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Published in:
Environmental Pollution

Publication date:
2020

NSAIDs detected in Iberian Avian Scavengers and Carrion after Diclofenac Registration for Veterinary use in Spain

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Abstract

Despite the now well recognised impact of diclofenac on vultures across the Indian subcontinent, this non-steroidal anti-inflammatory drug (NSAID) was registered in 2013 for livestock treatment in Spain, Europe's main vulture stronghold. We assessed the risk of exposure to diclofenac and nine other NSAIDs in avian scavengers in the Iberian Peninsula (Spain and Portugal) after the onset of diclofenac commercialization. We sampled 228 livestock carcasses from vulture feeding sites, primarily pig (n=156) and sheep (n=45). We also sampled tissues of 389 avian scavenger carcasses (306 Eurasian griffon vultures, 15 cinereous vultures, 11 Egyptian vultures, 12 bearded vultures and 45 other facultative scavengers). Samples were analysed by liquid chromatography with mass spectrometry (LCMS). Seven livestock carcasses (3.07%) contained NSAID residues: flunixin (1.75%), ketoprofen, diclofenac and meloxicam (0.44% each). NSAID residues were only detected in sheep (4.44%) and pig (3.21%) carcasses. Fourteen dead avian scavengers (3.60%) had NSAID residues in kidney and liver, specifically flunixin (1.03%) and meloxicam (2.57%). Flunixin was associated with visceral gout and/or kidney damage in three (0.98%) dead Eurasian griffons. To date, diclofenac poisoning has not been observed in Spain and Portugal, however, flunixin would appear to pose an immediate and clear risk. This work supports the need for well managed carrion disposal, alongside appropriate risk labelling on veterinary NSAIDs and other pharmaceuticals potentially toxic to avian scavengers.

Capsule: NSAIDs were present in livestock carrion and wild avian scavengers in Spain, but only flunixin was associated with visceral gout and/or kidney damage in three (0.98%) Eurasian griffons.

Keywords: Veterinary pharmaceutical, Europe, vultures, flunixin, poisoning.

1. Introduction

During the late-1990s and early-2000s, South Asian *Gyps* vulture populations collapsed (by up to 99.9%), almost leading to their extinction (Prakash et al., 2003). Demographic studies showed alarming rates of adult mortality in India, Pakistan and Nepal of white-rumped vulture (*Gyps bengalensis*), Indian vulture (*Gyps indicus*) and slender-billed vulture (*Gyps tenuirostris*) (Gilbert et al., 2006; Prakash et al., 2012). Based on consistent pathological findings in dead vultures (visceral gout with tubular nephrosis), dietary exposure to a toxicant was considered to be a plausible cause. Oaks et al. (2004) hypothesized that Asian vultures were being intoxicated with veterinary products used in livestock treatment and further investigation identified diclofenac as the potential driver. This was corroborated by a clear association between the presence of diclofenac residues in kidneys of dead vultures and visceral gout (Oaks et al., 2004; Oaks & Watson, 2011). To confirm diclofenac toxicity, experimental dosage studies were also conducted with non-releasable captive *Gyps* vultures, and these established a lethal dose (LD_{50}) between 0.098-0.225 mg kg^{-1} body weight (bw), with death occurring after ingesting carrion containing 0.007-0.94 mg kg^{-1} diclofenac (Oaks et al., 2004; Swan et al., 2006). Based on this data, Green et al. (2004) further estimated that just 0.13-0.75% of carcasses available to vultures in South Asia would need to contain a lethal dose of diclofenac to cause rapid population declines. In fact, carcass monitoring confirmed that 11.1-13.9% of carcasses available in India had detectable diclofenac residues, with levels in livers ranging between 0.01 and 10.1 mg kg^{-1} (Taggart et al., 2007, 2007b, 2009). Having identified this widespread problem in South Asia, Governments banned the manufacture and importation of diclofenac for veterinary use in India, Pakistan and Nepal in 2006 (and in 2010 in Bangladesh), which by 2011 decreased diclofenac positive carcasses by ~50% in India (Chaudhry et al., 2012; Prakash et al., 2012; Khan et al., 2013; Cuthbert et al., 2016). However, diclofenac was still being detected in carrion, and at lethal levels (Cuthbert et al., 2011, 2011b).

Identifying vulture-safe NSAIDs, that can also serve as alternatives to diclofenac for veterinary use, is key to successfully reducing risks to vultures. To date, meloxicam is the only available NSAID that has clearly been identified as vulture-safe (Swarup et al., 2007; Naidoo et al., 2008; Cuthbert et al., 2014). Meloxicam has been detected (below the limit of quantification) in two eggs from captive-reared bearded vultures (*Gypaetus barbatus*) but no adverse effects on reproduction have been observed (Zorrilla et al., 2018). Several other NSAIDs have also undergone robust vulture safety testing, but all have shown varying degrees of toxicity in *Gyps* vultures; these include ketoprofen (Naidoo et al., 2010, 2010b), carprofen (Cuthbert et al., 2007; Fourie et al., 2015), flunixin (Fourie et al., 2015; Zorrilla et al., 2015), nimesulide (Cuthbert et al., 2016) and aceclofenac, which is known to metabolize to diclofenac in vivo when administered to cattle (Galligan et al., 2016).

This risk scenario is however not just limited to South Asia. Diclofenac is currently authorized as a veterinary drug in certain African and European countries, and its toxicity has been demonstrated in native vulture species of these regions (Naidoo et al., 2009). In Europe, diclofenac was first registered for veterinary use in Spain, Italy, Estonia, Latvia and the Czech Republic in 2013. The Iberian Peninsula (Spain and Portugal) is particularly important in this context, as it hosts ~95% of all European vultures. Spain holds around 31000 breeding pairs of Eurasian griffon vultures (*Gyps fulvus*), >2500 pairs of cinereous vultures (*Aegypius monachus*), >1450 pairs of Egyptian vultures (*Neophron percnopterus*) and 125 pairs of bearded vultures (*Gypaetus barbatus*) (Del Moral, 2009, 2017; Margalida et al., 2014; Del Moral & Molina, 2018). Portugal hosts 500-1000 breeding pairs of Eurasian griffons, 5-8 pairs of cinereous vultures and 50-100 pairs of Egyptian vultures (BirdLife International, 2015). Due to concerns regarding risks to these important populations, the Spanish Agency of Medicines and the Ministry of Agriculture and Environment performed a risk assessment (AEMPS & MAGRAMA, 2014) and estimated that residues of diclofenac in carrion may cause 15-39

deaths of Eurasian griffons in Spain per year, which was in stark contrast to another study that calculated 715-6389 deaths to be plausible (Green et al., 2016).

Eleven NSAIDs are used for livestock treatment in Spain (CIMAVET, 2020), and most of these are now considered potentially toxic to *Gyps* vultures (Oaks et al., 2004; Cuthbert et al., 2007; Naidoo et al., 2010, 2010b; Fourie et al., 2015; Zorrilla et al., 2015). One of them, flunixin, has already been linked to wild vulture mortality in Spain (Zorrilla et al., 2015). However, until now, there have been no published monitoring data regarding NSAID residues in livestock carcasses and vultures in the Iberian Peninsula. The objectives of this work have therefore been [1] to quantify the presence of diclofenac and other NSAIDs in livestock carrion disposed of at supplementary feeding stations for vultures in Spain, [2] to assess NSAID residues (and potential poisoning) in avian scavengers found dead or moribund in Spain and Portugal between 2013 and 2019, and [3] to discuss the potential impact that NSAIDs may have on Iberian populations of avian scavengers.

2. Material and methods

2.1. Carrion sampling

Carrion sampling was performed by trained environmental technicians and agents at supplementary feeding stations in Castile and Leon, Valencia, Aragon, La Rioja, Asturias, Galicia, Navarra and Extremadura (Fig. 1). Sampled carrion were 156 pig, 45 sheep, 4 goat, 4 horse, 6 cow and 13 unknown (the species were not recorded) carcasses. During the first sampling period (in 2016; performed under project TEC0004566), muscle, liver and kidney were collected from 125 pig carcasses. In the second period (2018-2019; performed under project D16ZO-046-MAF-AvianScaven), liver and kidney were collected from the remaining

carcasses listed. Samples were collected into plastic zip-lock bags, frozen at -20 °C and sent to the Institute for Game and Wildlife Research (IREC) for NSAID analysis.

2.2. Avian scavenger sampling

We tested 389 avian scavengers from forensic cases admitted to wildlife rehabilitation centres in Spain and Portugal (Fig. 1). This included four vulture species corresponding to Eurasian griffons (n=306; 290 from Spain and 16 from Portugal), cinereous vultures (n=15; 13 from Spain and 2 from Portugal), bearded vultures (n=12 from Spain) and Egyptian vultures (n=11; 10 from Spain and 1 from Portugal). We also included 45 facultative avian scavengers from Spain corresponding to common buzzards (*Buteo buteo*, n=26), red kites (*Milvus milvus*, n=15), black kites (*Milvus migrans*, n=3) and booted eagle (*Hieraaetus pennatus*, n=1). Although booted eagle does not show scavenging habits, this bird was included in the analysis because it showed lesions of visceral gout.

The tissues collected and analysed at IREC were liver (n=384) and kidney (n=284). Sample collection was performed during two mortality monitoring programs. Since 2004, IREC has offered toxicological analyses of wildlife incidents to Spanish public administrations. In cases of suspected poisoning, during post-mortem examinations, veterinary staff from wildlife rehabilitation centres in Spain submitted liver and other samples (or whole carcasses) for toxicological analysis. Between 2013 and 2019, liver samples from avian scavengers (n=149) were collected through this analytical service and were tested for NSAID presence, among other toxic substances. Since 2017, liver and kidney sampling of avian scavengers (n=240) has also been carried out specifically for NSAID detection. These samples were taken during necropsies at wildlife rehabilitation centres (n=157), at the Universitat Autònoma de Barcelona (n=56) and at IREC (n=8) in Spain, and at two Portuguese wildlife rehabilitation centres (Centro de Estudos e Recuperação de Animais Selvagens (CERAS) and Centro de

Recuperação e Investigação de Animais Selvagens (RIAS) (n=19), whose samples were received by the University of Aveiro in Portugal. All samples were collected into polypropylene tubes or zip-lock bags and held frozen at -20 °C until analysis. Additionally, one Eurasian griffon which died in a wildlife rehabilitation centre in 2015 after iatrogenic poisoning with flunixin was analysed, but this case was not included in our statistical analysis and more details of the circumstances of this poisoning were given in Soler et al. (2016). The flunixin tissue levels detected in this vulture were used as a reference for confirmed poisoning.

2.3. NSAID Analysis

The veterinary NSAIDs registered in Spain (with number of commercial formulations for use in livestock) are ketoprofen (23), meloxicam (19), flunixin meglumine (14), acetylsalicylic acid (8), phenylbutazone (6), sodium salicylate (4), carprofen (4), metamizole (4), tolfenamic acid (3), diclofenac (2) and suxibuzone (1) (CIMAVET, 2020). The selected compounds tested were those initially covered by a method developed by Taggart et al. (2009) to monitor carrion from India, which included carprofen, diclofenac, flunixin, indomethacin, ketoprofen, meloxicam and naproxen. We also added tolfenamic acid, suxibuzone and phenylbutazone to include most of the NSAIDs registered in Spain for veterinary use in livestock according to the AEMPS database (CIMAVET, 2020). Indomethacin and naproxen were not registered in Spain but were also monitored as they were already covered by the method used. The only NSAIDs registered in Spain for veterinary use in livestock not included in this work were metamizole, acetylsalicylic acid and sodium salicylate (CIMAVET, 2020).

Analytical standards (Table S1) were acquired from Sigma-Aldrich and Supelco: carprofen (33975 Supelco), diclofenac disodium salt (D6899 Sigma-Aldrich), flunixin meglumine (F0429 Sigma-Aldrich), indomethacin (I8280 Sigma-Aldrich), ketoprofen (34016 Supelco), meloxicam hydrated sodium salt (M3935 Sigma-Aldrich), naproxen (N8280),

tolfenamic acid (T0535 Sigma-Aldrich), suxibuzone (S2400000 Sigma-Aldrich) and phenylbutazone (P8386 Sigma-Aldrich). Flunixin-d3 (34083 Supelco) was used as an internal standard.

The tissue extraction method was based on Taggart et al. (2009), with some modifications. First, 0.5 g of tissue was weighed into a polypropylene tube to which 2 mL of acetonitrile and 80 µL of flunixin-d3 (at 80 ng μL^{-1} in acetonitrile) were added. This mix was homogenized using an IKA-T8 homogenizer for 1 min. Between each sample, the homogenizer was thoroughly cleaned using Extran MA 01 solution (Merck), Milli-Q water and acetonitrile to avoid cross-contamination between samples. Once homogenized, the sample was sonicated for 5 min and then centrifuged at 1000 rcf for 5 min. Next, 1 mL of the supernatant was syringe-filtered through a 0.25 µm nylon filter into a 2 ml HPLC vial. The extract obtained was analysed immediately, or when this was not possible, stored at -20 ºC until analysis (for no longer than 24 h).

Muscle, liver and kidney samples from the first 125 pig carcasses and the liver of the first 10 forensic avian scavenger cases were analysed by liquid chromatography with electrospray ionization mass spectrometry (LC-ESI-MS) using an Agilent 1100 LC coupled to an Agilent 6110 single quadrupole MS following the method described by Taggart et al. (2009). For subsequent samples, we used ultra-high-performance liquid chromatography (UHPLC) with MS/MS time-of-flight mass spectrometry (LC-QTOF-MS; AB Sciex TripleTOFTM4600 System). Chromatographic separation was carried out using a Poroshell-120EC-C18 column (2.1 x 150 mm, 2.7 µm). Chromatography conditions were as follows: flow 0.5 mL min^{-1}; column temperature 40°C; gradient elution with (A) 0.1% formic acid in Milli-Q and (B) 0.1% formic acid in acetonitrile. Initial conditions were 40% phase A and 60% phase B for 1 min, then a 5 min linear gradient to 35% A and 65% B, followed by 100% B for 2 min, returning over 1 min to initial conditions. Injection volume was 5 µL and vials were kept cool at 4 °C in

the autosampler. The Q-TOF parameters were as follows: gas flow (CUR) at 20 psi, source 1 gas (GS1) at 40 psi, source 2 gas (GS2) at 40 psi, maximum temperature 400 °C (TEM), collision energy (CE) -35 V, propagation of collision energy (CES) 15 V and fragmentation potential (DP) -100 V. The molecular weights for the precursor ions and the three main fragmentation ions for the NSAIDs analysed in MRM (multiple reaction monitoring) mode with positive ionization are shown in Table S1. Quantification was performed using the most abundant fragment ion, with a fragmentation voltage of 50 to 500 V and a capillary voltage of 4500 V.

Calibrations were performed using diluted working solutions made up from stock solutions at 1 mg mL^{-1} for each NSAID. From these, mixed working solutions were prepared and kept at 4 °C until use. Mixed standards were made at concentrations of 50, 100, 200 and 400 ng mL^{-1} in a final volume of 1 mL whilst including 0.25 ng mL^{-1} of internal standard (flunixin-d3). Blank and fortified samples were also made using chicken liver (tissue surrogate) at NSAID levels of 50, 100 and 200 ng g^{-1}. These were processed daily in order to estimate the accuracy and precision of the analytical technique (% recovery ± RSD). We obtained recovery rates between 87% (for tolfenamic acid) and 129% (for suxibuzone) and RSD values ranged between 5.72 (for diclofenac) and 19.45 (for tolfenamic acid) (Table S2). Regression coefficients (R^2) in fortified calibration spikes were between 0.879 (for naproxen) and 0.989 (for meloxicam) (Table S2). Limits of quantification (LOQs) were established at 10 times the signal to noise ratio, and were between 0.0002 mg kg^{-1} (for flunixin) and 0.02 mg kg^{-1} (for naproxen) (Table S2).

2.4. Data analysis and interpretation

Detection frequency for each NSAID in carrion and avian scavengers was calculated and compared (i.e., between regions and species) using Fisher's exact tests with IBM SPSS

Statistics 24. To analyse the risk of intoxication by NSAIDs from carrion ingestion, we used median lethal dose (LD_{50}) information, where available. For diclofenac, we used the LD_{50} of 0.098-0.225 mg kg^{-1} bw calculated for white-rumped vulture by Swan et al. (2006). For flunixin, we used a lethal dose range of 1-4.5 mg kg^{-1} bw estimated for Rüppell's griffon vulture (*Gyps rueppellii*) and cinereous vulture (Cuthbert et al., 2007), and for ketoprofen 1.5-5 mg kg^{-1} estimated in Cape griffon vulture (*Gyps coprotheres*) and white-backed vulture (Naidoo et al., 2010b). Using the concentrations detected in carrion, we calculated the estimated theoretical exposure (ETE) in Eurasian griffon with a mean body weight of 7.4 kg and with an average daily intake of 1.2 kg of food (Donázar, 1993). These ETEs were used with the LD_{50} values to estimate toxicity exposure ratios ($TER=LD_{50}/ETE$) for each NSAID for *Gyps*. TERs were estimated using the minimum and maximum LD_{50} values noted above. This ratio is widely used to evaluate the first-tier risk of exposure to a chemical substance (such as a pesticide in birds) and must be <10 to represent an acute toxicity risk to wild birds (EFSA, 2009). In the case of diclofenac, we also calculated the per-meal probability of death in vultures feeding on the analysed carrion using the parameters of the dose-response curves for *Gyps* species (Swan et al., 2006).

NSAID concentrations in vulture tissues have been interpreted based on previous studies, which associated residues in vultures with adverse effects and/or mortality. Diclofenac levels of 0.05-0.64 mg kg^{-1} in kidney and flunixin levels of 2.7 mg kg^{-1} in liver and 6.5 mg kg^{-1} in kidney have been considered compatible with lethal poisoning by these NSAIDs in vultures (Oaks et al., 2004; Zorrilla et al., 2015). Likewise, the presence of visceral gout at post-mortem examination in birds with NSAID residues was considered additional evidence of NSAID intoxication (Oaks et al., 2004; Zorrilla et al., 2015; Cuthbert et al., 2016).

3. Results

3.1. Detection of NSAIDs in carrion and risk assessment for vultures

NSAID residues were detected in 3.07% (7/228) of all carrion tested (Table 1; Table S3). We detected 5 NSAID positive samples in pigs originating from intensive production, which represented 3.20% of the total pig carcasses analysed (n=156). Further, there were 2 positive sheep samples, representing 4.44% of the total sheep carcasses tested (n=45). Pig samples were positive for flunixin (n=2, 1.28%), diclofenac (n=1, 0.64%), ketoprofen (n=1, 0.64%), and meloxicam (n=1, 0.64%). Sheep were positive for flunixin (n=2, 4.44%) (Table 1). None of the carcasses of goat (n=4), horse (n=4), cow (n=6) or 'unknown' species had NSAID residues. However, these differences in prevalence between species were not statistically significant. Positive carrion were detected in three regions: Castile and Leon (2 of 35, 5.71%), Aragon (3 of 112, 2.68%), and Valencia (2 of 18, 11.11%) (see Fig. 1 and Table S4). There was a marginal significant difference between Aragon and Valencia (Fisher's test, p=0.051). Prevalence is also shown in more detail by provinces (Fig. S1).

The estimated acute TER value was well above 10 for all samples, except for the one positive to diclofenac and one positive to flunixin (Table 1). The diclofenac positive pig muscle with 0.171 mg kg^{-1} (ETE of 0.028 mg kg^{-1} bw) resulted in a per-meal probability of death for vultures of 25.4% or 0.8% using the relevant dose-response curves and with an LD_{50} of 0.098 or 0.225 mg kg^{-1} bw, respectively. However, it must be acknowledged that diclofenac residues in this muscle tissue were detected only at an injection point (Fig. S2), so, the real risk from this specific carcass was probably lower. The sheep liver with the highest level of flunixin had a TER value ranging between 0.22-1, so the risk of poisoning here was very high. The per-meal probability of death for vultures could not be calculated in this case because there is no available dose-response curve for flunixin in *Gyps* vultures.

3.3. Monitoring NSAIDs in dead avian scavengers including vultures

We observed that 3.60% (14/389) of individuals had detectable NSAID residues in liver and/or kidney (Table 2; Table S5). Eleven Eurasian griffons analysed (3.59%) were positive for NSAIDs, specifically meloxicam (n=7, 2.29%) and flunixin (n=4, 1.30%). Meloxicam was also detected in one Egyptian vulture (9.09%), one common buzzard (3.84%) and one black kite (33.33%) (Table S5). Concentrations ranged between 0.023-20.35 mg kg^{-1} for flunixin and between 0.033-2.44 mg kg^{-1} for meloxicam (Table 2). By region, the prevalence was highest in Castile and Leon (1/2, 50%), followed by Cantabria (1/12, 8.33%), Madrid (1/15, 6.67%), Aragon (4/85, 4.71%), Catalonia (6/133,4.51%) and Extremadura (1/28, 3.57%) (Fig. 1). However, prevalence was not significantly different between regions or between species. The situation in Castile and Leon may warrant further research given that one out of two animals had NSAID residues. Prevalence is also included in more detail by province (Fig. S1).

Post-mortem examinations showed that 10 out of 306 dead Eurasian griffons had degenerative lesions in kidney and/or liver (3.27%) and four of these presented extensive visceral gout (1.31%) (Fig. S2). Two of these cases (#2 and #4 in Table 2) also had elevated flunixin levels in tissues (20.35 mg kg^{-1} in the kidney and 11.32 mg kg^{-1} in the liver, and 4.91 mg kg^{-1} in liver, respectively). The other two Eurasian griffons with visceral gout had no detectable NSAID residues in their tissues. A third Eurasian griffon (found dead under a cliff with lesions of traumatism) also had 0.33 mg kg^{-1} of flunixin in liver and renal degeneration (#1 in Table 2), while a fourth Eurasian griffon with 0.023 mg kg^{-1} flunixin in liver had no lesions, gout or kidney damage on necropsy (#3 in Table 2). In addition to these wild birds, one Eurasian griffon that died in a wildlife rehabilitation centre was tested as it was suspected to have died from iatrogenic flunixin poisoning (Soler et al., 2016). This bird had 2.83 mg kg^{-}

299 [1] in liver and 0.44 mg kg^{-1} in muscle and visceral gout (#5 in Table 2); as such, these levels
300 were comparable with Eurasian griffons found dead in the field with this lesion.
301
302 **4. Discussion**
303 Residues of diclofenac and three other NSAIDs (flunixin, ketoprofen and meloxicam)
304 have been detected in livestock carcasses supplied to supplementary feeding stations for avian
305 scavengers in Spain. Diclofenac poisoning has not been detected in the avian scavengers tested,
306 but flunixin poisoning has been confirmed in three wild Eurasian griffons in which the presence
307 of the chemical was accompanied with visceral gout and/or renal damage.
308
309 *4.1. Risk assessment based on NSAID residues in carrion*
310 The first objective of the present study was to evaluate the risk of exposure to diclofenac
311 in avian scavengers in the Iberian Peninsula. Two commercial formulations of diclofenac have
312 been registered for veterinary use in livestock since 2013 in Spain (CIMAVET, 2020).
313 Diclofenac is not yet authorized in Portugal by the national authority (Direção-Geral de
314 Alimentação e Veterinária), despite a vote in favour of its use in the Portuguese Parliament in
315 January 2019. The carrion analyses performed here shows a potential risk of exposure to
316 diclofenac in Iberian avian scavengers because one pig carcass was found positive. Therefore,
317 the labelling of commercial diclofenac formulations, which includes warnings to avoid disposal
318 of carrion from treated animals for vulture feeding, is not being effective in all cases.
319 The pig carrion with diclofenac residues in muscle was possibly from an animal treated
320 more than 168 h before death, because residues in the muscle were limited to an area associated
321 with the likely injection point (Fig. S2), and no residues were detected in liver or kidney
322 (Naidoo et al., 2018). In Spain, diclofenac dosage for pig is specified at 2.3 mg kg^{-1} (1 mL per
323 20 kg bw of a solution with 46 mg mL^{-1} of diclofenac) administered intramuscularly in a three-

day treatment pattern, with no more than 3 mL injected in a single point. Therefore, a pig weighing 120 kg would need a daily dose of 276 mg, i.e., 138 mg in each of two injection points every day, resulting in six points after a 3-day treatment (AEMPS, 2018). The pharmacokinetics for diclofenac in pig indicate a 3.4 h elimination half-life and a maximum plasma level of 4.7 μg mL^{-1} at 0.5 h (AEMPS, 2018). In addition, experimental studies discussed in Green et al. (2006) describe a higher half-life in muscle (15 h) than in kidney and liver (6-8 h). In the case of this diclofenac positive pig carcass, acute poisoning could occur if scavengers consumed muscle from the injection sites, as has been observed for carprofen (Naidoo et al., 2018).

Green et al. (2004) estimated that just 0.13-0.75% of carcasses needed to contain a lethal level of diclofenac to explain (alone, without any other drivers) the rapid population declines seen for *Gyps* vultures in South Asia. We found that 0.64% of pigs tested positive to diclofenac, so the risk to Iberian avian scavengers exists. The relatively small number of carrion samples tested, alongside the fact that the single positive was from a pig with diclofenac residues at an injection site only, limits the possibility to perform a more robust risk assessment. However, the scenario observed here, on the Iberian Peninsula, is far from that seen in India where diclofenac residue prevalence prior to any legal ban was ~10% nationally, with certain states monitored with 22.3% diclofenac positive carcasses (Taggart et al., 2007).

In addition to diclofenac, we detected other NSAIDs in pig and sheep carcasses, specifically flunixin, ketoprofen and meloxicam. A risk to avian scavengers (according to TER calculations) was only noted in one sheep due to the high level of flunixin found. According to data reported from 2004-2018 in the Spanish Residue Research National Plan (PNIR; the focus of which is human food safety), two NSAIDs have been detected in samples obtained from slaughterhouses (both in 2016), specifically diclofenac in a horse (5.88%, 1/17) and flunixin in a cow (25%, 1/4) (PNIR, 2016). Flunixin and ketoprofen are both thought to be toxic to *Gyps*

vultures, causing visceral gout and rapid death mortality, although potentially at higher doses than for diclofenac (Cuthbert et al., 2007; Naidoo et al., 2010, 2010b; Zorrilla et al., 2015). Based on pharmacokinetic data, the two flunixin positive pigs here probably died >48 h after treatment (Buur et al., 2006) and the ketoprofen positive pig likely died >25 h after treatment (Mustonen et al., 2012). In terms of the two flunixin cases in sheep, the animal with the highest level (27.48 mg kg^{-1}) probably died quickly after treatment (within 5 h) due to the elevated levels in liver, while the second (at 0.297 mg kg^{-1} in liver) likely died 10 to 15 h after treatment (Cheng et al., 1998).

4.2. NSAID poisoning in Iberian avian scavengers

We have not detected cases of diclofenac poisoning in avian scavengers from the Iberian Peninsula to date, despite the previous estimations of mortality performed by AEMPS & MAGRAMA (2014) and Green et al. (2016). In the specific case of porcine livestock, AEMPS & MAGRAMA (2014) assumes that vultures consume 38413 intensively reared pig carcasses per year, of which, 0.14-0.24% could contain diclofenac residues. Based on this, and proposing different diclofenac concentration scenarios in carrion (0.1, 0.4 and 0.8 mg kg^{-1}) and time intervals between last diclofenac injection and carrion intake (0-3, 3-12 and 12-24 h), AEMPS & MAGRAMA (2014) estimated that the number of vultures that could die per year in Spain (from diclofenac in pig carcasses) would be between 4-7 individuals. This markedly contrasts with the estimations of Green et al. (2016) that calculated 364-4609 annual deaths of Eurasian griffons due to pig carcasses. The main difference between these studies is that Green et al. (2016) assumed that all carrion available (containing diclofenac residues) had the potential to be toxic, given that experimental studies have indicated marked interindividual variations. Mortality after exposure has been observed at doses as low as 0.007 mg kg^{-1} bw (Oaks et al., 2004; Swan et al., 2006). Although the only carcass in our study with diclofenac

residues would likely not pose a high risk to vultures, we used our 0.64% diclofenac prevalence value to recalculate the proportion of carcasses that could contain lethal levels for vultures in Spain and then refine the risk assessment. We can estimate that the probability of dying in the first 8 h after last treatment would be 4.76% (8 h/168 h) for all diclofenac treated animals, so the percentage of carrion with potentially lethal diclofenac levels would be 0.0476 × 0.64 = 0.03% (Table 3). Here we assume that probability of death at a determined time is constant throughout the 168 h period after treatment, during which diclofenac residues in tissues are above our limit of quantification. With this percentage (0.03%) and the number of swine carcasses available (38413, AEMPS & MAGRAMA, 2014), we can estimate the number of treated pigs with toxic levels (12) and the number of meals available to vultures from these carrion (1600). Following the approach of Green et al. (2016) (with the proportion of vultures killed by feeding on a contaminated pig treated 8 h before death), we can estimate that 78-600 vultures would die per year (with LD_{50} of 0.098 and 0.225 mg kg^{-1}, respectively), which is between the ranges given in previous estimations (Table 3). These estimates are based on LD_{50} data and dose-response curves showing that some individuals can be especially sensitive to diclofenac, so some mortality may occur at doses much lower than the median value.

In contrast with diclofenac, flunixin poisoning has been detected in three Eurasian griffons in this study, each showing visceral gout (Fig. S3) and/or kidney degeneration and flunixin residues in liver between 0.33 and 11.32 mg kg^{-1}. These residue levels are comparable with those detected in an iatrogenic flunixin poisoning in one Eurasian griffon, with 2.83 mg kg^{-1} of flunixin in liver and visceral gout (Soler et al., 2016); and, the case described by Zorrilla et al. (2015) of another Eurasian griffon with 2.7 mg kg^{-1} of flunixin in liver and visceral gout. Flunixin poisoning has also been described in two Rüppell's griffon vultures (*Gyps rueppellii*) and one white-backed vulture in captivity, with 0.016-0.039 mg kg^{-1} of flunixin in several tissues, who fed on flunixin contaminated beef with 31.35 mg kg^{-1} (Eleni et al., 2019). Flunixin

399 has also been linked to possible iatrogenic poisoning in other birds in captivity, including three
400 cinereous vultures, one Rüppell's griffon and one white-backed vulture, at exposure doses of
401 1-4.5 mg kg^{-1} (Cuthbert et al., 2007). Thus, our results clearly confirm that vultures are dying
402 due to flunixin in Spain, and the mortality observed here of 3 out of 306 Eurasian griffons
403 represents 0.98% of the studied cases (see Table S6). With 30946 breeding pairs of Eurasian
404 griffons in Spain, a productivity of 0.56 chicks/nest and considering a stable population
405 (natality≈mortality), we would estimate an annual mortality of 170 griffon vultures due to
406 flunixin poisoning.

407 Meloxicam residues were detected in seven vultures with evidence of traumatism,
408 electrocution, intoxication or suspected previous intoxication. None of the meloxicam positive
409 birds had visceral gout. Further, meloxicam is not thought to be a risk to vultures as extensive
410 vulture safety testing has taken place to demonstrate this (Swan et al., 2006b; Swarup et al.,
411 2007; Naidoo et al., 2008; Mahmood et al., 2010).

412 Finally, it should be noted that while visceral gout is a frequent lesion observed in
413 relation to NSAID poisoning in birds, confirmation must be attained using parallel chemical
414 analysis of kidney or liver tissues. Beyond NSAID poisoning, gout can also be caused by
415 metabolic disorders, dehydration, infectious etiology, renal damage or other nephrotoxic agents
416 (Echols, 2016).

417

418 **5. Conclusions**

419 The Eurasian griffon population has increased in Spain from 24541 to 30946 breeding
420 pairs from 2008 to 2018 (+21.16%; Del Moral & Molina, 2018), so at the moment, there is no
421 evidence of a population level impact of diclofenac use in livestock on this species.
422 Nevertheless, monitoring efforts to study causes of mortality in Iberian avian scavengers must
423 continue because of the observed risk posed by the potential disposal of diclofenac treated

carrion in the field or in supplementary feeding stations. The presence of diclofenac in one carcass indicates a failure in the formulation advisory systems in Spain (i.e., given on product labels) which states that diclofenac treated animal carrion should "never reach the trophic chain of wild animals". Likewise, the same recommendations should be applied to formulations of flunixin and ketoprofen marketed in Spain, and to that of any veterinary pharmaceutical known to be toxic to scavengers. For flunixin, levels capable of causing acute toxicity in vultures were clearly identified and as such changes to labelling/advice are certainly needed to protect these scavengers. In addition, this NSAID is not currently registered in Europe for veterinary use in sheep (EMEA, 2000), so these results clearly suggest that veterinary drugs have extra-label use. An effective risk assessment for veterinary drugs must always consider the possibility that these may enter wildlife food webs through a livestock carrion pathway. But also, farmers, veterinarians and wildlife technicians in charge of managing supplementary feeding stations or the disposal of carrion in the field must be aware of the risks that pharmaceutical treated livestock may represent for avian scavengers (Mateo et al., 2015; Zorrilla et al., 2015; Casas-Díaz et al., 2016).

Acknowledgments

The authors gratefully acknowledge support from regional governments and other institutions in Spain, specifically from Aragon, Catalonia, Extremadura (Acción por el Mundo Salvaje: AMUS), Asturias, Andalusia, La Rioja, Castile and Leon, Madrid, Cantabria, Navarra, the Balearic Islands, Valencia, Galicia, the Basque Country and Asturias. We would like to thank the veterinarians who carried out post-mortem examinations, for their assistance in tissue collection in the field and at wildlife rehabilitation centres. A special mention is given to Rafael Molina and Olga Nicolas for performing a high number of the vulture necropsies and submitting samples to IREC, specifically some key individuals within the intoxication case

group. We thank all the TRAGSATEC technicians and other staff from the regional governments who co-operated and contributed in terms of providing tissue samples from livestock carcasses from supplementary feeding stations.

Funding

The authors gratefully acknowledge funding from the Morris Animal Foundation within the project D16ZO-046-MAF-AvianScaven, TRAGSATEC through contract Ref. TEC0004566 and CGL2013–40975-R project from I+D+I National Plan funded by the Spanish Ministry of Economy and Competitiveness. We also acknowledge that the Portuguese samples were received due to FCT/MCTES financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020), through national funds.

CRediT author statement

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Marta Herrero-Villar: Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, **Roser Velarde:** Investigation, **Pablo R. Camarero:** Validation, Investigation, **Mark A. Taggart:** Writing - Review & Editing , **Victor Bandeira:** Resources, Funding acquisition, **Carlos Fonseca:** Resources, Funding acquisition, **Ignasi Marco:** Resources, Supervision, Project administration, Funding acquisition, **Rafael Mateo:** Conceptualization, Methodology, Resources, Writing-Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the present study.

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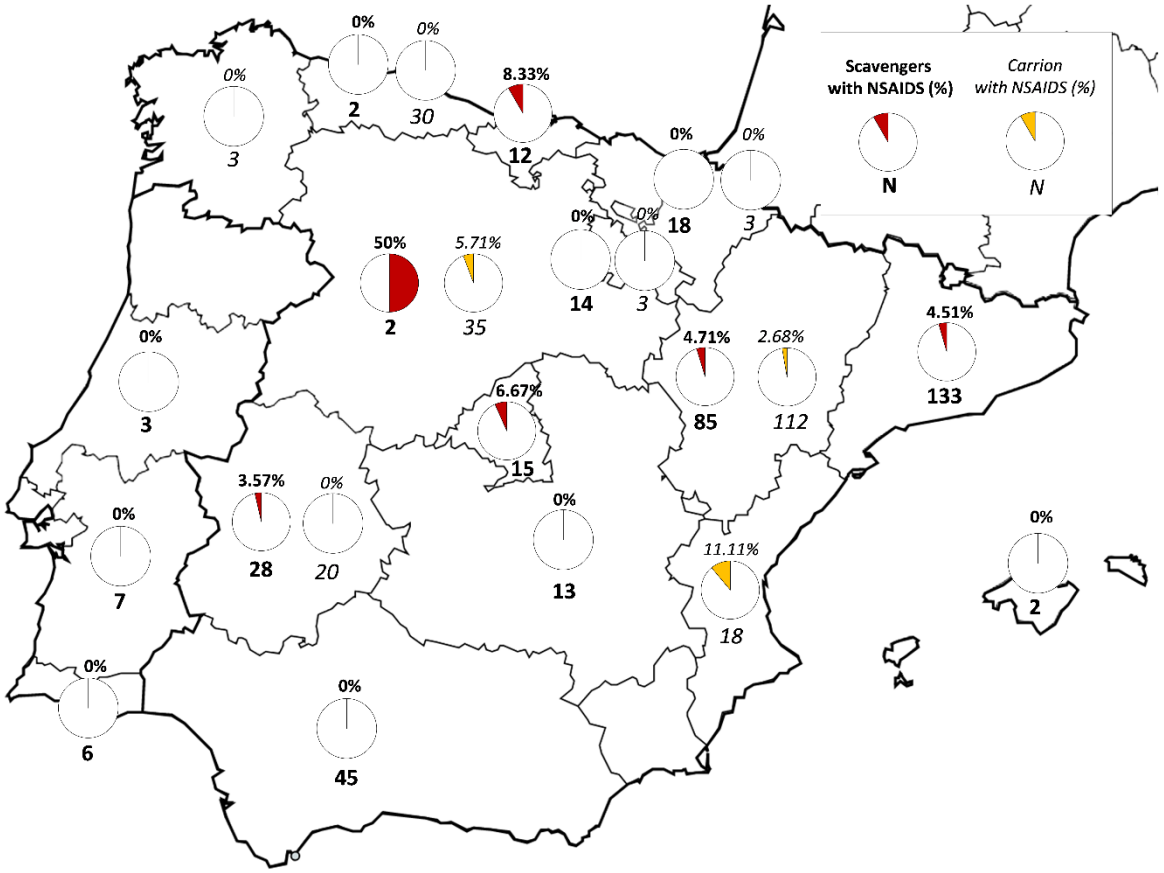
679 **Figure legend**

680

681 Fig. 1. Iberian Peninsula (Portugal and Spain) map with the distribution by regions of sampled carrions and avian scavengers and the percentage

682 of samples with NSAID residues.

683



684

685 Table 1. Concentrations of NSAIDs detected in positive carrions in Spain alongside a first-tier risk assessment based on toxicity-to-exposure ratios
686 (TERs) in vultures.

Carrion	Species	Year	Region	NSAID	Concentration in carrion (mg/kg)			ETE (mg/kg)	LD_{50} or observed LD (mg kg^{-1})		Acute TER	
					Muscle	Liver	Kidney		min	max	min	max
1	Pig	2016	Castile and Leon	Diclofenac	0.171	<LOQ	<LOQ	0.028	0.1[a]	0.2[a]	3.57	7.14
2	Pig	2018	Aragon	Ketoprofen	-	<LOQ	0.173	0.028	1.5[c]	5[c]	53.6	178.6
3	Pig	2016	Valencia	Meloxicam	<LOQ	0.023	<LOQ	0.378	-	-	-	-
4	Pig	2017	Valencia	Flunixin	<LOQ	0.004	<LOQ	0.001	1[b]	4.5[b]	1000	4500
5	Pig	2016	Castile and Leon	Flunixin	<LOQ	<LOQ	0.008	0.001			1000	4500
6	Sheep	2018	Aragon	Flunixin	-	27.5	-	4.55			0.22	1
7	Sheep	2018	Aragon	Flunixin	-	0.297	-	0.048			20.8	93.8

687 ETE: estimated theoretical exposure; LD_{50}: median lethal dose (LD_{50} only available for diclofenac); TER: toxicity exposure ratio; LOQ: limit of
688 quantification.

689 [a] Swan et al. 2006, [b] Cuthbert et al. 2007, [c] Naidoo et al. 2010b.

690

691 Table 2. Positive cases of NSAIDs with the region of origin, presumptive diagnosis, NSAID concentrations by tissue.

Bird	Species	Year	Region	Diagnosis	NSAID	Concentration (mg kg^{-1})		
						Liver	Kidney	Muscle
1	Eurasian griffon	2010	Aragon	Traumatism/ Renal degeneration	Flunixin	0.33	-	-
2	Eurasian griffon	2015	Madrid	Indeterminate/ Visceral gout	Flunixin	4.91	-	-
3	Eurasian griffon	2017	Extremadura	Feather disease	Flunixin	0.023	-	-
4	Eurasian griffon	2018	Catalonia	Indeterminate/Visceral gout	Flunixin	11.32	20.35	-
5	Eurasian griffon	2015	Extremadura	Iatrogenic[a]	Flunixin	2.83	-	0.44
6	Eurasian griffon	2018	Catalonia	Traumatism	Meloxicam	0.641	0.264	-
7	Eurasian griffon	2018	Catalonia	Traumatism	Meloxicam	0.159	0.231	-
8	Eurasian griffon	2019	Aragon	Traumatism/Pb intoxication	Meloxicam	2.44	<LOQ	-
9	Eurasian griffon	2019	Cantabria	Indeterminate	Meloxicam	1.84	-	-
10	Eurasian griffon	2019	Aragon	Suspected Pb intoxication	Meloxicam	1.06	-	-
11	Eurasian griffon	2019	Aragon	Suspected Pb intoxication	Meloxicam	0.887	<LOQ	-
12	Eurasian griffon	2019	Extremadura	Traumatism	Meloxicam	-	0.829	-
13	Black kite	2019	Catalonia	Indeterminate	Meloxicam	0.033	0.046	-
14	Egyptian vulture	2018	Castile and Leon	Carbofuran intoxication	Meloxicam	0.141	-	-
15	Common buzzard	2018	Catalonia	Electrocution	Meloxicam	0.838	1.245	-

692 LOQ: limit of quantification.
693 [a] Soler et al. (2016).
694

Table 3. Estimation of diclofenac treated pigs from intensive production with toxic levels for vultures, number of the corresponding toxic meals for vultures and number of vultures killed by diclofenac poisoning.

Study	Treated carcasses available to vultures (A)	Weight of carcass (kg) (B)	Total weight of treated carcasses available to vultures (kg) (C = A x B)	Treated meals available to vultures (80% of mass consumed by vultures, regular meal of 1.2 kg per vulture) (D = C x 0.8/1.2)	Vultures killed per year (F = D x proportion killed)[a]	
					Proportion killed	
					0.049	0.375
Present study	12	200	2400	1600	78	600
AEMPS	55-92	200	11122-18430	7415-12287	4	7
Green et al.					364-603	2781-4609

[a.]The number of dead vultures by feeding on meals containing a diclofenac concentration toxic for vultures was calculated following Green et al. (2016) with the proportion of killed vultures after feeding on a contaminated pig treated 8 h before death were 0.375 and 0.049. These proportions were obtained from the LD50 values of 0.098 and 0.225 mg kg^{-1} calculated by Swan et al. (2006) from the experimental data of Oaks et al. (2004).