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

Fabricio D. Cida,b,c,⁎, Noelia C. Fernándeza, María V. Pérez-Chacac,e, Rafael Parod, Enrique Caviedes-Vidal a,b,c,f, Juan G. Chediacka,b,c

a Laboratory of Biology “Prof. E. Caviedes Codella”, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de Los Andes 950, 5700 San Luis, Argentina
b Laboratory of Integrative Biology, Instituto Multidisciplinario de Investigaciones Biológicas de San Luis (IMIBIO-SL), Centro Científico Tecnológico San Luis, Consejo Nacional de Investigaciones Científicas y Técnicas, San Luis, Argentina
c Department of Biochemistry and Biological Sciences, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis, Argentina
d Department of Analytical Chemistry, Facultad de Ciencias, Universidad de Valladolid, Valladolid, Spain
f Laboratory of Morphophysiology, IMIBIO-SL, CONICET, Argentina

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
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 exposure 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 exposure (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 Pb exposure (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 heterophils/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.
stored for later histological studies. Samples were analysis. All carcasses were preserved for later 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-Grünewald-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 Gomez-Ramirez 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/1 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 7.4, 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 elicited 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 min; and after that added with 150 µL of H2O2, and kept at 90 °C for one hour. The resulting clear supernatant were 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. 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% (v/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. All regents were of analytical grade. 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
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 Pb doses 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 = $-0.015 \pm 0.003 \times$ Blood Pb (μg/dl) + 3.038 $(\pm 0.038)$, $F_{1,16}= 20.26$, $P < 0.001$, 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 ($F_{2,30} = 11.66$, $P < 0.01$), 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...
higher GST activities than EP15 and Control groups \( (F_{2,15} = 5.66, P < 0.05; \text{Fig. 3B}) \). 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 \( (F_{2,15} = 8.55, P < 0.01; \text{Fig. 3C}) \). No differences in lipid peroxidation (TBARS) were found between treatment and control groups \( (F_{2,15} = 0.017, P > 0.05; \text{Fig. 3D}) \). 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 kestrals (Falco sparverius) were found increased levels of ALAD activity in females compared to

![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).](image-url)
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; Chapavargas 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 platyrhynchos) 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 24h 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 levels (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 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 study. 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 index 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 (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 index 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.

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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