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

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26 **Abstract**

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

45

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

49

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

51

## 52 **1. Introduction**

53 During the late-1990s and early-2000s, South Asian *Gyps* vulture populations collapsed  
54 (by up to 99.9%), almost leading to their extinction (Prakash et al., 2003). Demographic studies  
55 showed alarming rates of adult mortality in India, Pakistan and Nepal of white-rumped vulture  
56 (*Gyps bengalensis*), Indian vulture (*Gyps indicus*) and slender-billed vulture (*Gyps*  
57 *tenuirostris*) (Gilbert et al., 2006; Prakash et al., 2012). Based on consistent pathological  
58 findings in dead vultures (visceral gout with tubular nephrosis), dietary exposure to a toxicant  
59 was considered to be a plausible cause. Oaks et al. (2004) hypothesized that Asian vultures  
60 were being intoxicated with veterinary products used in livestock treatment and further  
61 investigation identified diclofenac as the potential driver. This was corroborated by a clear  
62 association between the presence of diclofenac residues in kidneys of dead vultures and visceral  
63 gout (Oaks et al., 2004; Oaks & Watson, 2011). To confirm diclofenac toxicity, experimental  
64 dosage studies were also conducted with non-releasable captive *Gyps* vultures, and these  
65 established a lethal dose (LD<sub>50</sub>) between 0.098-0.225 mg kg<sup>-1</sup> body weight (bw), with death  
66 occurring after ingesting carrion containing 0.007-0.94 mg kg<sup>-1</sup> diclofenac (Oaks et al., 2004;  
67 Swan et al., 2006). Based on this data, Green et al. (2004) further estimated that just 0.13-  
68 0.75% of carcasses available to vultures in South Asia would need to contain a lethal dose of  
69 diclofenac to cause rapid population declines. In fact, carcass monitoring confirmed that 11.1-  
70 13.9% of carcasses available in India had detectable diclofenac residues, with levels in livers  
71 ranging between 0.01 and 10.1 mg kg<sup>-1</sup> (Taggart et al., 2007, 2007b, 2009). Having identified  
72 this widespread problem in South Asia, Governments banned the manufacture and importation  
73 of diclofenac for veterinary use in India, Pakistan and Nepal in 2006 (and in 2010 in  
74 Bangladesh), which by 2011 decreased diclofenac positive carcasses by ~50% in India  
75 (Chaudhry et al., 2012; Prakash et al., 2012; Khan et al., 2013; Cuthbert et al., 2016). However,  
76 diclofenac was still being detected in carrion, and at lethal levels (Cuthbert et al., 2011, 2011b).

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

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

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

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

115

## 116 **2. Material and methods**

117

### 118 *2.1. Carrion sampling*

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

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

128

## 129 2.2. Avian scavenger sampling

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

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

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

158

### 159 2.3. NSAID Analysis

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

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



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

179 The tissue extraction method was based on Taggart et al. (2009), with some  
180 modifications. First, 0.5 g of tissue was weighed into a polypropylene tube to which 2 mL of  
181 acetonitrile and 80  $\mu\text{L}$  of flunixin-d3 (at 80  $\text{ng } \mu\text{L}^{-1}$  in acetonitrile) were added. This mix was  
182 homogenized using an IKA-T8 homogenizer for 1 min. Between each sample, the homogenizer  
183 was thoroughly cleaned using Extran MA 01 solution (Merck), Milli-Q water and acetonitrile  
184 to avoid cross-contamination between samples. Once homogenized, the sample was sonicated  
185 for 5 min and then centrifuged at 1000 rcf for 5 min. Next, 1 mL of the supernatant was syringe-  
186 filtered through a 0.25  $\mu\text{m}$  nylon filter into a 2 ml HPLC vial. The extract obtained was analysed  
187 immediately, or when this was not possible, stored at  $-20\text{ }^{\circ}\text{C}$  until analysis (for no longer than  
188 24 h).

189 Muscle, liver and kidney samples from the first 125 pig carcasses and the liver of the  
190 first 10 forensic avian scavenger cases were analysed by liquid chromatography with  
191 electrospray ionization mass spectrometry (LC-ESI-MS) using an Agilent 1100 LC coupled to  
192 an Agilent 6110 single quadrupole MS following the method described by Taggart et al. (2009).  
193 For subsequent samples, we used ultra-high-performance liquid chromatography (UHPLC)  
194 with MS/MS time-of-flight mass spectrometry (LC-QTOF-MS; AB Sciex TripleTOFTM4600  
195 System). Chromatographic separation was carried out using a Poroshell-120EC-C18 column  
196 (2.1 x 150 mm, 2.7  $\mu\text{m}$ ). Chromatography conditions were as follows: flow 0.5  $\text{mL min}^{-1}$ ;  
197 column temperature  $40^{\circ}\text{C}$ ; gradient elution with (A) 0.1% formic acid in Milli-Q and (B) 0.1%  
198 formic acid in acetonitrile. Initial conditions were 40% phase A and 60% phase B for 1 min,  
199 then a 5 min linear gradient to 35% A and 65% B, followed by 100% B for 2 min, returning  
200 over 1 min to initial conditions. Injection volume was 5  $\mu\text{L}$  and vials were kept cool at  $4\text{ }^{\circ}\text{C}$  in

201 the autosampler. The Q-TOF parameters were as follows: gas flow (CUR) at 20 psi, source 1  
202 gas (GS1) at 40 psi, source 2 gas (GS2) at 40 psi, maximum temperature 400 °C (TEM),  
203 collision energy (CE) -35 V, propagation of collision energy (CES) 15 V and fragmentation  
204 potential (DP) -100 V. The molecular weights for the precursor ions and the three main  
205 fragmentation ions for the NSAIDs analysed in MRM (multiple reaction monitoring) mode  
206 with positive ionization are shown in Table S1. Quantification was performed using the most  
207 abundant fragment ion, with a fragmentation voltage of 50 to 500 V and a capillary voltage of  
208 4500 V.

209 Calibrations were performed using diluted working solutions made up from stock  
210 solutions at 1 mg mL<sup>-1</sup> for each NSAID. From these, mixed working solutions were prepared  
211 and kept at 4 °C until use. Mixed standards were made at concentrations of 50, 100, 200 and  
212 400 ng mL<sup>-1</sup> in a final volume of 1 mL whilst including 0.25 ng mL<sup>-1</sup> of internal standard  
213 (flunixin-d3). Blank and fortified samples were also made using chicken liver (tissue surrogate)  
214 at NSAID levels of 50, 100 and 200 ng g<sup>-1</sup>. These were processed daily in order to estimate the  
215 accuracy and precision of the analytical technique (% recovery ± RSD). We obtained recovery  
216 rates between 87% (for tolfenamic acid) and 129% (for suxibuzone) and RSD values ranged  
217 between 5.72 (for diclofenac) and 19.45 (for tolfenamic acid) (Table S2). Regression  
218 coefficients (R<sup>2</sup>) in fortified calibration spikes were between 0.879 (for naproxen) and 0.989  
219 (for meloxicam) (Table S2). Limits of quantification (LOQs) were established at 10 times the  
220 signal to noise ratio, and were between 0.0002 mg kg<sup>-1</sup> (for flunixin) and 0.02 mg kg<sup>-1</sup> (for  
221 naproxen) (Table S2).

222

#### 223 *2.4. Data analysis and interpretation*

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

226 Statistics 24. To analyse the risk of intoxication by NSAIDs from carrion ingestion, we used  
227 median lethal dose (LD<sub>50</sub>) information, where available. For diclofenac, we used the LD<sub>50</sub> of  
228 0.098-0.225 mg kg<sup>-1</sup> bw calculated for white-rumped vulture by Swan et al. (2006). For  
229 flunixin, we used a lethal dose range of 1-4.5 mg kg<sup>-1</sup> bw estimated for Rüppell's griffon vulture  
230 (*Gyps rueppellii*) and cinereous vulture (Cuthbert et al., 2007), and for ketoprofen 1.5-5 mg kg<sup>-1</sup>  
231 <sup>1</sup> estimated in Cape griffon vulture (*Gyps coprotheres*) and white-backed vulture (Naidoo et  
232 al., 2010b). Using the concentrations detected in carrion, we calculated the estimated  
233 theoretical exposure (ETE) in Eurasian griffon with a mean body weight of 7.4 kg and with an  
234 average daily intake of 1.2 kg of food (Donázar, 1993). These ETEs were used with the LD<sub>50</sub>  
235 values to estimate toxicity exposure ratios (TER=LD<sub>50</sub>/ETE) for each NSAID for *Gyps*. TERs  
236 were estimated using the minimum and maximum LD<sub>50</sub> values noted above. This ratio is widely  
237 used to evaluate the first-tier risk of exposure to a chemical substance (such as a pesticide in  
238 birds) and must be <10 to represent an acute toxicity risk to wild birds (EFSA, 2009). In the  
239 case of diclofenac, we also calculated the per-meal probability of death in vultures feeding on  
240 the analysed carrion using the parameters of the dose-response curves for *Gyps* species (Swan  
241 et al., 2006).

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

249

### 250 3. Results

251

#### 252 3.1. Detection of NSAIDs in carrion and risk assessment for vultures

253 NSAID residues were detected in 3.07% (7/228) of all carrion tested (Table 1; Table  
254 S3). We detected 5 NSAID positive samples in pigs originating from intensive production,  
255 which represented 3.20% of the total pig carcasses analysed (n=156). Further, there were 2  
256 positive sheep samples, representing 4.44% of the total sheep carcasses tested (n=45). Pig  
257 samples were positive for flunixin (n=2, 1.28%), diclofenac (n=1, 0.64%), ketoprofen (n=1,  
258 0.64%), and meloxicam (n=1, 0.64%). Sheep were positive for flunixin (n=2, 4.44%) (Table  
259 1). None of the carcasses of goat (n=4), horse (n=4), cow (n=6) or 'unknown' species had  
260 NSAID residues. However, these differences in prevalence between species were not  
261 statistically significant. Positive carrion were detected in three regions: Castile and Leon (2 of  
262 35, 5.71%), Aragon (3 of 112, 2.68%), and Valencia (2 of 18, 11.11%) (see Fig. 1 and Table  
263 S4). There was a marginal significant difference between Aragon and Valencia (Fisher's test,  
264  $p=0.051$ ). Prevalence is also shown in more detail by provinces (Fig. S1).

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

275

### 276 3.3. Monitoring NSAIDs in dead avian scavengers including vultures

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

288 Post-mortem examinations showed that 10 out of 306 dead Eurasian griffons had  
289 degenerative lesions in kidney and/or liver (3.27%) and four of these presented extensive  
290 visceral gout (1.31%) (Fig. S2). Two of these cases (#2 and #4 in Table 2) also had elevated  
291 flunixin levels in tissues (20.35 mg kg<sup>-1</sup> in the kidney and 11.32 mg kg<sup>-1</sup> in the liver, and 4.91  
292 mg kg<sup>-1</sup> in liver, respectively). The other two Eurasian griffons with visceral gout had no  
293 detectable NSAID residues in their tissues. A third Eurasian griffon (found dead under a cliff  
294 with lesions of traumatism) also had 0.33 mg kg<sup>-1</sup> of flunixin in liver and renal degeneration  
295 (#1 in Table 2), while a fourth Eurasian griffon with 0.023 mg kg<sup>-1</sup> flunixin in liver had no  
296 lesions, gout or kidney damage on necropsy (#3 in Table 2). In addition to these wild birds,  
297 one Eurasian griffon that died in a wildlife rehabilitation centre was tested as it was suspected  
298 to have died from iatrogenic flunixin poisoning (Soler et al., 2016). This bird had 2.83 mg kg<sup>-1</sup>

299 <sup>1</sup> in liver and 0.44 mg kg<sup>-1</sup> 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 (CIMA VET, 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<sup>-1</sup> (1 mL per  
323 20 kg bw of a solution with 46 mg mL<sup>-1</sup> of diclofenac) administered intramuscularly in a three-

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

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

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

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

357

#### 358 *4.2. NSAID poisoning in Iberian avian scavengers*

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



374 residues would likely not pose a high risk to vultures, we used our 0.64% diclofenac prevalence  
375 value to recalculate the proportion of carcasses that could contain lethal levels for vultures in  
376 Spain and then refine the risk assessment. We can estimate that the probability of dying in the  
377 first 8 h after last treatment would be 4.76% (8 h/168 h) for all diclofenac treated animals, so  
378 the percentage of carrion with potentially lethal diclofenac levels would be  $0.0476 \times 0.64 =$   
379 0.03% (Table 3). Here we assume that probability of death at a determined time is constant  
380 throughout the 168 h period after treatment, during which diclofenac residues in tissues are  
381 above our limit of quantification. With this percentage (0.03%) and the number of swine  
382 carcasses available (38413, AEMPS & MAGRAMA, 2014), we can estimate the number of  
383 treated pigs with toxic levels (12) and the number of meals available to vultures from these  
384 carrion (1600). Following the approach of Green et al. (2016) (with the proportion of vultures  
385 killed by feeding on a contaminated pig treated 8 h before death), we can estimate that 78-600  
386 vultures would die per year (with LD<sub>50</sub> of 0.098 and 0.225 mg kg<sup>-1</sup>, respectively), which is  
387 between the ranges given in previous estimations (Table 3). These estimates are based on LD<sub>50</sub>  
388 data and dose-response curves showing that some individuals can be especially sensitive to  
389 diclofenac, so some mortality may occur at doses much lower than the median value.

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

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

439

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### 472 **Declaration of Competing Interest**

473 The authors declare no competing financial interests or personal relationships that could have  
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475

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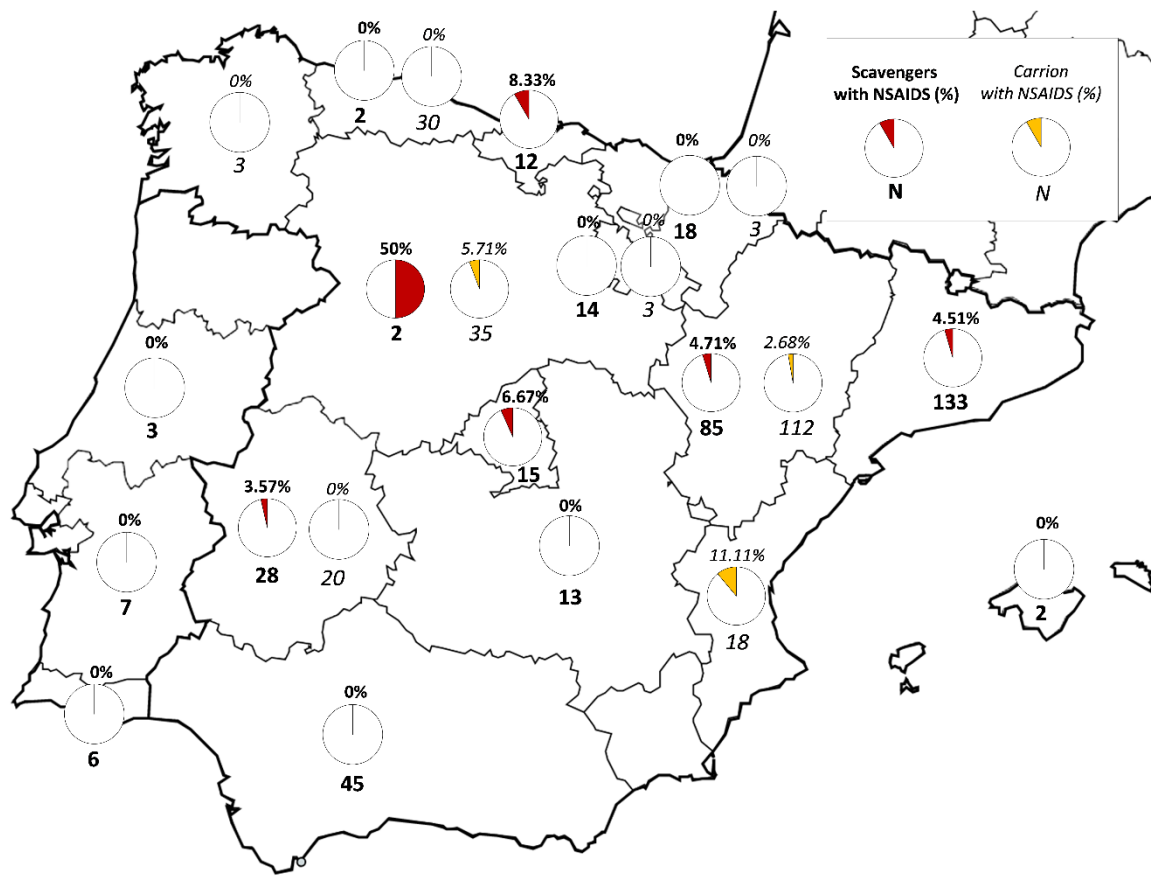
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679 **Figure legend**

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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.

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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 <sub>50</sub> or observed LD (mg kg <sup>-1</sup> )		Acute TER	
					Muscle	Liver	Kidney		min	max	min	max
1	Pig	2016	Castile and Leon	Diclofenac	0.171	<LOQ	<LOQ	0.028	0.1 <sup>a</sup>	0.2 <sup>a</sup>	3.57	7.14
2	Pig	2018	Aragon	Ketoprofen	-	<LOQ	0.173	0.028	1.5 <sup>c</sup>	5 <sup>c</sup>	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 <sup>b</sup>	4.5 <sup>b</sup>	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<sub>50</sub>: median lethal dose (LD<sub>50</sub> only available for diclofenac); TER: toxicity exposure ratio; LOQ: limit of  
 688 quantification.

689 <sup>a</sup> Swan et al. 2006, <sup>b</sup> Cuthbert et al. 2007, <sup>c</sup> 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 <sup>-1</sup> )		
						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 <sup>a</sup>	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 <sup>a</sup> Soler et al. (2016).

694



695 Table 3. Estimation of diclofenac treated pigs from intensive production with toxic levels for vultures, number of the corresponding toxic meals  
 696 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) <sup>a</sup>	
					Proportion killed	
Present study	12	200	2400	1600	78	600
AEMPS					4	7
Green et al.	55-92	200	11122-18430	7415-12287	364-603	2781-4609

697 <sup>a</sup>The number of dead vultures by feeding on meals containing a diclofenac concentration toxic for vultures was calculated following Green et al.  
 698 (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  
 699 were obtained from the LD50 values of 0.098 and 0.225 mg kg<sup>-1</sup> calculated by Swan et al. (2006) from the experimental data of Oaks et al. (2004).

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