Science of the Total Environment 536 (2015) 704–712

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

# Risk assessment of bearded vulture (*Gypaetus barbatus*) exposure to topical antiparasitics used in livestock within an ecotoxicovigilance framework

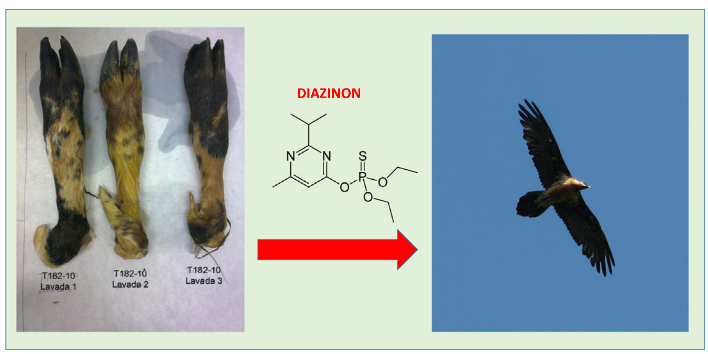
Rafael Mateo [a,*], Inés S. Sánchez-Barbudo [a], Pablo R. Camarero [a], José M. Martínez [b]

[a] Instituto de Investigación en Recursos Cinegéticos, IREC (CSIC, UCLM, JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain
[b] Departamento de Agricultura, Ganadería y Medio Ambiente, Dirección General de Desarrollo Sostenible y Biodiversidad, Zaragoza, Spain

## HIGHLIGHTS

- Antiparasitics used in livestock were found in dead bearded vultures.
- Diazinon was the most prevalent external antiparasitic detected in lamb feet.
- Toxicity–exposure ratio reveals potential risk of acute poisoning in worst-case scenarios.
- Estimated diazinon exposure may impair thermoregulation in bearded vultures.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 28 May 2015
Received in revised form 23 July 2015
Accepted 23 July 2015
Available online xxxx

Editor: D. Barcelo

*Keywords:*
Scavengers
Poisoning
Veterinary drugs
Topical antiparasitics
Organophosphates
Pyrethroids

## ABSTRACT

Between 2004 and 2013, 486 suspected scavenger poisoning cases, including 24 bearded vultures (*Gypaetus barbatus*), were investigated in the Pyrenees and surrounding areas in Spain as part of a monitoring programme regarding accidental and intentional poisoning of wildlife. Poisoning was confirmed in 36% of all analysed cases where scavenger species were found dead within the distribution range of bearded vultures. Organophosphates and carbamates were the most frequently detected poisons. Four of the bearded vulture cases were positive for the presence of topical antiparasitics (3 with diazinon and 1 with permethrin). These likely represented accidental exposure due to the legal use of these veterinary pharmaceuticals. In order to confirm the risk of exposure to topical antiparasitics in bearded vultures, pig feet (n = 24) and lamb feet (n = 24) were analysed as these are one of the main food resources provided to bearded vultures at supplementary feeding stations. Pig feet had no detectable residues of topical antiparasitics. In contrast, 71.4% of lamb feet showed residues of antiparasitics including diazinon (64.3%), pirimiphos-methyl (25.4%), chlorpyrifos (7.1%), fenthion (1.6%), permethrin (0.8%) and cypermethrin (27.8%). Washing the feet with water significantly reduced levels of these topical antiparasitics, as such, this should be a recommended practice for lamb feet supplied at feeding stations for bearded vultures. Although the detected levels of antiparasitics were relatively low (≤1 μg/g), a risk assessment suggests that observed diazinon levels may affect brain acetylcholinesterase and thermoregulation in bearded vultures subject to chronic exposure.



* Corresponding author.
*E-mail address:* rafael.mateo@uclm.es (R. Mateo).

http://dx.doi.org/10.1016/j.scitotenv.2015.07.109
0048-9697/

## 1. Introduction

The use of chemicals for productive activities can be a risk for the environment in general, and in particular, for wildlife (Guitart et al., 2010). Studies regarding the bioaccumulation and biomagnification of persistent organic pollutants (POPs) have demonstrated the importance of monitoring programmes which utilise top predators such as birds of prey (Gómez-Ramírez et al., 2014). However, the risk of released chemicals to wild animals is not only limited to POPs. Recognised differences in sensitivity to toxicants among animal species highlights the need for greater toxicovigilance of all chemical substances used in the environment (Hutchinson et al. 2014), since these can often be causative agents of wildlife mortality (Pain et al., 2004; Berny, 2007; Martínez-Haro et al., 2008). Recent studies have shown that toxicovigilance should also consider veterinary drugs that can be present in carcasses that are eaten by scavengers and opportunistic predators (Oaks et al., 2004; Taggart et al., 2009). In addition to *accidental poisoning* of wildlife (due to the legal use of pesticides or veterinary drugs), predatory species are also especially at risk of *intentional poisoning* due to human–wildlife conflicts. This occurs especially when predators attack livestock or game species with a high economic value (Whitfield et al., 2003; Wobeser et al., 2004; Venkataramanan et al., 2008; Richards, 2012). Accidental and intentional sources of poisoning can also be present at the same time in a geographic area and together represent a risk for wildlife conservation that has to be unequivocally identified and quantified if necessary corrective measures are to be effectively implemented (Mineau et al., 1999; Elliott et al., 2011).

One conservation measure which can be adopted to protect certain avian scavengers regards the use of supplementary feeding stations or so-called *vulture restaurants*; wherein, food security and food safety can potentially be better assured (Margalida et al., 2014). Such feeding stations have also reduced the impact of (for example) EU regulations against the free disposal of carcasses of livestock in the field (Margalida et al., 2010) and have also reduced the risk of poisoning due to illegal baits used to kill predators (Oro et al., 2008), and, of lead poisoning caused by ammunition residues left within shot carcasses of wild game animals (Mateo et al., 1997; Garcia-Fernandez et al., 2005; Hernández and Margalida, 2009a). However, these supplementary feeding stations may also alter the natural foraging behaviour of vultures and may represent a risk due to chronic exposure to veterinary drugs used in livestock (Oro et al., 2008; Taggart et al., 2009). As a result, various scavengers, including vultures, may be exposed to a range of potent/highly bio-active compounds — the implications of which remain very poorly characterized (Shore et al., 2014; Cuthbert et al., 2014). For example, poisoning due to topical antiparasitics has been described in relation to the use of anticholinesterasic compounds (Henny et al., 1987; Mineau et al., 1999); and across Asia, exposure to diclofenac (a non-steroidal anti-inflammatory drug — NSAID) through this pathway has caused the near global extinction of at least three species of Old World *Gyps* vultures (Oaks et al., 2004; Taggart et al., 2009; Cuthbert et al., 2014). Further, Zorrilla et al. (2015) also identified (in southern Spain) a suspected case of a griffon vulture being poisoned by flunixin (another anti-inflammatory drug used in livestock). These cases have highlighted the urgent need for far better pharmacovigilance and the requirement for improved, more comprehensive Life-Cycle Assessments for the myriad of pharmaceutical products currently in use (Shore et al., 2014).

Despite the observed impact of NSAIDs in Asia on scavengers, surprisingly little monitoring data currently exists regarding the fate of pharmaceuticals in the environment and their potential effects on higher wildlife. European vultures provide an obvious starting point for further work in this arena. As such, here, we present data regarding the exposure of bearded vulture (*Gypaetus barbatus*) to topical antiparasitics in north-east Spain. Bearded vulture is a species listed by the IUCN as Near Threatened and the European population is considered Endangered. The European population comprises of only 170 breeding units, 117 of them located in Spain (Margalida et al., 2014); with the core stronghold area being in Aragón in north-eastern Spain (Gómez de Segura et al., 2012). Several studies have compiled details regarding poisoning cases recorded for bearded vultures from the Pyrenees (Margalida et al., 2008; Berny et al., 2015), but antiparasitics have not been thoroughly considered. Between 2004 and 2013, we investigated cases of suspected poisoning in the Pyrenees and surrounding mountains of bearded vultures and other scavenging species. We considered the risk of exposure in bearded vulture to topical antiparasitics used to treat ovine livestock specifically because lamb feet are one of the main food resources consumed by bearded vultures in supplementary feeding stations; in addition, sheep carcasses are also consumed in the wild, within the wider landscape. The antiparasitic compounds and concentrations found in/on lamb feet were then used to assess the risk that these residues posed to bearded vultures. The risk of adverse effects that could compromise adult or nestling survival was evaluated in light of current toxicological knowledge with regard to the detected topical antiparasitics.

## 2. Materials and methods

### 2.1. Species and study area

Bearded vulture is a large raptor (mean weight: 5.79 kg) of the Accipitridae family, which feeds mostly on carcasses of mammals (95%), followed by birds (4%) and reptiles (1%). Medium sized mammals (especially sheep and goat, followed by Southern chamois *Rupicapra pyrenaica*, wild boar *Sus scrofa* and deer species) form the core of their diet (74%) (Margalida et al., 2009). The study area was the Spanish Pyrenees and the surrounding mountains where the bearded vulture is distributed (Fig. 1). Poisonings involving other scavengers in provinces where bearded vulture are present (Alava, Navarra, Zaragoza, Huesca, Lleida, Barcelona and Girona) were also studied to consider the prevalence of poisoning involving various types of toxicant and to compare results obtained with those for bearded vultures. Some of these cases were located outside of the normal distribution range for bearded vultures, but, the data may give an indication of the risk of poisoning if breeding or foraging range expansion occurred. The studied scavengers (facultative and opportunistic) were Egyptian vulture (*Neophron percnopterus*), griffon vulture (*Gyps fulvus*), cinereous vulture (*Aegypius monachus*), black kite (*Milvus migrans*), red kite (*Milvus milvus*), golden eagle (*Aquila chrysaetos*), Eurasian buzzard (*Buteo buteo*), red fox (*Vulpes vulpes*) and domestic dog (*Canis familiaris*).

### 2.2. Poisoning monitoring of bearded vulture and other scavengers

Cases of suspected poisoning of bearded vulture (n = 24) and other facultative or opportunistic scavengers (n = 462; Fig. S1) were submitted to our laboratory by Wildlife Rehabilitation Centres within the study area between 2004 and 2013. Samples of liver, gastric content, brain, pellets or carcass remains were taken by the veterinary staff of these Centres and were submitted with a report containing the more relevant clinical and necropsy findings.

### 2.3. Sampling of food supplied at feeding stations

Feet of lamb (n = 126) were sampled from five slaughterhouses (n = 102) and five supplementary feeding stations (n = 24). In order to study contaminant removal efficiency from the lamb feet, a washing procedure (with water) was utilised. Washed (n = 55) and unwashed (n = 47) lamb feet from the slaughterhouses were analysed. Lamb feet from supplementary feeding stations (n = 24) were all washed. Moreover, pig feet (n = 24) from slaughterhouses were also analysed (Fig. S2).

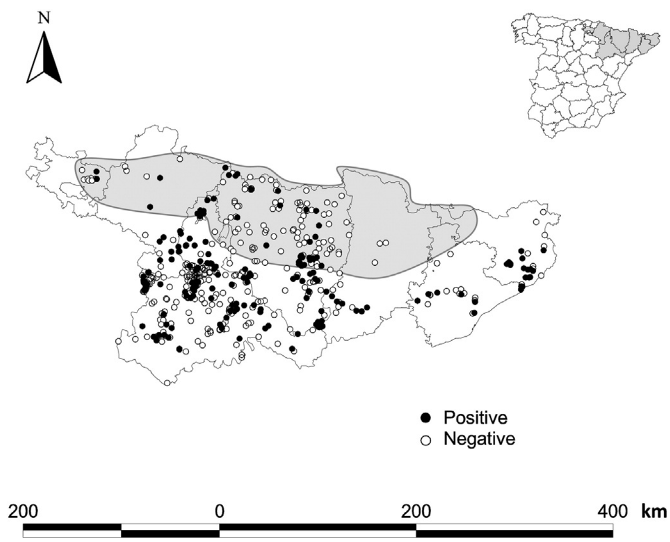



**Fig. 1.** Map of the provinces within the distribution range of the bearded vulture in the Pyrenees and results of the studied cases of poisoning (n = 486) in bird and mammal species of scavengers. Provinces from West to East: Alava, Navarra, Zaragoza, Huesca, Lleida, Barcelona and Girona. The shaded area of the large map corresponds to the approximate breeding range of bearded vultures in NE Spain.

*2.4. Toxicological analysis*

Cases of suspected poisoning were studied following the procedures described in Sánchez-Barbudo et al. (2012a, 2012b). Extraction/clean-up procedures involved a dichloromethane extraction, a gel-permeation chromatography (GPC) purification step, and then analyses with gas chromatography mass spectrometry (GC–MS) and/or liquid chromatography mass sprectrometry (LC–MS). Protocols were adapted from Brown et al. (1996) and covered most common acute neurotoxicants (i.e., organophosphates, carbamates, organochlorines, neonicotinoids, barbiturates, α-chloralose and strychnine) with limits of detection (LODs) in the order of 0.1–10 ng/g, depending on the chemical and type of sample involved. Based on initial results and, clinical signs and necropsy findings, other chemicals were also investigated. Anticoagulant rodenticides were analysed in cases where evidence of haemorrhages were observed (as described by Sánchez-Barbudo et al., 2012c). In cases with severe congestion or haemorrhages within the digestive tract, arsenic was determined by atomic absorption spectroscopy with graphite furnace (GF-AAS) after sample digestion in a microwave oven as described by Reglero et al. (2008). Lead exposure was also measured using GF-AAS on digested samples. In addition to chemical analysis, acetylcholinesterase (AchE) or cholinesterase (ChE) activities were measured in brain tissue and plasma, respectively, when these samples were available following the Ellman's method as described by Hill and Fleming (1982). In vitro AchE and ChE reactivations with buffer dilution (i.e., for carbamates) or 2-PAM addition (i.e., for organophosphates) (as described by Stansley, 1993) were used to support the chemical analysis.

The analysis of topical antiparasitics in lamb and pig feet was performed as described above for neurotoxicants with a solvent extraction, a GPC purification step and GC–MS analysis. Two extraction methods were used. Initially (method 1), 36 lamb feet and 24 pig feet (the distal 5 cm of the feet) were extracted by submersion in 100 ml of ethyl acetate with sonication for 10 min. The extract was evaporated, re-suspended in 1 ml of ethyl acetate:cyclohexane (50:50) and processed as described above (i.e., GPC purification followed by GC–MS analysis). Concentrations were expressed as ng/g of foot. As solvent consumption was high with this method, another procedure (method 2) was later used whereby a 5-cm height strip of skin from around each foot (just above the hoof) was extracted. The pieces of skin analysed had a surface area (mean ± SD) of 39 ± 7 $cm^2$ and a mass of 13.5 ± 3.3 g. The skin was extracted with sonication for 10 min with 15 ml of ethyl acetate and this process was repeated with additional volumes of 15 ml and 5 ml of ethyl acetate, consecutively. The three extracts were pooled and processed as described above (GPC purification and GC–MS analysis). In this case, the concentration of a topical antiparasitic is expressed as ng/g of skin. We must note that the concentration derived using both methods only approximated to the level of external contamination present on the feet, i.e., residues of antiparasitics may also have been absorbed into/beyond the skin tissue (as such, our data might be considered inherently conservative). Recovery using this analytical method, for the skin of lamb feet spiked with 125 ng/g, ranged between 60–97% for the detected organophosphorous and pyrethroid compounds. The average coefficient of variation within the replicate spiked samples (n = 3) was 13%. The concentrations measured in the lamb feet samples were corrected according to obtained recoveries.

*2.5. Risk assessment procedure and data analysis*

A risk assessment regarding the exposure of bearded vultures to topical antiparasitics used in livestock was performed using the observed concentrations in animal feet supplied at supplementary feeding stations. The energetic (food) requirements of bearded vultures are 417–535 Kcal/day (334–428 g) at 30 °C and 478–615 Kcal/day (382–492 g) at 0 °C (Donázar, 1993). Margalida et al. (2012) estimated that 75% of bearded vulture diet is based on bones and meat with energetic values of 161 and 140 Kcal/100 g, respectively. According to these values, they estimated that each pair would consume 341 kg of food per year (467 g of food/bird/day). We have used the figure of 492 g of food per day and the mean weight of the species as 5.79 kg to calculate the Estimated Daily Intake (EDI) with regard to the mean and maximum concentrations of antiparasitics detected in whole lamb feet. The values detected in unwashed samples from the slaughterhouse were taken as the potential exposure level in the field/wild, although these were probably conservative since lambs should not be treated with antiparasitics within 15 days of slaughter (i.e., sheep or lambs that die in the field may well have much higher levels). Values were then employed to calculate the Toxicity-Exposure Ratio (TER) with respect to different toxicity values in birds (Shore et al., 2005; EFSA, 2009). One of these toxicity values was the Hazardous Dose 5 ($HD_5$) for the detected organophosphorous and pyrethroid compounds as estimated for wild birds (Mineau et al., 2001). The $HD_5$ is defined as the Median Lethal Dose ($LD_{50}$) at the 5th percentile of the species sensitivity distribution — i.e., it is most relevant to bird species that are particularly sensitive to a particular toxicant (Mineau et al., 2001). The second toxicity value considered in the risk assessment was based on the sublethal effect that organophosphorous compounds can have on thermoregulation at a dose equivalent to 5% of the $LD_{50}$ (Rattner and Franson, 1984; Grue et al., 1997; Hill, 2003). If we consider the $HD_5$ as a precautionary $LD_{50}$ for the bearded vulture, the potential adverse effect dose on thermoregulation ($ED_{th}$) could then occur at 5% of the $HD_5$. These values were then used to calculate the respective TER at the $HD_5$ ($TER_{HD}$) and the $ED_{th}$ ($TER_{th}$). A risk for bearded vultures was considered relevant when these values were below trigger values of 10 (for acute toxicity) or 5 (for long-term toxicity), respectively (Shore et al., 2005; EFSA, 2009).

The percentage of positive determinations in cases of suspected poisonings were compared between species with Yate's $\chi^2$ test. Fisher's exact test was also used to compare the occurrence of topical antiparasitics on feet between washed and unwashed samples. Antiparasitic concentrations were also compared between methods and between washed and unwashed samples with Mann–Whitney tests. Significance was set at $p \leq 0.05$. Statistical analyses were performed with IBM SPSS Statistics 19.

## 3. Results

### 3.1. Poisons used in the distribution range of bearded vultures in Spain

Poisoning was confirmed in 36% of analysed cases involving scavenger species found dead within the provinces inside the distribution range of bearded vultures in NE Spain (Fig. 1). The occurrence of confirmed poisoning was highest in Egyptian vulture (76.7%), red kite (62.1%) and other medium sized raptors, whereas lower values were found in bearded vultures (16.7%) and other large raptors (Fig. 2; Table S1). Significantly higher occurrences were found in Egyptian vulture (Yate's $\chi^2 = 20.1$, $p < 0.001$), red kite (Yate's $\chi^2 = 12.2$, $p < 0.001$), Eurasian buzzard (Yate's $\chi^2 = 4.2$, $p = 0.042$) and dog (Yate's $\chi^2 = 8.7$, $p = 0.003$) than in bearded vultures. Anticholinesterasic compounds (organophosphates and carbamates) accounted for 70% of the confirmed cases of poisoning in the study area (Fig. S3). The most frequently detected chemicals were carbofuran (n = 35), fenthion (n = 16), aldicarb (n = 14), methamidophos (n = 13), demeton (n = 12) and monocrotophos (n = 10). Other 33 chemicals were detected in lower frequency (n < 10) (Table S1).

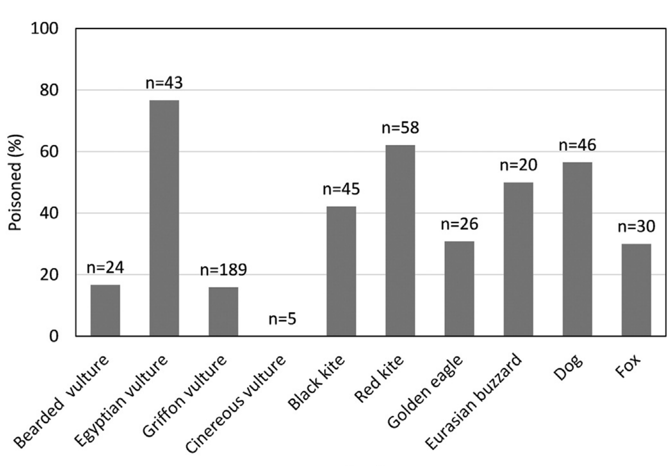



**Fig. 2.** Percentage of scavengers diagnosed as poisoned in Spanish provinces where bearded vulture is present.

### 3.2. Suspected poisoning of bearded vultures

The analytical results regarding suspected poisoning of bearded vultures have been summarized in Table 1. The types of toxicants detected in bearded vultures were more frequently associated with likely *accidental poisoning* (i.e., via the use in livestock of topical antiparasitics) in comparison to other species which were more affected by the likely *intentional poisoning* used to kill predators (whereby restricted or banned pesticides/poisons like carbofuran, aldicarb and strychnine are used) (Table S1).

One of the dead bearded vultures (ref. 019/10) had diazinon (12 ng/g) in its gastric content and another one (ref. 218/11) had permethrin (56 ng/g). Lamb feet associated with the death of another bearded vulture (ref. 165/13) were positive for diazinon (48.5 ng/g) and a pellet collected in a failed nest (ref. 183/09) also contained diazinon (28.5 ng/g). Brain AChE activity in the bearded vulture with diazinon in its gastric contents (ref. 019/10) was 16.8 μmol/min/g, which is lower than the activity found in another bearded vulture which was shot (ref. 156/06) and had 23.5 μmol/min/g. However, AchE in this diazinon-exposed vulture was not reactivated after 2-PAM addition or buffer dilution of the brain homogenate. The bearded vulture 165/13 was a captive bird fed with lamb feet that were also used to feed vultures in the field. This bird showed similar clinical signs and necropsy lesions (abnormal posture and visceral congestion) as other birds found dead in the field, including the gastric content diazinon positive bird ref. 019/10. Another bearded vulture found sick (ref. 245/08) showed octinoxate (a sunscreen product) in its blood (not quantified), but, it survived and was later released. This unexpected detection may have resulted from the accidental ingestion by the vulture of a sunscreen product (e.g., a lipstick or skin cream) or may be due to cross-contamination during blood sampling. Tetramethrin was also found in bearded vulture 157/06, but, this was considered possible cross-contamination as this pyrethroid was used against flies within the necropsy room at the Wildlife Rehabilitation Centre from which it originated.

### 3.3. Antiparasitics in lamb feet used to feed wild bearded vultures

Pig feet had no residues of topical antiparasitics. In contrast, the pooled data for all the lamb feet (independent of site of collection, analytical method or washing procedure) showed that 71.4% of samples had residues of antiparasitics including diazinon (64.3%), pirimiphos-methyl (25.4%), chlorpyrifos (7.1%), fenthion (1.6%), permethrin (0.8%) and cypermethrin (27.8%). Significant differences were found in terms of the occurrence of antiparasitics among the slaughterhouses ($\chi^2_4 = 42.5$, $p < 0.001$) and the supplementary feeding stations ($\chi^2_4 = 10.4$, $p = 0.034$). This was probably due to differences in treatment regimes used on farms from which the lambs came (Fig. 3). It seems that antiparasitics occurred more frequently in samples from slaughterhouses in mountainous areas when compared to those in the valley; with prevalence values up to 95% in Sabiñanigo and Huesca (Fig. 3).

Detailed data regarding the occurrence and concentration of topical antiparasitics in whole feet or skin samples are shown in Table S2. The effect of washing was analysed separately for each type of sample. Washing with water significantly reduced the occurrence of chlorpyrifos in the whole lamb feet analysed with method 1 (Fisher's exact test, $p < 0.001$) and pirimiphos-methyl in skin analysed with method 2 ($p = 0.031$; Table S2). Washing also reduced the levels of diazinon, chlorpyrifos and cypermethrin in lamb feet analysed with method 1 (Mann–Whitney tests, all $p \leq 0.003$; Fig. 4) and pirimiphos-methyl in skin analysed with method 2 ($p = 0.022$; Table S2).

### 3.4. Risk assessment for livestock antiparasitic exposure in avian scavengers

The assessment of the risk posed by topical antiparasitics used in livestock to bearded vultures (Table 2) reveals that the $TER_{HD}$ was 6.7

**Table 1**
Studied cases of suspected poisoning in bearded vultures from the Spanish Pyrenees.

| Ref/Y[a] | RT[b] | Age/Sex[c] | TFD[d] | Anamnesis and necropsy findings[e] | Toxicology[f] |
|---|---|---|---|---|---|
| 124/06 | No | —/— | <7 | Traumatism | Negative (brain AChE:15.08 μmol/min/g) |
| 152/06 | Yes | J/— | >120 | Abnormal posture, dead blowflies | Negative |
| 153/06 | – | N/— | <7 | Haemorrhagic pneumonia, hepatitis | Negative |
| 154/06 | Yes | Ad/ | >30 | Abnormal posture, possible electrocution | Negative |
| 155/06 | Yes | 6y/M | 1 | Abnormal posture, visceral congestion | Negative (brain AChE: 16.7 μmol/min/g; Pb: 0.04 μg/g w.w. liver) |
| 156/06 | Yes | 2y/ | 3 | Shot, cachexia | Negative (brain AChE: 23.5 μmol/min/g) |
| 157/06 | Yes | Ad/ | >120 | Abnormal posture, old fractures | Negative (tetramethrin probably from cross-contamination) |
| 158/06 | No | —/— | >120 | Old fractures | Negative |
| 113/07 | No | Ad/ | 15 | Abnormal posture in nest | Negative |
| 169/07 | No | N/ | 15 | Predation | Negative |
| 229/07 | No | Ad/M | <7 | Electrocution | Negative |
| 149/08 | No | Ad/F | 0 | Traumatism, visceral congestion | Negative |
| 230/08 | No | ? | 0 | Traumatism | Negative (brain AChE: 19.0 μmol/min/g; Pb: 0.04 μg/g w.w. blood) |
| 245/08 | Yes | J/ | – | No lesions, found alive in water | Octinoxate (Pb: 4.5 μg/dl blood) |
| 056/09 | Yes | J/F | <7 | Traumatism | Negative (Pb: 0.08 μg/g w.w. liver) |
| 105/09 | No | Ad/F | 0 | Found alive, electrocution | Negative (Pb: 0.06 μg/g w.w. liver) |
| 181/09 | No | ?? | – | No lesions, found alive in water | Negative (plasma ChE: 608 mU/ml, 725 mU/ml with 2-PAM) |
| 183/09 | – | —/— | – | Pellet in a failed nest | Diazinon: 28.5 ng/g pellet |
| 019/10 | No | Ad/F | 3 | General congestion | Diazinon: 11.9 ng/g gastric content (brain AChE: 16.8 μmol/min/g; 0.32 μg/g w.w. liver; bromadiolone 1 ng/g liver) |
| 111/11 | Yes | J/— | >30 | Probable traumatism | Negative (Pb: 0.46 μg/g bone) |
| 204/11 | No | Ad/F | 60 | Traumatism | Negative |
| 218/11 | Yes | 1y/ | 1 | Traumatism | Permethrin: 56.2 ng/g liver |
| 230/12 | No | 5y | ? | Traumatism | Negative |
| 165/13[g] | ? | ? | ? | Abnormal posture, congestion | Diazinon (48.5 ng/g lamb feet) |

[a] IREC reference number with last two digits of the year (from 2006 to 2013).
[b] Radiotracking.
[c] N: nestling, J: juvenile, Ad: adult, #y: number of year, M: male, F: female.
[d] Time from death to collection.
[e] Most significant anamnesis data and necropsy findings for diagnosis.
[f] Relevant results of the toxicological analyses: Negative to GC–MS, HPLC-DAD, LC–MS and/or GF-AAS, AChE: acetylcholinesterase, ChE: cholinesterase.
[g] Bird dead in captivity.

for diazinon, which is below the trigger value of 10 commonly used in acute dietary risk assessments. This suggests the need for higher tier evaluation (such as field monitoring or captive exposure studies). In terms of the $TER_{th}$, this value was 0.3, hence the risk of impaired thermoregulation was highly possible under the exposure conditions considered in this risk assessment (Table 2).

## 4. Discussion

The intentional use of poison to kill predators, especially wolf (*Canis lupus*) and fox, is still widespread in Spain. This is also the case in remote mountainous areas of Northern Spain where some emblematic species have their last Iberian, or even Western European, strongholds

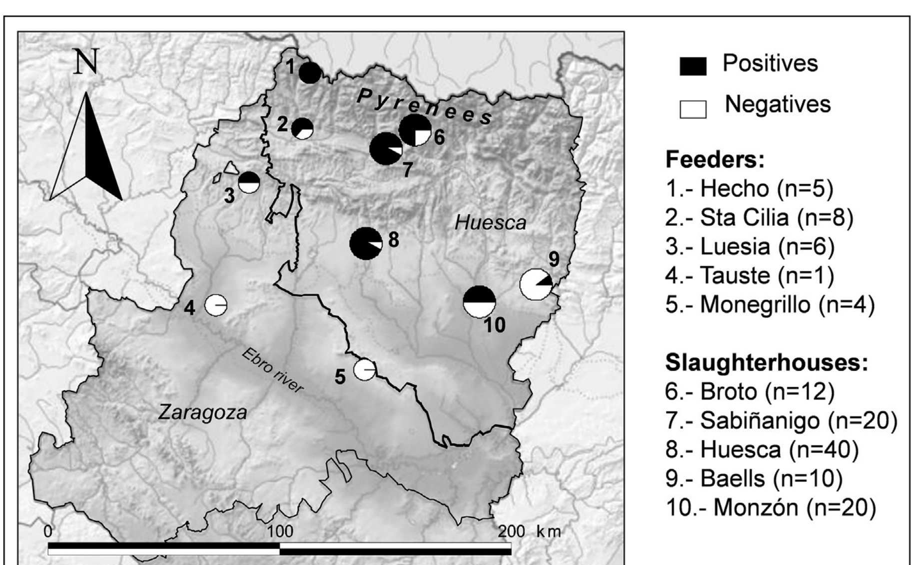



**Fig. 3.** Occurrence of antiparasitics in lamb feet from supplementary feeding stations and the supplying slaughterhouses in the provinces of Huesca and Zaragoza (Aragón).

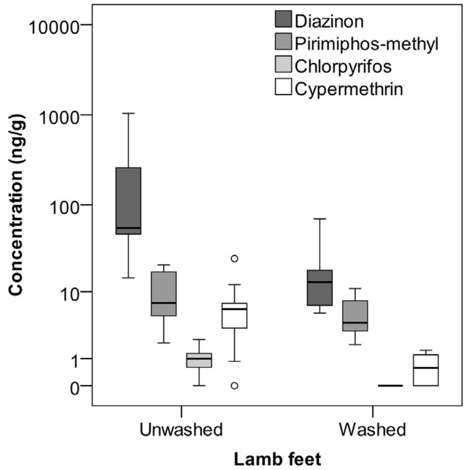



**Fig. 4.** Box plot diagram (median, 25–75th percentiles and range) of the concentrations of topical antiparasitics in lamb feet washed (n = 10) and unwashed (n = 10) with water. Diazinon, chlorpyrifos and cypermethrin showed a significant reduction in residue levels after the washing procedure (Mann–Whitney tests, all p ≤ 0.003).

(i.e., bearded vulture, brown bear (*Ursus arctos*) and wolf). Bearded vultures, as well as other avian scavengers, can also be poisoned *accidentally* if they consume livestock carcasses that contain residues of veterinary drugs. Here, we have found that topical antiparasitics used widely on sheep can represent a significant hazard for bearded vultures in the Pyrenees (in addition to the intentional use of poisons).

### 4.1. Intentional poisoning of wildlife in the Pyrenees area

The most frequently detected compounds in poisoned animals in the study area were organophosphates, closely followed by carbamates (Table S1; Fig. S3). These results contrast with other studies conducted in other regions of Spain where the use of poison is more clearly related with game protection, i.e., where carbamate insecticides, mostly carbofuran and aldicarb, predominate (Martínez-Haro et al., 2008; Hernández and Margalida, 2008, 2009b; Sánchez-Barbudo et al., 2012b). Until recently, strychnine was also frequently used in Spain (Guitart et al., 1999; Motas-Guzmán et al., 2003; Martínez-López et al., 2006), but, its ban as a rodenticide in 1994 has made it more difficult to use this product for illegal purposes. However, restrictions or bans on some pesticides have not eliminated the risk posed by their illegal use as poisons. Indeed, most poisoning in the last decade in Spain has been caused by pesticides that were restricted to use by authorized personnel only (Martínez-Haro et al., 2008). It is also worth pointing out that in the present study there are still cases of endrin poisoning in foxes, even though endrin (an organochlorine) was totally banned more than two decades ago (Martínez-Haro et al., 2008).

The use of poison in Spain and, elsewhere (Mateo-Tomás et al., 2012; Richards, 2012; Ogada, 2014), has long been associated with the killing and persecution of predators that supposedly threaten valuable natural resources used by humans, mostly, game and livestock species. In Spain, the illegal use of poison in relation to game protection is frequent in areas where small game is important to hunters (Hernández and Margalida, 2008); the main predator (and therefore poisoning target) being the fox (Márquez et al., 2013). This is not however the case in the Pyrenees. The most important game species here are large ungulates, which are far less affected by foxes. Another reason for the illegal use of poison is to protect livestock, and especially sheep, against wolf attacks (and to a lesser extent, fox attack). Again, the Pyrenees is not a highly conflictive area in this regard, at least in contrast with the Cantabrian Mountains in Northern Spain (Mateo-Tomás et al., 2012) where wolf is more common. The wolf has not yet expanded its stronghold range from Northwest Spain into the Northeast (Gortázar et al., 2000; Echegaray and Vilà, 2010). Likewise, few wolves from the Italian population have become established in the Catalan Pyrenees (Lampreave et al., 2011).

### 4.2. Is intentional or accidental poisoning most common in bearded vultures?

Poisoning has been confirmed in bearded vulture at a similar frequency to that in griffon vulture, but, it was much lower than in other medium sized scavengers like Egyptian vultures and kites (Fig. 2; Table S1) that have been severely affected by the illegal use of poison (Hernández and Margalida, 2009b; Sánchez-Barbudo et al., 2012b). Differences in the percentage of confirmed poisonings among avian scavengers were less marked in a recent study of cases from the French side of the Pyrenees (range: 18–33%; Berny et al., 2015).

The types of toxicants detected in our cases indicate that bearded vultures are more frequently affected by accidental poisoning due to the use of organophosphates as topical antiparasitics in ovine livestock (Table 1), which is the main food source for this vulture (Margalida et al., 2009). Organophosphates are in general highly toxic compounds, and in our study area, especially in the Aragon region, they have been the most frequently used illegal poisons against wildlife (Sánchez-Barbudo et al., 2012b). However, the low concentrations we detected here in bearded vultures and, the risk assessment performed with lamb feet, seems to indicate that many poisonings in bearded vultures in the Pyrenees were *accidental* and likely associated with the use of topical antiparasitics in livestock (Table 1). A previous study of cases of poisoning in bearded vultures from the Spanish Pyrenees (for the period 1955–2006) described a high incidence of alkaloids (i.e. strychnine) followed by organophosphates and carbamates (Margalida et al., 2008). On the French side of the Pyrenees, two (out of eight) cases of confirmed poisoning in bearded vultures were caused by anticholinesterasic compounds (Berny et al., 2015).

**Table 2**
Risk assessment regarding lamb feet ingestion in bearded vultures performed with residue levels of topical antiparasitics in unwashed whole lamb feet from slaughterhouses (n = 10).

| Compound | Residues[a] (ng/g) mean (max) | EDI[b] (mg/kg b.w.) mean (max) | $HD_5$[c] (mg/kg b.w.) | $TER_{HD}$[d] mean (max) | $ED_{th}$[e] (mg/kg b.w.) | $TER_{th}$[f] mean (max) |
|---|---|---|---|---|---|---|
| Diazinon | 198 (1,039) | 0.017 (0.088) | 0.6 | 35.1 (6.7) | 0.0295 | 1.8 (0.3) |
| Pirimiphos-methyl | 9.6 (50.1) | 0.001 (0.004) | 13.5 | 16,556 (3,173) | 0.6755 | 828 (159) |
| Chlorpyrifos | 1 (1.3) | 0.0001 (0.0001) | 3.8 | 44,235 (34,027) | 0.188 | 2212 (1701) |
| Cypermethrin | 7.3 (23.8) | 0.001 (0.002) | 579.2 | 933,360 (286,283) | – | – |

[a] Residues detected in the unwashed lamb feet collected at the slaughterhouse.
[b] EDI: Estimated Daily Intake. Calculated based on a food intake by bearded vultures of 0.492 kg/day and a body mass of 5.79 kg.
[c] $HD_5$: Hazardous Dose established as the $LD_{50}$ at the 5th percentile in the species sensitivity distribution (Mineau et al., 2001).
[d] $TER_{HD}$: Toxicity–exposure ratio calculated as $HD_5$/EDI. Values <10 can be considered of concern for bearded vulture protection.
[e] $ED_{th}$: Effect dose considering thermoregulation impairment as endpoint and estimated as 5% of the $HD_5$ This level has been estimated based on the observation that anticholinesterasics can reduce the core temperature by 2 °C in homoeothermic animals with a single exposure of about 5% the $LD_{50}$.
[f] $TER_{th}$: Toxicity–exposure ratio calculated as $ED_{th}$/EDI. Values <5 can be considered of concern for bearded vulture protection.

The illegal use of poison is a known handicap for this species. Indeed, the current reintroduction project for bearded vulture in Sierra Cazorla (Southern Spain) has been threatened by cases of aldicarb poisoning since 2008 (CAPMAA, 2013). More recently, one bearded vulture from the reintroduction project in the Picos de Europa (Cantabrian Mountains in NW Spain) was found dead and it was positive for the presence of aldicarb in its stomach (authors own unpublished data). It should be noted that bearded vultures disappeared from most of their breeding range in Spain during the 20th century. At one point, the only breeding population was in the Pyrenees; this probably indicates that (historically at least) the use of poison has been less in this area, perhaps due to the absence of wolf for many years (Ruiz-Olmo and Aguilar, 1995).

*4.3. Risk assessment of topical antiparasitic exposure in avian scavengers*

The risk assessment performed here with the EDI of diazinon in bearded vultures indicates a potential effect on their thermoregulation and a need of further research to confirm acute poisoning. The detection of diazinon as the only toxicological evidence in three potential poisoning cases involving bearded vultures may indicate that this possibility exists. In the case of a Near Threatened species such as the bearded vulture, the use of the $HD_5$ (Mineau et al., 2001) is appropriate because it is one of the most conservative methods available and is based on Species Sensitivity Distributions (SSDs), which consider the effect of body weight scaling and phylogeny (Luttik et al., 2005). We have used $HD_5$ values to calculate its ratio with the calculated EDI of each antiparasitic in order to obtain a corresponding TER (EFSA, 2009). The $TER_{HD}$ derived from our mean diazinon concentration was 35.1, which indicates a low risk of acute poisoning. However, the $TER_{HD}$ was 6.7 at the maximum exposure dose used here for diazinon (Table 2). This is below the trigger value of 10 used in an acute risk assessment to decide if further refinement is needed — based on more accurate data and field work (EFSA, 2009). This suggestion of risk is also supported by the observation here of several cases of bearded vultures found dead solely with evidence of diazinon exposure (Table 1). Moreover, one captive bearded vulture fed with exactly the same lamb feet as are supplied at supplementary feeding stations died with similar clinical signs and necropsy findings to those birds found dead in the field. This evidence suggests that (1) bearded vultures may be more sensitive to diazinon than the 5th percentile of even the most sensitive species, i.e., the HD of bearded vulture may be $<HD_5$, and/or (2) the diazinon concentration in some lamb feet could well exceed the values detected here.

In terms of the other antiparasitics found in lamb feet here, the risk of poisoning due to acute exposure seems lower. In the case of pirimiphos-methyl, the detected concentrations were relatively low and its $HD_5$ (13.5 mg/kg b.w.) is much lower than that of diazinon. Chlorpyrifos represent a higher risk because of its low $HD_5$ value (3.8), but, the residue levels detected were far lower than for diazinon. In the case of fenthion, which was detected in two samples of lambs foot skin only, the $HD_5$ is also low (0.83; Mineau et al., 2001), but, it was detected at both low frequency and at low concentrations. The two detected antiparasitics from the pyrethroid family have much higher $HD_5$ values (579 and 3,127 mg/kg b.w. for cypermethrin and permethrin respectively; Mineau et al., 2001) when compared to the organophosphorous compounds. As such, their estimated risk is low. However, high levels of cypermethrin were detected in some samples (Table S2) and one bearded vulture was found dead with 56.2 ng/g of permethrin in its liver (Table 1).

When we evaluate the risk of acute exposure that can produce sublethal endpoints, such as the inhibition of brain AchE or an impairment in thermoregulation, the obtained results were more critical. A single exposure to an organophosphorous compound at a dose equivalent to 5% of the $LD_{50}$ for that compound can inhibit brain AchE and reduce core temperature by 2–6 °C in homoeothermic animals (Rattner and Franson, 1984; Grue et al., 1997; Hill, 2003; Gordon, 1994). Moreover, thermoregulation impairment can also increase mortality due to organophosphate exposure under excessive heat (Rattner et al., 1987). By taking $HD_5$ as a precautionary $LD_{50}$ for bearded vulture, the adverse effect on thermoregulation could occur at 5% of this value (which is the $ED_{th}$ value calculated in Table 2). The obtained $TER_{th}$ value indicates a significant risk of impaired body thermoregulation caused by diazinon at the observed residue levels found in lamb feet (1.8 and 0.3 with mean and maximum residue levels, respectively). This could be especially relevant for nestlings, because many species are not fully homoeothermic for 1–3 weeks after hatching (Hill, 2003). Moreover, nests of bearded vultures in the Pyrenees are located at altitudes of up to 2150 m (mean ± SD: 1387 ± 363 m), where laying starts in December/January (average is 6th January) and hatching occurs on average between 21 February and 3 March, when ambient temperatures can fall well below 0 °C (Margalida et al., 2003, 2012). Moreover, nestling survival could be also compromised because parental care behaviour of adults exposed to AchE inhibitors may be altered (Grue et al., 1982; Hill, 2003). This may be highly relevant for a species in which incubation is usually interrupted by parents for < 5% of the time to prevent the risk of embryo mortality due to hypothermia or predation (Margalida et al., 2012). Here, we have selected the thermoregulation endpoint, but, other adverse effects on flight performance and migration have also been observed in pigeons (*Columba livia*) with diazinon at doses as low as 0.25 mg/kg b.w. (Brasel et al., 2007).

In addition to the risk of acute exposure to antiparasitics, we must highlight the risk of chronic exposure due to the high frequency of detection of these products in lamb feet (up to 100% with diazinon and pirimiphos-methyl in unwashed whole feet collected at slaughterhouses). Little information exists about the potential long-term effects in wild birds of exposure to low levels of organophosphates, but, experimental work with chickens exposed to 2 μg/g of monocrotophos in diet has shown adverse effects on serum ChE, metabolism and on immune function (Garg et al., 2004). Another observed long-term effect regards changes in feeding behaviour produced by organophosphates (i.e., parathion). This can occur at levels below those inducing outward illness or even those that depress brain AChE activity, possibly via a mechanism of conditioned food aversion related to sublethal toxicity (Nicolaus and Lee, 1999).

It should be noted that the risk assessment performed here considered external contamination of the whole lamb foot because bearded vultures usually ingest everything, including the hoof. Our derived values may be considered conservative because they do not fully account for any dermally absorbed antiparasitic. However, dermal absorption of diazinon has been estimated to be just 4% (APVMA, 2014) because the permeation rate of diazinon into an animal skin is smaller than the desquamation rate (Sugino et al., 2014). Therefore, it seems likely that the residue levels for diazinon detected here are indeed likely to be representative and not overly conservative.

*4.4. Strategies for risk reduction*

The first measure adopted by regional authorities in our study area has been to begin washing lamb feet provided at supplementary feeding stations for bearded vultures. We have observed that this measure can certainly reduce (significantly) residue levels, although these were not completely eliminated (Fig. 4; Table S2). This is an appropriate strategy for all lamb feet provided at supplementary feeding stations, but, the risk will continue to persist if/when vultures feed on ovine carcasses in the field. Moreover, adult sheep in the field may have higher levels than lamb sampled in the slaughterhouse (dependant of applied dose and time since treatment). Diazinon treated livestock raised for meat marketed for human consumption must be withheld for a safety period of 15 days prior to being slaughtered, but, the persistence of diazinon in/on wool is actually longer than three months (Wilkinson, 1980). In the UK, the surveillance of veterinary treatment residues in 643 sheep kidney fat samples in 1999 showed the presence of diazinon and propetamphos in 11 and 9 samples, respectively, with levels as high as 150 ng/g in the case of diazion (VMD, 2000). Such information highlights the need for further monitoring of antiparasitics in livestock carcasses left in the field for vultures or supplied at feeding stations; especially given that recent epizootics of mange in wild ungulates in Northern Spain (Oleaga et al., 2008) may result in higher use of antiparasitics in sheep and goat in future.

## 5. Conclusions

Bearded vultures, like other scavengers, face risks due to the intentional and illegal use of poisons used to kill predators. However, the present study reveals that other sources of highly toxic compounds, such as topical antiparasitics used in livestock, could be an as yet overlooked cause of mortality and breeding impairment in this endangered species. The washing protocol used by authorities for lamb feet supplied at supplementary feeding stations for bearded vultures would seem to be partially (but not totally) effective in terms of reducing residue levels of antiparasitics significantly. Moreover, vultures may also be exposed when they consume fallen treated livestock outside supplementary feeding stations. In this case, toxicovigilance and monitoring of residue levels in carcasses found in the field could be informative. Further, the use of pyrethroids as a less toxic alternative to organophosphates (Rattner and Franson, 1984) could be an appropriate measure to help reduce apparent risks to bearded vultures. At this time, as diazinon is still authorized as a veterinary medicine in the EU, education of farmers, veterinarians and animal health authorities regarding less toxic alternatives for the treatment and prevention of external parasites could be a first step which may also help to reduce the risk posed to Europe's remaining endangered bearded vultures.

## Acknowledgements

This study was funded by a grant of the Spanish Program of Research and Development (CGL2013-40975-R) and a contract with the Biodiversity Service of the Gobierno de Aragón (RB82125, RB29049, RB044077, RB14087, RB24105, RB34047, RB44050, RB54098). The Generalitat de Catalunya (Ref. 060469060004), Gobierno de Navarra and Diputación Foral de Álava also collaborated with the sampling and funding. The Fundación para la Conservación del Quebrantahuesos (Bearded Vulture Conservation Foundation) is especially acknowledged for their important collaboration. We thank M.A. Taggart for reviewing the English grammar.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.07.109.

## References

APVMA, 2014. Sheep ectoparasiticides. Reconsideration of registration of selected sheep ectoparasiticide products and approvals of their associated labels. Australian Pesticides and Veterinary Medicines Authority, Kingston, Australia (69 pp.).

Berny, P., 2007. Pesticides and the intoxication of wild animals. J. Vet. Pharmacol. Ther. 30, 93–100.

Berny, P., Vilagines, L., Cugnasse, J.M., Mastain, O., Chollet, J.Y., Joncour, G., Razin, M., 2015. VIGILANCE POISON: Illegal poisoning and lead intoxication are the main factors affecting avian scavenger survival in the Pyrenees (France). Ecotoxicol. Environ. Saf. 118, 71–82.

Brasel, J.M., Collier, A.C., Pritsos, C.A., 2007. Differential toxic effects of Carbofuran and Diazinon on time of flight in pigeons (*Columba livia*): potential for pesticide effects on migration. Toxicol. Appl. Pharmacol. 219, 241–246.

Brown, P., Charlton, A., Cuthbert, M., Barnett, L., Ross, L., Green, M., Gillies, L., Shaw, K., Fletcher, M., 1996. Identification of pesticide poisoning in wildlife. J. Chromatogr. A 754, 463–478.

CAPMAA (Consejería de Agricultura, Pesca y Medio Ambiente de Andalucía), 2013. El ADN permite resolver un caso de veneno en Andalucía. Quercus 323, 60–61.

Cuthbert, R.J., Taggart, M.A., Prakash, V., Chakraborty, S.S., Deori, P., Galligan, T., Kulkarni, M., Ranade, S., Saini, M., Sharma, A.K., Shringarpure, R., Green, R.E., 2014. Avian scavengers and the threat from veterinary pharmaceuticals. Roy. Soc. Philos. Trans. B 369, 20130574.

Donázar, J.A., 1993. Los Buitres Ibéricos. Biología y Conservación. J.M. Reyero Editor, Madrid, Spain ( 258 pp.).

Echegaray, J., Vilà, C., 2010. Noninvasive monitoring of wolves at the edge of their distribution and the cost of their conservation. Anim. Conserv. 13, 157–161.

EFSA (European Food Safety Authority), 2009. Risk assessment for birds and mammals. EFSA J. 7, 1438.

Elliott, J.E., Bishop, C.A., Morrissey, C.A. (Eds.), 2011. Wildlife Ecotoxicology: Forensic Approaches, Emerging Topics in Ecotoxicology 3. Springer, New York (466 pp.).

Garcia-Fernandez, A.J., Martinez-Lopez, E., Romero, D., Maria-Mojicam, P., Godinom, A., Jimenez, P., 2005. High levels of blood lead in griffon vultures (*Gyps fulvus*) from Cazorla Natural Park (Southern Spain). Environ. Toxicol. 20, 459–463.

Garg, U.K., Pal, A.K., Jha, G.J., Jadhao, S.B., 2004. Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. Int. Immunopharmacol. 4, 1709–1722.

Gómez de Segura, A., Martínez, J.M., Alcántara, M., 2012. Population size of the endangered bearded vulture *Gypaetus barbatus* in Aragon (Spain): an approximation to the Pyrenean population. Ardeola 59, 43–55.

Gómez-Ramírez, P., Shore, R.F., van den Brink, N.W., van Hattum, B., Bustnes, J.O., Duke, G., Fritsch, C., García-Fernández, A.J., Helander, B.O., Jaspers, V., Krone, O., Martínez-López, E., Mateo, R., Movalli, P., Sonne, C., 2014. An overview of existing raptor contaminant monitoring activities in Europe. Environ. Int. 67, 12–21.

Gordon, C.J., 1994. Thermoregulation in laboratory mammals and humans exposed to anticholinesterase agents. Neurotoxicol. Teratol. 16, 427–453.

Gortázar, C., Herrero, J., Villafuerte, R., Marco, J., 2000. Historical examination of the status of large mammals in Aragon, Spain. Mammalia 64, 411–422.

Grue, C.E., Powell, G.V.N., McChesney, M.J., 1982. Care of nestlings by wild female starlings exposed to an organophosphate pesticide. J. Appl. Ecol. 19, 327–335.

Grue, C.E., Gibert, P.L., Seeley, M.E., 1997. Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: thermo-régulation, food consumption, and reproduction. Am. Zool. 37, 369–388.

Guitart, R., Mañosa, S., Guerrero, X., Mateo, R., 1999. Animal poisonings: the 10-year experience of a veterinary analytical toxicology laboratory. Vet. Hum. Toxicol. 41, 331–335.

Guitart, R., Sachana, M., Caloni, F., Croubels, S., Vandenbroucke, V., Berny, P., 2010. Animal poisoning in Europe. Part 3: Wildlife. Vet. J. 183, 260–265.

Henny, C.J., Kolbe, E.J., Hill, E.F., Blus, L.J., 1987. Case histories of bald eagles and other raptors killed by organophosphorus insecticides topically applied to livestock. J. Wildl. Dis. 23, 292–295.

Hernández, M., Margalida, A., 2008. Pesticide abuse in Europe: effects on the Cinereous vulture (*Aegypius monachus*) population in Spain. Ecotoxicology 17, 264–272.

Hernández, M., Margalida, A., 2009a. Assessing the risk of lead exposure for the conservation of the endangered Pyrenean bearded vulture (*Gypaetus barbatus*) population. Environ. Res. 109, 837–842.

Hernández, M., Margalida, A., 2009b. Poison-related mortality effects in the endangered Egyptian vulture (*Neophron percnopterus*) population in Spain. J. Wildl. Res. 55, 415–423.

Hill, E.F., 2003. Wildlife toxicology of organophosphorus and carbamate pesticides. In: Hoffman, D.J., Rattner, B.A., Burton, G.A., Cairns, J. (Eds.), Handbook of Ecotoxicology. Lewis Publishers, Boca Raton, FL, USA, pp. 281–312.

Hill, E.F., Fleming, W.J., 1982. Anticholinesterase poisoning of bird: field monitoring and diagnosis of acute poisoning. Environ. Toxicol. Chem. 1, 27–38.

Hutchinson, T.H., Madden, J.C., Naidoo, V., Walker, C.H., 2014. Comparative metabolism as a key driver of wildlife species sensitivity to human and veterinary pharmaceuticals. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 20130583.

Lampreave, G., Ruiz-Olmo, J., García-Petit, J., López-Martín, J.M., Batille, A., Francino, O., Sastre, N., Ramírez, O., 2011. El lobo vuelve a Catalunya: historia del regreso y medidas de conservación. Quercus 302, 16–25.

Luttik, R., Mineau, P., Roelofs, W., 2005. A review of interspecies toxicity extrapolation in birds and mammals and a proposal for long-term toxicity data. Ecotoxicology 14, 817–832.

Margalida, A., Garcia, D., Bertran, J., Heredia, R., 2003. Breeding biology and success of the Bearded Vulture *Gypaetus barbatus* in the eastern Pyrenees. Ibis 145, 244–252.

Margalida, A., Heredia, R., Razin, M., Hernández, M., 2008. Sources of variation in mortality of the Bearded Vulture *Gypaetus barbatus* in Europe. Bird Conserv. Int. 18, 1–10.

Margalida, A., Sánchez-Zapata, J.A., Eguía, S., Arroyo, A.B.M., Hernández, F.J., Bautista, J., 2009. Assessing the diet of breeding bearded vultures (*Gypaetus barbatus*) in mid-20th century in Spain: a comparison to recent data and implications for conservation. Eur. J. Wildl. Res. 55, 443–447.

Margalida, A., Donázar, J.A., Carrete, M., Sánchez-Zapata, J.A., 2010. Sanitary versus environmental policies: fitting together two pieces of the puzzle of European vulture conservation. J. Appl. Ecol. 47, 931–935.

Margalida, A., Garcia, D., Bertran, J., 2012. Els voltors a Catalunya: biologia, conservació I sintesi bibliogràfica. Grup d'estudi i Protecció del Trencalós, El Pont de Suert, Spain (158 pp.).

Margalida, A., Colomer, M.A., Oro, D., 2014. Man-induced activities modify demographic parameters in a long-lived species: effects of poisoning and health policies. Ecol. Appl. 24, 436–444.

Márquez, C., Vargas, J.M., Villafuerte, R., Fa, J.E., 2013. Risk mapping of illegal poisoning of avian and mammalian predators. J. Wildl. Manag. 77, 75–83.

Martínez-Haro, M., Mateo, R., Guitart, R., Soler-Rodríguez, F., Pérez-López, M., María-Mojica, P., García-Fernández, A.J., 2008. Relationship of the toxicity of pesticide formulations and their commercial restrictions with the frequency of animal poisonings. Ecotoxicol. Environ. Saf. 69, 396–402.

Martínez-López, E., Romero, D., María-Mojica, P., Navas, I., Gerique, C., Jiménez, P., García-Fernández, A.J., 2006. Detection of strychnine by gas chromatography–mass spectrometry in the carcase of a Bonelli's eagle (*Hieraaetus fasciatus*). Vet. Rec. 159, 182–183.

Mateo, R., Molina, R., Grífols, J., Guitart, R., 1997. Lead poisoning in a free ranging griffon vulture (*Gyps fulvus*). Vet. Rec. 140, 47–48.

Mateo-Tomás, P., Olea, P.P., Sánchez-Barbudo, I.S., Mateo, R., 2012. Alleviating human-wildlife conflicts: identifying the causes and mapping the risk of illegal poisoning of wild fauna. J. Appl. Ecol. 49, 376–385.

Mineau, P., Fletcher, M.R., Glaser, L.C., Thomas, N.J., Brassard, C., Wilson, L.K., Elliott, J.E., Lyon, L.A., Henny, C.J., Bollinger, T., Porter, S.L., 1999. Poisoning of raptors with organophosphorus and carbamate pesticides with emphasis on Canada, U.S. and U.K. J. Raptor Res. 33, 1–37.

Mineau, P., Baril, A., Collins, B.T., Duffe, J., Joerman, G., Luttik, R., 2001. Pesticide acute toxicity reference values for birds. Rev. Environ. Contam. Toxicol. 170, 13–74.

Motas-Guzmán, M., María-Mojica, P., Romero, D., Martínez-López, E., García-Fernández, A.J., 2003. Intentional poisoning of animals in Southeastern Spain: a review of the veterinary toxicology service from Murcia, Spain. Vet. Hum. Toxicol. 45, 47–50.

Nicolaus, L.K., Lee, H., 1999. Low acute exposure to organophosphate produces long-term changes in bird feeding behavior. Ecol. Appl. 9, 1039–1049.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633.

Ogada, D.L., 2014. The power of poison: pesticide poisoning of Africa's wildlife. Ann. N. Y. Acad. Sci. 1322, 1–20.

Oleaga, A., Casais, R., González-Quirós, P., Prieto, M., Gortázar, C., 2008. Sarcoptic mange in red deer from Spain: improved surveillance or disease emergence? Vet. Parasitol. 154, 103–113.

Oro, D., Margalida, A., Carrete, M., Heredia, R., Donázar, J.A., 2008. Testing the goodness of supplementary feeding to enhance population viability in an endangered vulture. PLoS ONE 3, e4084.

Pain, D.J., Gargi, R., Cunningham, A.A., Jones, A., Prakash, V., 2004. Mortality of globally threatened Sarus cranes *Grus antigon* from monocrotophos poisoning in India. Sci. Total Environ. 326, 55–61.

Rattner, B.A., Franson, J.C., 1984. Methyl parathion and fenvalerate toxicity in American kestrels: acute physiological responses and effects of cold. Can. J. Physiol. Pharmacol. 62, 787–792.

Rattner, B.A., Becker, J.M., Nakatsugawa, T., 1987. Enhancement of parathion toxicity to quail by heat and cold exposure. Pestic. Biochem. Physiol. 27, 330–339.

Reglero, M.M., Monsalve-González, L., Taggart, M.A., Mateo, R., 2008. Transfer of metals to plants and red deer in an old lead mining area in Spain. Sci. Total Environ. 406, 287–297.

Richards, N., 2012. Carbofuran and wildlife poisoning: global perspectives and forensic approaches. Wiley-Blackwell, Chichester, UK (277 pp.).

Ruiz-Olmo, J., Aguilar, A., 1995. Els Grans Mamífers de Catalunya i Andorra. Lynx Edicions, Barcelona, Spain (246 pp.).

Sánchez-Barbudo, I.S., Camarero, P.R., García-Montijano, M., Mateo, R., 2012a. Possible cantharidin poisoning of a great bustard (*Otis tarda*). Toxicon 59, 100–103.

Sánchez-Barbudo, I.S., Camarero, P.R., Mateo, R., 2012b. Intoxicaciones intencionadas y accidentales de fauna silvestre y doméstica en España: diferencias entre Comunidades Autónomas. Rev. Toxicol. 29, 20–28.

Sánchez-Barbudo, I.S., Camarero, P.R., Mateo, R., 2012c. Primary and secondary poisoning by anticoagulant rodenticides of non-target animals in Spain. Sci. Total Environ. 420, 280–288.

Shore, R.F., Crocker, D.R., Akcakaya, H.R., Bennett, R.S., Chapman, P.F., Clook, M., Crane, M., Dewhurst, I.C., Edwards, P.J., Fairbrother, A., Ferson, S., Fischer, D., Hart, A.D., Holmes, M., Hooper, M.J., Lavine, M., Leopold, A., Luttik, R., Mineau, P., Moore, D.R., Mortenson, S.R., Noble, D.G., O'Connor, R.J., Roelofs, W., Sibly, R.M., Smith, G.C., Spendiff, M., Springer, T.A., Thompson, H.M., Topping, C., 2005. Case Study Part 1: how to calculate appropriate deterministic long-term toxicity to exposure ratios (TERs) for birds and mammals. Ecotoxicology 14, 877–893.

Shore, R.F., Taggart, M.A., Smits, J., Mateo, R., Richards, N.L., Fryday, S., 2014. Detection and drivers of exposure and effects of pharmaceuticals in higher vertebrates. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 20130570.

Stansley, W., 1993. Field results using cholinesterase reactivation techniques to diagnose acute anticholinesterase poisoning in birds and fish. Arch. Environ. Contam. Toxicol. 25, 315–321.

Sugino, M., Todo, H., Suzuki, T., Nakada, K., Tsuji, K., Tokunaga, H., Jinno, H., Sugibayashi, K., 2014. Safety prediction of topically exposed biocides using permeability coefficients and the desquamation rate at the stratum corneum. J. Toxicol. Sci. 39, 475–485.

Taggart, M.A., Senacha, K.R., Green, R.E., Cuthbert, R., Jhala, Y.V., Meharg, A.A., Mateo, R., Pain, D.J., 2009. Analysis of nine NSAIDs in ungulate tissues available to Critically Endangered vultures in India. Environ. Sci. Technol. 43, 4561–4566.

Venkataramanan, R., Sreekumar, C., Kalaivanan, N., 2008. Malicious carbofuran poisoning of a Leopard (*Panthera pardus*) in Sandynallah reserve forest, India. J. Wildl. Rehabil. 29, 15–17.

VMD, 2000. Annual Report on Surveillance for Veterinary Residues in 1999. Veterinary Medicines Directorate, UK (92 pp.).

Whitfield, D.P., McLeod, D.R.A., Watson, J., Fielding, A.H., Haworth, P.F., 2003. The association of grouse moor in Scotland with the illegal use of poisons to control predators. Biol. Conserv. 114, 157–163.

Wilkinson, F.C., 1980. The uptake of dipping fluid and persistence of diazinon on shower-dipped sheep. Aust. Vet. J. 56, 561–562.

Wobeser, G., Bollinger, T., Leighton, F.A., Blakley, B., Mineau, P., 2004. Secondary poisoning of eagles following intentional poisoning of coyotes with anticholinesterase pesticides in western Canada. J. Wildl. Dis. 40, 163–172.

Zorrilla, I., Martinez, R., Taggart, M.A., Richards, N., 2015. Suspected flunixin poisoning of a wild Eurasian Griffon Vulture from Spain. Conserv. Biol. 29, 587–592.