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Highlighted article

# Metal levels in the bones and livers of globally threatened marbled teal and white-headed duck from El Hondo, Spain

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ABSTRACT

El Hondo is a key area for marbled teal and white-headed duck. We present Pb, Cu, Zn, Se, and As data for bone and liver in birds found dead between 1996 and 2001. Several metals were higher in adult white-headed ducks than in marbled teal. They were higher in female than in male white-headed ducks, and did not differ with sex in marbled teal, but did by age. Lead in liver of adults was influenced by Pb shot ingestion, which was detected in 21% of marbled teal and in 71% of white-headed duck. No marbled teal had liver levels indicative of Pb poisoning, while 86% of white-headed ducks did. Selenium, Zn, and Cu were elevated in 13%, 7%, and 39% of birds, respectively. Whilst Pb shot poses the greatest threat to these species, further work should assess exposure via plants, invertebrates, water, and sediments for other metals, and investigate possible sub-lethal effects.



## 1. Introduction

Global populations of marbled teal (*Marmaronetta angustirostris*) and white-headed duck (*Oxyura leucocephala*) have suffered from historic and ongoing long-term declines due to a widespread loss of habitat, and are now globally endangered. The marbled teal is currently classed as Vulnerable by the IUCN (Birdlife International, 2006a). Prior to 1991 the global population was estimated at 34,000–40,000 birds, but recent data suggests just 14,000–26,000 now remain. White-headed duck is classed as Endangered by the IUCN, having undergone a 50% decline in its global population in the 10–15 years prior to 2006. The global population in 1991 was around 19,000 birds but this probably stands at less than 10,000 now (Green and Anstey, 1992; Birdlife International, 2006b).

Whilst global populations of these species have declined, Spanish populations of marbled teal have been relatively stable, and white-headed duck in Spain has increased over the last 20–30 years (Birdlife International, 2006a, b). A key stronghold for these species in Spain is El Hondo, a wetland in the Valencian Autonomous Community designated as a Ramsar site (Troya and Bernués, 1990), Natural Park and Special Protection Area (SPA). At any one time, this area has often held most of the European population of one or both species, making it hugely important (Green and Navarro, 1997; Torres and Moreno-Arroyo, 2000; Madroño et al., 2004). However, this wetland is fed by the Segura River watershed, which due to agricultural pollution, is one of the most eutrophic in Spain (Cobelas et al., 1992). El Hondo is similarly eutrophic, and low rainfall and high summer temperatures also cause seasonally high salinity in the wetland (Rodrigo et al., 2001; Colmenarejo et al., 2007). Further, the wetland has experienced periodic episodes where avian mortality has increased markedly (1690 birds in 1999 for example; León-Quinto et al., 2004), but the reason for this remains unclear.

Lead poisoning from residual Pb shot used in hunting has long been a significant cause of mortality for a range of waterbird species in El Hondo, and many other Spanish wetlands

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(Mateo et al., 1998, 2001). Waterbirds consume residual Pb shot deposited in sediments of wetlands that have been used for hunting when they mistake the shot for grit particles (and possibly seeds) that they use in their gizzard to aid in the physical breakdown of food items (Mateo et al., 2000; Figuerola et al., 2005). Wetlands in Valencia, including El Hondo, have been shown to contain some of the highest densities of sedimentary shot reported globally. Mateo et al. (1998) recorded 163 Pb shot $m^{-2}$ in El Hondo; and the prevalence of Pb shot ingestion in *Oxyura* sp. (*O. leucocephala* and *O. jamaicensis*) shot by hunters in Valencian wetlands was 32% (Mateo et al., 2001). Svanberg et al. (2006) found that 71% of white-headed duck and 20% of marbled teal found dead in El Hondo between 1996 and 2001 had ingested Pb shot, and using Pb isotope analysis showed that the main source of Pb for these birds in El Hondo was spent shot. Epizootics of Pb poisoning in greater flamingos (*Phoenicopterus roseus*) have frequently occurred in El Hondo (Mateo et al., 1997).

Waterfowl may also be exposed to pollutants that are deposited in and associated with sediments in wetlands, as they consume sediment when taking food. This is especially true for species feeding on benthic invertebrates or plants, rather than for species that feed on items within the water column or floating on the water surface. For example, Beyer et al. (1999) reported that soil ingestion by herbivorous grazing ducks was 3%, whilst granivorous filtering species ingested $<1\%$. In certain scenarios metals may also become concentrated on submerged plant surfaces within the water column (Beyer and Day, 2004), or on plant material within soil or sediments such as plant roots, rhizomes and bulbs, which are also food components for some waterfowl (Taggart et al., 2005).

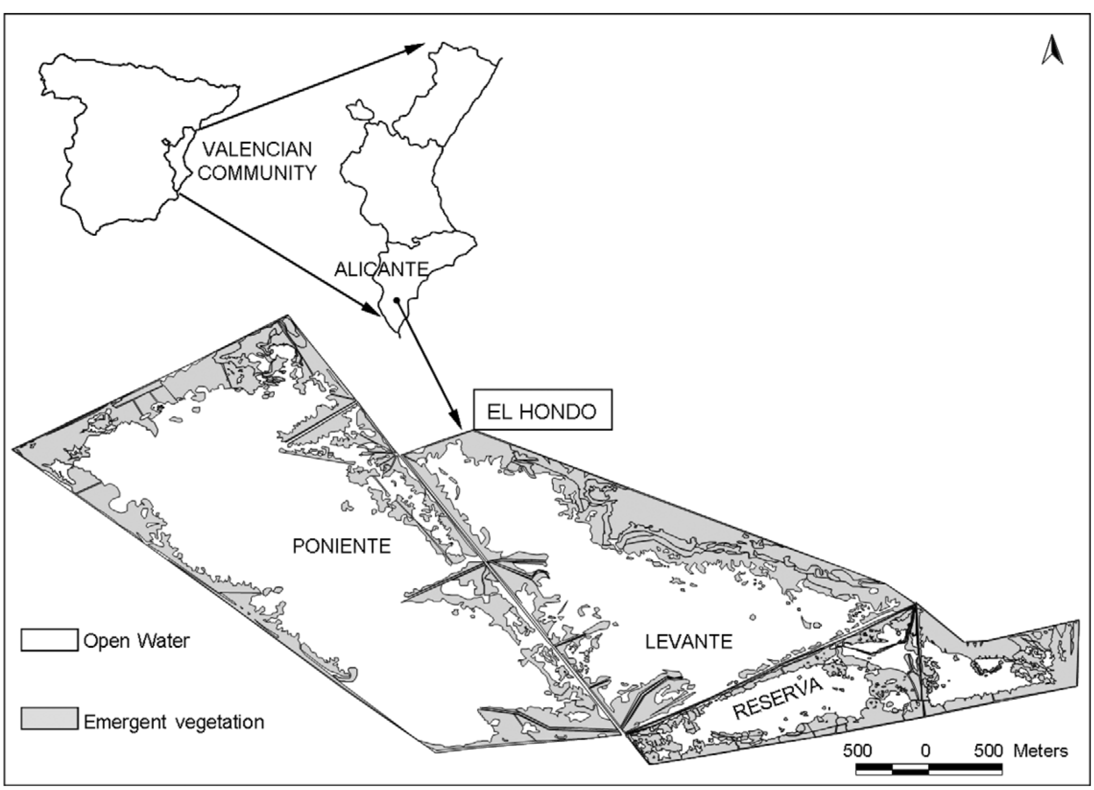
Despite existing information regarding Pb shot ingestion and Pb in tissues of white-headed duck and marbled teal from El Hondo (Mateo et al., 2001; Svanberg et al., 2006), there are no previous studies regarding exposure to a wider range of metals in these species in El Hondo, or elsewhere. Here, data on As, Se, Cu, Zn, and Pb in white-headed duck and marbled teal bone and liver is presented, for birds found dead in El Hondo between 1996 and 2001. Liver and bone were used in order to give an indication of both short- and long-term exposure. Data are analysed and discussed in terms of toxicity towards these species, and in relation to differences found by sex, age, species of bird, and in light of whether or not the birds were found to have ingested Pb shot. Whilst most of the tissue samples used were the same as those used by Svanberg et al. (2006) who studied Pb isotopes in detail, the data presented here are substantially different in terms of both their content and focus.

## 2. Methods

### *2.1. Source of birds studied*

Birds were collected dead or moribund in El Hondo (38°11′N, 00°45′W), Spain, between 1996 and 2001 (Map 1). The birds were not the same individuals as those in Mateo et al. (2001), but were those utilised in Svanberg et al. (2006) plus an additional 18 marbled teal. Thirty-nine marbled teals (from a total of 85), and all 36 white-headed ducks studied were fully grown (considered as adults from hereon). Twenty-nine small marbled teal chicks ($<8$ days old) and 17 juveniles (defined as 8–100 days old) were studied (using a modified age class based on Green, 1998b).

All birds were frozen shortly after collection and sent to the Centre for the Study and Protection of Nature, El Saler, Valencia, for necropsy. Cause of death for all adult and juvenile birds studied was reported as traumatism due to illegal shooting, lead poisoning, or unknown. All marbled teal chicks studied died after becoming trapped in concrete-lined irrigation ditches that run through El Hondo (Svanberg et al., 2006). Birds were sexed by careful visual inspection of the reproductive organs. The presence or absence of Pb shot in the gizzard was determined by X-ray in




**Map 1.** Map of El Hondo, in Alicante, South Eastern Spain (38°11′N, 00°45′W, 1650 ha in extent).

**Table 1**
Detection limits and mean percentage recovery with relative standard deviation, of spikes and certified reference materials

| | As | Pb | Zn | Se | Cu |
|---|---|---|---|---|---|
| Spike recovery % ($n = 5$) | 92±6 | 104±4 | 93±5 | 82±3 | 95±4 |
| Detection limit (in tissue, $n = 6$) | 0.004 | 0.281 | 2.26 | 0.010 | 1.79 |
| Bone standard value (% recovery, $n = 5$) | 0.006[a] (77±15) | 1.335[b] (87±7) | 147[b] (95±3) | 0.13[a] (75±8) | 0.8[a] (nd) |
| Liver standard value (% recovery, $n = 5$) | 0.05[a] (62±8) | 0.129[b] (nd) | 127[b] (99±7) | 0.73[b] (80±10) | 160[b] (98±6) |

Bone meal standard (SRM1486); bovine liver standard (SRM1577b)—standard/detection limit values given in $mg\,kg^{-1}$.
nd shown where the standard value is less than or equal to the detection limit.
[a] Uncertified value data are for information only.
[b] Certified value.

34 marbled teal and 34 white-headed duck adults. Samples of liver and/or bone (femur and/or humerus) were removed and stored in plastic vials. Since most birds were found some time after death, a number were partially decomposed and hence livers could not be sampled for all birds. Livers were available for all white-headed ducks, but only 36 of 85 marbled teal.

### 2.2. Analytical methods

In total, 85 marbled teal bones and 36 livers, plus 35 white-headed duck bones and 36 livers were analysed. Livers were analysed fresh (wet weight), whilst bones had marrow removed and were then dried at 60 °C. Approximately 0.25 g of liver or bone (humerus or femur) was removed using a stainless-steel scalpel or stainless-steel bone cutters, weighed to an accuracy of $\pm 0.001$ g, then placed in an acid washed borosilicate digest tube. For chicks, the whole bone was used, which commonly weighed less than 0.25 g. To each digest tube, 2.5 ml of analytical grade 70% nitric acid was added, the tubes covered, and left overnight to digest at room temperature (~20 °C). The following day, 2.5 ml of analytical grade 30% hydrogen peroxide was added and the digest tubes heated in a heating block, up to a final temperature of 120 °C. This temperature was then maintained for 4 h, until the sample was fully digested. The digest solution was decanted into 15 ml polypropylene (PP) centrifuge tubes, and made up to 10 ml with deionised water. These solutions were stored under refrigerated conditions until analysis.

Exactly the same procedure was performed for blank samples (replacing tissue with 1 ml of deionised water), spiked samples (using certified 1000 $mg\,l^{-1}$ stock solutions of metals provided by Fisher Chemicals) and for samples of bone reference material (National Institute of Standards and Technology (NIST), Standard Reference Material (SRM), 1486 bone meal), and liver reference material (NIST, SRM 1577b bovine liver).

Copper and Zn in the digests were determined using a Perkin-Elmer atomic absorption spectrometer (AAS) 100; arsenic and Se were determined using a Perkin-Elmer Hydride Generation AAS 300 system; Pb was determined using a Perkin-Elmer Graphite Furnace AAS 3300 system. Arsenic was analysed after pre-reduction with a solution of 10% potassium iodide, 10% hydrochloric acid, and 5% ascorbic acid; while Se was analysed after acidification with 10% hydrochloric acid. All liver data are presented in $mg\,kg^{-1}$ wet weight (WW), while bone data are presented as $mg\,kg^{-1}$ dry weight (DW).

### 2.3. Statistics and quality control

Statistical analyses were conducted in Minitab Version 13.32, on log-transformed data. Values below the limit of detection (LOD) were considered as one half of the relevant LOD. Differences in metal concentrations between species, by age, sex, and in relation to Pb shot ingestion, were studied using one-way ANOVAs. The variation in metal concentrations were analysed using generalised linear models (GLMs), the results of which are expressed as eta-squared (where $\eta^2$ is the sum of squares for the effect, divided by the total corrected sum of squares). Included in the models were species, age (for marbled teal only: chick, juvenile, or adult), sex, and whether or not birds were found to have ingested Pb shot, as factors. Where information was sufficient, interactions among factors were studied. Models were run which included both species, and separately, by species. Correlation matrices were generated for adult birds, controlling for species and sex, in order to identify possible links between the concentrations within the two tissues, and relationships between them.

Recovery of elements from spikes, bone meal (SRM 1486) and bovine liver (SRM 1577b) certified reference material, is reported in Table 1. Detection limits (back transformed to tissue concentrations) were based on three times the standard deviation of six blanks ran at intervals during the analysis of samples.

## 3. Results

There were significant differences in Zn, Se, and Pb in liver and bone, and Cu in liver, between adult marbled teal and white-headed ducks (Table 2). Levels of these metals, in both tissues, were consistently higher in the white-headed ducks than in the marbled teals. The GLM results (Table 3, model[a]) further showed that for Zn, Se, Pb, and Cu in bone, and for Zn and Pb in liver, there was a significant interaction between species and sex, but not between species and Pb shot ingestion (interaction term not significant in model[b]). Further, none of the marbled teal studied here would be considered to have been clinically poisoned by Pb ($>6\,mg\,kg^{-1}$ WW in liver) but 31 of 36 (86.1%) white-headed duck fall into this category.

In marbled teal adults, metal levels were generally found to be independent of sex (Table 4), although a significant effect was found for Cu in bone in the GLM results in an analysis including both sexes and just two age classes, juveniles and adults (Table 3, model[d]). Although significant differences between sexes were not found for marbled teal, in general, mean metal levels were higher in males than in females in this species. In contrast, there were statistically significant differences between adult male and female white-headed ducks in relation to Zn and Pb in liver, and Zn, Pb, Se, and Cu in bone (Table 4). Levels of these metals were all significantly higher in females than they were in males.

Levels of metals could not be analysed with respect to age class for white-headed ducks (all samples were from adults). However, for marbled teal significant differences between ages were found for all metals in bone, and for Cu and As in liver (Table 5). In liver, for both Cu and As, concentrations increased with age, as was the case for Pb in bone. In contrast, As, Zn, Se, and Cu were significantly higher in bones of younger birds than adults.

The presence of Pb shot in the gizzard of adult birds was related to significantly higher Pb in the liver of white-headed duck

**Table 2**
Geometric mean (range) and 95% CIs of concentrations (mg kg$^{-1}$) of metals in liver and bone of adult marbled teal and white-headed duck from El Hondo, Spain

| Liver | N | As~ | Zn*** | Se* | Pb*** | Cu*** |
|---|---|---|---|---|---|---|
| Marbled teal | 18 | 0.012 (nd-0.136) | 54.5 (31.1–120.3) | 1.47 (0.37–3.39) | 0.45 (nd-4.78) | 41.9 (3.3–105.4) |
| *Marmaronetta angustirostris* | | 0.008–0.019 | 46.1–64.4 | 1.10–1.97 | 0.28–0.74 | 28.9–60.8 |
| White-headed duck | 36 | 0.009 (nd-0.034) | 110.4 (32.1–229.7) | 2.08 (1.01–3.97) | 16.37 (nd-56.97) | 156.5 (14.1–751.0) |
| *Oxyura leucocephala* | | 0.007–0.012 | 95.9–127.1 | 1.86–2.34 | 10.18–26.33 | 118.8–206.0 |
| Bone | N | As~ | Zn* | Se** | Pb*** | Cu~ |
| Marbled teal | 39 | 0.015 (nd-0.073) | 126.5 (98.9–211.9) | 0.60 (0.13–1.60) | 5.19 (0.54–328.33) | 3.0 (nd-43.9) |
| *Marmaronetta angustirostris* | | 0.011–0.020 | 118.7–134.9 | 0.49–0.74 | 3.19–8.45 | 2.5–3.5 |
| White-headed duck | 35 | 0.015 (nd-0.256) | 156.6 (101.5–192.9) | 0.86 (0.28–1.81) | 91.75 (1.22–419.16) | 2.7 (nd-7.3) |
| *Oxyura leucocephala* | | 0.010–0.021 | 149.7–163.8 | 0.76–0.98 | 59.63–141.15 | 2.4–3.0 |

* $<0.05$, ** $<0.01$, *** $<0.001$, ~not significantly different by species, using one-way ANOVA.
nd—below limit of detection (LOD). Values below LOD were replaced by half the LOD in statistics.

**Table 3**
Results of GLMs (general linear models) for marbled teal and white-headed duck from El Hondo, Spain (proportion of variance in concentrations as explained by partial effects of different factors, and expressed as $\eta^2$)

| | Liver | | | | Bone | | | |
|---|---|---|---|---|---|---|---|---|
| | Zn | Se | Pb | Cu | Zn | Se | Pb | Cu |
| *All adults* | | | | | | | | |
| Species[a] | 0.396***w | 0.103*w | 0.620***w | 0.359***w | 0.259**w | 0.227**w | 0.567***w | ~ |
| Sex[a] | ~ | ~ | ~ | 0.076*m | ~ | ~ | ~ | ~ |
| Species × sex[a] | 0.111** | ~ | 0.062** | ~ | 0.168*** | 0.061* | 0.075** | 0.126* |
| Species[b] | 0.393***w | ~ | 0.604***w | 0.340***w | 0.284**w | 0.269**w | 0.566***w | ~ |
| Pb shot[b] | ~ | ~ | 0.055*i | ~ | ~ | ~ | ~ | ~ |
| *White-headed duck adults* | | | | | | | | |
| Sex[c] | 0.261*f | ~ | 0.225*f | ~ | 0.369***f | 0.198**f | 0.396***f | ~ |
| Pb shot[c] | ~ | ~ | ~ | ~ | ~ | ~ | ~ | ~ |
| *Marbled teals (juvenile and adult)* | | | | | | | | |
| Age[d] | ~ | ~ | ~ | ~ | ~ | ~ | ~ | 0.177*a |
| Sex[d] | ~ | ~ | ~ | ~ | ~ | ~ | ~ | 0.172*m |

Arsenic models produced no significant results and are not shown.
* $<0.05$, ** $<0.01$, *** $<0.001$, ~ means not significant. Superscripts relate to which group had the higher metal values; white-headed ducks[w], female[f], male[m], with ingested shot[i], adult[a]. Values below LOD were replaced by half the LOD in statistics.
Separate models are dilineated by the lines within the table and the superscript letters. Not all model options were possible due to a lack of overlap between certain factors. In model[b] interaction terms between species × Pb shot were not significant, as was the case for sex × Pb shot in model[c], and age × sex in model[d].

**Table 4**
Geometric mean and 95% CIs of concentrations (mg kg$^{-1}$) of metals in liver and bone of adult male and female marbled teal and white-headed duck from El Hondo, Spain

| Marbled teal | Liver | | | Bone | | |
|---|---|---|---|---|---|---|
| | Female (9) | Male (9) | *p* | Female (16) | Male (22) | *p* |
| As | 0.014 (0.008–0.023) | 0.013 (0.006–0.027) | ~ | 0.013 (0.008–0.021) | 0.014 (0.009–0.020) | ~ |
| Zn | 47.6 (40.6–55.7) | 64.3 (47.4–87.3) | ~ | 134.4 (123.5–146.2) | 147.5 (135.3–160.7) | ~ |
| Se | 1.49 (1.00–2.21) | 1.73 (1.23–2.43) | ~ | 0.52 (0.39–0.68) | 0.63 (0.48–0.84) | ~ |
| Pb | 0.30 (0.18–0.51) | 0.73 (0.31–1.73) | ~ | 3.95 (2.05–7.62) | 6.03 (3.06–11.89) | ~ |
| Cu | 30.3 (16.3–56.1) | 54.0 (38.9–74.9) | ~ | 2.5 (2.1–3.1) | 3.4 (2.6–4.4) | ~ |
| White-headed duck | Female (26) | Male (9) | | Female (25) | Male (9) | |
| As | 0.010 (0.008–0.013) | 0.007 (0.004–0.013) | ~ | 0.014 (0.010–0.021) | 0.012 (0.007–0.020) | ~ |
| Zn | 124.6 (112.9–137.5) | 75.5 (50.2–113.4) | ** | 164.6 (158.4–171.1) | 136.6 (124.5–150.0) | *** |
| Se | 2.03 (1.79–2.30) | 2.09 (1.59–2.75) | ~ | 0.96 (0.85–1.09) | 0.65 (0.48–0.89) | ** |
| Pb | 24.81 (18.68–32.95) | 5.12 (1.11–23.55) | ** | 143.90 (108.94–190.08) | 23.30 (7.84–69.27) | *** |
| Cu | 130.9 (107.5–159.4) | 230.4 (94.9–559.4) | ~ | 2.9 (2.6–3.2) | 2.2 (1.8–2.8) | * |

* $<0.05$, ** $<0.01$, *** $<0.001$, ~means not significantly different by sex, using one-way ANOVA.
Values below LOD were replaced by half the LOD in statistics.
*n* values are given in brackets after sex.

(mean = 24.86 versus 6.35 mg kg$^{-1}$; $p<0.05$; one-way ANOVA) but not marbled teal when controlling for species. The presence of Pb shot was not significantly related to bone Pb in either species. Neither was the presence of Pb shot significantly related to the concentration of any of the other metals studied. Whilst presence of Pb shot was a significant factor in the GLM for Pb in liver when including adults of both species (Table 3, model[b]), in white-headed ducks alone it was not a significant factor (Table 3, model[c]). Here, sex appeared to be the dominant factor influencing liver Zn and Pb, and bone Zn, Se, and Pb. For Pb, this effect is likely to be related to the fact that there is a higher prevalence of Pb shot ingestion in females (80%) than in males (44%; the reverse was true for marbled teal) although the interaction term in this model also showed no significant effects. Seven of 34 (21%) marbled teal adults were found to have Pb shot in the gizzard, and in all these cases, only 1 shot was present. Twenty-four of 34 (71%) white-headed duck adults had shot in the gizzard, 16 had 1 shot, 3 had 2, 1 had 3, 2 had 5, and 2 had 6. The number of Pb shot was not correlated with any of the metal levels in either tissue in marbled teal alone and was only weakly correlated to liver As in white-headed duck alone ($r = 0.340$, $p < 0.05$). However, in an analysis combining both species, the number of Pb shot in the gizzard correlated with Pb and Zn in liver ($r = 0.424$, $p < 0.01$; $r = 0.368$, $p < 0.01$, respectively) and Pb in bone ($r = 0.434$, $p < 0.001$).

Correlation matrices (Table 6) revealed the presence of significant relationships between a number of metals in both tissues, and between both tissues. However, since the duration/intensity of exposure to these metals is not known, these correlations should be interpreted with a degree of caution. Nevertheless, notably, correlations between Pb and Zn within and between tissues were clearly present in white-headed ducks, but not in marbled teals (see Fig. 1; where the overall $r^2 = 0.421$).

## 4. Discussion

### *4.1. Differences in metal levels between species*

For most metals, white-headed ducks had higher levels in liver and bone than marbled teal (Table 2), and our results suggest that these differences are not specifically related to the presence or absence of Pb shot in the gizzard (with the exception of Pb levels in liver). Since the differences seen are expressed in bone as well as liver, the trends noted reflect a long-term difference in exposure and/or degree of accumulation of these metals. Exposure will predominately be via the food and water sources utilised by these species, and via the ingestion of non-food items such as sediment and grit (and Pb shot). Marbled teal tend to feed at the water surface (Green, 1998a) and compared to other European ducks its diet contains a particularly high proportion of seeds (Green and Sánchez, 2003). Seeds comprised 72% of material ingested by adults in El Hondo (Fuentes et al., 2004), and these were mainly *Scirpus litoralis* seeds eaten as they floated on the water, while invertebrates made up 21% of gut contents. In contrast, in white-headed duck (plus north American ruddy duck (*O. jamaicensis*) and hybrids of the two species) from Spain (mainly from El Hondo), invertebrates constituted 73% of gut contents, with benthic chironomid larvae and pupae being the most common food item (Sanchez et al., 2000). This constitutes an important difference in feeding behaviour. White-headed ducks feed at depth (Green et al., 1999) by diving and are more likely to consume both Pb shot, and perhaps importantly in terms of long-term continuous exposure to metals other than Pb, sediment particles associated with benthic fauna. Likewise, benthic invertebrates in themselves may contain elevated metal levels as they have the potential to bioaccumulate all of the metals studied here (Flinders, 2006). In contrast, marbled teal are less exposed to all of these potential sources of metals because of their feeding behaviour. They also tend to ingest smaller grit particles than white-headed ducks, and hence, less Pb shot (Mateo et al., 2001).

**Table 5**
Geometric mean and 95% CIs of concentrations ($mg\,kg^{-1}$) of metals in liver and bone of marbled teal of various age groups in El Hondo, Spain

| Liver | Adult (18) | Juvenile (5) | Chick (13) | *p* |
|---|---|---|---|---|
| As | 0.012 (0.008–0.019) | 0.005 (0.002–0.010) | 0.004 (0.002–0.006) | ** |
| Zn | 54.5 (46.1–64.4) | 50.3 (45.2–56.0) | 52.8 (47.4–58.9) | ~ |
| Se | 1.47 (1.10–1.97) | 1.14 (0.58–2.26) | 1.39 (1.25–1.54) | ~ |
| Pb | 0.45 (0.28–0.74) | 0.26 (0.10–0.67) | 0.26 (0.16–0.42) | ~ |
| Cu | 41.9 (28.9–60.8) | 37.0 (25.4–53.9) | 7.3 (5.8–9.1) | *** |
| **Bone** | **Adult (39)** | **Juvenile (17)** | **Chick (29)** | ***p*** |
| As | 0.013 (0.010–0.018) | 0.007 (0.004–0.012) | 0.030 (0.016–0.056) | ** |
| Zn | 143.3 (134.4–152.7) | 167.6 (154.8–181.4) | 200.5 (212.9–188.8) | *** |
| Se | 0.60 (0.49–0.73) | 0.96 (0.77–1.20) | 1.58 (1.23–2.01) | *** |
| Pb | 5.41 (3.33–8.81) | 1.51 (0.75–3.04) | 1.08 (0.70–1.66) | *** |
| Cu | 3.0 (2.6–3.6) | 2.8 (2.0–3.8) | 4.6 (3.5–5.9) | * |

*<0.05, **<0.01, ***<0.001, ~ means not significantly different by age, using one-way ANOVA.
Values below LOD were replaced by half the LOD in statistics.
*n* values are given in brackets after age group.

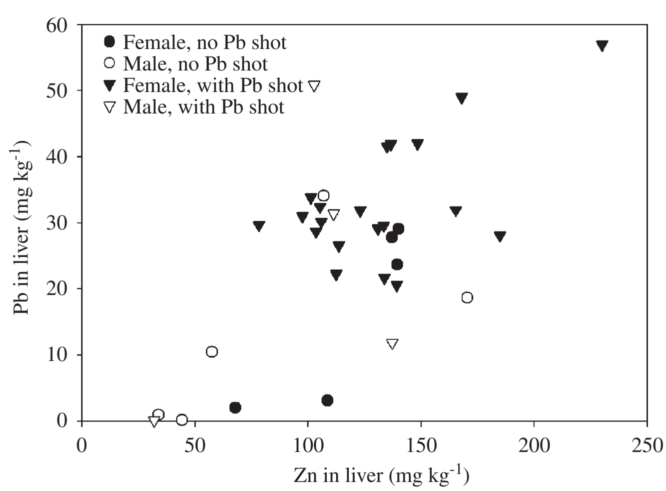



**Fig. 1.** Liver Pb and Zn concentrations in adult white-headed duck from El Hondo in relation to sex, and whether or not birds were found to have ingested Pb shot in the gizzard at necropsy.

**Table 6**
Results of correlation matrices for adult birds, showing where significant correlations were present when controlling for species and sex

| | Liver | Bone | Liver:bone |
|---|---|---|---|
| Marbled teal[males] | | Zn:As**; Zn:Se* | Se:Se***; (Cu:Se*) |
| Marbled teal[females] | Se:Cu***; (As:Se, Cu**) | As:Cu** | Se:Se**; Cu:Se*; As:Cu*; (As:Se*) |
| White-headed duck[males] | Zn:As, Se, Pb**; Se:Pb, Cu* | Cu:As, Zn* | As:Zn**; Cu:As, Cu*; Zn:Pb**; Pb:Pb**; Se:Pb*** |
| White-headed duck[females] | Pb:Zn** | Pb:Zn** | (Cu:Pb*) |

Correlations in brackets were negative (*p* values, *<0.05, **<0.01, ***<0.001).

### 4.2. Differences in metal levels between sexes

Whilst the differences in most metal levels between species are not generally well related to Pb shot ingestion, they may be linked to sexual differences (Tables 3 and 4). While white-headed ducks show higher levels of metals than marbled teals, female white-headed ducks commonly had significantly higher metal levels than males, and the reverse was true for marbled teal (although in this species differences were not significant). Sanchez et al. (2000) found no significant difference between the diet of male and female white-headed duck in Spain, although such differences can not be ruled out as they are common in ducks (Krapu and Reinecke, 1992). Gómez-Serrano and Rioja (2004) found that female white-headed ducks in El Hondo commonly ingested more Pb shot than males which indicates that there are at least some differences in feeding behaviour. In this study, 7% of female and 32% of male marbled teal had ingested Pb shot, while 80% of female and 44% of male white-headed duck had done so. This trend fits the trend in metal concentrations observed here by sex, assuming Pb shot ingestion would also cause, directly or indirectly, elevated tissue levels of metals other than Pb. However, the statistics provide unconvincing evidence that this is the case since the presence of Pb shot is unrelated to the levels of all but Pb in liver, and since consistent correlations between Pb and the other metals are not shown, especially for marbled teal.

Although Pb shot ingestion may be playing an underlying role in terms of differences in levels of metals other than Pb in white-headed duck by sex, it is also worth considering alternative scenarios. It remains possible that females are ingesting more metals because of dietary differences. The differences we see could also be related to the way in which, and the rate at which, metals are metabolised, become partitioned into different tissues, and are excreted by the two sexes (based on underlying hormonal and genetic controls). For example, metallothioneins (MTs) form important complexes with many metals in birds and are thought to act to reduce the toxicity of otherwise free metal ions. Significant differences have been noted in the degree of synthesis of MTs by sex in some studies (Debacker et al., 2001), but others have seen no measurable difference between the sexes (Geffard et al., 2007). In female birds, metal turnover in bone is more rapid than it is in males and levels may therefore reflect a shorter period of exposure than they do in males. This is related to increased turnover of calcium stored in bones which is actively re-metabolised during periods of egg laying when calcium requirements are elevated. Female birds are unique amongst vertebrates in that they produce medullary bone within their long bones, which fills the space normally taken up by marrow with a weave of bone "spicules" or spikes. This can then act as a reservoir for minerals required during periods of egg laying (Dacke et al., 1993). Adult birds in this study were collected all year round, within and outside the breeding season, however, Zulauf-Fischer et al. (2006) has noted that birds may also lay down medullary bone outside the breeding season. Differences in the rate of metabolism and excretion of metals may also be affected by the differential production of melanin-based plumage coloration between the sexes, in that the increased expression of certain coloration may be an indication of an increased ability to excrete certain metals to generate particular feather colours (Niecke et al., 2003). In a review of metal levels in vertebrates (Burger, 2007), 30 of 43 studies showed levels tended to be higher in females than in males, and in waterbirds Taggart et al. (2006) also found higher As, Zn, and Pb in livers from females than from males. In contrast, Braune et al. (2005) found higher levels of Hg, Cu, and Se in male long-tailed ducks (*Clangula hyemalis*) than in females, and Warren et al. (1990) determined the same trend for Cr, Ni, Pb, and Cu in blue-winged teal (*Anas discors*). As yet, however, such mechanisms and controls over variations in metal concentrations observed by sex in numerous studies are still poorly understood.

### 4.3. Differences in metal levels between age groups

In marbled teal, patterns in metal levels were noted in relation to age, whereby liver As and Cu, and bone Pb increased with advancing age. In the case of Pb in bone, this increase is likely to reflect cumulative exposure to Pb, due to long-term consumption of Pb shot (Svanberg et al., 2006). Similar trends in relation to the accumulation of Pb in bone with advancing age have also been noted elsewhere (Merchant et al., 1991), whilst other studies have not seen age-related trends (Merendino et al., 2005). Chicks (in this case $<8$ days old) are unlikely to consume Pb shot, however, with advancing age and size, the likelihood of ingesting shot may increase, and bone tends to act as a long-term deposition site for Pb (Pain, 1996). Conversely, the low level of Cu in chick liver may reflect an increased turnover and requirement for this essential element during the earliest developmental stages. Swaileh and Sansur (2006) also observed accumulation of Cu in liver with advancing age in house sparrow (*Passer domesticus*). For As, there is a marked elevation in chick bones in comparison to older birds, as was also noted in Taggart et al. (2006). Arsenic (specifically arsenate) is a chemical analogue of phosphate, a primary component in bone, and perhaps in chicks the increased requirement and metabolism of phosphate as bones grow rapidly is reflected as a depletion in chick liver As and an enhancement in chick bone As (as arsenate is mistaken for phosphate in apatite, the mineral constituent of bone). Zinc, Se, and Cu in bone all decreased with advancing age in this study, which again may reflect a rapid deposition of essential elements at an early life stage, followed by a more efficient turnover of these elements in later life and a net dilution in bones as they grow. Taggart et al. (2006) also noted lower bone Zn in adult waterbirds compared to young birds, while Agusa et al. (2005) noted the same trend for Se in black-tailed gulls (*Larus crassirostris*).

### 4.4. Metal levels in relation to toxicity thresholds

Pain (1996) considered background concentrations of Pb in liver tissue to be $<2\,mg\,kg^{-1}$ (WW), and in bone to be $<10\,mg\,kg^{-1}$ (DW). The ecotoxicological effects of elevated lead in birds are well described (Burger, 1995; Burger and Gochfeld, 2000; De Francisco et al., 2003). Here, one of 36 (2.8%) marbled teals had liver Pb levels above this (at $4.78\,mg\,kg^{-1}$, but without having Pb shot in the gizzard), whilst 5 of 19 (26.3%) had Pb shot in their gizzards. In bone, 11 of 85 (12.9%) marbled teals had Pb above $10\,mg\,kg^{-1}$ (at $15.56–328.33\,mg\,kg^{-1}$), but 30.0% had Pb shot in the gizzard. In total, 8 of 50 (16.0% of all ages) marbled teals studied had Pb shot in their gizzard. For white-headed duck, the relationship between the presence of Pb shot and elevated Pb was more clear. For 36 livers, 33 (91.7%) were from birds with liver Pb levels $>2\,mg\,kg^{-1}$ ($2.02–56.97\,mg\,kg^{-1}$), and 74.2% were found to have 1 or more Pb shot in the gizzard. In bone, 32 of 35 (91.4%) birds had bone Pb levels above $10\,mg\,kg^{-1}$ ($11.97–419.16\,mg\,kg^{-1}$) and 73.3% had Pb shot in the gizzard. While none of the marbled teals studied would be considered to have been clinically poisoned by Pb ($>6\,mg\,kg^{-1}$ WW in liver), in the case of white-headed duck, 31 of 36 (86.1%) animals studied would fall into this category. For Spain, Mateo et al. (2001) determined that 20% and 80% of marbled teal and white-headed ducks, respectively, had lethal liver Pb levels upon death; and, as suggested by this study, Mateo et al. (2001) also noted that Pb poisoning (from Pb shot) was more likely to be a cause of mortality for white-headed duck than for marbled teal. Svanberg et al. (2006) has also shown using

Pb isotopes that Pb shot is probably the main source of Pb for these species in El Hondo. The relationship between the presence of Pb shot and tissue levels is unclear in this study for marbled teal. In the case of white-headed duck, the apparent relationships may be significant but weak simply because such a high proportion of animals had Pb shot in the gizzard and/or high tissue levels. For marbled teal, however, this is not the case, and in certain individuals high liver Pb is present without ingested shot, and visa-versa. Such birds may have died very quickly after shot ingestion (before tissue levels rose) or may have expelled shot that had caused high tissue levels (in feces) before they died. Likewise, significant differences between species may exist in terms of susceptibility to poisoning with Pb shot (Kendall et al., 1996). Here, 26% of marbled teal had ingested Pb shot, yet liver Pb levels were consistently low. This may reflect an ability to minimise Pb uptake and toxicological effect (perhaps via increased synthesis of MTs), or conversely, marbled teal may be highly susceptible to Pb and may die very quickly after exposure.

For Se, an essential trace element for animals, 9 of 72 (12.5%) of all birds studied had levels in liver $>3\,\mathrm{mg\,kg^{-1}}$, considered by Heinz (1996) to be high enough to potentially impair reproduction in female birds. All of these birds were adults, 7 of 9 were white-headed ducks and 4 of these 7 were females (all others, including the 2 marbled teal, were male). The source of selenium for these birds is unknown. In the western USA, elevated selenium has been found to occur naturally in fine-grained marine sedimentary rocks from the Late Cretaceous. Agricultural irrigation practices in areas overlying these rocks are known to have caused Se laden water to enter wetland areas and very significant toxic effects on wildlife have occurred (Presser and Ohlendorf, 1987; Naftz et al., 1993; Presser et al., 1994). The underlying geology near El Hondo is based on cenozoic and meso-cenozoic clay and limestone deposits (Jorge and Fumanal, 1996) but we are unaware of any detailed information on levels of Se in plants, invertebrates, waters, soils or sediments in El Hondo (or in the surrounding agricultural land). Selenium levels in Pb shot are negligible (Mann et al., 1994; Rasberry, 1998) and the GLM data does not indicate that the ingestion of Pb shot is directly related to Se levels. Selenium and Pb are weakly correlated (at $p<0.05$, Table 6) in the livers of white-headed duck males (and between liver and bone in the same group).

For Cu (also essential), 28 of 72 (38.9%) animals had liver levels above $100\,\mathrm{mg\,kg^{-1}}$ WW, and all but 1 of these was a white-headed duck. This is above the level that has, in a study of Canada geese (*Branta canadensis*), indicated acute Cu poisoning (187–323 mg $\mathrm{kg^{-1}}$ DW, Henderson and Winterfield, 1975). All these animals were adults, and the 5 with the highest levels were males with over $400\,\mathrm{mg\,kg^{-1}}$ WW (approximately $1400\,\mathrm{mg\,kg^{-1}}$ DW). The maximum level noted here was over $750\,\mathrm{mg\,kg^{-1}}$ WW (approximately $2625\,\mathrm{mg\,kg^{-1}}$ DW). In comparison to previous studies these levels are certainly quite elevated, but not unprecedented. Mateo and Guitart (2003) reported a DW range between 0.84 and $599\,\mathrm{mg\,kg^{-1}}$ for 13 waterfowl species from 5 Spanish wetlands, while Hernández et al. (1999) reported 2.05–1298 $\mathrm{mg\,kg^{-1}}$ DW in livers of birds affected by the Aznalcóllar mine spill in 1998. Likewise Taggart et al. (2006) reported 7.3–743 $\mathrm{mg\,kg^{-1}}$ WW liver Cu in birds just 2–3 months after the same mine spill, and in Japan, Horai et al. (2007) has noted particularly high levels of Cu, comparable to those noted here, in grey heron (*Ardea cinerea*; up to $4970\,\mathrm{mg\,kg^{-1}}$ DW) and intermediate egret (*Egretta intermedia*; up to $2420\,\mathrm{mg\,kg^{-1}}$ DW). In the correlation matrices (Table 6), there were no relationships found between Cu and Pb (except for a negative one between liver Cu and bone Pb in white-headed duck females) which again indicates that Cu exposure is not related to Pb shot. This may indicate that there is a significant alternative source of Cu in this system, or that white-headed duck has an elevated requirement or tolerance for Cu, as observed in other waterfowl species (Mateo and Guitart, 2003). It is unclear at this stage what the cause of the highly elevated liver Cu concentrations are, but further work is advised to identify whether there is an underlying geological reason, an agricultural or industrial source of Cu pollution near El Hondo, or whether key invertebrate food items are accumulating Cu to unusual levels. Alternatively, perhaps white-headed duck males have a propensity for accumulating Cu, and this may be in association with elevated MTs induced to reduce the toxic effects of the elevated Pb these birds are being exposed to.

Five of 72 (6.9%) birds had Zn levels above $150\,\mathrm{mg\,kg^{-1}}$ WW (around $525\,\mathrm{mg\,kg^{-1}}$ DW), although there is no consensus in the literature as to the level of Zn indicative of poisoning. Carpenter et al. (2004) reported that a trumpeter swan (*Cygnus buccinator*) that appeared to have died of Zn poisoning had $154\,\mathrm{mg\,kg^{-1}}$ WW in liver, and as such, a threshold guideline of $150\,\mathrm{mg\,kg^{-1}}$ WW may actually be conservative. Likewise, Levengood et al. (1999) and Sileo et al. (2003) found signs of zinc poisoning in birds with liver concentrations as low as 473 and $280\,\mathrm{mg\,kg^{-1}}$ DW, respectively. For Zn, our results suggest that for white-headed duck at least, the increase in Zn may be linked in some way to Pb exposure (i.e., to Pb shot). Lead and Zn were positively correlated in white-headed duck livers of both sexes, and in bones of females (see results plotted in Fig. 1 for liver). However, no such relationships were observed for marbled teal. Zinc exposure is probably not directly related to Pb shot exposure because Pb shot would normally contain only trace amounts of Zn (Mann et al., 1994; Rasberry, 1998). Whether Pb is inducing increased production of MTs which are then accumulating Zn is a question for further work. Moreover, the geochemical behaviour of weathered fragments of Pb shot in sediment may also be important since particles could potentially act as concretion sites for other metals. Upon ingestion metal-rich oxide coatings on shot fragments may well be preferentially soluble in the digestive tract.

In terms of non-essential arsenic, levels below $0.5\,\mathrm{mg\,kg^{-1}}$ WW might be considered normal (Geode, 1985), and none of the birds studied here reached this level. Likewise As does not appear to be entering the food chain in association with Pb shot since no relationships were seen between Pb shot ingestion and As, or Pb and As levels in the correlation matrices (Table 6). This is despite the fact that As has historically been added to Pb shot at levels of up to 0.2% to enhance sphere formation of lead during manufacture (Mann et al., 1994).

## 5. Conclusions

Although our study did not use a random population sample (since it utilised birds that were found dead), it can perhaps give a reasonable indication as to the likelihood of these species dying or suffering ill effects from metals encountered within El Hondo. For both species, but for white-headed duck in particular, the ingestion of Pb shot is very common and as a result, levels of Pb in tissue of this species are abnormally high in the vast majority of birds studied. Since El Hondo is a haven within Europe for this endangered species this is of particular conservation concern. The use of Pb shot was banned in Ramsar sites such as El Hondo in October 2001 (Mateo and Guitart, 2003; although this ban was not fully implemented until 2005 in the wetlands of the Valencia region) but a legacy of historic use still remains in the sediments of many of Spain's important wetlands. Efforts are being made to clean-up El Hondo in terms of residual Pb Shot (Bonet et al., 2004), and such remediation undertaken elsewhere (Dávila and Domínguez, 1999) has apparently made a difference to Pb shot ingestion rates for certain waterfowl (Green et al., 2003; Mateo et al., 2006, 2007).

Whilst Pb may be the most important metal-related threat to marbled teal and white-headed duck in El Hondo, for a significant percentage of individuals, Cu, Zn, and Se levels are also elevated in tissues. There is currently a lack of data regarding the level of other metals associated with weathered Pb shot fragments present in sediments, and likewise, the possibility remains that increased exposure to Pb shot may be triggering elevated synthesis of MTs and that these are then causing increased levels of Zn, Cu, and Se to accumulate in the livers of these birds. Beyond Pb shot, further monitoring of water, sediment quality, and food sources (plant parts and invertebrates) is also recommended in El Hondo so that exposure pathways for these metals can be considered in more detail given the importance of this area for these two endangered species, and for other waterbirds.

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