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Lead exposure affects health indices in free-ranging ducks in Argentina

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Abstract Numerous experiments under controlled conditions and extensive investigation of waterfowl die-offs have demonstrated that exposure to lead from spent gunshot is highly detrimental to the health of waterfowl. However, few studies have focused on examining the more subtle sub-lethal effects of lead toxicity on ducks in non-experimental settings. In our study, the health of ducks exposed to varying amounts of lead under natural conditions was assessed by correlating individual lead exposure with relevant indices of health. Based on hunter-killed wild ducks in Argentina, we measured spleen mass, body condition, examined bone marrow smears, and determined Ca and P in bone tissue. In free-ranging live-trapped ducks we determined basic hematology and aminolevulinic acid dehydratase activity. Using multivariate analyses, we found that, when controlling for the potential confounding effect of site type, year, duck species, body mass and age, lead levels in the liver were negatively associated with body condition and spleen mass. Spleen mass was also lower in ducks with higher lead levels in their bones. In live ducks, high blood lead levels were associated with low packed cell volume and red cell morphologic abnormalities. These findings suggest that, despite the lack of recorded lead-induced mortality in the region, lead exposure results in less conspicuous but still significant impacts on the health of ducks, which could have serious implications for their conservation. Moreover, this evidence further supports the need for urgently banning lead shot in the region.

Keywords Argentina · Effects · Health · Hunting · Lead shot · Waterfowl · Wetlands

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Introduction

Historically, lead poisoning in waterfowl has been associated with the absorption of damaging levels of lead into body tissues from ingested spent shot pellets (Friend et al. 2009). Lead exposure has long been proven to cause physiological, neurological, immunological and behavioral problems affecting waterfowl fitness and survival (Bates et al. 1968; Rocke and Samuel 1991; Flint and Grand 1997; Eisler 2000; Franson and Pain 2011). However, most of this information stems from experimental studies under controlled conditions and extensive investigation of

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waterfowl mortality (Bono and Braca 1973; Clemens et al. 1975; Zwank et al.1985; Mateo et al. 1997).

Even though the sublethal effects of lead toxicity on the health of wild birds have been demonstrated (e.g. body weight loss, anaemia) (Finley et al. 1976; Pain and Rattner 1988; Pain 1989; Hohman et al. 1990; Franson and Pain 2011) extremely little is known about these more subtle impacts under natural conditions (Pokras and Kneeland 2009).

Clinical signs and the outcome of lead poisoning vary depending on the degree (temporal and quantity) of exposure (Friend 1999; De Francisco et al. 2003; Gasparik et al. 2012). While lead toxicity is obvious when it results in death, less conspicuous adverse effects can be harder to identify and quantify (Friend 1999). Decreased food consumption, progressive loss of body mass (the keel becomes prominent), bright green droppings, weakness, lethargy, ataxia, convulsions, paralysis of legs or wings, and inability to fly, swim or dive are common observations in acutely poisoned waterfowl (Lumeij 1985; Friend 1999; De Francisco et al. 2003). Necropsy findings in intoxicated birds include emaciation, absence of fat, impaction of esophagus or proventriculus, prominent gallbladder, bright green staining of the vent area, and presence of lead shot in the gastrointestinal tract (Locke and Thomas 1996; Friend 1999). The effects of lead poisoning can also be detected by changes in blood characteristics in live animals. The inhibition of delta-aminolevulinic acid dehydratase (ALAD) enzyme activity (Finley et al. 1976; Scheuhammer 1987; Pain 1989), results in impaired heme synthesis, and consequently, anemia. Abnormalities in red cell morphology like hypocromasia (pallor indicating a lack of hemoglobin), anisocytosis (unequal size), poikilocytosis (abnormally shaped), polychromasia (variation in red cell coloration), decreased packed cell volume (PCV), and a high number of immature red blood cells can also be associated with lead-induced anemia (Bates et al. 1968; Bono and Braca 1973; O'halloran et al. 2008; Campbell 2012). In addition, developmental defects in the erythroid line in bone marrow were reported in experimental studies in ducks (Bates et al. 1968).

Likewise, lead exposure causes significant alterations in bone mineralization (Smits et al. 2005; Gangoso et al. 2009; Álvarez Lloret et al. 2014). Calcium (Ca) and phosphorus (P) are the mineral constituents of bones, and together with organic components, they provide rigidity, elasticity and resistance to bone tissue (Turner 2002; García-Garduño and Reyes-Gasga 2006). Lead can directly compete with Ca in bones by modifying the activity of osteoblasts and osteoclasts, or indirectly by altering renal function and vitamin D metabolism (Pounds et al.1991; Smits et al. 2007; Álvarez Lloret et al. 2014). This may result in an altered mineral composition, reflected by a lower Ca/P ratio, and affecting bone characteristics (Pounds and Rosen 1986).

Furthermore, immunosuppressive effects have been linked to lead exposure (Faith et al. 1979; Grasman and Scanlon 1995; Fair and Myers 2002), and might be an additional factor in reducing bird survival due to impaired resistance to infection. An experimental study in ducks exposed to lead showed a decline in white blood cells and spleen mass (Bates et al. 1968; Rocke and Samuel 1991).

For decades, lead shot ingestion was an important cause of waterfowl mortality in the United States and Europe (U.S. Fish & Wildlife Service 1990; Mateo 2009). Prior to lead shot banning, several tons of spent lead shot discarded in heavily hunted areas resulted in large quantities of lead ingested and accumulated in waterbird tissues (Franson and Pain 2011).

In South America, despite the massive use of lead ammunition, there are no confirmed reports of waterfowl mortality due to lead poisoning. Waterfowl hunting practices in Argentina over the past twenty years have been characterized by high and concentrated intensity and the exclusive use of lead ammunition (Zaccagnini 2002). As expected, sound evidence of exposure to lead in several duck species in these hunting hotspots has been documented (Ferreyra et al. 2009, 2014), yet mortality events have not been recorded. However, the probability of failure to detect carcasses is very large, as poisoned ducks seek refuge before dying (Friend 1999).

This scenario provides a unique opportunity to assess the effects of chronic exposure to lead from spent ammunition in free-ranging waterfowl under natural conditions. Therefore, the main objective of the current study was to assess changes in generic health indices and other relevant variables of wild ducks from hunting hotspots in Argentina following cumulative lead intake from spent shot.

Materials and methods

Study area

This study took place in natural wetlands of Santa Fe and Corrientes provinces, where duck hunting is permitted for 3–4 months each year (May to July/August). This region is an important waterfowl wintering area along a main migratory route in Argentina, the Paraná River flyway (Capllonch et al. 2008). The study area is a mosaic of wetlands and rice fields which attract at least 25 species of ducks (Blanco et al. 2006; Lesterhuis 2011).

The selected study sites were separated by 40–80 km, one on the west margin of the Paraná river (Santa Fe), the other on the east side (Corrientes) (Fig. 1). In 2011, ducks were sampled in Santa Fe Province. In 2012, high temperatures and drought delayed duck migration to Santa Fe

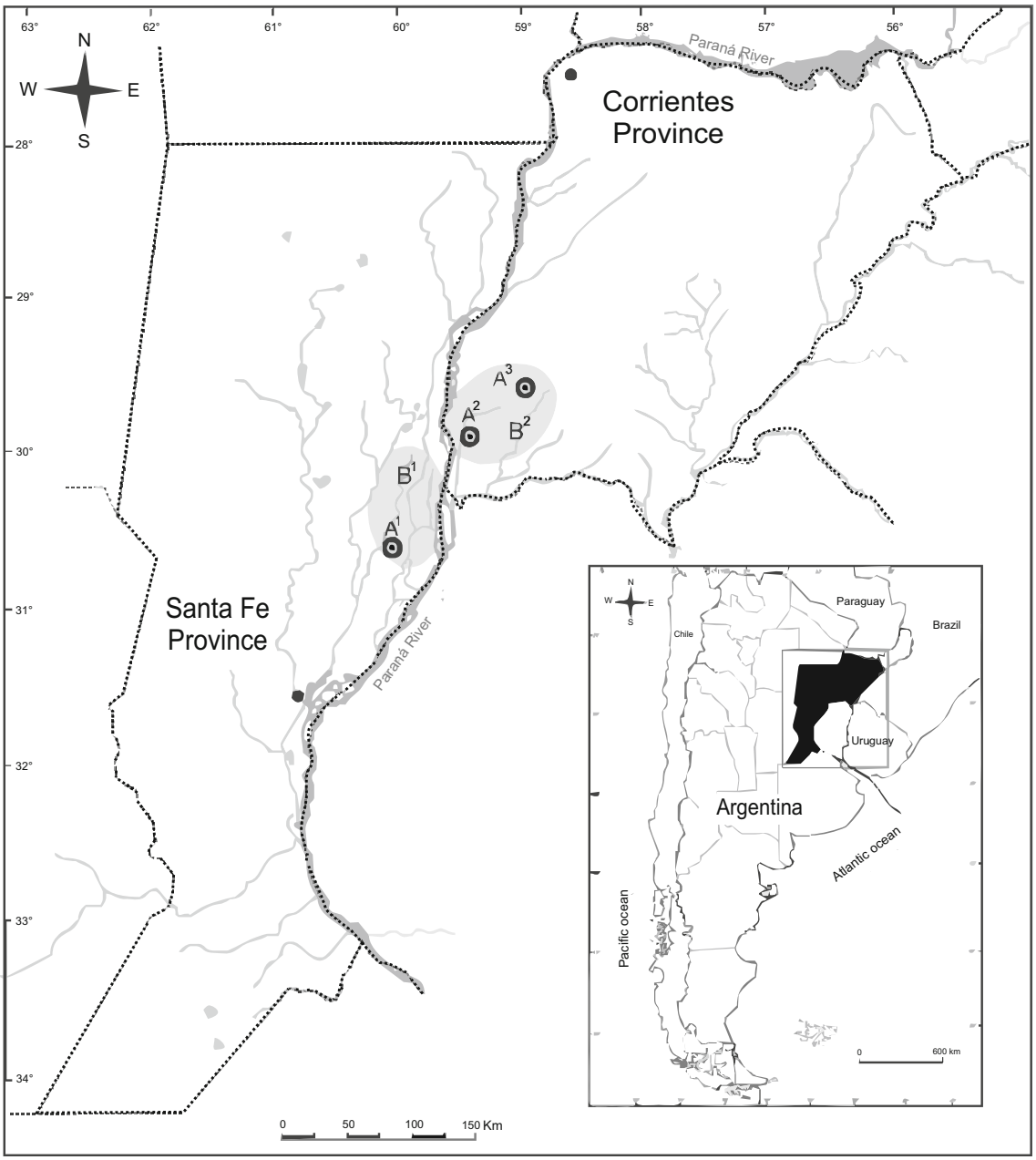

Fig. 1 Map of Argentina showing our study sites in Corrientes and Santa Fe provinces (in *black*, map *insert*). Live duck sampling via corral traps took place at locations A^1, A^2 and A^3. Hunter-killed ducks were received from hunting areas B^1 and B^2

diverting our sampling efforts to the neighboring Corrientes Province where waterfowl were available. Within each site, samples were collected from two site types, some from the islands that are part of the Paraná River system, and others from landlocked water bodies (lagoons, marshes, artificial reservoirs).

Samples from hunter-killed ducks

During the hunting seasons of 2011 and 2012, we opportunistically necropsied and sampled 415 ducks, including 103 whistling ducks (*Dendrocygna bicolor*), 134 white-faced tree ducks (*Dendrocygna viduata*), 103 rosy-billed pochards (*Netta peposaca*), 57 Brazilian ducks (*Amazonetta brasiliensis*), and 18 black-bellied whistling ducks (*Dendrocygna autumnalis*) donated by licensed hunters. For each individual, body mass, sex, age class (juvenile or adult), were recorded, and the spleens were weighed (Digital scale Ohaus Pioneer, precision: 0.001 g). Body condition was estimated by measuring the angle of the keel, as in flying birds it is a sensitive and objective measure of investment in skeletal muscle (Nyeland et al.

2003). Reduced keel angles are one of the most frequently reported findings in waterfowl mortality due to lead (Friend 1999). Also, 187 bone marrow smears were performed. The whole gastrointestinal tract was collected to assess lead pellet ingestion, and 225 bone samples were collected for Ca/P ratio determination. Samples of liver and bone were collected and snap frozen immediately in liquid nitrogen for lead measurement.

Samples from live-captured ducks

Samples were collected from 96 live ducks, 24 in Santa Fe (May 2011) and 73 in Corrientes (June, July and August 2012). These included six Brazilian ducks, 17 black-bellied whistling-ducks, one whistling duck, 68 white-faced tree ducks, and four rosy-billed pochards. The capture protocol used was approved by the Institutional Animal Care Use Committee (IACUC) of the Wildlife Conservation Society. Birds were captured using corral traps, baited with corn at night and checked for capture during the morning.

Trapped ducks were handled with care and held in clean cotton fabric bags until processed within an average of 99 min (range 20–220). Once ducks were measured and sampled, they were identified with a temporary leg band and immediately released at the site of capture. No injuries resulted from trapping and handling procedures.

From each live animal we recorded body mass, age class (juvenile or adult), sex (where possible), and keel angle (as described above). Blood samples (between 2.5 and 4 ml) were collected by venipuncture of the basilic or superficial plantar metatarsal vein, with 3–5 ml plastic syringes and 25 G heparinized needles. Blood samples were kept cool following extraction and then a fraction was preserved frozen in liquid nitrogen (2 to 2.5 h from extraction) for lead concentration and ALAD activity determination (see laboratory methods below).

Laboratory methods

Packed cell volume (PCV) was determined from fresh blood samples in microhematocrit tubes centrifuged for 20 min at 1,100×*g* (Mobilespin, model 128, Cardinal Health, Dublin, Ohio, USA). Plasma total solids were measured using a hand-held refractometer (Schulco, Toledo, OH). Red cells and granulocytes (heterophils and eosinophils) were counted using Erythro-Pette kit® (Exotic Animal Solutions, Inc. Hueytown, AL, USA) and Eopette kit® (Exotic Animal Solutions, LLC. Melbourne FL, USA), respectively. The morphology of blood cells was examined in 52 blood smears stained with Diff Quick® (Jorgensen Laboratories, Inc. Loveland, CO, USA). Upon overall blood smear assessment using low power scanning microscopy, the degree of morphological abnormalities were further investigated and graded through high power magnification assessments. The general appearance of blood cells was classified as normal (cells with typical overall appearance), slightly abnormal (a small proportion of the cells of the smear had morphological changes, including poikilocytosis, hypochromasia, anisocytosis), and clearly abnormal (atypical overall appearance of the smear and the cells due to moderate to severe morphological changes). Blood cells were also classified into scores (ranked 1–3) for degrees of hypochromasia, anisocytosis, poikilocytosis and polychromasia. All blood smear assessments were performed by the same person. ALAD activity was determined by a private laboratory ("Laboratorio de Medicina", Buenos Aires, Argentina), following Burch and Siegel (1971).

Ca and P concentrations in bone were determined by inductively coupled plasma-atomic emission spectrometry (ICP-OES; Shimadzu 9000, Shimadzu Corporation, Kyoto, Japan), following 200.7 EPA standards (U.S. Environmental Protection Agency), at the Chemical Analysis Laboratory-LANAQUI, Centro de Recursos Renovables de la Zona Semiárida, (CONICET, Universidad Nacional del Sur, Bahia Blanca, Argentina). Detection limits for Ca and P in bones were 0.09 mg/kg (dw) and 0.55 mg/kg (dw), respectively.

Lead exposure

The analysis of lead exposure in the ducks included in this study was described in detail in Ferreyra et al. (2014). Briefly, ingestion rates were evaluated using radiographs of each gastrointestinal tract, followed by confirmation by dissection and lead pellet recovery. Lead concentration in frozen tissues (bone, liver and blood) was determined by inductively coupled plasma-atomic emission spectrometry (ICP-OES). For further details, refer to Ferreyra et al. (2014).

Data analysis

We obtained descriptive statistics including mean, geometric mean (only for lead concentrations), standard deviation, and range (lowest and highest value) for each parameter evaluated. To explore changes in generic health indices of wild ducks according to lead exposure, we used linear models (LM; Crawley 2007) and ordinal logistic regression models (OLRM; Armstrong and Sloan 1989) with the statistical software package R v. 2.15.2 (R Development Core Team 2012). The significance level used (α) was 0.05. LM were used for response variables that were normally distributed or that could be transformed to approximate normality by appropriate exponentiation; for response variables that were ordinal (e.g. anisocytosis

score), we used OLRM. The independent variables of interest were the levels of lead in liver and bone for hunter-killed ducks, and in blood for live-trapped ducks. The models built for hunter-killed ducks included environmental and host factors that were considered potential confounders, namely species, sex, body mass, age class, site type (inland/island), and year/Province (2011/Santa Fe and 2012/Corrientes). The final models only included potential confounders that were important for the model goodness of fit. Model selection was carried out by stepwise elimination of unimportant terms using Akaike information criterion (AIC) (Akaike 1994). A single term was not retained in the model if its inclusion did not reduce AIC by at least 2 units. An analysis of outliers was conducted in the final models measuring Cook's distances to verify that no single observation had an exaggerated relative influence on the outcome (i.e. no Cook's distance should be >0.5) (Kim and Storer 1996).

The sample size for live-trapped ducks was too small (N = 94) to allow for multivariate analysis including several factors, and therefore regression models (either LM or OLRM) only controlled for duck species.

Results

Descriptive statistics of generic indices of health and lead concentrations from waterfowl in Argentina hunting hotspots are detailed in Table 1.

In hunter-killed ducks, when adjusting for the potential confounding effect of site type, year, species, body mass and age, the keel angle was negatively associated with lead levels in liver (Table 2), whereas there was no relationship with lead in bone. For every one ppm increase of lead in liver, the keel angle decreased 0.135° on average.

The mass of the spleens were negatively associated with lead levels in bone and in liver (Table 3). On average, a duck with 10 ppm of lead in liver had a spleen 6.2 % lighter than one with undetectable levels, whereas one with 10 ppm of lead in bone had a spleen 1.64 % lighter. Lead concentrations were not associated with Ca and P, and no abnormalities were found in bone marrow smears (all p values > 0.1).

In live individuals, blood lead levels were negatively correlated with PCV (LM coefficient = 1.291; SE = 0.526; $p = 0.0161$) (Fig. 2). However, there was no significant correlation between blood lead levels and red blood cell counts ($p = 0.389$). The degree of abnormality in red cell morphology was greatest in ducks with high blood lead levels (OLRM coefficient = 4.952; SE = 1.727; $p = 0.003$) (Fig. 3a). Similarly, high blood lead levels were found positively associated with anisocytosis (OLRM coefficient = 0.864; SE = 0.326; $p = 0.0055$) (Fig. 3b), poikilocytosis (OLRM coefficient = 0.685; SE = 0.294; $p = 0.012$) (Fig. 3c) and polychromasia (OLRM coefficient = 0.893; SE = 0.321; $p = 0.0038$) (Fig. 3d).

There were no significant associations between lead in blood and the rest of the variables measured in live individuals, namely keel angle, ALAD enzyme activity, granulocytes, and total solids (all p values > 0.1). No significant association was found between bone lead and Ca/P.

Discussion

Little is known about the sub-lethal effects of chronic lead exposure on duck health under natural conditions. Here we report significant impact of lead on generic indices of health, as evidenced by poor body condition, reduced spleen mass, and hematological abnormalities compatible with lead toxicosis, in naturally exposed ducks that were not presenting overt clinical signs or gross pathological findings compatible with lead poisoning.

A strong negative association between the keel angle and liver lead levels indicated that lead exposure was associated with reduced pectoral muscle mass, as is commonly found in lead-poisoned ducks (Friend 1999). Several studies found reduced body mass in waterfowl exposed to sub-lethal lead levels (Hohman et al. 1990; Locke and Thomas 1996; Friend 1999; Franson and Pain 2011). This emaciation is thought to result from chronic lead exposure, which causes anorexia (Friend, 1999; De Francisco et al. 2003; Rodriguez et al. 2010).

Experimental studies by Bates et al. (1968), Rocke and Samuel (1991) and McMurry et al. (1995), reported that spleen mass was reduced by lead exposure. We found a negative association between spleen mass and lead levels in liver and bone of dead ducks, which strongly suggests that this effect is also occurring under natural conditions. Smaller spleens may be an indication of reduced investment in lymphoid tissue. Some studies have shown that individuals with smaller spleens were less immune-competent (John 1994; Møller and Erritzøe 1998; Ardia 2005). However, more studies are needed to elucidate the mechanisms underlying the reduced spleen mass of ducks exposed to lead, as well as to measure the effects on functional outputs of immune response and their long term consequences on fitness (Grasman 2002).

Bone deformities in stork chicks were caused, at least partially, by heavy metal exposure (Smits et al. 2005). Álvarez Lloret et al. (2014) reported a reduction in the degree of bone mineralization and an increase in the crystallinity of bone mineral with increasing lead bone concentrations in a wild population of red-legged partridge (*Alectoris rufa*), and it was reported that vultures exposed to lead showed disrupted bone mineralization (Gangoso et al. 2009). We failed to find a significant association

Table 1 Descriptive statistics of studied variables and lead concentrations in blood, liver and bone from live-captured and hunter-killed ducks from Argentina hunting hotspots

Variables/species	*Dendrocygna bicolor* (n = 1)	*Dendrocygna viduata* (n = 45–67)	*Netta peposaca* (n = 4)	*Amazonetta brasiliensis* (n = 5–6)	*Dendrocygna autumnalis* (n = 13–17)
Live ducks					
PCV[a] (%)	50	45.34 ± 4.65 (30–55)	45.38 ± 4.03 (40–49.50)	42.6 ± 5.69 (34–48.5)	44.21 ± 4.26 (36–50)
Keel angle[a] (°)	133	108.54 ± 11.27 (85–144)	101.12 ± 12.39 (87–112.5)	111.5 ± 11.64 (97–127)	106.47 ± 14.06 (83.5–146)
Erythrocytes[a] (cells/µl × 10^6)	2.19	3.07 ± 0.84 (1.05–5.80)	2.89 ± 0.60 (2.20–3–0.64)	2.72 ± 0.57 (2.20– 3.51)	3.04. ± 1.07 (1.00–5.32)
Granulocytes[a] (cells/µl × 10^3)	NA	17.31 ± 6.60 (4.98–35.04)	NA	NA	15.6 ± 7.45 (4.82–33.84)
ALAD[a] (µm/h/ml)	NA	0.422 ± 0.13 (0.099–0.647)	NA	NA	0.498 ± 0.174 (0.119–0.737)
Total Solids (g/dl)	5.6	5.4 ± 0.47 (4.2–6.6)	5.3 ± 0.65 (4.4– 5.9)	5.3 ± 0.42 (4.8– 5.9)	5.8 ± 0.43 (5.1– 6.8)
Lead in blood[b] (mg/kg dry weight)	0.31	0.21 ± 2.59 (ND–5.75)	ND	ND	0.19 ± 2.66 (ND–4.71)
	Dendrocygna bicolor (n = 33–103)	*Dendrocygna viduata* (n = 76–134)	*Netta peposaca* (n = 42–100)	*Amazonetta brasiliensis* (n = 56–57)	*Dendrocygna autumnalis* (n = 17–18)
Hunter-killed ducks					
Spleen weight[a] (g)	0.60 ± 0.28 (0.16–1.48)	0.43 ± 0.21 (0.07–1.50)	0.70 ± 0.81 (0.17–7.58)	0.25 ± 0.17 (0.05–1.27)	0.36 ± 0.1 (0.18–0.54)
Keel angle[a] (°)	127.64 ± 13.09 (104–147)	123.83 ± 13.13 (93–148)	144.3 ± 18.61 (114–180)	138.97 ± 10.19 (106–162.5)	131.56 ± 9.36 (112–150)
Ca[a] (%)	19.8 ± 2.2 (14.8–27.5)	18.1 ± 2.5 (10.4–23.3)	18.7 ± 2.2 (13.7–23.4)	16.6 ± 2.5 (11.2–20.80	18.6 ± 2.7 (12.1–21)
P[a] (%)	9.5 ± 1 (7.6–12.9)	9.4 ± 0.9 (6.3–11.8)	9.1 ± 1.1 (5.3–10.8)	8.85 ± 1.1 (6.10–10.80	9.45 ± 0.7 (8.0–10.90)
Lead in liver[b] (mg/kg dry weight)	0.39 ± 5.87 (ND–196)	0.29 ± 3.22 (ND–35.10)	0.33 ± 4 (ND–38.5)	0.28 ± 2.75 (ND–16.9)	0.2 ± 2.14 (ND–0.79)
Lead in bone[b] (mg/kg dry weight)	5.49 ± 5.52 (0.33–388.5	3.40 ± 3.37 (0.36–89.7)	3.39 ± 4.38 (0.30–143.2)	2.35 ± 2.55 (0.30–389)	3.86 ± 3.05 (1.10–34.40)
Pellet ingestion (%)	7.77	12	24.27	5.26	5.55

n number of samples (range), *ND* below detectable limits, *NA* not analyzed

[a] Mean ± SD (range); [b] Geometric mean ± GSD(geometric standard deviation) (range)

between Ca/P and lead concentration in bones from hunted ducks, but this should not be considered evidence of lack of effect. Given the large variance present in our data because ducks in our sample varied greatly in genotype, phenotype and environmental influence, our statistical power was limited. On the other hand, more complex techniques than the simple determination of absolute mineral levels may be necessary to assess the degree of bone mineralization (Chappard et al. 2011).

As expected, we found lower PCV values in live ducks with greater blood lead levels, yet red blood cell counts were not significantly associated with lead. This inconsistency may be due to lead intoxication causing microcytic anemia (Bates et al. 1968; Geens et al. 2010), as PCVs are affected not only by the number of red cells, but also by their size (Campbell 2012). In addition, there was a strong positive association between red cell morphological abnormalities and lead levels in blood. Increased severity of hypocromasia, anisocytosis, poikilocytosis and polychromasia was strongly associated with blood lead levels. By inhibiting ALAD and heme synthetase enzymes, lead causes a reduction in total blood hemoglobin concentration

Table 2 Linear model showing the association between lead concentrations in liver and the keel angle of hunter-killed ducks

Model:
Response = Keel angle
Independent variables = *lead in liver* + site type + year/province + species[a]

Term	Coefficients	Standard error	*p* value
Intercept	122.68	2.324	<0.0001
Lead in liver	−0.135	0.050	0.0069
Site type$_{(island)}$[b]	8.522	2.065	<0.0001
Year/province$_{(2012)}$[c]	4.070	2.001	0.0426
Species$_{(D.\ viduata)}$[d]	−3.411	1.716	0.0475
Species$_{(N.\ peposaca)}$[d]	9.966	1.710	<0.0001
Species$_{(A.\ brasiliensis)}$[d]	3.794	2.831	0.1809
Species$_{(D.\ autumnalis)}$[d]	2.946	3.608	0.4146

The independent variable of interest is italicised

[a] Age and body mass were removed from the model because they were not important for its goodness of fit

[b] Simple contrasts—reference level: inland (the coefficients reflect comparison with inland)

[c] Simple contrasts—reference level: 2011 (the coefficients reflect comparison with 2011)

[d] Simple contrasts—reference level: *D. bicolor* (the coefficients reflect comparison with *D. bicolor*)

and anemia. Lead may also produce anemia by disrupting red blood cell production, resulting in polychromasia and anisocytosis (Coles 1986). Morphological alterations in red blood cells might result in increased hemolysis and, consequently, contribute to anemia (Pattee and Pain 2003; Mateo et al. 2003; Mitchell and Johns 2008). These changes in red cell morphology have been previously reported in birds intoxicated with lead (Bates et al. 1968; Bono and Braca 1973; O'halloran et al. 2008; Campbell 2012).

A common indicator of lead poisoning in live vertebrates is the inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity (Finley et al. 1976; Scheuhammer 1987; Pain 1989). Although we failed to find a significant association between blood lead levels and ALAD, we did detect what might be the consequences of ALAD inhibition, namely low PCVs and defective red cells (see above) (Bates et al. 1968; Bono and Braca 1973; Campbell 2012). ALAD may remain inhibited in blood several weeks after exposure and it is likely that ducks that have recovered from acute exposure do not present detectable lead levels in their bloodstream, yet ALAD may still be inhibited (Finley and Dieter 1978). This would undermine the ability to find a statistically significant association between current blood lead levels and ALAD activity. Another source of error with the use of ALAD is the large variability in the baseline levels of this enzyme's activity in unexposed individuals (Pain 1989). The use of an ALAD ratio method greatly reduces this source of error (Scheuhammer 1987; Pain 1989), but this could not be performed in our study due to lack of capacity at local laboratories.

As previously reported by Ferreyra et al. (2009; 2014) and shown in Table 1, ducks in our study area regularly ingested lead pellets, had high levels of lead in their blood, and accumulated lead in their livers and bones. Furthermore, lead levels often surpassed toxicity thresholds at which clinical poisoning is expected (Franson and Pain 2011). Despite these levels of exposure, we only observed clinical signs consistent with lead poisoning in three (3/94, 3.2 %) live-captured individuals with blood lead levels of 0.65, 0.73 and 5.75 mg/kg. These animals showed difficulty in flying when released, and remained still in the vegetation with labored breathing for approximately 15 min until recovery (Ferreyra et al. 2014). Lead exposure may increase susceptibility to stress (Rattner et al. 1989), and handling ducks with high blood lead levels may have favored the signs observed. Although local outfitters reported occasional sightings of ducks unable to fly, during our time in the field since 2007, we failed to document events of duck morbidity and mortality, and to our knowledge there are no extant reports of lead-induced waterfowl die-offs in the region.

Notwithstanding, our results strongly suggest that the absence of noticeable indicators of lead poisoning should not be interpreted as lack of impact on the duck's health. Lead toxicity effects may be subtle and not readily visible, but not harmless. Ducks with greater exposure to lead had poorer body condition, reduced spleen mass, and alterations in their blood cells. Those conditions are indicative of allostatic overload, lesser immunocompetence, and reduced aerobic capacity, which are likely to have a detrimental effect on fitness, reproduction and survival of individuals

Table 3 Linear model showing the association between lead concentrations (in liver and bone) and the spleen weight of hunter-killed ducks

Model:
Response = Spleen weight$^{0.1}$ ~
Independent variables = *lead in liver* + species[a]

Term	Coefficients	Standard error	*p* value
Intercept	0.944	0.0045	<0.0001
Lead in liver	−0.0006	0.0001	0.00068
Species$_{(D.\ viduata)}$[b]	−0.034	0.006	<0.0001
Species$_{(N.\ peposaca)}$[b]	0.001	0.006	0.8538
Species$_{(A.\ brasiliensis)}$[b]	−0.082	0.007	<0.0001
Species$_{(D.\ autumnalis)}$[b]	−0.044	0.011	0.00019

Model:
Response = Spleen weight$^{0.1}$ ~
Independent variables = *lead in bone* + species[a]

Term	Coefficients	Standard error	*p* value
Intercept	9.439e−01	5.046e−03	<0.0001
Lead in bone	−1.373e−04	6.725e−05	0.0418
Species$_{(D.\ viduata)}$[b]	−3.335e−02	6.354e−03	<0.0001
Species$_{(N.\ peposaca)}$[b]	2.590e−03	6.740e−03	0.7010
Species$_{(A.\ brasiliensis)}$[b]	−8.057e−02	7.708e−03	<0.0001
Species$_{(D.\ autumnalis)}$[b]	−4.235e−02	1.219e−02	0.0005

The independent variable of interest is italicised

[a] Year/province, site type, age and body mass were removed from the final models because they were not important for the goodness of fit

[b] Simple contrasts—reference level: *D. bicolor* (the coefficients reflect comparison with *D. bicolor*)

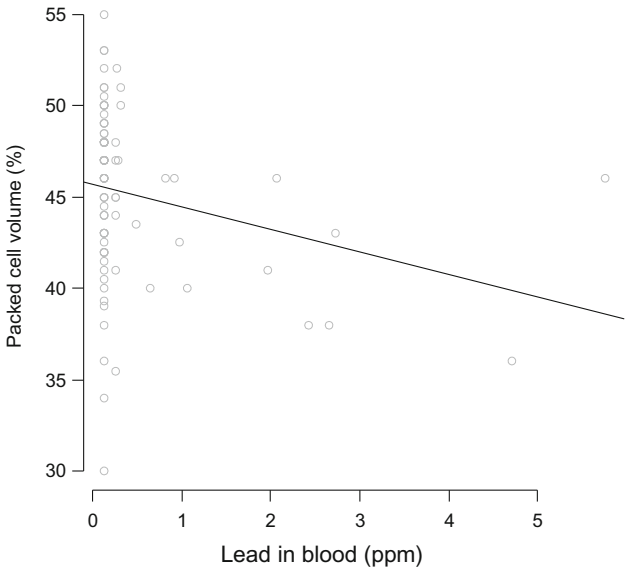

Fig. 2 Relationship between packed cell volume and concentrations of lead in blood in live-captured ducks. On average, for every one unit (ppm) of increase in lead concentration, packed cell volume decreased 1.3 units (%)

(Beldomenico and Begon 2010; McEwen and Wingfield 2010). Although further studies should explore the ecological meaning (i.e. impact on fitness and survival) of the sublethal effects of lead poisoning found in these duck populations, it has been established in other wildlife species that physical deterioration, reduced immunological investment, and functional disorders weaken and affect the fitness and survival of animals. This occurs by a reduction in their ability to evade predators and cope with infection (Beldomenico and Begon 2010), as well as their capacity to perform normal behaviors, such as reproduction (Tranel and Kimmel 2009) and migration (Jordan and Bellrose 1951). Because lead pellets are available virtually to all individuals in the duck community studied, it is expected that these effects ultimately have a significant impact on population dynamics. This may occur gradually, accompanying the chronic pace of lead shot ingestion, and thus making the detection of mortality very unlikely. There is great potential for "selection" bias as diseased birds tend to hide in vegetation and are therefore less likely to be hunted or captured (Friend 1999). An experimental study that assessed the accuracy of documenting waterfowl die-offs estimated the detection efficacy for ducks that died of lead poisoning to be 0 % (Stutzenbacher et al. 1986). This is a particularly important problem given many of the wetlands in our study area are highly inaccessible and relatively isolated, therefore limiting opportunities for observation of deceased ducks before they are found by predators or scavengers.

The wetlands in our study area are an international hotspot for duck hunting, an activity that has grown considerably since the 1990 s (Zaccagnini and Venturino 1992; Zaccagnini 2002). Even though in comparison to Europe and North America massive use of lead shot in Argentina is relatively recent (last two decades), our studies over the past seven years have shown that nearly 15 tons of spent lead are added to wetlands annually. This has resulted in sediment pellet densities up to 77 units/m^2 (between 0 and 15 cm deep) (Romano et al. unpublished data), matching some of the most polluted areas in Europe (Mateo 2009). Here we show evidence of the direct impact of discarded lead on waterfowl health.

Besides the impact on ducks, lead might have cascading implications for ecosystem dynamics. Moreover, considering the vast agricultural activity that takes place in our study area, it is possible that other contaminants, such as pesticides, could be additively or synergistically contributing to the problem.

In this high risk scenario, curtailing the use of lead shot is urgent. Beyond waterfowl, other species are

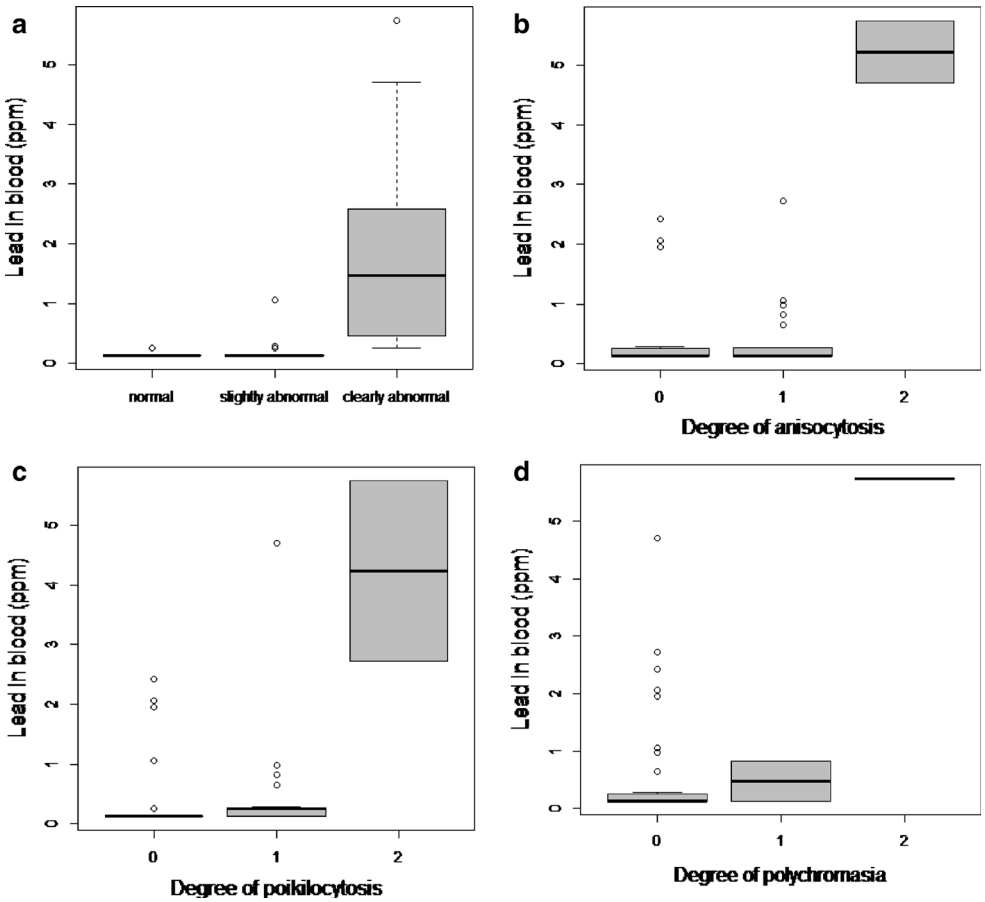

Fig. 3 Relationship between concentrations of lead in blood of live-captured ducks and **a** degree of abnormality in red cell morphology, **b** anisocytosis, **c** poikilocytosis, and **d** polychromasia. In all cases associations between abnormalities and blood lead levels were highly significant and of great magnitude

susceptible to poisoning from spent lead shot, including those in predator–prey systems (Friend et al. 2009). In Europe and North America at least 17 and 9 raptor species, respectively, have been poisoned by eating prey contaminated with lead bullets or ammunition (Mateo 2009; Tranel and Kimmel 2009). Therefore, replacement of lead with non-toxic ammunition will directly benefit the health of many species inhabiting these ecosystems as well as surrounding human populations (Ferreyra et al. 2014).

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Conflict of interest The authors declare that they have no conflict of interest.

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