematocrits were significantly lower in house sparrows exposed to doses above 7.0 µg Pb g^{-1} animal day^{-1} ($F_{5,24} = 5.09$, $P < 0.01$; Fig. 1C) than controls. Hemoglobin concentration was only significantly reduced in birds exposed to the highest dose of Pb acetate (14.0 µg Pb g^{-1} animal day-1; $F_{5,24} = 7.40$, $P < 0.01$; Fig. 1D). The H/L index, a stress proxy, did not exhibit statistically significant differences among treatment and control groups ($F_{5,24} = 0.46$, $P > 0.05$; Fig. 1E), as well as, no apparent changes were detected neither in food and water consumption or body mass (data not shown).

3.2. Effects of Pb on house sparrow exposed for 15 or 30 days

In Experiment 2, where house sparrows were exposed to Pb via drinking water, blood Pb levels were significantly increased after 15 days compared to controls, though no further differences were observed between 15 and 30 days of treatment (Fig. 2A). Inhibition of ALAD activity was similar between the groups exposed for 15 and 30 days to Pb. A decrease of ~35% of the ALAD activity when compared the mean value of both treatment groups and the controls ($F_{2,30} = 11.66$, $P < 0.01$; Fig. 2B). A statistically significant negative linear relationship was found between blood Pb levels and ALAD enzyme activity (log ALAD Activity = –0.015 (± 0.003) x Blood Pb (µg/dl) + 3.038 (± 0.038), $F_{1,16} = 20{,}26$, $P < 0.001$, correlation coefficient $r = -0.75$, $r^2 = 0.56$). Hematocrit (Hct) and hemoglobin ([Hb]) were not significantly altered by Pb treatments ($P > 0.05$; Fig. 2C and D). H/L index for both, 15 and 30 days exposure time treatments, increased significantly compared to controls ($F_{2,30} = 6{,}66$, $P < 0.01$), but no differences were observed between treatment groups (Fig. 2E). The hematological parameters measured were similar between genders ($P > 0.05$). Similar to Experiment 1, no significant differences were observed in food and water consumption or body weight between control and treated animals (data not shown).

Activity of CAT after 15 days of treatment was similar to controls, but decreased in the animals exposed during 30 days to Pb ($F_{2,15} = 9.30$, $P < 0.01$; Fig. 3A). The animals of EP30 group displayed

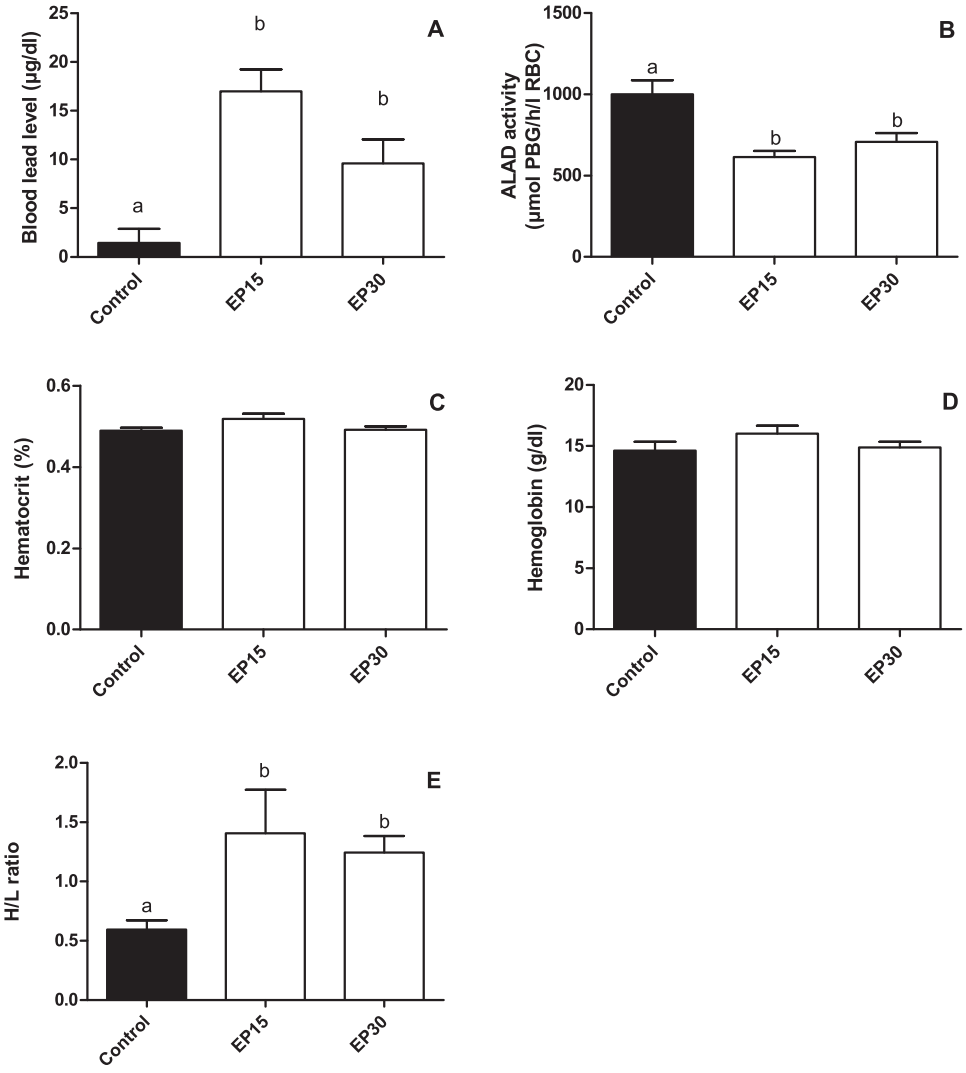

Fig. 2. Effects of different exposure period (15 and 30 days, EP15 and EP30 respectively) to the same Pb dose in house sparrow on (A) blood Pb levels, (B) red blood cell ALAD activity, (C) hematocrit, (D) hemoglobin, and (E) H/L ratio. Values are expressed as mean ± SE; n = 11 animals per experimental group, except for blood Pb levels that was n = 6 birds per group. Different letters indicate significant differences between groups (Tukey's test, $p < 0.05$).

higher GST activities than EP15 and Control groups ($F_{2,15} = 5.66$, $P < 0.05$; Fig. 3B). No significant differences were found among the treatments and control group in GR activity, although it was higher in the longest Pb-exposed group (EP30) than in shorter exposed birds ($F_{2,15} = 8.55$, $P < 0.01$; Fig. 3C). No differences in lipid peroxidation (TBARS) were found between treatment and control groups ($F_{2,15} = 0{,}017$, $P > 0.05$; Fig. 3D). No relationships were found between blood Pb levels or ALAD enzyme activity and oxidative stress parameters.

4. Discussion

4.1. Basal hematological parameters and ALAD activity

Basal hematological parameters (hematocrit and hemoglobin) analyzed in this study were similar to those previously described as baseline data for house sparrows by other authors (Puerta et al., 1995).

To our knowledge, this is the first study that report the blood ALAD values for house sparrows. The average constitutive levels of ALAD activity determined in control house sparrows (not exposed to Pb) was 834.8 (µmoles PBG/h/l RCB) in Experiment 1, and 1221.5 (µmoles PBG/h/l RCB) in Experiment 2 (Figs. 1 and 2 respectively). Control levels of ALAD activity were similar to those informed in nestlings of some birds of prey species, such as, *Hieraaetus pennatus*, *Accipiter gentilis* and *Buteo buteo* (range: 537.2–852.0 µmoles PBG/h/l RCB; (Martínez-López et al., 2004), and higher than in eagle owl (*Bubo bubo*) nestlings (155.67 ± 116.33 µmoles PBG/h/lRCB; (Gómez-Ramírez et al., 2011). However, our the constitutive levels of ALAD activities in house sparrow were also lower than the levels reported for other bird species, such as, ~3000 µmoles PBG/h/lRCB in domestic pigeons (*Streptopelia risoria*), 4737 µmol PBG/h/l RBC in a nestling of American kestrel (*Falco sparverius*), and 1505.79 µmoles PBG/h/lRCB in common quails (*Coturnix coturnix*) (McFarland, 2005; Scheuhammer, 1987a; Stone et al., 1977). The observed range of the basal ALAD activities in birds underpin a broad interspecies variation, similar to that observed in mammals (400–2370 µmol PBG/h/l RBC) and reptiles (456–2480 µmol PBG/h/l RBC) (McFarland, 2005; Scheuhammer, 1987b). The optimal pH range of blood ALAD enzymatic activity found in house sparrows was similar to that of other avian, reptile and mammal species (McFarland, 2005; Scheuhammer, 1987b).

Sex differences in blood ALAD were not apparent in house sparrows exposed and not exposed to Pb. Likewise, several authors (Finley et al., 1976; Scheuhammer, 1987a) fail to find sex differences of blood ALAD activity in other bird species (*Anas platyrhynchos*, *Aythya valisineria, Streptopelia risoria*). Although, in American kestrels (*Falco sparverius*) were found increased levels of ALAD activity in females compared to

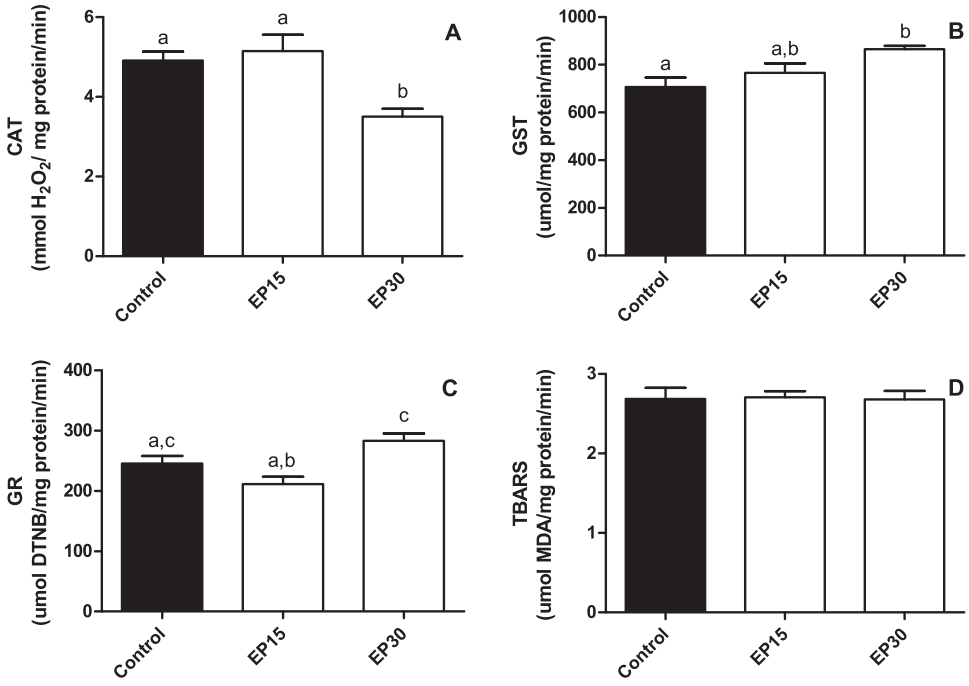

Fig. 3. Effects of different exposure period (15 and 30 days, EP15 and EP30 respectively) to the same Pb dose in house sparrow on liver enzymes activity (A.- CAT = Catalase, B.- GST = Glutathione S-transferase, C.- GR = Glutathione reductase) and lipid peroxidation (D.- TBARS = thiobarbituric acid reactive substances). Values are expressed as mean ± SE, n = 6 animals per experimental group Different letters indicate significant differences between groups (Tukey's test, $p < 0.05$).

males (Franson et al., 1983). More studies are urged to have a better understanding about this topic.

4.2. Pb Exposure - Effect on hematological parameters

Our results showed that blood Pb concentration increases similarly among treated subjects during different time-periods, regardless of the time-length of the Pb-exposure. Ghorbe et al. (2001), as well, found that chronic administration of Pb acetate during different time-periods (15, 30, 45, 60 or 90 days) induced a similar increase of Pb concentration in rat blood. Blood Pb concentration reflects a dynamic equilibrium between the rate of absorption of the heavy metal by the organism and the rate of accumulation in the tissues, because after absorption, it circulates in the blood and from there is deposited in bones and soft tissue (Ghorbe et al., 2001; Pain et al., 2009). Furthermore, the drinking water Pb acetate concentration chosen was adequate to recreate the blood Pb levels recent reported in house sparrow inhabiting different parts of the world (Bounagua et al., 2014; Chandler et al., 2004; Chapa-Vargas et al., 2010). These highlights the importance of this work, because it reflects the physiological effects under the current conditions of Pb contamination.

As mentioned previously, there are interspecific differences in the basal activity of ALAD, however despite these differences, it is always possible to compare the relative degree of inhibition of the enzyme activity when individuals (of any species and taxonomic group) are exposed to Pb (Buekers et al., 2009). Like most studies, we observed a negative relationship between blood Pb concentration and ALAD enzyme activity (Martinez-Haro et al., 2011; Scheuhammer, 1987a). The use of the ALAD enzyme as an useful and sensible biomarker of Pb exposure is supported by numerous studies in birds and other animals that show that even low doses diminish the activity of this enzyme (Beyer et al., 2000; Hoffman et al., 1981). In house sparrow, the greater blood ALAD inhibition was ~82%, that stabilized between the highest doses (≥ 7.0 µg Pb g^{-1} animal day^{-1}). However, in other bird species [mallards (*Anas platyryhnchos*) and Canada geese (*Branta canadensis*)] exposed to Pb were found that ALAD activity decreased more than in house sparrow, reaching values as high as 90–97% inhibition (Beyer et al., 2000; Hoffman et al., 2000a, 2000b; Mateo and Hoffman, 2001). Although, those birds had lower or similar blood Pb levels (0.68 − 2.56 ppm) than those observed in house sparrows exposed to the highest Pb dose. The differences observed in ALAD inhibition could be related with the different life stages of birds, because mallards and Canada geese were juveniles and house sparrows were adults.

In mammals and birds it has been observed that a severe and prolonged inhibition of ALAD can cause anemia, with considerable reduction of the hematocrit and hemoglobin concentration (Eisler, 1988). However, the observed effects have varied substantially between species depending on the degree and time of exposure. In our work, the sparrows exposed to the maximum dose of Pb (14.0 µg Pb g^{-1} animal day^{-1}) for 5 days had a significant increase of blood Pb levels (mean 233.5 µg/dl) with a reduction (82%) of the enzymatic activity of ALAD and associated to a significant reduction in the hematocrit and hemoglobin concentration. Hoffman et al. (1981) reported in *Haliaeetus leucocephalus* after 24hs of lead shot ingestion, a similar ALAD activity reduction (80%) also associated to a significant reduction in the hematocrit and hemoglobin, although the mean blood Pb levels (80 µg/dl) were lower than the observed in our study. This finding was not apparent in the case of *Falco sparverius* individuals, in which, high blood Pb levels (0.37–33.00 ppm, mean 3.94 ppm) and 80% inhibition of ALAD was observed after an exposure of Pb (54 ppm) in the diet for at least 5 months, but no adverse effects on hematocrit and hemoglobin (Franson et al., 1983). Hoffman et al. (2000a), (2000b) also fail to find a unique association between a decrease in ALAD activity and hemoglobin and hematocrit when they exposed mallards and Canada geese to the same Pb-contaminated sediment in diet (828 µg/g). Both species demonstrated a high ALAD activity depression (over 90%), but only Canada geese exhibited a significant reduction of mean values of hemoglobin and hematocrit, although this species had lower blood Pb levles (1.61 ppm) than those (2.56 ppm) observed in mallards.

Several reports in different species have shown that stress increases the H/L index by increasing the number of heterophils and/or diminishing the number of lymphocytes in circulating blood, therefore an increasing number of laboratory and wildlife studies are using it use as a proxy to detect stress events. (Davis et al., 2008). In our study, H/L index was not significantly different in house sparrows exposed for a short period of time (5 days; Experiment 1) versus controls, despite the fact that a dose-response was observed that produced high levels of blood Pb. However, when animals were exposed to Pb via drinking water for longer periods (15 and 30 days: Experiment 2) H/L index increased significantly (from 0.6 to 1.4). These results were unexpected,

because the blood Pb levels found in animals chronically exposed were much lower than those found in birds acute exposed. Thus, the acute and chronic Pb exposure produce different H/L index response. A possible explanation of the difference between short and long term exposures, that remains to be tested in the future, is that intermittent Pb exposure (one pulse per day) during five days may be a too short exposure time for the immune system to fully react at those concentrations, while chronic exposure via drinking water is a steady stress condition. In comparison to studies, in Mourning doves (*Zenaida macroura*) that had high blood Pb levels (7.4 ppm) after 4 days of shot lead ingestion, an increase of H/L ratio (from 0.35 to 0.47) was observed (Plautz et al., 2011), which represent a continuously high Pb exposure. It is interesting to note that long-term exposure to low Pb concentration in house sparrows produced a greater effect on H/L ratio than the observed in mourning doves, although the sparrows had lower blood Pb levels than those found in this doves. Similar to our results, no sex differences were found in this study (Plautz et al., 2011). However, Japanese quails (*Coturnix coturnix*) exposed to Pb showed that H/L ratio depends on the type of administered diet; a trend of elevated H/L indexes in Pb-dosed groups fed with corn compared to quails fed poultry-feed was observed (Grasman and Scanlon, 1995). Apparently, H/L index variation pattern in birds exposed to Pb is influenced by several variable, such as, exposure time, doses and diet composition.

4.3. Pb exposure - Oxidative stress

In the present study, we found that a long Pb exposures produced changes in antioxidant enzymes activity, but not lipid peroxidation. Several studies documented Pb-induced changes of oxidative stress parameters in birds, although as previously analyzed variables, species specificity, types and levels of pollution and experimental designs greatly influence the variation (Koivula and Eeva, 2010).

Experiment 2 tested the increment of liver GST enzyme activity by Pb. In this study, only the longest Pb exposure to a constant dosing produced an apparent effect on GST compared to the control group. This result may suggest that house sparrows have some defense against ROS provided by a constitutive level of the enzyme activity (spare capacity) and that beyond certain ROS concentrations a protective increase of GST response is triggered. (Fig. 3). Chick embryos exposed to Pb acetate during development also exhibited an increase of the GST activity in liver (Somashekaraiah et al., 1992). However, the liver GST activity did not exhibit apparent changes in young (first 6 wk) of two species of waterfowl (Canada geese and mallards) exposed to Pb (1.7, 414 and 828 μg/g of diet) (Mateo and Hoffman, 2001).

As for CAT, another liver enzyme related to oxidative stress, we observed in house sparrows after 30 days exposed to Pb (Experiment 2), a significant decrease of the activity. An explanation for this observation is that the structure of CAT have heme/iron groups used to participate in the reaction that this enzyme catalyze, and as previously described (see Introduction), heme biosynthesis is affected by Pb (Gurer and Ercal, 2000). Our results confirmed those found in Eurasian Eagle Owl chicks (*Bubo bubo*) living in different scenarios of heavy metal exposure, where the blood CAT activity was inversely related with blood Pb concentrations and other single metals (Cd and Cu) (Espin et al., 2014). On the contrary, exposure to Pb did not change the CAT activities in Japanese quail (*Coturnix coturnix japonica*) (Paskova et al., 2011). A different effect of Pb on CAT was reported by Berglund et al. (2007); they found that the activity of this enzyme was positively related to Fe concentration itself or combined to Pb liver levels in pied flycatcher (*Ficedula hypoleuca*) nestlings dwelling metal contaminated environments in northern Sweden. In mammals, the effect of Pb on CAT activity are in both directions, upmodulation (Pande and Flora, 2002) and downmodulation (Sandhir and Gill, 1995).

The GR enzyme did not show significant differences between the Pb-exposed and unexposed in this study (Fig. 3). Likewise, no differences in GR activity was reported in mallards and Canada geese exposed to Pb (Hoffman et al., 2000a, 2000b; Mateo et al., 2003; Mateo and Hoffman, 2001). However, GR activity was reduced in liver of chick embryos during development exposed to Pb acetate (Somashekaraiah et al., 1992). Rats exposed to Pb also was displayed an inhibition of liver GR (Sandhir and Gill, 1995). It has been postulated that Pb can interact with the disulfide group of the active site of this enzyme, blocking its activity (Gurer and Ercal, 2000).

The modification in TBARS levels has been used as a parameter of damage to membrane lipids. Pb concentration in drinking water in Experiment 2 did not show an effect on lipid peroxidation of liver. Two no exclusive explanation may be posed, our Pb-dosing was insufficient to produce lipid peroxidation and/or that the antioxidant capacity of these animals was enough to keep TBARS levels constant. In concordance with our findings, no lipid peroxidation was observed in livers of Japanese quail males (*Coturnix coturnix japonica*) exposed during 10 days to six ammunition lead shots Pb (total 1.5 g) (Osičková et al., 2014). Opposite results to previous are reported as well, a significant increase of lipid peroxidation was detected in liver of Canada geese and mallards juveniles and nestlings exposed to Pb contaminated sediments (414 and 828 μg/g of diet) (Hoffman et al., 2000b; Mateo et al., 2003; Mateo and Hoffman, 2001). Strikingly, an increase of lipid peroxidation was observed in livers of mallard ducklings exposed to Pb-contaminated sediment (828 μg/g of diet), but in the same experiment no differences in TBARS were observed between control group and ducklings feed with clean sediment containing Pb acetate equivalent to the Pb in the sediment diet (Hoffman et al., 2000a). In this sense, we used a much lower concentration in drinking water (12.4 ppm), so perhaps we did not observe lipid peroxidation in sparrows. Although, as mentioned, different responses of lipid peroxidation were found in birds exposed to the same dose of Pb, or this parameter did not vary in quails with high exposure to Pb (six ammunitions). Thus, as for previous analyzed parameters affected by Pb, oxidative stress enzymes analyzed and lipid peroxidation depends on a large matrix of interacting factors, as well.

5. Conclusions

In conclusion, we document effects of Pb exposure on a variety of common physiology traits in an urban wild species, the house sparrow. The blood ALAD enzyme activity was inversely related to Pb dosing and blood Pb levels. Hemoglobin and hematocrit levels were relatively stable, only small changes were observed in these hematological parameters with the highest Pb doses together with greater ALAD inhibition. The heterophil/lymphocyte index was not Pb dose dependent, when the exposure time was short, but was sensitive to chronic exposure to low environmental Pb concentration. We found a modification in the levels of antioxidant enzyme in liver, increased GST activity and decreased CAT activity, without lipid peroxidation. Based on our results and bibliographic revision, we can conclude that house sparrows are an excellent bioindicator for urban Pb pollution and that sensible biomarkers to use are blood ALAD enzyme activity.

Finally, to evaluating these parameters in wild birds is important to consider that ecological resource and animal conditions (nutritional requirements, immunological state, and reproductive stage among others parameters) have temporal and spatial changes, as well as the fact that animals are not exposed only to Pb but to a complex mixture of pollutants, which can also vary.

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