Increasing incidence of barbiturate intoxication in avian scavengers and mammals in Spain

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ABSTRACT

Pharmaceuticals are considered emerging contaminants in terms of impacts on wildlife. One chemical group of concern is euthanasia agents used in veterinary medicine. Here we present data on the occurrence of barbiturate intoxication using samples collected from 2004 to 2020 of suspected wildlife and domestic animal poisoning cases in Spain (n = 3210). Barbiturate intoxication was seen in 3.4% (45/1334) of the total number of confirmed intoxicated animals. Barbiturates were detected in 0.2% (1/448) of baits containing detectable poisons. The most frequently detected barbiturate was pentobarbital (42/45, 93.3%), but we also detected phenobarbital, barbital, and thiopental (2.2% prevalence for each). Avian scavengers were most frequently affected by barbiturate intoxication (n = 36), especially Eurasian griffon vultures (Gyps fulvus) (n = 28). Median pentobarbital concentrations detected in intoxicated griffon vultures was 27.3 mg kg⁻¹ in gastric content and 38.1 mg kg⁻¹ in liver, which highlights the acute effect of the chemical soon after ingestion. At least two large intoxication events affecting griffon vultures were related to the consumption of carcasses from euthanized livestock. We also found phenobarbital in a prepared bait linked to the intoxication of one Eurasian buzzard (Buteo buteo). This study highlights the need for stronger regulation of barbiturates to avoid secondary intoxications due to improper disposal of euthanized livestock.

1. Introduction

Pharmaceuticals are considered emerging contaminants, especially in relation to their impacts on wildlife, and veterinary drugs (in particular) are expected to be a hazard for avian scavengers (Arnold et al., 2013). The main route of exposure to avian scavengers is through the consumption of livestock carcasses, which in many cases have been treated with pharmaceuticals shortly before death (Shore et al., 2014; Taggart et al., 2016). By way of a dramatic example, >99% of the populations of three native Gyps vulture species in Asia have been extirpated due to diclofenac intoxication in recent years (Prakash et al., 2003). Diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) can be present in carcasses of treated livestock at levels that can be toxic for vultures scavenging on them (Oaks et al., 2004; Zorrilla et al., 2015; Herrero-Villar et al., 2020, 2021). In addition to NSAIDs, other veterinary drugs used in livestock treatment can also be toxic to avian scavengers (Shore et al., 2014; Mateo et al., 2015; Casas-Díaz et al., 2016). One such group is euthanizing agents, which can produce secondary intoxications in scavengers feeding on euthanized animals (Hayes, 1988; Thomas, 1999; O’Rourke, 2002). Among these agents, barbiturates are the main chemical group used for this purpose, and pentobarbital is the active substance most frequently used (Wells et al., 2020). Pentobarbital is a highly toxic chemical, with an intravenous median lethal dose (LD₅₀) of 33, 72 and 75 mg kg⁻¹ in rabbits, cats and wild birds, respectively (Schafer, 1972; PubChem, 2020).

Poisoning is one of the most common causes of mortality in birds of prey worldwide (McClure et al., 2018; Mateo-Tomás et al., 2020), and barbiturates cause a significant portion of these incidents. In a large-scale study of mortality causes in 4407 large eagles in the USA, Russell and Franson (2014) found that up to 25.6% (762/2980) of bald eagle (Haliaeetus leucocephalus) and 8.2% (117/1427) of golden eagle (Aquila chrysaetos) deaths were due to poisoning. Among these,

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barbiturate intoxication (specifically pentobarbital) represented 4.3% (33/762) of poisoning cases and 1.1% (33/2980) of total mortality in bald eagle, while in golden eagle this was 2.6% (3/117) and 0.2% (3/1427), respectively (Russell and Franson, 2014). Additionally, pentobarbital intoxication was described in other birds of prey, including Cooper’s hawk (Accipiter cooperii), red-shouldered hawk (Buteo lineatus) and red-tailed hawk (Buteo jamaicensis) (Russell and Franson, 2014). Barbiturate intoxication has also been described in birds of prey, including Cooper’s hawk (Accipiter cooperii), red-shouldered hawk (Buteo lineatus) and red-tailed hawk (Buteo jamaicensis) (Russell and Franson, 2014). Barbiturate intoxication has also been described in birds of prey in Europe, with pentobarbital and phenobarbital detected in obligate and facultative avian scavengers from Spain and France, including Eurasian griffon vulture (Gyps fulvus), Egyptian vulture (Neophron percnopterus), and red kite (Milvus milvus) (Hernandez and Margalida, 2009; Wells et al., 2020). In a previous study in Spain, Sanchez-Barbudo et al. (2012b) found that 0.54% of wildlife intoxication cases were caused by barbiturates (4/734). Moreover, barbiturates were detected in 1.11% (2/179) of baits involved in wildlife poisoning incidents (Sanchez-Barbudo et al., 2012b). This finding was relevant, because it showed that barbiturates were being illegally used to prepare baits to kill predators. Therefore, barbiturate intoxication in scavengers can occur via two types of exposure: [1] through the consumption of carcasses of pets or livestock incorrectly disposed of in the field, at supplementary feeding stations (i.e., for vultures), or at landfills frequently visited by scavengers; and [2] by the ingestion of poisoned baits prepared for the lethal control of predators.

Here, we report data from a long-term study of barbiturate intoxications in wildlife in Spain. The objectives are [1] to describe cases of accidental and intentional barbiturate intoxication in wildlife in Spain between 2004 and 2020, and [2] to perform a risk assessment of the improper disposal of animals euthanized with barbiturates and likely impacts on avian scavengers in Europe.

2. Materials and methods

The Institute for Game and Wildlife Research (IREC) offers a toxicological analysis service to Spanish public administrations for suspected wildlife intoxications, additionally including some domestic animals found dead in the field. Samples provided through this service are kept under custody at IREC and results of forensic investigations usually have legal implications in Court cases. Over a 16-year period (from April 2004 to July 2020), a total of 3210 animals and 876 suspected poisoned baits or other food items involved in animal poisonings (including one foal carcass associated with the deaths of 8 Eurasian griffon vultures) were analysed by this service for barbiturates (among other types of chemicals; Sánchez-Barbudo et al., 2012a; López-Perea et al., 2015, 2019; Descalzo et al., 2021).

2.1. Animal carcass sampling

Animal tissues used for barbiturate analysis were liver (n = 1854) and gastric content (n = 2217) from 3210 individuals. In the case of extremely decomposed carcasses, we also analysed the content of the celomic cavity which includes remains of the gastrointestinal tract and other viscera (n = 536). Samples were taken during necropsies performed by trained technicians and veterinary staff from the wildlife rehabilitation centres in the region of origin, or by the veterinary staff at IREC. Animals were collected in the following Spanish regions: Andalusia (n = 8), Aragon (n = 885), Asturias (n = 116), Cantabria (n = 122), Castilla and Leon (n = 34), Castilla-La Mancha (n = 1159), Catalonia (n = 1854), and Madrid (n = 2217). The sample distribution is shown in Fig. 1.
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wildlife species (n = 1667), other birds (n = 497), carnivorous mammals (n = 837), other mammals (n = 131) and other wildlife species (n = 78). Animals analysed corresponded to 2790 indi-

viduals of wildlife species and 420 individuals of domesticated animal species. Samples were collected in zip lock bags or polypropylene flasks. All samples were stored at −20 °C until analysis.

2.2. Bait sampling

Bait sampling was performed in the field by trained wildlife officers when a wildlife mortality incident was investigated, and collected samples were submitted to IREC for toxicological analysis. Baits (n = 876) were collected in the following Spanish regions: Aragon (n = 149), Asturias (n = 35), Cantabria (n = 26), Castilla and Leon (n = 3), Castilla-La Mancha (n = 454), Catalonia (n = 48), Balearic Islands (n = 17), La Rioja (n = 34), Madrid (n = 74), Navarra (n = 21), the Basque country (n = 12), and from an unreported region (n = 3) (Fig. 1).

2.3. Forensic toxicological analysis

Forensic toxicological analysis included compound screening (using different analytical techniques) which covered the most common toxic substances involved in intentional and accidental intoxications in wildlife and domestic animals. Three types of analyses were performed to detect: (Group 1) strychnine, anticholinesterase pesticides, organo-

chlorines, neonicotinoids, pyrethroids and other acute-action neurotoxic compounds such as barbiturates and α-chloralose (Brown et al., 1996); (Group 2) anticoagulant rodenticides (Sánchez-Barbudo et al., 2012a; López-Perea et al., 2015); and (Group 3) toxic metals and metallo-

doids (Reglero et al., 2008; Descalzo et al., 2021). The compounds in group 1 were investigated in all cases, and depending on the case history and necropsy findings, groups 2 and 3 were also analysed. Anticoagulant rodenticides (group 2) were analysed when haemorrhages had been detected at necropsy (Sánchez-Barbudo et al., 2012a). For group 3, lead was analysed in birds of prey commonly susceptible to lead intoxication (Descalzo et al., 2021), and arsenic was analysed when gastrointestinal haemorrhages had been detected at necropsy (Sajan et al., 2010).

The analysis of acute-action neurotoxic compounds (group 1) including barbiturates, was performed following the method developed by Brown et al. (1996) with some modifications (Sánchez-Barbudo et al., 2012b). Briefly, 1 g of sample (liver, gastric content, tissues remaining in decomposed and/or predated carcasses, and bait) was weighed and homogenised in a mortar with 10 g of anhydrous sodium sulphate. The homogenate was placed in a 30 mL tube with a Teflon cap and 15 mL of dichloromethane was added. The tube was then subjected to horizontal agitation for 10 min and then sonicated for 5 min. The extract was then filtered (Whatman prepleated filter paper, 595 1/2) into a 100 mL heart-shaped glass flask. The same extraction was repeated with 5 mL of dichloromethane and the supernatant combined with the previous extract. The extract was then evaporated in a rotavapor at 40 °C over 60 min. The MS was maintained at 230 °C and the pressure at 31.5 kPa. Helium flow was 34.9 mL min⁻¹. Initial column oven temperature was 50 °C, increasing at 5 °C min⁻¹ up to 310 °C over 60 min. The MS was maintained at 230 °C and at a voltage of 70 V. Compound identification was carried out using retention time and full-scan mass spectrum data for each compound.

The barbiturates monitored in this study were those marketed in Spain for veterinary use, although the full-scan GC-MS method also permitted the detection of other barbiturates. These compounds are regulated by the Spanish Agency for Medicinal Products and Medical Devices (AEMPS). There are only three active substances authorized in Spain (with number of marketed formulations shown): pentobarbital (5), phenobarbital (4) and thiopental (2) (CIMAVEY, 2020), to which we also added barbital. Analytical standards were acquired from Sigma Aldrich, phenobarbital (compound reference 1,507,002), phenobarbital (1524001), barbital (B0300000), thiopental (1,661,002) and pentobarbital-d5 (52,944,66-8). Stock solutions were prepared at 1 mg mL⁻¹ in methanol for each barbiturate, from which we prepared working solutions at 10 ng mL⁻¹ in ethyl acetate for each compound (stored at 4 °C). We then prepared mixed standards from the working solutions at 0.5, 1 and 2 ng mL⁻¹ for each barbiturate in ethyl acetate and added pentobarbital-d5 as an internal standard at 0.75 ng mL⁻¹. These mixed standards were processed daily to estimate accuracy and precision of the technique together with spiked samples. Recoveries were obtained using fortified liver samples (1 g, n = 7) with a 2 mg kg⁻¹ spike. We obtained recovery rates between 69.4% for barbital and 101.3% for phenobarbital. Regression coefficients (R²) for calibration curves were all above 0.9989 (Table S4). We established limits of quantification (LOQs) at 10 times the signal-to-noise ratio, and LOQs were between 0.02 mg kg⁻¹ for pentobarbital and 0.39 mg kg⁻¹ for phenobarbital (Table S4).

The analysis of group 1 (other than barbiturates), group 2 and 3 chemicals/elements have been described elsewhere (Sánchez-Barbudo et al., 2012a; López-Perea et al., 2015, 2019; Descalzo et al., 2021), hence these results are not fully detailed in this study (but only given at the chemical group level).

2.4. Data analysis

Statistics and data interpretation were performed with IBM SPSS Statistics 24. Prevalence values of barbiturate intoxication were calculated for geographical regions and years. The differences in the prevalence of barbiturate intoxication between years, regions and animal groups (mammals or birds) were tested with generalized linear models with binomial distribution and logit link function using barbiturate intoxication as a binary logistic response variable (with or without barbiturate intoxication) and with the years, regions and animal groups as fixed factors. Pairwise comparisons between years and regions were performed with least square difference tests. Moreover, the linear regression coefficient was calculated to study the temporal trend of the observed prevalence by events and animals. Paired Wilcoxon rank tests were used to compare barbiturate concentrations in the gastric content and the liver of the same animal (tested for Eurasion griffon vultures and for all animals).

We used European Food Safety Authority (EFSA, 2009) guidance to perform a first-tier risk assessment for acute oral toxicity of pentobarbital for Eurasion griffon vultures and Egyptian vultures based on toxicity exposure ratios (TERs) (Table 1). First, we calculated the estimated theoretical exposure (ETE) using concentrations detected in carrion in this study and the maximum single dietary intake for both obligate avian scavengers (as described by Donázar, 1993). These...
Intakes were 1.2 kg for a Eurasian griffon vulture with an average mass of 7.4 kg, and 0.5 kg for an Egyptian vulture with an average mass of 2 kg. We used the ratio between LD₉₀ values (described in the literature for pentobarbital) and the ETE values to estimate TERs. This ratio must be below 10 to discount a risk of acute oral toxicity (Table 1). As there is no LD₉₀ calculated for pentobarbital in avian scavengers, we used the LD₉₀ value of 75 mg kg⁻¹ obtained for wild red-winged blackbirds (Agelaius phoeniceus) (Scafer, 1972). We also included pentobarbital concentrations in carcases of domestic animals reported in the literature (Anderson et al., 1979; Campbell et al., 2009; Wells et al., 2020) to calculate corresponding TER values and compare these with those obtained using data from carcases analysed here.

3. Results

3.1. Epidemiological relevance of barbiturate intoxication

Intoxication was confirmed as the cause of the death in 1334 (41.6%) of the 3210 animals analysed (Fig. 1). Barbiturate intoxication was confirmed in 45 of those 1334 animals (Table 1, Table S2), which represents 3.4% of intoxicated animals and 1.4% of all analysed animals. This places barbiturates as the fifth most prevalent cause of confirmed intoxications, after carbamates (n = 715, 53.6%), organophosphates (n = 245, 18.4%), coumarins (n = 169, 12.7%) and strychnine (n = 74, 5.5%) (Fig. 2). The occurrence of barbiturate intoxication among confirmed animal intoxications by geographical region ranged from 0.6% in Castilla-La Mancha to 34.5% in Navarra (see Fig. 1 for full breakdown of prevalence among all animals analysed and confirmed intoxication by region). The prevalence detected varied among regions (p < 0.001), being significantly higher in Navarra than in the other regions (Fig. 1). The prevalence of barbiturate intoxication varied among years (p < 0.001) and increased over time (r² = 0.543, p < 0.001), especially since 2017. Prevalence in 2018–2019 (678–9.42%) was significantly higher than in 2004–2016 (0–0.96%, Fig. 3). The difference between years was also significant when it was calculated for events instead of individuals (p < 0.001). Prevalence did not differ between birds and mammals.

In the case of suspected poisoned baits or food involved in animal poisoning cases, 448 (51.1%) of them (n = 876) contained toxic compounds. Barbiturates were found to be present in 1 bait deliberately prepared with chicken meat to poison predators and in 1 carcass of a foal associated with a large intoxication event involving Eurasian griffon vultures (in which at least 8 birds were known to have died and the presence of barbiturate residues was confirmed in all of them). These two cases represent 0.2% each of the positive samples of bait or food investigated. In the case of bait, this places barbiturates below other frequently used chemicals such as carbamates (n = 292, 65.2%), organophosphates (n = 71, 15.8%), arsenic (n = 19, 4.2%), strychnine (n = 13, 2.9%), metaldehyde (n = 11, 2.5%), organochlorines and coumarins (n = 10, 2.2%, each), pyrethroids (n = 8, 1.8%), α-chloralose (n = 5, 1.1%), paracetamol (n = 4, 0.9%) and ethylene glycol (n = 3, 0.7%).

The most frequently detected barbiturate in animal cases was pentobarbital (n = 42), which represented 93.3% of barbiturate intoxications. Phenobarbital, barbital and thiopental were detected in one animal each (2.2% each). The foal carcass associated with the large intoxication of Eurasian griffon vultures (mentioned above) contained pentobarbital. In the case of the bait, phenobarbital was the detected compound.

According to the information available for each intoxication event, cases were classified as suspected accidental intoxication (i.e., ingestion of a carcass of a euthanized animal) or intentional poisoning (i.e., ingestion of a bait prepared with a barbiturate formulation). Accidental barbiturate intoxication was suspected in 35 of the animal cases (77.8%), with all of them corresponding to avian obligate or opportunistic scavengers. These animals were collected from 21 intoxication events. The number of animals submitted for analysis in each of these events ranged between 1 and 8. The highest (8 individuals) related to a single event involving Eurasian griffon vultures. However, we cannot rule out the possibility of intentionality for some of these avian scavenger intoxications.

In two cases (one involving a Eurasian buzzard, and one a red squirrel (Sciurus vulgaris)), there was clear evidence of intentional barbiturate poisoning (4.4% of animals) because of the presence of a bait containing the barbiturate or the information gathered by the environmental agents in charge of the investigation. The circumstances of death were unclear in another eight intoxication events affecting mammals, but deliberate barbiturate poisoning was the most probable cause of intoxication (17.8% of animals). All these suspected deliberate poisoning events affected single individuals.

3.2. Barbiturate concentrations and lesions in intoxicated animals

Pentobarbital concentrations in gastric content were not significantly different than in liver in intoxicated animals (Table 2). Median (range) values in Eurasian griffon vulture, the best represented species, was 27.3 (0.12–344) mg kg⁻¹ in gastric content and 38.1 (0.20–164) mg kg⁻¹ in liver. Phenobarbital, thiopental and barbital were detected in only one specimen each, at lower concentrations (Table 2). The most frequently detected lesion observed at post-mortem examination in positive cases for pentobarbital was a generalized congestion of the internal tissues, which was found in 54.5% of the positive avian scavengers (n = 18) and 50% of the positive mammals (n = 4).
3.3. Barbiturate concentrations in euthanized animals and risk assessment for avian scavengers

The mean pentobarbital concentration in muscle from the three samples from the foal carcass associated with the death of 8 Eurasian griffon vultures was 80.9 mg kg\(^{-1}\) (range = 0.49 – 130 mg kg\(^{-1}\)). The ETE calculated with the highest concentration detected in carrion (130 mg kg\(^{-1}\)) for Eurasian griffon vulture and Egyptian vulture was 21.1 mg kg\(^{-1}\) body weight (bw) and 32.5 mg kg\(^{-1}\) bw, respectively (Table 3). Therefore, the estimated TERs were 3.55 for Eurasian griffon vulture and 2.31 for Egyptian vulture. TER Values < 10 may indicate a risk for exposed individuals. Similarly, TER values calculated using pentobarbital concentrations in cases described in the literature (Anderson et al., 1979; Campbell et al., 2009; Wells et al., 2020) and in the present study would range between 0.1 and 5.17 (Table 3).

Table 2
Barbiturate concentrations (mg kg\(^{-1}\)) detected in gastric content, liver, and other tissues of intoxicated wild and domestic animals.

<table>
<thead>
<tr>
<th>Barbiturate</th>
<th>Species</th>
<th>N</th>
<th>Gastric content</th>
<th>Liver</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>Median</td>
<td>Min-Max</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>Griffon vulture</td>
<td>28</td>
<td>27</td>
<td>27.3</td>
<td>0.12–344</td>
</tr>
<tr>
<td></td>
<td>Egyptian vulture</td>
<td>2</td>
<td>1</td>
<td>17.3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Spanish imperial eagle</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Cinereous vulture</td>
<td>1</td>
<td>1</td>
<td>5.81</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red kite</td>
<td>2</td>
<td>2</td>
<td>41.9</td>
<td>2.50–81.3</td>
</tr>
<tr>
<td></td>
<td>Red fox</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Stone marten</td>
<td>1</td>
<td>1</td>
<td>3.72</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>European badger</td>
<td>1</td>
<td>1</td>
<td>15.29</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red squirrel</td>
<td>1</td>
<td>1</td>
<td>46.6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Wild boar</td>
<td>1</td>
<td>1</td>
<td>0.12</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>2</td>
<td>2</td>
<td>216</td>
<td>–</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Eurasian buzzard</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Barbital</td>
<td>Spanish imperial eagle</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Thiopental</td>
<td>Dog</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

LOQ: limit of quantification.

\(^{a}\) This dog was also positive for strychnine (2.02 mg kg\(^{-1}\)) and probably died due to this compound.

\(^{b}\) This dog was also positive for metaldehyde, and the thiopental concentration is given in \(\mu\)g mL\(^{-1}\).

Table 3
Pentobarbital concentrations detected in carrion samples in literature and in the present study, alongside a first-tier risk assessment based on toxicity exposure ratios (TERs) in griffon vulture and Egyptian vulture.

<table>
<thead>
<tr>
<th>Carcass species</th>
<th>Tissue</th>
<th>Concentration in carrion (mg kg(^{-1}))</th>
<th>(\text{LD}_{50}) (mg kg(^{-1}))</th>
<th>Griffon vulture</th>
<th>Egyptian vulture</th>
<th>Reference for tissue concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ETE (mg kg(^{-1}))</td>
<td>Acute TER</td>
<td>ETE (mg kg(^{-1}))</td>
<td>Acute TER</td>
<td></td>
</tr>
<tr>
<td>Horse (foal)</td>
<td>Muscle</td>
<td>130</td>
<td>75(^{a})</td>
<td>21.1</td>
<td>3.55</td>
<td>32.5</td>
</tr>
<tr>
<td>Calf</td>
<td>Soft tissue</td>
<td>300</td>
<td>75</td>
<td>48.7</td>
<td>1.54</td>
<td>75</td>
</tr>
<tr>
<td>Goat</td>
<td>Soft tissue</td>
<td>3000</td>
<td>75</td>
<td>486</td>
<td>0.15</td>
<td>750</td>
</tr>
<tr>
<td>Dog</td>
<td>Liver</td>
<td>89.6</td>
<td>75</td>
<td>14.5</td>
<td>5.17</td>
<td>22.4</td>
</tr>
<tr>
<td>Chicken</td>
<td>Not reported</td>
<td>200</td>
<td>75</td>
<td>32.4</td>
<td>2.31</td>
<td>50</td>
</tr>
</tbody>
</table>

ETE: estimated theoretical exposure; \(\text{LD}_{50}\): median lethal dose; TER: toxicity exposure ratio.

\(^{a}\) \(\text{LD}_{50}\) is estimated for red-wing blackbirds (\textit{Agelaius phoenicus}) (Schafer, 1972).

\(^{b}\) Extracted from the Veterinary Poisons Information Service (UK).
4. Discussion

The evidence here of barbiturate intoxication together with the detection of these compounds in prepared baits and accessible livestock carcasses, confirms that euthanizing drugs can reach wildlife food webs in Spain. Our results point out that barbiturates are posing a threat to avian (and other) scavengers in Europe, and therefore there is a need for more active vigilance, specific regulations, and training for relevant stakeholders and actors. In a previous study in Spain, Sanchez-Barbudo et al. (2012b) reported that 0.54% of total intoxication cases in wildlife in Spain were due to barbiturates. Here, barbiturate intoxication reached 3.4% of confirmed intoxication cases. This increase in recent years (mainly between 2018 and 2020), suggests barbiturates are now potentially an increasing threat for avian (and other) scavengers.

4.1. Barbiturate intoxication of avian scavengers in Spain

The most frequently affected group of species here were avian scavengers, and in particular the Eurasian griffon vulture. Mortality due to accidental pentobarbital intoxication represented 5.9% of cases in griffon vultures tested (n = 25/473), and so was the third most common cause of mortality after intoxication with all other chemicals (48.0%) and trauma/tis (11.4%) (Table S3). This mortality is similar to that reported by Russell and Franson (2014) for bald eagles (4.3%) and golden eagles (2.6%) in the USA. The most frequently detected barbiturate in vultures was pentobarbital, with concentrations in gastric content samples ranging from 0.12 to 344 mg kg⁻¹. Similar pentobarbital concentrations have also been reported in France in one intoxication event involving 7 griffon vultures, in which three birds showed concentrations of 8.5, 116 and 146 mg kg⁻¹ in their gastric content (Gedoux, 2010). Barbiturate intoxication in avian scavengers has been reported before in France and Spain, but not always alongside barbiturate residue concentration data (Gedoux, 2010; Marfa-Mojica et al., 2017; Wells et al., 2020). The presence of pentobarbital in both the stomach and liver of these intoxicated animals (Table S2) highlights the swift and acute toxic effect of this pharmaceutical, which explains why (in many cases) these scavengers are found dead around the euthanized animal carcass.

We have also reported barbiturate intoxication here in other avian scavenger species in Spain. We detected pentobarbital in two Egyptian vulture juveniles. One of them found dead in the nest and one in another event found at the bottom of a cliff below the nest (this intoxication event also included another dead nestling), suggesting this barbiturate can reach nestlings through parental feeding. In addition, we found pentobarbital residues in one cinereous vulture that was found dead by electrocution, which may indicate that neurological impairment caused by barbiturate exposure can increase the risk of death due to other causes, especially traumas and electrocutions. We also found pentobarbital residues in two red kites, one of them with high levels (81.3 mg kg⁻¹ in gastric content and 154 mg kg⁻¹ in liver) and another found next to a landfill, with a lower concentration (2.50 mg kg⁻¹ in gastric content). Pentobarbital intoxication in red kites has been reported before in the UK (Wells et al., 2020). This confirms the risk of barbiturate intoxication in opportunistic scavengers like kites. We also detected pentobarbital and barbital in the vomit or gastric content of two Spanish imperial eagles, another opportunistic scavenger. Barbital is not actually even authorized in Spain for veterinary use, even though it has been reported previously at trace levels in one carrion sample in Spain (Aldegue et al., 2009).

Symptoms of barbiturate intoxication include comatose condition, paralysis, and ataxia, which may vary depending on the dose ingested, and other intrinsic factors which may vary by species (Martin and Mallock, 1987; Bischoff et al., 2011; Campbell et al., 2009). These signs of intoxication were reported in one Spanish imperial eagle from the present study, which showed 40.4 mg kg⁻¹ of pentobarbital in vomit. This bird survived after two-months of intensive treatment in a wildlife rehabilitation centre. This individual was tagged with a GPS tracker and released on the April 29, 2020, after complete recovery, and seven months later (November 2020) it was still alive (information provided by E. Crespo, El Chaparrillo Wildlife Rehabilitation Centre, JCCM). Long treatment times have also been necessary in bald eagles intoxicated with barbiturates (Viner et al., 2016). The main observed lesion in the intoxicated animals here was tissue congestion, which has not been highlighted in cases of barbiturate intoxication before. Some individuals presented good corporeal condition and food remained in the oral cavity, which has been described as a common sign in barbiturate intoxication cases, as well as for other acute poisoning incidences (Thomas, 1999).

4.2. Accidental intoxication or deliberate poisoning?

Barbiturate intoxication in vultures and opportunistic scavengers has often been related to the consumption of carcasses of euthanized animals. In the present study we found that at least two intoxication events were related to domestic animals euthanized with pentobarbital: a foal carcass containing an average pentobarbital concentration of 80.9 mg kg⁻¹ (in muscle) and a cow carcass in which barbiturate levels were not quantified. We also registered two more pentobarbital intoxication events affecting 7 griffon vultures found dead near supplementary feeding stations, but we do not have further information/data on the carcass they fed on. Most of the cases involving griffon vultures here were from Navarra (67.9%), so a concerted effort must be made in that region to control improper disposal of livestock euthanized with barbiturates. It is also possible that euthanized pets are illegally disposed of in landfills along with domestic waste – and again, vultures and other opportunistic scavengers searching for food in these environments (Serrano, 1999) may then become intoxicated.

We also found evidence of intentional use of barbiturates (laced into poisoned baits) to kill predators. One Eurasian buzzard, with phenobarbital in plasma and faeces (Table 2), was possibly poisoned by deliberate use of this barbiturate to poison predators (even though not necessarily buzzards) given that chicken meat bait (containing 226 mg kg⁻¹ of phenobarbital) was also found close to the buzzard (this individual survived after treatment). This compound is registered in Spain for epileptic seizure treatment, and it is marketed in tablets that must be supplied to a pet (dogs) by an owner. Therefore, this drug is available to be mixed within bait to deliberately poison wildlife. The fact that other barbiturates are strictly restricted for veterinary use (CIMAVET, 2020) likely makes them somewhat less accessible compared to other lace chemicals (i.e., pesticides) used for illegal bait preparation. However, such restriction may not completely eliminate the risk of misuse, since many restricted or banned pesticides (i.e., carbofuran and aldicarb) have been frequently involved in animal poisonings in Spain in recent years (Martínez-Haro et al., 2008; Ruiz-Suárez et al., 2015).

The detection of barbiturates in some intoxication cases involving wild and domestic mammals may also indicate deliberate use of these drugs as a poison; however, providing clear evidence of intentionality can often be complicated and difficult. Here, pentobarbital was detected in one stone marten (Martes foina), which was captured in a cage trap, and one red squirrel from an event in which two individuals died and were suspected of being poisoned intentionally. In another event, pentobarbital was detected in the liver of two red foxes (Vulpes vulpes) found dead near each other and in one European badger (Meles meles) found next to a path. In addition, we have reported pentobarbital concentrations in two dogs and thiopeptoral in one dog. None of these dogs had been euthanized or treated before death with a barbiturate (according to information given by the vets and pet owners who supplied these animals for testing). One of these dogs also showed a higher pentobarbital concentration in gastric content (429 mg kg⁻¹) than in liver (89.6 mg kg⁻¹), which clearly indicates oral barbiturate exposure. Nevertheless, secondary intoxication has also been described previously in domestic dogs and cats (Campbell et al., 2009; Kaiser et al., 2010; Wells et al., 2020), so the risk from improper disposal of euthanized...
animals can affect mammalian scavengers.

4.3. Risk assessment based on pentobarbital residues detected in carrion

The TER values estimated here based on pentobarbital detected in a foal carcass and a dog liver (from the present study) and literature levels for calf, goat and chicken (Anderson et al., 1979; Campbell et al., 2009; Wells et al., 2020) were all below or slightly above 5. Following EFSA (2009) guidance, this chemical present in the food of avian scavengers could therefore represent a risk under acute exposure scenarios. The detection here of pentobarbital concentrations in gastric content from avian scavengers at levels similar or above those detected in carrion confirms the risk of intoxication in these species.

Carrion provided for avian scavengers at supplementary feeding stations (or left in the field in priority conservation areas) is regulated in Spain (RD 1632/2011). Livestock in Spain is usually slaughtered using non medicated procedures (AVMA, 2020), but barbiturates are commonly used for pet euthanasia because this is considered good animal welfare practice (BOE, 2020). Horses are frequently considered avian scavengers. Pentobarbital dosage in a horse is 100 mg kg\(^{-1}\) bw (1 mL/5 kg bw from a 500 mg mL\(^{-1}\) formulation). Therefore, for a foal weighing 100 kg, 10,000 mg of pentobarbital is administered for euthanasia in an intravenous continuous bolus until achieving death in 5–10 s (AEMPS, 2019). The half-life of pentobarbital varies among species (~1 h in small ruminants, ~2–7.5 h in cats and ~7–12.5 h in dogs), but at high euthanasia doses it would cause death before any significant metabolism occurs (AEMPS, 2019). Post-mortem degradation is also likely to be limited given its persistence in carcasses and the environment (Kaiser et al., 2010; Schwarz et al., 2013; Bagsby et al., 2018). Soil surrounding carcasses of euthanized horses (after decomposition) has shown concentrations between 22.5 and 77.5 mg kg\(^{-1}\) for up to 367 days (Payne et al., 2015). As pentobarbital tends to have a uniform distribution throughout a treated organism (with a higher concentration detected in liver) (AEMPS, 2019), ~100 mg kg\(^{-1}\) could be present throughout the carcass (soft tissue) of an euthanized horse. Such a dose would yield a TER of 4.63 for griffon vultures and 3 for Egyptian vultures, i.e., very similar to the values calculated here (in Table 3).

5. Conclusions

These results clearly point to the need for stricter regulation of barbiturates, especially after euthanasia and in terms of carcass disposal. In particular, pentobarbital would be of greatest current concern. Product labelling of this compound includes a warning stating that the ingestion of euthanized animals (by other animals) can lead to secondary intoxication and mortality. It is also noted on the labelling that these compounds persist in carcasses post-mortem and that drug stability remains even at high temperatures. Appropriate labelling is also considered important for reducing risks posed by other veterinary drugs, such as NSAIDs, that can also negatively impact avian scavengers (Herrero-Villar et al., 2020, 2021). As a recommendation, euthanized animals should never be made available to wildlife in the field (i.e., in landfills or at supplementary feeding stations) in order to prevent the clear risk of secondary intoxication events (Campbell et al., 2009; Payne et al., 2015). Moreover, the deliberate use of barbiturates to illegally kill predators has been reported here. This misuse of barbiturates for illegal purposes must be acknowledged and investigated, and potentially, stronger regulations regarding their veterinary use should be implemented.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117452.

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