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VIGILANCE POISON: Illegal poisoning and lead intoxication are the main factors affecting avian scavenger survival in the Pyrenees (France)

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ABSTRACT

A specific surveillance program has been set up to monitor avian scavenger populations in the French Pyrenean Mountains, hosting a high proportion of the French populations. The two main purposes of the study were to identify all causes of death and to investigate poisoning cases. All 170 birds found dead during the 7-year program were submitted to full necropsy, X-Ray, parasitological investigations and consistent analytical toxicology screenings (Cholinesterase inhibitors, anticoagulant rodenticides, organochlorine insecticides, Pb, Cd). Over the study period, 8 Bearded Vultures, 120 Griffon Vultures, 8 Egyptian Vultures and 34 Red kites were eventually collected. Mortality events were often multifactorial, but poisoning was by far the most common cause of death (24.1%), followed by trauma/fall (12%), bacterial diseases and starvation (8%) and electrocution (6%). Illicit use of banned pesticides was identified as a common cause of poisoning (53% of all poisoning cases) and lead poisoning was also identified as a significant toxicant issue (17% of all poisoning cases). Lead isotopic signature could be associated primarily with ammunition. Last, a positive association between trauma and lead contamination was detected, indicating that lead could be a significant contributor to different causes of death. These results urge for severe restrictions on the use of lead ammunition to prevent scavengers from detrimental exposure.

1. Introduction

In Europe, wildlife species suffer from severe anthropic pressure, most notably predator and scavenger species.

In the French Pyrenees, scavenging birds of prey are mostly located in the western part of the mountain range, where major sheep herds can still be found. Scavenger species may feed on dead cattle and sheep, which provide a complementary food source to resources from wild ungulates. The vulture populations on the French Pyrenees have benefited both from populations still well present in northern Spain in the 1970s and from increasing protection measures in France (Terrasse, 1992; Razin and Bretagnolle, 2002).

The central part of the Pyrenees is a natural barrier to all migratory bird species flying to Africa or wintering in Spain, and most of them cross the mountain range at selected passes (Hilgerloh et al., 1992), providing abundant food sources for birds of prey during migration. Among those species, the wood pigeon *Columba palumbus* suffers from severe hunting in fall during migration across the Pyrenees (Jean, 1997; Rouxel and Czajkowski, 2004). Unrecovered bodies (as well as bodies of local game species not collected such as wild ungulates, partridges etc.) may represent an important seasonal food source for some scavenging bird species (Cramp and Simmons, 1980; Margalida and Bertran, 1997; Mateo-Thomas and Olea, 2010; Margalida 2010).

Scavenging birds may sometimes be perceived as “undesirable” species, because of their feeding habits and some reports of vultures feeding on moribund animals (Choisy, 2013). They may also

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be unexpected victims of local use of poisons to eradicate unwanted species such as water voles or even the brown bear (Choisy, 2013). As a consequence, poisoning cases have been identified since 1992 (Berny and Gaillet, 2008) in France in Red kites (*Milvus milvus*), but also in Griffon Vultures (Razin – personal communication) and this has resulted in the development of a program to survey mortality and identify causes of death in order to suggest conservation actions to the French Government responsible for the management of European conservation programs. Similarly, in Spain, there is ample evidence that illicit poisoning and accidental lead exposure from carcasses of game species is very common in scavenging birds of preys (including vulture species) (Margalida, 2012; Margalida et al., 2013). Figures for Spain include at least 53 Bearded Vultures (*Gypaetus barbatus*), 366 Egyptian Vultures (*Neophron percnopterus*) and 2877 Eurasian Griffon Vultures (*Gyps fulvus*) from 1990 to 2010 (Margalida, 2012). No such data could be found for scavenging birds of prey in France.

The objectives of the present study were to determine the causes of death of Pyrenean avian scavengers, and specifically to investigate toxicant exposure as a potential threat for the survival of endangered birds species involved in major European conservation programs.

2. Material and methods

2.1. Study area

The French Pyrenees Mountains stretch over 350 km between the Atlantic Ocean and the Mediterranean see across *ca* 10,000 km^2. It is constituted of abrupt mountain areas with a piedmont area. The highest peaks reach over 3000 m and are separated by deep valleys, in which livestock breeding (pastoralism) and wood production have profoundly affected the landscape. The piedmont area is more devoted to agriculture (livestock breeding, corn production in the center and western parts, orchards and vineyards in the eastern part). Industrial activities are poorly developed in very localized areas. In the XIXth century lead, iron, copper, zinc and silver mining existed and may result in persisting environmental contamination (Cottard et al., 2003; Gourdon 1858; Milian, 2004) cf Fig. 3.

2.2. Selection of bird species and population estimates

Among a wide variety of birds of prey, Bearded Vulture, Griffon Vulture, Egyptian Vultures and Red kite are locally abundant and represent 72%, 57%, 77% and 15–20% of non-migratory French populations respectively (Razin, 2012; Lecuyer and Neouze, 2012; Ponchon et al., 2012; Ballereaud and Tlahoëts, 2012; Mionnay, 2004; Pinaud et al., 2009) (Table 1). The Ligue pour la Protection des Oiseaux (LPO, Bird Life) coordinates a network of volunteers, public institutions (National Parks, National Game and Wildlife Agency, National Forestry Agency), local nature protection associations, hunting associations etc. to monitor Bearded Vultures, Griffon Vultures, Egyptian Vultures and Red kite populations for the Ministry of Environment (Razin, 2002).

Griffon Vulture is the most abundant of the three local vulture species (Bearded Vulture, Griffon Vulture, Egyptian Vulture), markedly in the western part of the Pyrenees (it was not found on the eastern/Mediterranean part before 2008) (Razin et al., 2009). Its diet (mostly remains of carcasses of wild and domestic ungulates) is partially shared with the three other species. The Bearded Vulture is highly specialized and its diet is made of bones (80%) from ungulates. The rest of its diet is constituted of carcasses of birds, reptiles and mammals (rodents, mustelids). The Bearded Vulture is the last scavenger to feed in a group of scavenging birds (Terrasse and Terrasse, 1974). Because Bearded Vultures may be confused with Griffon Vultures, they are potentially exposed to the same threats, mostly from illicit poisoning (Margalida et al., 2001). The diet of the Egyptian Vulture is more diversified. It feeds on remains of ungulates left by other vulture species, on small mammals, on birds, on insects and on waste (Cramp et Simmons, 1980). The Red kite has an opportunistic diet. It may feed on remains left by other scavengers and also hunt small rodents (among which the water vole *Arvicola terrestris* (Cœurdassier et al., 2011), bird species, earthworms, and consume organic wastes issued from intensive breeding units (poultry, rabbits, pigs) or waste management sites (synthesis in Cramp and Simmons, 1980). Because of their diet, the last two species are potentially more exposed to insecticides or rodenticides used in agriculture than the other 2 species.

The Griffon Vulture population is monitored on a 5-year basis by the same team. All cliffs, identified colonies are monitored, birds counted and breeding success and an annual population check is performed on a specific sub group in the Ossau Natural Reserve (Razin et al., 2008). The Red kite population is monitored during the migration season at selected passes, wintering areas, in breeding areas and through national surveys (Urcun and Filippi-Condaccioni, 2009; Riols, 2009; Bretagnolle et al., 2009).

Bearded Vultures and Egyptian Vultures are classified Endangered in France, the Red kite is considered vulnerable and the Griffon Vulture is classified as minor preoccupation (UICN France et al., 2011). All 4 species are subject to national conservation programs. These figures do not take into account migratory birds. The Bearded Vulture is sedentary (Margalida et al., 2013). The Egyptian Vulture migrates across the Sahara, but population numbers were taken during the presence of birds in spring and summer (maximum population, Kobierzycki, 2012). The Red kite is a partial migratory bird, with most northern European populations wintering in Spain and in the Pyrenees. In winter, large wintering dormitories may be seen in the French Pyrenees, representing up to 55% of the total French wintering population (3892 birds counted out of a total of 7052 identified during the same time-

Table 1
Age, gender and geographic origin of avian scavenger species collected between 2005 and 2012 (GB : *Gypaetus barbatus*, MM : *Milvus milvus*, GF : *Gyps fulvus*, NP : Neophron percnopterus) – Estimated populations in the Pyrenees and in France.

Species*	Adult	Immature	Juvenile	Unknown	F	M	Unknown	64[a]	65[a]	31[a]	09[a]	11[a]	66[a]	Pyrenees[b]	France[b]	Ref
GB (8)	6	1	1		5	2	1	3	5	–	–	–	–	33	46	Razin2012
MM (34)	29	1	4		14	15	5	17	9	6	1	1	–	350–604	2330–3020	Balleraud2012
GF (120)	29	26	60	5	37	49	34	79	16	3	6	15	1	832	1462	Lecuyer2012
NP (9)	5	2	2		1	3	5	3	–	3	–	3	–	72	93	Ponchon2012
Proportion					33.5	40.6	26.4	59.9	17.5	7.0	4.0	11.1	0.5			

* Total number of birds for each species in parentheses.
[a] Numbers refer to the French administrative “départements” from West to East (see also Fig. 1).
[b] Estimated population in 2012.

period in France, Riols, 2013); the Griffon Vulture is non-migratory but may undergo large-scale seasonal movements. Young Bearded Vultures and Griffon Vultures may be somewhat erratic and be detected far from their original nest. As a consequence, birds from the Northern Pyrenees (France) may leave the country while exogenous birds may be observed in France and other individuals stay at their original breeding site (Terrasse, 2006). Because the Griffon Vulture is a scavenger and locally abundant it was included as a surrogate / sentinel species to increase the likelihood of identifying causes of death in other birds of prey notably the more critical Bearded Vulture, and have a more complete evaluation of the environmental quality of the Pyrenees inhabited by Bearded Vultures all year round. Therefore, including several species sharing the same environment and diet in the program gave us a better chance to determine the various causes of death and their potential impact on species with smaller populations (Bearded Vultures, Egyptian Vultures and even Red kites).

2.3. Body collection

Birds included in the program were either found dead by naturalists during surveys in the mountains, or by the personnel of the Pyrenees National Park, or by personnel of the National Game and Wildlife Agency during their routine surveillance activities, or in bird rescue centers. This network is an opportunistic, passive network of collection. Open areas between 200 and 2000 m altitude were covered with regular visits from network participants. These areas are used for human activities on a regular basis, including pastoralism. Most avian scavengers live and breed between 200 and 2000 m in this part of France, with the exception of the Bearded Vulture, which breed as high as 2550 m altitude. As a consequence, the mountains area between 200 and 2000 m is of major importance for the life of these species. Surveillance and care of birds occurs all year round. Each bird of prey found dead was declared and collected as soon as possible by an authorized person (SAGIR network,[1] including the Parc National des Pyrénées, LPO and Union des Centers de Soins) and transferred to the referent veterinarian of the National Conservation Programs for necropsy (DVM Lydia Vilagines). Each bird was sent with a detailed record, weighed and underwent external clinical examination and X-Ray before necropsy.

2.4. Necropsy and X-ray

X-ray was used to detect bone lesions, some systemic diseases, and the presence/absence of lead bullets from shooting or prey contamination. During necropsy, the gastric and intestinal tract was subjected to macroscopic and microscopic parasitological investigations. The liver, kidneys, crop and gizzard content were collected and kept frozen (−20 °C) for further toxicological investigations. Whenever possible (body condition) complementary histopathological or bacteriological investigations were conducted to establish a proper diagnosis. Because the objectives of the program were to investigate poisoning cases, priority was given to toxicological analyses, conducted on all birds.

Toxicological investigations were selected on the basis of personal experience (Berny and Gaillet, 2008; Joncour, 1986; Lafontaine et al., 1990), on the basis of the SAGIR network experience and on the basis of the international literature available on poisoning issues in birds of prey (Pattee et al., 1990; Hernandez, 2005; Margalida et al., 2008; Berny and Gaillet, 2008; Hernandez and Margalida, 2009b; Nam and Lee, 2009; Knott et al., 2009; Rodriguez-Ramos et al., 2009; Grund et al., 2010). Local environmental issues also gave rise to specific investigations such as the illicit use of lindane (Gibert and Appolinaire, 2004), the ancient mining sites (Gourdon, 1858, Cottard et al., 2003) or heavy hunting activity in the western Pyrenees with lead (Jean, 1997; Rouxel and Czajkowski, 2004; Hernandez and Margalida, 2008; Gangoso et al., 2009; Hernandez and Margalida, 2009a; Mateo, 2009), but also other pollutions such as Cd, Organochlorine (OC, Ramade 2007). A national database was used to identify potential industrial pollution sites (http://www.irep.ecologie.gouv.fr/IREP/index.php) consulted on March and December 2013.

2.5. Toxicological analyses

All toxicological analyses were performed at the Toxicology Laboratory of Vetagro Sup Lyon, reference laboratory for wildlife suspected poisoning cases under the French Sanitary Surveillance System (SAGIR) (Berny and Gaillet, 2008).

2.5.1. Metals

Lead and cadmium were analyzed by Graphite Furnace Atomic Absorption Spectrometry (Mazet et al., 2005; Lemarchand et al., 2010) on grinded liver/kidney after digestion in 50% sulfuric acid at 700 °C overnight. The results were expressed in mg kg^{-1} (dry weight). Limits of detection were 20 μg kg^{-1} (Pb, Cd). Each sample was run in triplicate. Certified samples were used for quality control (CRM185R certified values and 95% confidence interval for Pb, Cd, Hg from LGC Standards, Molsheim, France). Blank vessels and daily spiked samples were used to assess recovery (>90%). Metal concentrations were determined using 5-point calibration curves ($r^2 > 0.99$). The limit of detection was determined as 3 × the SD of blank (Strapáč et al., 2012). For statistical purposes, any value below the limit of detection was set at 1/2 the limit of detection.

Lead isotopic determination was performed on liver and kidney samples of exposed animals, as an indicator of the potential source of lead exposure: lead from ammunition (Tsuji et al., 2008), industrial pollution (Meharg et al., 2002) or lead from (ancient) mining activity (Laperche et al., 2004). All isotopic analyses were conducted with ICP-MS, as already published (Pain et al., 2007). Standard NIST SRM981 with a certified isotopic composition of Pb^{206}, Pb^{207}, and Pb^{208} was used. Spiked samples, blanks and certified samples were used in all series of analyses. Recoveries were >91%.

2.5.2. OC

OCs were analyzed by means of Gas-chromatography with Electron Capture Detection (GC-ECD) on liver samples. Samples were extracted twice with acetone:hexane (25/75, 30 mL). Dry residues were dissolved in hexane and purified with H_2SO_4 (SO_3 7%). After centrifugation and phase separation, the supernatant was used for residue analysis. Limits of quantification were between 5 and 10 ng g^{-1} (wet weight) for individual OCs. Cod liver oil (BCR349) certified material was used as a regular quality control (Lemarchand et al., 2010). Spiked samples were used to assess recovery (>92%).

2.5.3. OP

Gizzard or liver samples were analyzed. Organophosphate (OP) and Carbamate (CA) pesticides (Aldicarb, Benfuracarb, Dichlorvos, Carbofuran, 3-Hydroxy-Carbofuran, Mevinphos, Phorate, Phorate oxon, Phorate sulfone, Methiocarbe, Terbufos, Diazinon, Disulfoton, Chlorpyriphos methyl, Chlorpyriphos ethyl, Fenitrothion, Pyrimiphos methyl, Malathion, Fenthion, Parathion, Methidathion, Disulfoton sulfone, Triazophos) concentrations were determined

[1] Members of the SAGIR network http://www.oncfs.gouv.fr/Reseau-SAGIR-ru105 have an individual authorization to collect dead animals of protected species for diagnostic purposes

by GC/MS in SIM mode using 4 specific ions for each compound (OP+carbofuran and methiocarb) or by HPLC (CA) according to (Lemarchand et al., 2012). Limits of detection were 0.025 mg kg^{-1} by GC–MS and 1 mg kg^{-1} by HPLC. Spiked samples were used in each series of analysis (recoveries between 76% and 104%).

2.5.4. Anticoagulant rodenticides (AR)

Analyses for anticoagulant rodenticide (8 compounds marketed in France: bromadiolone, chlorophacinone, difenacoum, difethialone, warfarin, coumatetralyl, brodifacoum, flocoumafen) exposure were performed using high-performance liquid chromatography according to Lemarchand et al. (2010), Berny et al. (2006) and Giraudoux et al. (2006). Briefly, liver samples were extracted using acetone:ethanol (8/2) and analyzed using reverse-phase HPLC with both UV (diode-array) and fluorescence detection. Limits of detection were 0.02 mg kg^{-1} wet weight for all anticoagulants tested.

2.5.5. Result interpretation

For each bird, diagnosis was based on clinical and necropsic (including histopathology and radiographic examinations) evidence as well as analytical results. An animal is considered "exposed" when tissue residue levels are below known toxic threshold values (laboratory's experience and published data) and in the absence of clinical or necropsic signs compatible with poisoning; a bird is considered "poisoned" when tissue residue levels are above the known toxic threshold values and when clinical or necropsic evidence is present, sometimes confirmed by epidemiological evidence (bait for instance).

In wild animals found dead, the apparent cause(s) of mortality may be associated with aggravating factors, including moderate toxic exposure. In most instances, death may be the result of several combined direct or indirect factors and these causality factors had to be classified:

1. In case of sudden death by electrocution, trauma or shooting, these death causes were considered as the primary cause of death.
2. When several events may have a synergistic effect and lead to death, the apparent cause of death may only be the result of a severe preceding pathological status. Under such circumstances, trauma, fall at fledging and other "obvious" causes of death were not retained as the primary cause of death when clinical, necropsic or analytical evidence suggested that an underlying pathological process was debilitating the animal (for instance septicemia resulting in trauma or fall of a debilitated animal).
3. Unknown cases may be considered when the apparent cause of death does not match other findings on the bird (fall from a cliff for an experienced, adult bird)

For the following toxicants, some threshold values were considered based on available literature or analytical capabilities of the lab:

1. Lead environmental exposure was considered positive when liver or kidney [Pb] > 2 μg/g dry weight based en maximum residue limits (0.5 μg/g wet weight, i.e. 1.5 μg/g dry weight) recommended for domestic and game species.
2. Lead sub-clinical or clinical poisoning was confirmed for liver

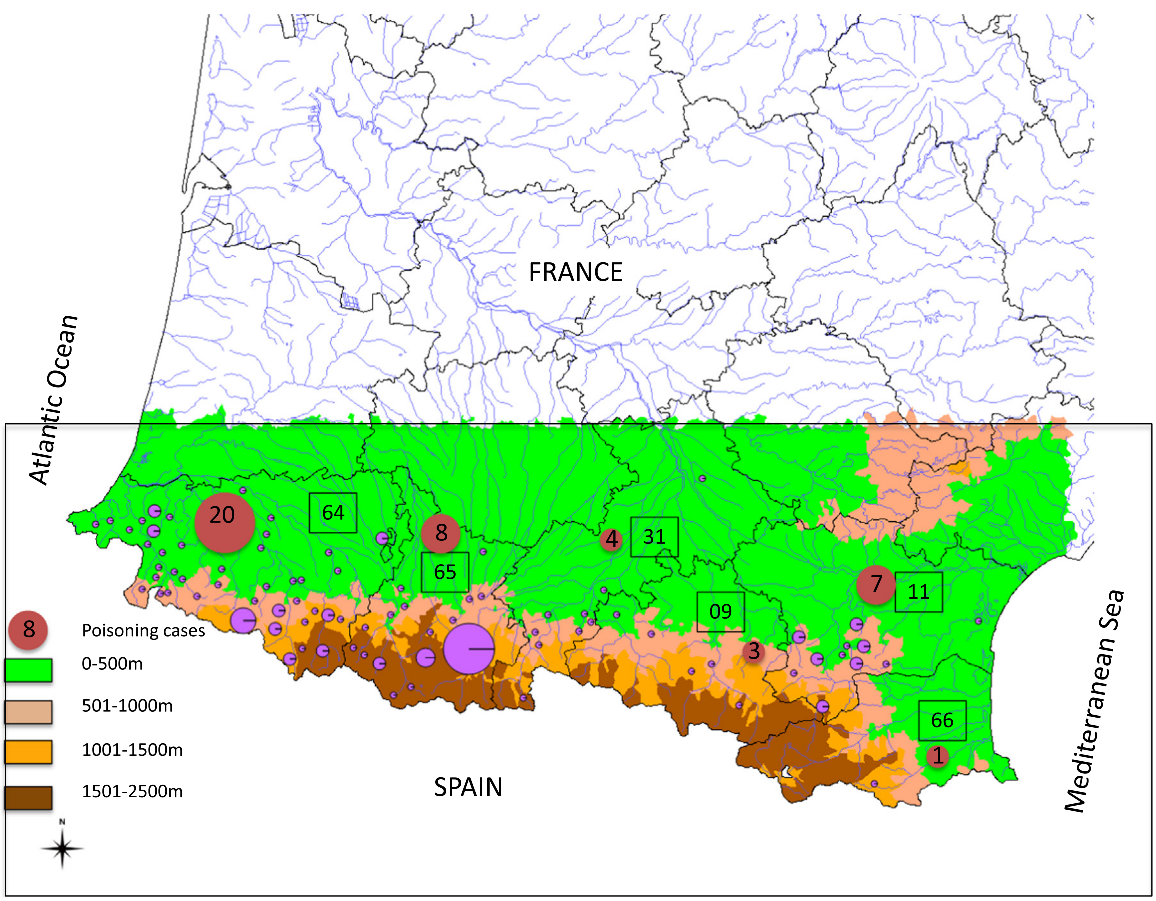

Fig. 1. Geographic distribution of mortality events for birds of prey in the Pyrenees (cases/township) Purple circles are proportional to the number of birds Dead (max=8) ((2005–2012, n=170; brown circles are proportional to the number of poisoning cases) Scale 1:1100000. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

or kidney [Pb] > 6 μg/g or 15 μg/g (dry weight) respectively (Pain et al., 2007)
3. Cadmium > 2 μg/g (dry weight) in the liver or kidney was considered as exposure, based on maximum residue limits (0.5 μg/g wet weight, i.e. 1.5 μg/g dry weight) recommended for domestic and game species.
4. In case of multiple toxicant exposures, the value closest to known toxic threshold was considered as the primary cause of poisoning

Whenever possible, all analytical results were discussed after consideration of demographic data (species, age, gender), biological data (diet, behavior) and environmental risk factors (local exposure).

2.5.6. Statistical analyses

The Kruskall-Wallis test was used for multiple comparisons and post-hoc pairwise comparisons were performed using the pairwise Wilcoxon rank sum test with Bonferroni adjustment. Linear regression analyses were conducted after log transformation for Pb and Cd concentrations in order to approximate a normal distribution using Pearson's product moment correlation. Normality of residuals was checked by means of residuals vs fitted distribution analysis and QQ-plot of residuals. Statistics were performed using *R* (R Core Team, 2012).

3. Results

3.1. General data – temporal distribution

A total of 170 birds (8 Bearded Vultures, 9 Egyptian Vultures, 34 Red kites, 119 Griffon Vultures) could eventually be included in the study between 2005 and 2012. Based on the population estimates (Table 1) there was a significantly lower proportion of Red kites than expected, but this was not observed for the three vulture species. The overall sex ratio was 1.20 (M/F), with no significant difference between species ($p > 0.1$). The gender and age distribution are described in Table 1. As could be anticipated from their mean residence time in the Pyrenees, mostly adults were retrieved for all species, except for Griffon Vultures: in this eventuality, juveniles were mostly discovered at the fledging period in the surroundings of the breeding colonies.

The majority of carcasses were collected in Dept 64 (60%) where a large majority (up to 90% on given years) of the nesting Griffon Vulture population is found (Griffon Vulture represent 70% of all birds collected), and where the majority of wintering Red kites are observed (Source LPO, http://rapaces.lpo.fr/milan-royal, consulted on March 2014); Dept 65 stand second in terms of bird collection (17%), but first for Bearded Vultures (43% of Bearded Vulture pairs nest in this Dept). The remaining 4 depts account for 23% of carcasses collection.

Overall, the collection of cases was quite homogeneous across years ($p > 0.05$), ranging from a low 13 cases in 2008 and 2010, to a high of 29 cases in 2011 (mean = 21.2 cases/year).

Fig. 1 describes the geographical distribution of carcasses retrieved along the Pyrenees Mountains. Most cases were discovered on the western part of the mountain range.

3.2. Causes of death by species

Table 2 displays the causes of mortality after grouping (infectious disease/malnutrition; poisoning; trauma with infrastructures; falls; electrocution; shooting/mutilation/hit; undetermined). No significant differences could be detected between species for the proportion of mortality causes.

Overall, poisoning cases are by far the most common causes of death (25% of all cases), followed by falls and collisions (12.3%), diseases (infectious and malnutrition 11.6%), then by a group comprised of shooting, blows and mutilation (11.2%), electrocution (7%). No statistical difference could be identified between species, except for a lower frequency of cholinesterase inhibitor poisoning frequency in Griffon Vultures (Table 2). The cause of death could not be identified in 34% of the cases.

Over the study period, anthropogenic direct causes of death accounted for 51% of all causes of death (combined shooting, mutilation, poisoning, electrocution and trauma with electric cables and cars). The most common non-anthropogenic cause of death in Griffon Vultures was related to falls of young animals (at fledging).

Among infectious diseases, pneumonitis was the most common, sometimes associated with aerosacculitis (3GF, 1MM, 1GB), enteritis (3GF including one case of *E. coli* infection), cachexia with terminal sepsis (7 Griffon Vultures, mostly juveniles and 1 Red kite), *Staphylococcus sp*. Septicemia in 1 Bearded Vulture and one case of nephritis in a Griffon Vulture.

Fig. 2 displays a geographical distribution of individual lead exposure cases (Log[Pb]liver). As can be seen on the map, most cases were retrieved from the western part of the mountain range where most of the birds live. Some of the highest concentrations were also detected in this part of the mountain range, where intensive hunting occurs along the major passes. Identified lead sources (industry, mining) are indicated and do not overlap with the distribution of lead poisoning or exposure cases in birds.

The relative distribution of poisoning and exposure cases is quite homogeneous across the mountain range ($p > 0.1$), from a low 43% in the western border (Dept 64) to a high 58% in the eastern part (Dept 11). A significantly higher proportion of anthropogenic causes of death (shooting, blows, mutilation) is detected in the western Pyrenees (16%). Electrocution cases are homogeneous along the mountain range.

Table 2
Cause of death by species.

Species	Infectious disease + malnutrition	Poisoning (cholinesterase inhibitors)	Poisoning (other organic chemicals)	Lead poisoning	Collision	Falls	Electrocution	Shooting	Undetermined	Detection of AR residues
GB (8)	3	2	–	–	1	–	1	1	1	–
GF(119)	13	13	12[a]	3	15	3	4	13[b]	45	5
MM(34)	2	5	1	4	–	1	5	5	11	-
NP (9)	1	3	–	–	–	1	2	–	2	2
Total (%)	19 (11.6%)	23(13.5%)	13 (7.6%)	7 (4.1%)	16 (9.4%)	5 (2.9%)	12 (7.0%)	19 (11.2%)	58 (32.7%)	7 (4%)

[a] Including one case with 9 vultures and pentobarbital.
[b] Including 4 cases of deliberate destruction.

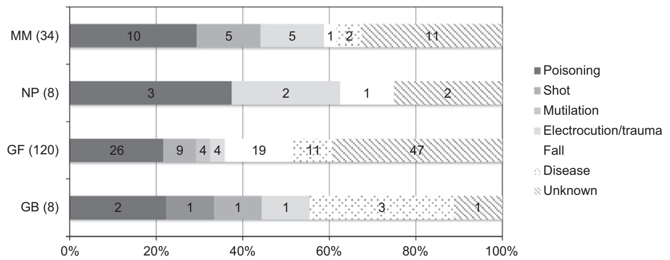

Fig. 2. Primary cause of mortality in Vultures (gray: anthropic origin). Egyptian Vulture: *Neophron percnopterus*, Griffon Vulture: *Gyps fulvus*, Red kite: *Milvus milvus*, Bearded Vulture: *Gypaetus barbatus*Numbers indicate the number of animals. Mutilation: voluntary.

3.3. Poisoning and exposure to toxicants

Poisoning accounted for 22-37% of the causes of death in the 4 species included in this study. The products involved included OP/CA, Pb and some euthanasia products (9 Griffon Vulture). Cholinesterase inhibitor poisoning was detected in 23 birds (2 Bearded Vultures, 3 Egyptian Vultures, 5 Red Kites, 13 Griffon Vultures) as a result of illicit use of these banned products (see Table 2) and was the single most important cause of poisoning. Carbofuran was the most common pesticide detected (18 cases), followed by aldicarb (3 cases). Lead was responsible for acute poisoning in 7 birds (4 Red Kites and 3 Griffon Vultures), but overall Pb concentrations $>2\,mg\,kg^{-1}$ dry weight (liver) were detected in 25% of all birds, and Pb concentrations $>6\,mg\,kg^{-1}$ dry weight (liver) in 9% of all birds (see Table 2).

Organochlorine insecticides were detected but usually at very low levels, considered not clinically significant. DDT was almost never detected and only DDE was present in most samples, at environmentally common levels. Some birds presented higher levels of lindane (1 Griffon Vulture poisoned), but generally below toxic threshold (22% of the birds had detectable residues of lindane).

Table 3 presents the median Pb and Cd concentrations in liver and kidney samples. Red Kites had significantly higher liver and kidney Pb ($p=0.0049$) and Cd concentrations ($p<0.0001$) as compared with all other species. Red Kites had significantly higher Pb and Cd residues than Bearded Vultures and Griffon Vultures, but not than Egyptian Vultures. Griffon Vulture lead and cadmium residues were not different from residues in Bearded Vultures.

There was a significant age effect, with juvenile having significantly lower concentrations of both Pb and Cd in liver and kidney tissues.

Embedded lead shots were identified in 20 birds (8 Griffon Vultures, 11 Red Kites and 1 Bearded Vulture). There was also a significantly higher concentration of Pb in the kidney or liver in birds with one or more lead bullet on the X-Ray, as compared with birds without embedded lead shots $p<0.001$). There was a significantly higher concentration of liver and kidney [Pb] in birds found dead of trauma and/or electrocution vs other cause of death ($p<0.001$), and also a significantly higher concentration of Cd in the liver of birds with embedded lead shots.

Lead isotopic ratios are presented in supplementary material. There were no statistically significant differences between [Pb] in the liver or kidney across species. Isotopic ratios $Pb^{206/207}$ and $Pb^{206/208}$ were lower for birds with an identified shot at X-Ray ($p<0.05$). Fig. 3 presents the geographical distribution of lead poisoning cases with local, identified lead sources (ancient mines, industry emissions) and Fig. 4 presents lead isotopic ratios in birds together with bibliographic references for various European lead sources, including local mines.

3.4. Temporal distribution of poisoning cases

Carbofuran poisoning cases were detected mostly during spring and summer, between mid-april and mid-september (18 out of 23); aldicarb and mevinphos cases were detected in the summer (all 3 cases), Pb poisoning cases were identified in the fall and winter (all 7 cases) and pentobarbital poisoning cases were all observed after cattle field-euthanasia in the summer ($n=9$). Overall, no annual trend could be detected for poisoning cases ($p>0.10$).

3.5. Geographical distribution of poisoning cases

Poisoning and exposure cases with Pb represent the most significant toxicant issue in the Pyrenees. Poisoning cases occurred across the Mountain range (from west to east: 20 cases in dept 64, 8 cases in dept 65, 4 cases in dept 31, 7 cases in dept 11, 3 cases in dept 09 and 1 case in dept 66) but the proportions were not different from overall case collection (Table 1 and Fig. 1). A single but massive poisoning case occurred with pentobarbital in Dept 65 (7 Griffon Vultures found dead). Cd exposure cases are mostly described in Red Kites in Dept 64 (10 cases) and in Dept 65 (2 cases). Lindane exposure cases were described in Dept 64 (12 cases) mostly.

Table 3
Concentration of Pb, Cd, and proportion of birds with detectable residues of Lindane (HCH), DDT or cholinesterase inhibitor (OP/CA) in vultures species in the Pyrenees. (GB : *Gypaetus barbatus*, MM : *Milvus milvus*, GF : *Gyps fulvus*, NP : *Neophron percnopterus*)

Species[a]	Pb liver	Pb kidney	Cd liver	Cd kidney	HCH[b]	DDT[b]	OP/CA*
GB (8)	**0.56** (0.16–16.11)	**0.75** (0.10–2.76)	**0.26** (0.04–0.66)	**0.49** (0.11–1.22)	**25%** (ND–0.34)	**0%** (ND)	**25%**
MM (34)	**1.38**** (0.02–159.03)	**2.56**** (0.09–189.00)	**0.92**** (0.05–2.31)	**1.79**** (0.02–13.17)	**26%** (ND–0.98)	**3%** (ND–0.19)	**15%**
GF (119)	**0.98** (0.02–21.50)	**0.86** (0.08–34.02)	**0.20** (0.03–1.24)	**0.27** (0.02–3.89)	**18%** (ND–5.50)	**5%** (ND–0.27)	**11%**
NP (9)	**0.84** (0.23–3.27)	**1.07** (0.90–3.74)	**0.07** (0.02-0.12)	**0.22** (0.13–0.31)	**25%** (ND–4.51)	**20%** (ND–0.19)	**33%**
Ad (68)	**1.08** (0.03–159.0)	**1.18** (0.08–189.0)	**0.32** (0.02–2.31)	**0.58** (0.02–4.1)			
Imm (27)	**1.30** (0.18–6.60)	**1.10** (0.23–7.50)	**0.23** (0.05–1.24)	**0.29** (0.02–3.89)			
Juv (53)	**0.89** (0.11–19.80)	**0.72**** (0.10–7.91)	**0.17**** (0.02–1.50)	**0.23**** (0.02–13.17)			
Collision (43)	**1.52**** (0.22–16.11)	**2.44**** (0.10–21.70)	**0.26** (0.02–2.20)	**0.42** (0.02–13.2)			
No collision (116)	**0.84** (0.02–159.0)	**0.86** (0.08–189.0)	**0.21** (0.02–2.21)	**0.34** (0.02–6.42)			
Shot (20)	**1.24**** (0.5–159.0)	**2.68**** (0.73–189.0)	**0.53** (0.02–2.31)	**1.44**** (0.02–4.10)			
No shot (139)	**0.92** (0.02–67.81)	**0.92** (0.08–144.0)	**0.22** (0.02–2.31)	**0.31** (0.02–13.17)			

Values are given as median and (range) in $\mu g\,g^{-1}$ dry weight (metals) or $\mu g\,g^{-1}$ wet weight (Lindane, DDT).

* Percent poisoning cases per species.
** Significant difference ($p<0.05$).
[a] Total number of birds for each species in parentheses.
[b] Limit of detection : 0.1 $\mu g\,g^{-1}$.

4. Discussion

Scavenging birds feeding on carcasses and remains of dead animals occupy a privileged place in the food web to serve as bioindicators of environmental issues (Helander et al., 2009)

4.1. Demographic, seasonal and geographical data

4.1.1. Demographic data

The collection of dead birds was similar for all vulture species, but significantly lower for Red Kites. This may be related to the difference in population monitoring strategies as well as a smaller size for Red Kites, making it more difficult to detect (Ward et al., 2006). Indeed, Bearded Vultures and Egyptian Vultures are closely watched and there is an exhaustive count of the populations (Razin, 2012; Ponchon et al., 2012). Similarly, Griffon Vulture populations are well known and all colonies are monitored on a regular basis (Lecuyer and Neouze, 2012). On the contrary, Red Kite populations are widespread in France, wintering in different areas (not specifically in the Pyrenees, Mionnay, 2004, Bretagnolle et al., 2009), with both migratory and wintering birds. As a consequence, population estimates are less accurate.

Despite a small number of animals included in the study (7 Bearded Vultures), the age class is representative of the local population in the French Pyrenees, with a small proportion of young animals present (young, juveniles) (Razin, 2001). Margalida et al. (2008) did not find differences between sex and age groups, in terms of primary cause of death in Bearded Vultures in Spain. Young Egyptian Vultures migrate to sub-Saharan Africa and only fly back to Europe as adult birds to nest (Cramp and Simmons, 1980). It may be coincidental, but the number of Bearded Vultures and Egyptian Vultures collected was similar and, although the population of Egyptian Vultures is twice as big, they spend 50% of their time out of Europe. For Red Kites, the sex ratio is balanced (1.1) and most likely reflects the natural situation, although no specific data could be found to confirm this hypothesis. For Griffon Vultures, the sex ratio appears balanced, considering the high proportion of birds for which the gender could not be determined (poor body condition). The proportion of juveniles is high, but similar to other studies in France and the distribution of mortality cases does not differ between age groups as described in other studies (Bosé et al., 2007). In wild-hatched groups Bosé et al. (2007) found a sex ratio at ringing equal to 1:1. According to these

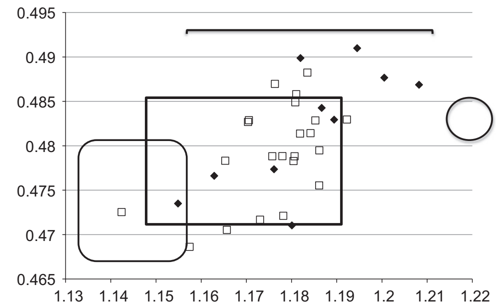

Fig. 4. Lead isotope ratios in birds of prey (liver) and from various published sources. Open squares: [Pb]liver > 2 μg g^{-1} (dry weight). Rectangle: lead in European ammunition (Thomas et al., 2009); Round-edge rectangle: lead in Pyrenean ancient mines (Cardellach et al., 1996); Circle: lead in Basq county (Monna et al., 2004); line: lead in US ammunition (Lambertucci et al., 2011). Leaded gas (< 1.08 on X axis) could not be represented on this graph.

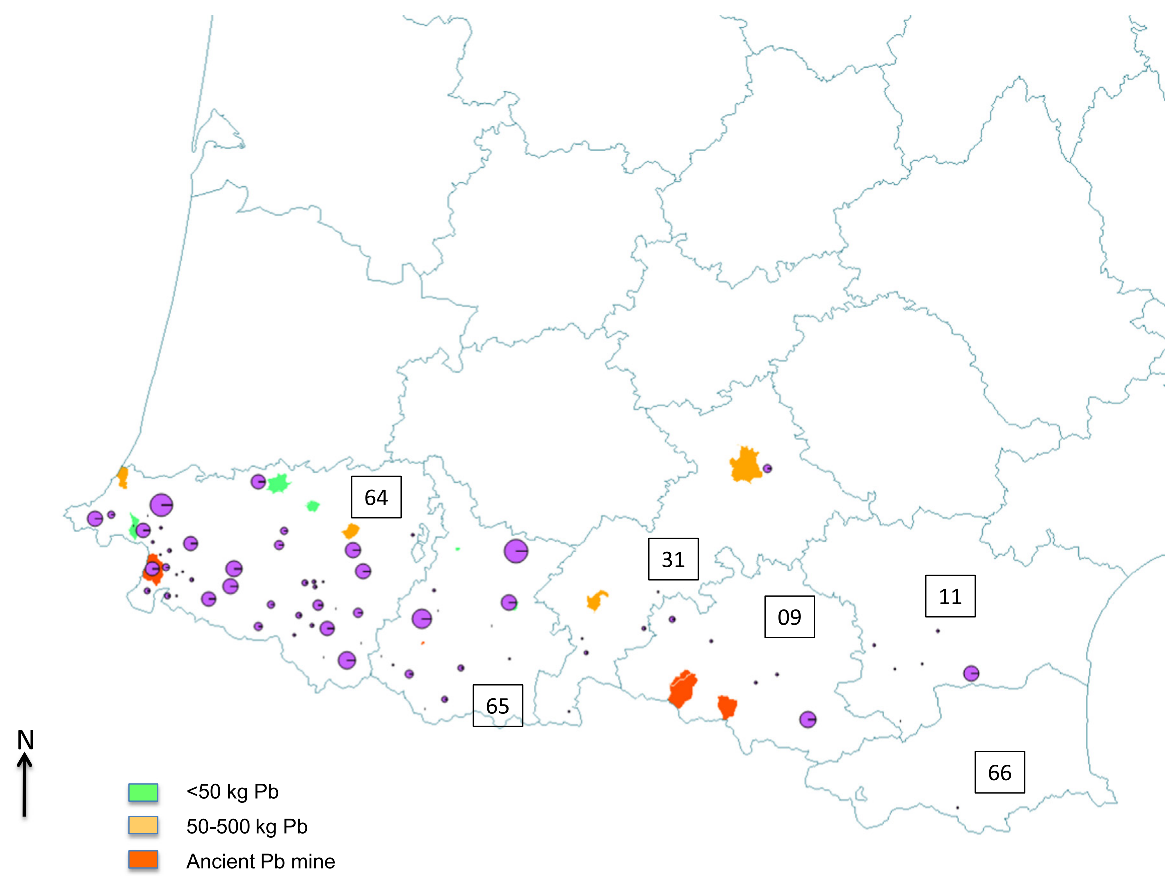

Fig. 3. Sources of lead (emission in kg/annuum) and distribution of liver lead concentrations detected in birds of prey in the French Pyrenees. Purple circles represent $\log_{10}$[Pb]+1 in the liver of birds. Purple circles are proportional to Log[Pb]liver. Max: 159 μg g^{-1} dry weight. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

authors, absence of sex bias in birth, mortality and dispersal could be explained by low competition between sexes and equal investment in reproduction by males and females. The bird distribution (gender, age groups) appears similar to published data regarding sedentary or migratory populations of raptors (Newton, 1979).

4.1.2. Seasonal and geographical data

Municipalities where several birds were collected (4–8) are major breeding areas for Griffon Vultures and the geographical origin of birds is consistent with bird distribution in the Western Pyrenees, as monitored by the regular bird watching system (Razin et al., 2009) and the presence of the Pyrenees National Park. Other focal points in the central and eastern parts of the mountain range are consistent with the lower density of birds (Source LPO, http://rapaces.lpo.fr/milan-royal, consulted on March 2014 for the Red Kite). Because the number of Bearded Vultures and Egyptian Vultures collected is low, no significant quantitative conclusion should be drawn, but these data provide at least some qualitative evidence.

The seasonal presence of Red Kites is well reflected in the period of discovery of bodies: 32% fly to Spain in fall or start wintering locally, 32% stay in the winter, 36% are present during spring and summer (breeding season, sedentary birds). For Griffon Vultures, birds are collected all year round, with an increased proportion of animals detected in the summer (48%), corresponding to the dangerous / difficult fledging period of juveniles (Eliotout, 2007).

4.2. Causes of mortality

4.2.1. Identified biases

Traumas are described in all 4 species, all across the mountain range, all year round. Usually speaking, trauma is associated with power lines. No significant association with age/experience could be identified. Only in Bearded Vultures did we fail to identify electrocution cases, but these birds are never observed on power lines, contrarily to Red Kites and Egyptian Vultures (for which this cause of mortality is important). Similarly, regular surveillance of breeding sites facilitates the collection of birds found dead (Griffon Vulture), especially juveniles, found in a high proportion as compared with similar studies in scavenging birds of prey (Rideout et al., 2011). Funding limitations, as well as poor body condition did not permit regular histopathological or bacteriological examination of all birds and may lower the proportion of infectious diseases in the identified causes of death.

Very few birds have been detected with a systemic pathological problem. Malnutrition in young animals increases the likelihood of developing other pathological disorders and dehydration was also usually associated with severe uricemia, urate crystals, with concomitant visceral gout, joint and kidney lesions responsible for increased severity of the disease (Beynon et al., 1996).

4.2.2. Season

More than 50% of the carcasses with detectable lindane residues were found during the summer. This season is also known for transhumance and 86% of all herds are located in Dept 64 (western Pyrenees). There is evidence that illicit pesticides can still circulate in this part of the country, across the border, as was recently demonstrated (http://www.oncfs.gouv.fr/IMG/communique_trafic%20dejoue_produits%20phytopharmaceutiques.pdf)

Lead poisoned birds were detected during fall and winter, the major hunting season, especially in the western Pyrenees (Dept 64/65) where hunting is concentrated around several passes known for migration (168,000 pigeons killed, 1,552,000 estimated shots, 1 hunting site every 2 km-Jean, 1997). It has been estimated that, between 1979 and 1984, for 100 shots, 11.3 pigeons were killed, 6.4 were injured and never found, thereby remaining available for scavenging birds (Sagot and Tanguy Le Gac, 1985). The National Hunting Federation states that, during the hunting season for wild ungulates (Aug 15–Feb 28), this part of France is the first region for the number of hunters.

http://www.chasseurdefrance.com/Decouvrir-la-chasse/Qui-sont-les-chasseurs/Les-Chasseurs-qui-sont-ils.html consulted in Dec 27, 2013).

4.2.3. Acute poisoning cases

There are very few published data for wild birds of prey in Europe. The trend appears quite similar however: plant protection products (pesticides) especially cholinesterase inhibitor insecticides and AR account for the majority of poisoning cases (Berny, 2007). In Red kite, poisoning was identified in almost 80% of the birds sent to the wildlife disease surveillance network in France (Berny and Gaillet, 2008). Since only birds found dead and suspected of poisoning were submitted for analysis, the proportion of poisoning cases was probably overestimated. The present study, however, confirms this trend on birds collected spontaneously with no a priori selection. It is also quite similar to Spanish data identifying poisoning cases as a major threat in many scavenger species (Margalida, 2012), and a real concern that illicit poisoning was responsible for the quasi-extinction of Bearded Vulture in Europe (Margalida et al., 2013)

A specific study in Bearded Vultures confirms the high prevalence of poisoning cases in this species in Spain, either after accidental or illicit exposure (Margalida et al., 2008). A major concern in terms of conservation is the high proportion of birds found with detectable residues at environmentally critical levels: 50% are associated with acute poisoning cases, 50% are exposed and this exposure can be considered as a risk factor of concomitant disease. These results can be compared with studies on the Californian Condor (*Gymnogyps californianus*). Indeed, in this species, anthropogenic causes of death account for most of the identified causes of death and poisoning cases are common or sometimes the single most common cause of death (depending on the age group) (Rideout et al., 2011).

As sometimes described in other parts of Europe, summer pastures are shared with wildlife and inappropriate euthanasia techniques may result in death of scavenging birds, despite regular training and information of local actors (Gedoux, 2010; Joncour, 2011).

Several studies illustrated in Guitart et al. (2010) show that illicit poisoning is very common in Red Kites and in Griffon Vultures in Spain. Indeed, Martínez-Haro et al. (2008) and Hernandez and Margalida (2009b), for instance, describe cholinesterase inhibitor insecticides (carbofuran and aldicarb) as a major problem in Spain, despite complete ban of these pesticides in Europe since 2008. These authors consider that the lack of specific surveillance on the presence/distribution of these products after ban is a major concern. This is of particular concern since adult mortality is highly detrimental to the survival of threatened and long-lived species as pointed out by Hernández and Margalida (2008) and especially (Margalida et al., 2014) who demonstrated that adult mortality, including illicit poisoning, could greatly affect the survival of Bearded Vultures.

Although commonly detected, lindane is found at relatively low concentrations, and it is reasonable to consider that the former contamination accident described earlier in this region has less impact on the bird population today, and that the illicit use of this insecticide decreased over the years (Gibert and Appolinaire, 2004). The presence of lindane was not significantly associated with any of the other causes of mortality. Similarly, DDT (mostly present as its ultimate metabolite DDE, banned in 1972 in Europe) is only present at low levels. There is still debate, however, on the potential impact of these low-level contaminations on the long-term survival of a species locally. Tenan et al. (2012), for instance, showed that poisoning cases (representing 43–76% of mortality events in vulnerable age groups in Red Kite populations in Majorca, Spain) could be responsible for a 20% decrease in bird population demography. Because, locally in Spain, the species productivity is high (1.83 young per breeding pair per year), overall the population still grows. The authors, however, clearly point out the risk for less productive species or populations, which is the case for Bearded Vultures (0.4 Razin, personal data), Griffon Vultures (0.6, Razin et al., 2008), and Egyptian Vultures (0.7-http://rapaces.lpo.fr/vautour-percnoptere/reproduction consulted in March 2015) in the Pyrenees and also Red Kites locally (only 1.12 young/breeding pair/year, LPO, Milan info 2013, http://rapaces.lpo.fr/milan-royal consulted in March 2014).

Overall Cd exposure appears low, Cd exposure is mostly detected in Red Kites and cannot be related to ungulate carcasses (the other bird species would be affected as well). There is evidence that Red Kites may consume earthworms (Cramp and Simmons, 1980), known to accumulate Cd in their tissues (Burgat, 1990). More than air pollution, which would affect all species (Lucia et al., 2009), it is suspected that soil contamination may result from regular use of phosphate fertilizers in pasture, known to contain high concentrations of Cd (Ramade, 2007).

4.2.4. Lead poisoning and exposure

One of the most critical issues identified in this study is the potential exposure of birds of prey to Pb. It appears to be an emerging problem in Bearded Vulture populations in Europe, as a consequence of Pb cumulative, long-term toxicity. Because of their diet, the Bearded Vulture gastric physiology is adapted to the digestion of bones and is highly acidic and this favors lead absorption (http://www.4vultures.org/2015/02/09/bearded-vulture-monitoring-report-for-the-french-alps-life-gyphelp/ consulted in March 2015). Only one other study provides information with respect to Bearded Vultures (Hernandez and Margalida, 2009a). In this Spanish study, Pb contamination appeared higher, but the total number of French Pyrenean Bearded Vultures is too low to be significant ($N=3$). In Red Kites, Pain et al. (2007) also showed a high prevalence of Pb exposure resulting from lead bullets, but to a lesser extent than in our study. In Spain, lead concentrations measured in the Spanish imperial eagle (*Aquila adalberti*) were somewhat lower than the levels detected in France, but the authors identified a significant positive association with hunting of large game species (Rodriguez-Ramos et al., 2009). In the same region, there is evidence that migratory waterfowl are highly exposed to lead (>100 μg/g wet weight, Lucia et al., 2009) as a result of hunting. Overall, there is evidence of lead bullet exposure in the 4 bird species in Europe: Red Kites in the UK (Pain et al., 2007), Griffon Vultures and Egyptian Vultures in Spain (Garcia-Fernandez et al., 2005; 2008; Gangoso et al., 2009; Hernandez and Margalida, 2009b) and Bearded Vultures in the Pyrenees, in the Alps and in Andalousia (Spain) (Hernandez, 2005; Hernandez and Margalida, 2009a). Both fragmentation and dispersion of lead bullets in preys may favor indiscriminate ingestion by large scavenging birds of prey (Knott et al., 2009) and the very low gastric pH is certainly responsible for rapid Pb dissolution and absorption (Fisher et al., 2006). It is also generally accepted that birds of prey are highly susceptible to Pb effects (Pattee et al., 2006), although some species differences can be observed (Carpenter et al., 2003). The Californian Condor reintroduced population is only surviving thanks to the provision of uncontaminated food sources and enforced protection (Finkelstein et al., 2010). Fisher et al. (2006) consider that Pb exposure may endanger species with a poor reproductive potential, by decreasing survival rates in adults. As a consequence, such a threat could really hamper the survival of Bearded Vultures in France, if strong protection measures and lead bullet restrictions of use are not considered.

One of the original finding of our study is the strong positive association between the presence of a bullet on the X-ray and a higher lead concentration in the liver and kidney of exposed birds. Furthermore, lead isotopic ratios are in favor of an anthropogenic origin of lead in birds, namely lead bullets. Indeed, the isotopic ratios measured in the study by Helander et al. (2009) ($Pb^{206/208}$ and $Pb^{206/207}$) on white-tailed sea eagles or by Pain et al. (2007) on Red Kites were very similar to our own findings (for instance: $Pb^{206/207}$ median: 1.170). In these studies, local projectiles displayed a pattern of isotopic ratios compatible with lead-bullet exposure. Our median isotopic ratios for $Pb^{206/207}$ (1.178) is in the median range for European lead bullets (1.12–1.18) according to Pain et al. (2007) or Thomas et al. (2009), or even American bullets (Lambertucci et al., 2011). On the contrary, studies on local mines (Cardellach et al., 1996) in the central Pyrenees describe values far from the birds tested (with less than 10% of the birds falling in the interval for environmental lead in local mines). Furthermore, birds with higher lead concentrations had lead isotopic ratios clearly distinct from these environmental sources. Comparison was also considered with ancient mines in the Western Pyrenees (Monna et al., 2004). Their median ratio for $Pb^{206/207}$ was close to 1.12 and the other ratios provided ($Pb^{204/206}$ and $Pb^{204/208}$) had ranges different from those obtained for the birds in this study. Last, few values have been published for Pb in leaded gasoline (Helander et al., 2009). This source of lead is not available any more and the ratio $Pb^{206/207}$ (1.14) was also quite different from the ratio measured in birds. A recent study in Switzerland also detected lead exposure in Golden eagles (*Aquila chrysaetos*), with isotopic ratios similar to the isotopic signature of lead ammunition and clearly distinct from the isotopic signature of their preys (Madry et al., 2015).

A spatial analysis of large game hunting and vultures feeding areas in Spain shows a definite superposition of these areas (Mateo, 2009). It is therefore our conclusion that scavenging birds in the French Pyrenees are exposed to lead from hunting, not only via food contamination but also via prolonged diffusion from embedded lead bullets (birds who survived a shot as analyzed here).

We also demonstrated the existence of a significant association between lead concentration and the proportion of trauma and/or electrocution, with 75% of all birds with trauma/electrocution below the usual threshold for environmental exposure (<6 μg/g dry weight) (Pain et al., 2007). This raises questions about the health status of the bird population and sub-acute/indirect effects of toxicant exposure at levels lower than expected. There is already evidence that Pb exposure may have some effect on the flying capacity of birds (Mathiasson, 1992). Experimental studies have also demonstrated the neurotoxic potential of Pb in bird species (Mazliah et al., 1989; Burger and Gochfeld, 2005; Bana, 2004), impairing both locomotor activity and learning capabilities of the exposed animals. There is also evidence that chronic Pb poisoning will cause flying incapacity or limitation (McLelland et al., 2011). It is our conclusion that long-term, low level Pb exposure (even at levels <6 μg/g dry weight) could, therefore, reduce the capacities of birds of prey to avoid obstacles or learn how to avoid them, as suspected also by Rideout et al. (2011).

The last significant trend observed in this study is that some bird species may be considered as indicators of exposure for others, with smaller, limited populations. We conclude that the Griffon Vulture is a good surrogate species for the Bearded Vulture (both in terms of causes of death and contaminant load). Working with Griffon Vultures, whose populations are larger and in better shape, can help provide statistically significant data, which could not be obtained in Bearded Vultures, despite potential species susceptibility differences (Carpenter et al., 2003). Similarly, but with smaller populations, it seems that Red Kites could be a good surrogate for Egyptian Vultures.

In Europe, major conservation programs have been set up for the Pyrenean Birds of Prey. There is evidence showing that the Bearded Vulture is endangered as a result of anthropogenic disturbances (Arroyo and Razin, 2006), including lead exposure (Hernandez and Margalida, 2009a). This study shows that pesticide and Pb represent the most important identified cause of death in this long-lived species. Complete ban of these toxic products would certainly be beneficial for the survival of the remaining population, provided there is strict control of local use in the field (Martínez-Haro et al., 2008). In Bearded Vultures, Egyptian Vultures and Red Kites, with either limited populations, and low reproductive potential, collisions and trauma may hamper their survival. A thorough review of the interactions between identified threats and adaptative management actions on the survival of scavenging birds of prey has been provided for the Cinereous Vulture (*Aegypius monachus*) in Spain (Moreno-Opo and Margalida., 2014) and shows the necessity of careful adaptation of protective measures to identified threats for a given population. Demonstration of the high frequency of Pb exposure and associated locomotor/behavioral disorders may also highlight previous studies showing a high proportion of electrocution / trauma, which could, in fact be attributed to high Pb exposure. As a consequence, the use of Pb in hunting projectiles has a major impact on the long-term probability of survival of large birds of prey species in the Pyrenees. Replacing lead with other metals should be considered both to give these species a better likelihood of survival and maintain effective bullets for hunting (Trinogga et al., 2013; Margalida et al., 2013).

Capsule abstract

Illicit use of pesticides and lead poisoning appear as the single most frequent causes of death in endangered vulture species in France

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.04.003.

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