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RESEARCH ARTICLE

Trace Elements (Pb, Zn, Cu) in Blood of Mute Swan (*Cygnus olor*) from the Isonzo River Nature Reserve (Italy)

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ABSTRACT

Lead concentrations in blood of 45 specimens of mute swan from the molting area of the Isonzo River Mouth Nature Reserve (Italy) were determined in two consecutive years (2006-2007), some birds were neck ringed to identify their homing behavior. The second sampling included whole body X-ray radiography and Cu and Zn plasma analyses to investigate the health impact of putative Pb exposure. X-ray images of all investigated specimens did not show any radiopacity due to the ingestion of metal bodies. Lead levels (0.08-0.44 μg/ml) were in the range of those reported for swans living in unpolluted or slightly polluted environments and excluded acute intoxication, as confirmed by clinical investigation. Zinc concentrations ranged between 2.93 and 7.59 μg/ml and were one order of magnitude higher than Cu concentrations (0.21-0.42 μg/ml). The negative correlation between Pb and Zn concentrations could be indicative of adverse health effects caused by chronic lead exposure. To our knowledge this is the first study reporting Pb, Zn and Cu blood levels, X-ray radiographies and data on the origin of swan populations.

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INTRODUCTION

In birds non essential trace metals like Pb, Hg and Cd and essential metals, namely Cu, Zn and Fe, can cause toxic effects when they reach threshold concentrations (Carpenè *et al.*, 1995; Nakade *et al.*, 2005; Carpenè *et al.*, 2006). At the molecular level, Pb toxicity is mainly due to inhibition of δ-aminolevulinic acid dehydratase (ALAD) and subsequent impairment of haeme biosynthesis. It has also long been recognized that Pb pellets can be a cause of waterfowl mortality. Pb poisoning in swans is mainly caused by ingestion of lead fishing weights and spent shotgun shells (Nakade *et al.*, 2005; O'Connell *et al.*, 2009; Naz and Javed, 2012). Humans can also be exposed to the widespread use of Pb when waterfowl meat accounts for a high percentage of dietary protein as in Greenland (Johansen *et al.*, 2006). Little information is available on Pb concentration in tissues of mute swan in Italy, but wild fowl presenting spent shotgun in the gizzard and saturnism symptoms have been reported (Ancora *et al.*, 2008).

Legislation restricting the use of Pb for fishing weights and hunting ammunition has been introduced in several countries since the 1980s. As a consequence, Pb has been replaced by other metals such Cu, Zn, W and Fe, which in turn can cause toxic effects. Unlike Pb poisoning, it is more difficult to recognize Cu, Zn and Fe toxicity because their blood and plasma concentrations are tightly controlled by sophisticated homeostatic mechanisms. On the other hand, when high rates of metal exposure overcome physiological control mechanisms, tissue damage is observed, e.g. pancreatic lesions, as documented in several studies on waterfowl (Sileo *et al.*, 2003; Van der Merwe *et al.*, 2011). Naturally occurring Cu poisoning in birds is rare, but a natural mass outbreak of Cu poisoning in mute swan has been reported (Kobayashi *et al.*, 1992).

The Isonzo estuary is connected to several brackish wetlands and shallow marine tidal mudflats that have long been exploited by hunters. Today, the river-mouth lies in a nature reserve and a Natura 2000 site and constitutes a sanctuary for waterfowl, and holds the largest mute swan

(*Cygnus olor*) population in Italy with up to 840 specimens (November, 2011) forming a unique huge flock or, more often, split into a number of smaller wedges. High numbers are commonly recorded in the summer, when many birds gather in this safe area with abundant food (mostly eel-grass *Zostera noltii*) during the molting period. The numbers recorded can be slightly lower in the beginning of autumn, to increase again with the arrival of birds from North-Eastern Europe or other Northern Adriatic areas.

This research is part of our studies addressed to investigate trace elements in wild vertebrates (Carpenè *et al.*, 1995; Carpenè *et al.*, 2006; Andreani *et al.*, 2008; Zaccaroni *et al*., 2011). The aims of this paper were to: (1) determine blood Pb concentrations in mute swan by atomic absorption spectrometry; (2) investigate the presence of radiopaque particles in the gizzard and small intestine by X-ray radiography; (3) determine Zn and Cu levels in plasma and evaluate possible Pb interactions. The substitution of Pb pellets with new brands made of Zn or other metals requires knowledge of normal plasma concentrations of essential trace metals to obtain useful information on these parameters in healthy specimens.

To our knowledge, this is the first paper to report blood Pb and plasma Zn and Cu levels of wild mute swans identified with tracking collars, which could be useful for tracking their homing behavior.

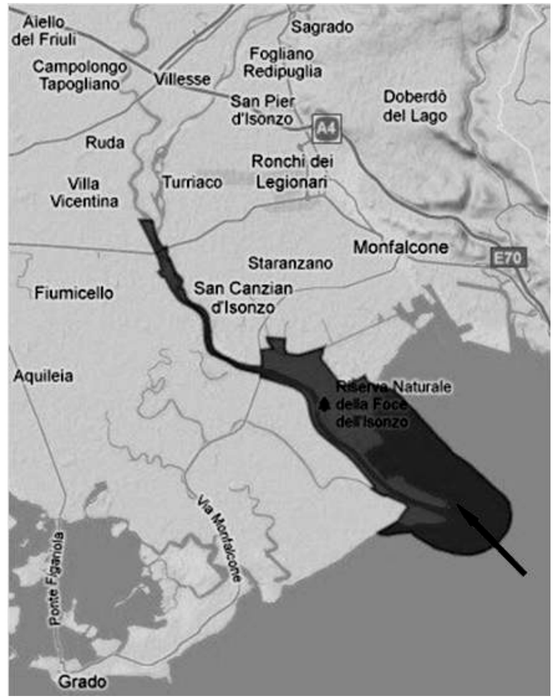

Fig. 1: The arrow at the bottom of the dark area indicates the blood sampling location for mute swans captured at the Isonzo River Mouth Nature Reserve (Italy).

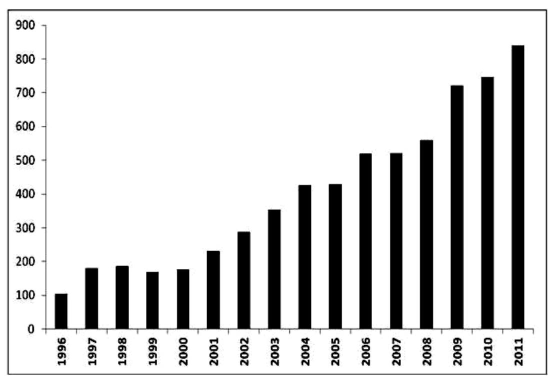

Fig. 2: The increasing trend, between 1996 and 2011, of the mute swan population at the Isonzo River Mouth Nature Reserve is reported.

MATERIALS AND METHODS

Study area: Mute swans were caught at their molting site at the Isonzo River-mouth, Northern Adriatic (Longitude 13° 30' E; Latitude 45° 45' N) (Fig. 1); the vast mudflats are partially covered by *Zostera noltii*, which is substituted by *Zostera marina* and/or *Cymodocea nodosa* in deeper non tidal areas. Moulting grounds are open to the Adriatic Sea and the Gulf of Trieste, with a number of small sandy and/or gravel islets that are regularly used by swan flocks as roosting sites (Perco *et al.,* 2008).

Birds and sample collection: The common technique employed to catch the birds is similar to that traditionally implemented in England, using a hook (with rounded tip) attached to a long pole.

Swans were caught during the annual moult at the beginning of August for two consecutive years (2006 and 2007) when the birds are mostly flightless and it is easy to catch living swans without causing any trauma and obtain blood samples and x-ray images. Birds were treated according to the Italian guidelines for animal welfare legislation. The population, still increasing (Fig. 2), is constituted mainly of adult swan spread in vast areas, many flying back to Central or Northern Europe up to the Baltic.

Swans were clinically inspected, weighted, sexed and aged according to size and the plumage that is white in the adult and grey in immature and first year birds. In both years the birds were marked with a metal tarsus ring and a white plastic collar with an alpha-numerical code. The 2006 swan sampling comprised 19 adult males (10.65-9.00 kg), 6 adult females (8.60-6.60 kg), 2 immature males (9.80-9.40 kg) and 4 immature females (8.20-7.00 kg). The 2007 sampling comprised 17 adult males (12.00-10.00 kg), 16 adult females (10.00-6.50 kg), 4 immature males (10.70-10.47 kg) and 4 immature females (9.75-7.75 kg).

Blood samples (2 ml) were taken from the brachial vein using a needle 21 Gx ½ applied to a syringe for metal analysis (Sarstedt, Germany). Each blood sample was transferred and separated in two eppendorf tubes. One tube containing whole blood was used for Pb analysis, the other was centrifuged at 2000 rpm to obtain the plasma for Zn and Cu analysis. For Pb analyses we obtained respectively 21 samples in 2006 and 25 in 2007, while for Zn and Cu 19 samples were analyzed only in 2007.

X-ray radiography: Before release the swans were positioned for conscious radiography of the gizzard using a high frequency portable radiological unit equipped with a computed radiograph. The beam parameters were: 65 kVp and 10 mAs. Whole body images were obtained by manual positioning for the lateral view.

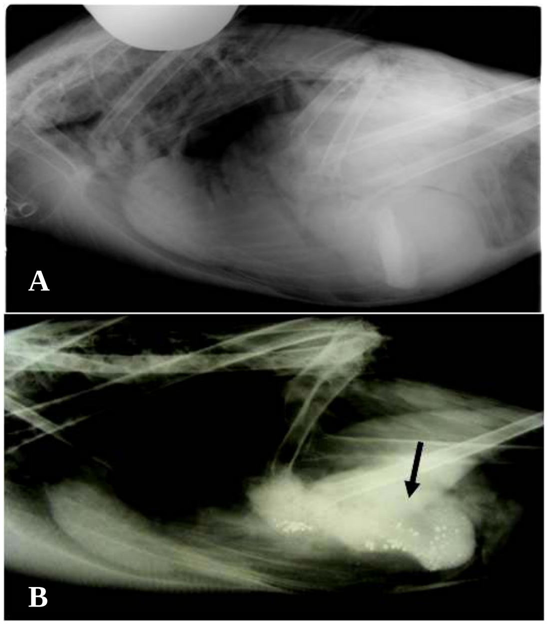

Fig. 3: Abdominal radiographs of two specimens of mute swan without (a) or with ingested shotgun pellets (b), the arrow indicates their position in the gizzard. The radiograph of the intoxicated swan was obtained from the clinical collection of the Department of Veterinary Medical Sciences.

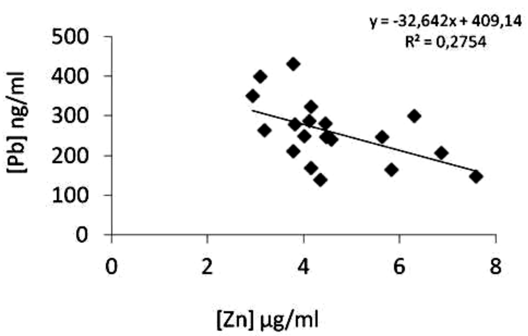

Fig. 4: Scatter plot and regression line between blood Pb and plasma Zn concentrations in 19 mute swans sampled during 2007 (significant negative correlation, P<0.05).

Table 1: Plasma Zn and Cu concentrations in specimens (n=19) of mute swans sampled at the Isonzo Nature Reserve during 2007. Metal concentrations are expressed in µg/mL of plasma.

Metal	Mean±S.D.	Median	Range
Zn	4.64±1.27	4.22	2.93-7.59
Cu	0.29±0.06	0.30	0.21-0.42

Metal analysis: Blood Pb concentrations were determined by atomic absorption spectrophotometry with a flameless atomizer attachment. The accuracy of the analysis was verified with a certified bovine blood reference containing 0.416 µg Pb/mL (ERM-CE195, Geel, Belgium), the limit of detection (LOD) was 3 ng/mL. Plasma Zn and Cu concentrations were determined by direct aspiration into the flame of atomic absorption spectrophotometer after tenfold dilution of plasma with bi-distilled water. LOD was 5 ng/mL for Zn and 10 ng/mL for Cu.

Pb concentrations were expressed in ng/mL or µg/mL of total blood; Zn and Cu concentrations were expressed in µg/mL of plasma and were reported as mean±SD. A simple conversion to enable comparison with earlier studies is obtained by multiplying by 207 the values expressed in µmol/L giving the equivalent value in µg/L.

Statistics: All statistical calculations were carried out using MedCalc ®. The normality of data was checked by D'Agostino Pearson test. As the data were not normally distributed, non-parametric Mann–Whitney U and Kruskal–Wallis tests were used throughout. The correlation between the two essential trace metals Zn, Cu and Pb was determined by linear fit y=ax+b. The significance was set at P<0.05.

RESULTS

Clinical inspection indicated that all the investigated specimens of mute swans (*Cygnus olor*) did not present saturnism symptoms and were healthy. No X-ray images of investigated specimens showed any radiopacity due to the ingestion of metal bodies (Fig. 3a).

Metal concentrations in plasma of mute swan are listed in Table 1. Pb concentrations refer to whole blood whereas those of Zn and Cu refer to plasma. Lead concentrations ranged between 0.08 and 0.28 µg/mL in mute swans sampled in 2006 while a wider range (0.14-0.44 µg/mL) was found for specimens sampled in 2007. During the first sampling, only one bird exceeded the 0.25 µg/mL exposure threshold, while 44% of mute swans from the second year of sampling showed concentrations higher than the threshold. Blood Pb concentrations in birds sampled in 2006 were significantly lower (P<0.001) than those measured in birds sampled in 2007.

Zinc and copper concentrations were measured in plasma of mute swans only in 2007 (Table 1). Zinc concentrations ranged between 2.93 and 7.59 µg/mL and were one order of magnitude higher than Cu concentrations, which varied between 0.21 and 0.42 µg/mL. A significant inverse correlation (P<0.05) was found for Zn and Pb concentrations (Fig. 4), while Cu and Pb were not significantly correlated (data not shown).

DISCUSSION

This study determined the blood Pb concentrations in mute swans during two consecutive years. Our results on Pb levels (0.08-0.44 µg/mL) are in the range or slightly more elevated than those reported for swans living in unpolluted environments (Perrins *et al.*, 2003; Day *et al.*, 2003) and exclude acute intoxication as confirmed by radiography and clinical examination. In addition, the radiographies exclude other kinds of shot pellets (Zn, Cu, Fe, W). Data on blood Pb levels are currently lacking for Italian avifauna, whereas Pb concentrations in tissues have been reported. In flamingos acutely poisoned due to the ingestion of Pb shots, liver presented the highest metal concentrations, reaching a value of 361.29 µg/g dry weight (Ancora *et al.*, 2008). By contrast, low hepatic Pb concentrations have been reported in species from non-polluted environments (Zaccaroni *et al.*, 2011).

In the majority of swans we found blood Pb concentrations lower than the threshold of 0.25 µg/mL (1.21 µmol/L) indicated by Perrins *et al.* (2003); this is a level lower than that set at 0.40 µg/mL (1.93 µmol/L) used by past workers (Sears and Hunt, 1991). In Eurasian eagle owl (*Bubo bubo*) adverse Pb effects based on ALAD inhibition may occur when metal levels are present at 0.15 µg/mL (Gomez-Ramirez *et al.*, 2011). On the other hand, according to Buekers *et al.* (2009) the association between blood Pb concentration and systemic toxicity was set at a higher level for birds (0.71 µg/mL) with respect to mammals (0.18 µg/mL). A recent study in mice reported that low levels of Pb exposure alter normal gene expression in peripheral blood cells, indicating that probably there is no safe level of Pb exposure (LaBreche *et al.*, 2011).

The Pb concentrations exceeding the threshold limit found in some specimens in our study could be related to a previous ingestion of Pb shotgun pellets and their subsequent digestion or elimination through the feces. However, elevated Pb levels may be related to sources of exposure other than shot ingestion, such as the dietary intake of Pb rich feed or contaminated sediments. Specimens of young mute swans from Chesapeake Bay, USA fed a commercial diet had a mean of 0.19±0.008 µg/g blood Pb. When a diet containing Pb-contaminated sediments was used, blood Pb concentrations rose to 3.2±0.76 µg/g (Day *et al.*, 2003).

A wide range of blood Pb concentrations has been reported in different species of swans. Perrins *et al.* (2003) reported values ranging from a minimum of 0.02 µg/mL in UK mute swans from sites thought likely to be “clean” to a maximum of 30 µg/ml in birds from Pb-polluted areas. A value as high as 57.21 µmol/L (11.8 µg/mL) was reported in a mute swan with severe Pb poisoning (Cousquer *et al.*, 2006); the bird was rescued by a specific treatment with EDTA and antibiotic therapy. According to Ochiai *et al.* (1992) and Nakade *et al.* (2005) whooper and tundra swans with ingested Pb pellet in Japan had Pb blood concentrations between 3.0-6.3 µg/mL and 2.5-6.7 µg/g respectively. In Nakade *et al.*’s study (2005) five whooper swans died due to the severity of intoxication, while only one bird with a level of 2.9 µg/g survived; after a clinical treatment with CaEDTA and activated charcoal for 64 days blood Pb concentrations decreased to 0.09 µg/g.

The radiographic investigation did not disclose any metal body and the comparison with a positive case of a mute swan (Figure 3b) confirmed the reliability of the analysis of Pb blood levels. On the other hand, clinical diagnosis of Pb poisoning is not always correlated with high blood levels of Pb, and the lack of radiodense pellets does not rule out Pb toxicosis (Routh, 2000). Due to the relatively short half-life of Pb in blood (13 days in pigeons), blood is often used as a source of biomarkers for recent Pb exposure (Buekers *et al.*, 2009). Pellet degradation took approximately 30 days in mallards (*Anas plathyrhynchos*) experimentally intoxicated and a Pb peak was observed in blood between 10 and 20 days (Rodriguez *et al.*, 2010).

We exclude changes in environmental Pb contamination of Isonzo Nature Reserve as a cause of the higher blood Pb concentrations measured in swans during 2007 compared to 2006. In this respect, we cannot rule out that the main cause is due to the wintering location of sampled specimens, because 50% of them come from different areas of Europe and their frequencies change over the years, as evidenced by the identification of neck ringed specimens.

During the second year we also measured Zn and Cu concentrations in the plasma; Zn levels were one order of magnitude higher than those of Cu, reflecting the widespread presence of this essential element in biological systems. Data on essential trace elements in plasma/serum of swans are lacking, with the exception of Zn (11.2 µg/mL) and Cu (0.17 µg/mL) reported for one specimen of trumpeter swan (*Cygnus buccinator*) intoxicated by Zn (Carpenter *et al.*, 2004).

A significant negative correlation was found between blood Pb and plasma Zn concentrations in mute swans, even though Zn concentrations are closely regulated due to an efficient molecular strategy of homeostasis based on a fine regulation of transporters and the induction of metallothionein.

Conclusion: In conclusion, we exclude acute Pb intoxication even if the negative correlation observed between Pb and Zn concentrations could be indicative of chronic environmental exposure to low doses of the toxic metal. Accordingly, a preliminary investigation in humans demonstrated that children with higher Pb blood levels had significantly lower concentrations of plasma Zn consistent with cognitive deficiency (Hubbs-Tait *et al.*, 2007). Altered behavior due to chronic Pb exposure cannot be excluded in birds and could have a dramatic impact on flocks of migrating birds.

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