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Short Communication

APPARENT TOLERANCE OF TURKEY VULTURES (*CATHARTES AURA*) TO THE NON-STEROIDAL ANTI-INFLAMMATORY DRUG DICLOFENAC

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Abstract—The nonsteroidal anti-inflammatory drug diclofenac is extremely toxic to Old World *Gyps* vultures (median lethal dose ~0.1–0.2 mg/kg), evoking visceral gout, renal necrosis, and mortality within a few days of exposure. Unintentional secondary poisoning of vultures that fed upon carcasses of diclofenac-treated livestock decimated populations in the Indian subcontinent. Because of the widespread use of diclofenac and other cyclooxygenase-2 inhibiting drugs, a toxicological study was undertaken in turkey vultures (*Cathartes aura*) as an initial step in examining sensitivity of New World scavenging birds. Two trials were conducted entailing oral gavage of diclofenac at doses ranging from 0.08 to 25 mg/kg body weight. Birds were observed for 7 d, blood samples were collected for plasma chemistry (predose and 12, 24, and 48 h and 7 d postdose), and select individuals were necropsied. Diclofenac failed to evoke overt signs of toxicity, visceral gout, renal necrosis, or elevate plasma uric acid at concentrations greater than 100 times the estimated median lethal dose reported for *Gyps* vultures. For turkey vultures receiving 8 or 25 mg/kg, the plasma half-life of diclofenac was estimated to be 6 h, and it was apparently cleared after several days as no residues were detectable in liver or kidney at necropsy. Differential sensitivity among avian species is a hallmark of cyclooxygenase-2 inhibitors, and despite the tolerance of turkey vultures to diclofenac, additional studies in related scavenging species seem warranted.

Keywords—Diclofenac   Scavenging birds   Species sensitivity   Vultures

INTRODUCTION

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) used by veterinarians for the treatment of inflammation, fever, and pain in domestic livestock. This drug appears to have been the principal cause of a population crash of Old World vultures (genus *Gyps*) in India, Pakistan, and Nepal [1–5]. Vultures unintentionally ingested diclofenac when scavenging carcasses of livestock that had been treated with this drug shortly before death. This is perhaps the only well-documented instance of a veterinary or human drug resulting in an adverse population-level response in nontarget free-ranging wildlife.

Based upon observational and experimental data, the median lethal dose (LD50) of diclofenac in Oriental white-backed vulture (*Gyps bengalensis*) was estimated to be 0.098 mg/kg body weight (95% CI, 0.027–0.351 mg/kg), and 0.225 mg/kg (95% CI, 0.117–0.432 mg/kg) when an outlier was excluded [1,6]. Oral dosing of Eurasian (*G. fulvus*) and African (*G. africanus*) vultures with diclofenac at 0.8 mg/kg body weight has been shown to evoke a substantial increase in plasma uric acid, which is indicative of altered renal function, and dosed birds succumb within 48 h [6]. Based upon lethality data, diclofenac can be categorized as extremely toxic to *Gyps* vultures. Pathological signs in affected vultures include visceral

gout (grossly visible urate deposits on internal organs) that is the result of kidney failure [1]. Acute necrosis of the proximal convoluted tubules in affected individuals is severe [7]. Diclofenac is known to be a potent inhibitor of cyclooxygenase-2 and prostaglandin synthetase. From a mechanistic perspective, it has been hypothesized that impaired production of prostaglandins E_2 and I_2 may alter smooth muscle control of the renal portal valve and shunt blood from the renal cortex; this would result in ischemia and necrosis of the proximal convoluted tubules [7]. Similar pathology has also been reported in domestic fowl (*Gallus gallus*), although they seem to be considerably more tolerant to diclofenac (median lethal dose estimate of 9.8 mg/kg when administered intramuscularly) [8].

Although veterinary use of diclofenac in India is being phased out and an alternative that is not toxic to *Gyps* vultures has been identified (meloxicam [9]), widespread sale and veterinary and human use of diclofenac continues on a global scale [10,11] (http://www.birdlife.org/news/news/2007/11/africa_diclofenac.html; accessed 19 December 2007). At present there are no data on diclofenac-induced mortality in free-ranging birds of prey in North and South America. While New and Old World vultures are not closely related, data on diclofenac toxicity to New World vultures is warranted for several reasons. Notably, simple extrapolation of diclofenac toxicity data for *Gyps* vultures (order Falconiformes family Accipitridae) may not adequately assess risk in New World vultures (order Ciconiiformes family Cathartidae) due to taxonomic and

genetic distinctions; diclofenac is easy to manufacture, inexpensive, and readily accessible in many undeveloped countries for treatment of livestock; seven species of vultures are found in North and South America and numerous reports in developed countries document poisoning of raptors that fed on animal carcasses treated with veterinary drugs (barbiturates) or exposed to pesticides [12–16]; carcass burial and disposal practices are less sophisticated on ranches with large open range, in remote regions, and in undeveloped countries, increasing the likelihood of exposure; modeling studies suggest that even the rare presence of contaminated carcasses can have significant population effects on vultures [5]; comparative toxicological aspects of nonsteroidal anti-inflammatory drugs are poorly known in birds [11,17–19]; and there is growing concern for the ecotoxicological effects of veterinary pharmaceuticals released from animal feed lot operations into streams and estuaries [20,21]. Finally, other currently used NSAIDs, including carprofen and flunixin, have been associated with mortality, gout, and/or renal failure in some scavenging birds outside of the *Gyps* genus [11], and thus their toxicity would also merit urgent investigation should diclofenac prove extremely toxic to New World vultures. For these reasons, and given worldwide conservation concerns for scavenging birds (e.g., California condor [*Gymnogyps californianus*] and bald eagle [*Haliaeetus leucocephalus*]) that may be unintentionally exposed to NSAIDs, a study was undertaken examining diclofenac toxicity to turkey vultures (*Cathartes aura*) as an initial step in determining sensitivity among species potentially at risk.

MATERIALS AND METHODS

Animals

The present study was conducted at The Center for Birds of Prey in Charleston, South Carolina, USA, during April to June of 2007. During dosing trials ambient temperature ranged from approximately 10.6 to 32.2°C. Captive adult turkey vultures (n = 7 males and 4 females) that were not candidates for release (improperly healed shoulder and wing fractures) were obtained from various rehabilitation facilities. Birds were housed individually in outdoor pens (10–12 feet wide × 10–12 feet long × 10 feet high; pressure-treated lumber with vinyl-coated wire, and a roof for shade) and fed 150 to 200 g skinned rats (Rodentpro, Evans, IN, USA) or whole chicks (Mike Dupuy's Hawk Food, Silver Spring, MD, USA) supplemented with Vionate® (Gimborn US, Atlanta, GA, USA) and calcium on a daily basis between 10:00 AM and noon. A bowl containing fresh water was present in each pen. Birds were acclimated to housing and husbandry activities for several weeks, and were handled and weighed at least weekly.

Physical exams, hematology, plasma clinical chemistries, and electrophoresis (Advanced Well Bird Exam; Comparative Pathology Laboratory, University of Miami, Miller School of Medicine, Miami, FL, USA) indicated that birds were in generally good health. Plasma samples were screened for West Nile Virus exposure using enzyme-linked immunosorbent assay and by plaque reduction neutralization assay on vero cells at a starting dilution of 1:20 and using a 90% neutralization rule [22]. Notably, two vultures had been exposed to West Nile Virus in the past. Results for another bird were equivocal for West Nile Virus and St. Louis encephalitis, and positive for flavivirus exposure; this individual had elevated serum gamma globulin and plasma aspartate and alanine aminotransferase

activities, but was ultimately included in trial 2 as plasma enzyme activities had declined substantially.

Diclofenac-dosing solutions

Because of difficulties in the importation of the veterinary formulation of diclofenac used in the Indian subcontinent, technical grade sodium diclofenac (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt; >99% purity; Chemical Abstract Service no. 15307-79-6) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 2.5 mg/ml stock solution was prepared by dissolving sodium diclofenac in distilled water on a stirring hot plate at 30°C. Dosing solutions were prepared by addition of various quantities of this stock into distilled water in volumetric flasks. Nominal concentrations ranged from 0.16 to 25 mg sodium diclofenac/ml, and were analytically verified (recovery averaged 104.9%; methods described below). Solutions were administered in a volume of 0.5 ml/kg body weight, and nominal doses ranged from 0.08 to 12.5 mg/kg body weight. Due to limits of solubility, the greatest dose, 25 mg/kg body weight, was administered in twice the volume (1 ml/kg body wt).

Dosing and sampling procedures

Regurgitation of stomach contents is an acknowledged problem when conducting studies with vultures. In advance of dosing trials, each bird was handled and sham-dosed on several occasions with water (0.5 ml/kg body wt) orally administered (behind the glottis) using a 2.5 ml luer lock syringe (Hamilton Company, Reno, NV, USA) with an 18 gauge 3.8 cm long ball point intubation needle (Popper and Sons, New Hyde Park, NY, USA).

For study trials, each bird was weighed and a 1 to 2 ml brachial venipuncture sample for blood chemistry and diclofenac residues was collected between 8:00 and 10:00 AM (time 0). Birds were then fed, and food scraps were removed from pens in the late afternoon 16 h before dosing. On the day of dosing, each vulture was captured, briefly restrained (<2 min), orally dosed, and released, at staggered 15-min intervals between 8:00 and 10:20 AM, and then fed 4 h after dosing. Birds were observed for signs of intoxication at hourly intervals during daylight and at 4-h intervals during the night for 2 d, and then at 4-h intervals during day light for five additional days. Body weight was determined and a 1 to 2 ml blood sample was collected at 12, 24, and 48 h, and 7 d postdosing. Birds were then lightly anesthetized with isoflurane and euthanized with pentobarbital. These procedures had been evaluated and approved by the Institutional Animal Care and Use Committees of The Center for Birds of Prey and the Patuxent Wildlife Research Center (Beltsville, MD, USA).

Euthanized birds were tagged for identification, placed in doubled plastic bags, chilled in a freezer and express shipped in a cooler lined with freezer packs to the National Wildlife Health Center (Madison, WI, USA). A detailed necropsy (external and internal examination, weights of liver and kidney, fixation of major organs in 10% neutral buffered formalin) was conducted on each bird within 48 h of euthanasia. Liver from each bird was tested for bacteria using standard aerobic culture techniques on blood agar. Kidney was cultured for virus using vero cell cultures to isolate viruses [23]. Subsamples of liver and kidney were placed in chemically clean jars (ICHEM Research, New Castle, DE, USA) and frozen at −10°C for subsequent diclofenac residue analysis. Portions of formalin-fixed liver, kidney, brain, trachea, lung, heart, digestive tract, en-

docrine organs, and spleen were processed for histopathology using standard methods, sectioned at 5 μm, stained with hematoxylin and eosin (Histology Laboratory of the Department of Pathobiology, College of Veterinary Medicine, University of Wisconsin, Madison, WI, USA), and examined by light microscopy.

Analytical methods

Dosing solutions, blood plasma, and liver and kidney samples were shipped on dry ice to the University of Aberdeen (UK) for diclofenac analysis. Dosing solutions were gently heated to redissolve diclofenac. Dosing solutions and blood plasma samples were vortex mixed with acetonitrile, while liver and kidney samples were homogenized in acetonitrile. All samples were filtered and quantified by liquid chromatography–electrospray ionization mass spectrometry using an Agilent 1100 series instrument (1946D; Agilent Technologies, Stockport, UK). Details of this procedure have recently been published [24]. For tissue samples the limit of quantification was 10 μg/kg and the limit of detection was 4 μg/kg.

Blood samples were express shipped and analyzed at the Avian and Wildlife Laboratory of the Division of Comparative Pathology (University of Miami, FL, USA). Initial health checks included complete blood counts [25], serum electrophoresis [26], hematocrit and a chemistry panel (enzymes: alanine aminotransferase, amylase, aspartate aminotransferase, creatine phosphokinase, lactic dehydrogenase, lipase, and gamma glutamyl transferase; electrolytes and minerals: sodium, potassium, carbon dioxide, calcium, and phosphorus; other analytes: glucose, blood urea nitrogen, uric acid, creatinine, cholesterol, triglycerides, and total protein) using dry-slide reagents and a Vitros 250 Chemistry System (Ortho Diagnostics, Rochester, NY, USA) (Kossoff S, Bladow R, Luya M, Cray C. 2001. *Association of Avian Veterinarians Newsletter and Clinical Forum*, Sept–Nov, 2001, pp 6–7). Only hematocrit and the 19 analyte blood chemistry panel were conducted on samples collected during the dosing trials.

Statistical analysis

Relations among administered dose, body and organ weight change, and plasma diclofenac concentrations were examined using Pearson product-moment correlation. Approximation of diclofenac plasma half-life and its elimination constant were obtained using a simple first-order kinetic model (http://www-users.med.cornell.edu/~spon/picu/calc/halfcalc.htm).

Dose–responses, and temporal change in the dose–response relations, were investigated for 20 blood chemistry constituents using a repeated measures model with controls for baseline differences between the two trial periods. The data for each blood chemistry constituent consist of measurements, y_i i = 1, 2, . . . , 80. Our model was that

$$y_i = \text{Bird}_{B(i)} + (\alpha_{j(i)} + \beta_{j(i)}\text{Treatment}_i) + k_i\text{Trial} + \epsilon_i$$

where $B(i)$ identifies the bird associated with measurement i, $B(i)$ = 1, 2, . . . , 11. Index $j(i)$ describes the measurement time, with $j(i)$ = 0, 1, 2, 3, 4 for measurement times −24, 12, 24, 48, and 168 h from dosing. Covariate k_i = 0 for the original trial, and k_i = 1 for the second trial. Thus the model includes bird specific normal random effects (Bird), a time-specific linear regression on treatment level (α + β Treatment), a change in level associated with the two trial occasions (Trial), and a mean zero normal error term (ϵ).

This model is easily fit using a Bayesian approach with the program WinBUGS [27]. Standard vague priors for regression coefficients and variance components were employed.

RESULTS AND DISCUSSION

To test the hypothesis that turkey vultures and Old World vultures (Oriental white-backed, Eurasian, and African) are equally sensitive to diclofenac, data on its lethality to the Oriental white-backed vultures (LD50 of 0.098–0.225 mg/kg) [1,6] were used to select doses at and well-above levels presumed to evoke toxicity. Compared to sham-dosed controls (n = 1 male, 1 female), no overt signs of toxicity were apparent when turkey vultures were administered diclofenac at 0.08 mg/kg (n = 1 female), 0.25 mg/kg (n = 1 female, 2 males), 0.8 mg/kg (n = 1 female, 2 males), and 2.5 mg/kg body weight (n = 1 male), and all birds survived the 7-d trial. Rather than sacrifice all of the vultures used in this trial, the decision was made to euthanize only half (i.e., one sham-dosed, one dosed at 0.25 mg/kg, two dosed at 0.8 mg/kg, and the single bird administered 2.5 mg/kg) and re-expose remaining birds to greater concentrations of diclofenac.

Three weeks following the initial study, a second trial was conducted with previously dosed birds plus an additional vulture. Treatments included sham-dosed (n = 1 male not included in trial 1), and vultures dosed with diclofenac at 2.5 mg/kg (n = 1 female previously sham-dosed in trial 1), 8 mg/kg (n = 1 female and 1 male previously dosed with 0.08 and 0.25 mg/kg, respectively), and 25 mg/kg body weight (n = 2 males previously dosed with 0.25 or 0.8 mg/kg body wt). Notably, diclofenac concentrations given to redosed birds were 31.25 to 100 times the quantity administered in trial 1. No overt signs of toxicity were observed during trial 2, which included diclofenac doses exceeding its LD50 in *Gyps* vultures by more than two orders of magnitude.

Over the 7-d time course of trials 1 and 2, slight fluctuations in body weight were noted (ranging from +0.72 to −6.38%) compared to predosing weight (time 0), although changes were not associated (p > 0.05) with dosage level of diclofenac. At necropsy, neither liver to body weight nor kidney to body weight ratios were related (p > 0.50) to dose of administered diclofenac.

Necropsies indicated that turkey vultures were in good flesh and had moderate amounts of subcutaneous fat. With rare exception (e.g., occasional enlarged spleen, one instance of healed hepatic fracture), their internal organs were grossly unremarkable. Histopathological examination revealed multifocal areas of cytoplasmic vacuolation of renal subcortical cells, and mild periportal inflammation and hepatocellular vacuolation, in all vultures regardless of diclofenac dose. Scant dark deposits associated with granulomatous inflammation were observed in a few collecting tubules and fewer distal convoluted tubules in one of two tissue sections from one vulture that received 25 mg diclofenac/kg body weight. This was suggestive of urate deposits reminiscent of a past dehydration event rather than diclofenac intoxication. Clearly, no lesions were seen in any of the turkey vultures that would have contributed to death other than those associated with isoflurane–pentobarbital euthanasia. This is in direct contrast to observations of visceral gout [1,3,6] in several species of *Gyps* vultures naturally exposed to or experimentally treated with diclofenac, and the urate aggregates and acute necrosis of the proximal convoluted tubules [7] observed in naturally exposed Oriental white-backed vultures.

No diclofenac residues were detected in turkey vulture liver

Table 1. Plasma diclofenac concentration in turkey vultures in trials 1 and 2[a]

Dose (mg/kg)	Trial	n	Plasma diclofenac (ng/ml)				
			−24 h	12 h	24 h	48 h	7 d
0	1, 2	3	—	—	—	—	—
0.08	1	1	—	7	—	—	
0.25	1	3	—	12 (6, 13, 17)	—	—	—
0.8	1	3	—	66 (40, 62, 96)	6.3 (4, 5, 10)	—	—
2.5	1, 2	2	—	326 (76, 577)	118 (14, 221)	— (–, 82)	—
8	2	2	—	937 (420, 1454)	227 (94, 360)	17 (11, 23)	—
25	2	2	—	13,845 (6350, 21340)	513 (245, 781)	330 (16, 645)	—

[a] Values are mean (individual values); — = not detected.

or kidney 7 d after administration of doses ranging up to 25 mg/kg body weight. This is in contrast to *Gyps* vultures that died with detectable diclofenac in liver and kidney within 2 d of its administration [6]. Diclofenac was detected in blood plasma of turkey vultures up to 48 h after administration in trials 1 and 2, and was apparently cleared by day 7 (Table 1). When data for turkey vultures in trial 1 and 2 were combined in a regression analysis, plasma diclofenac concentrations were dose dependent at 12 h [$r = 0.92$; $p < 0.001$, $n = 16$; $\log_{10}$(plasma diclofenac) $= -1.062 + 0.088(\log_{10}$dose)] and at 24 h [$r = 0.74$; $p < 0.025$, $n = 16$; $\log_{10}$(plasma diclofenac) $= -1.796 + 0.065(\log_{10}$dose)]. Using plasma diclofenac values at 12 and 48 h after administration of 8 and 25 mg/kg, and assuming steady state conditions, a rough estimate of the plasma half-life (mean ± standard deviation) of diclofenac in turkey vultures was 6.04 ± 1.33 h with an elimination constant of 0.12 ± 0.03. This half-life is markedly greater than the 1-h half-life estimated from a detailed pharmacologic study in domestic fowl, but less than the 14- to 18-h half-life estimated for *Gyps* vultures [8].

Based upon previous observations of elevated plasma uric acid concentration and alanine aminotransferase activity in *Gyps* vultures administered 0.8 mg diclofenac/kg body weight [6], we focused greatest attention on these analytes. Using data from both trials 1 and 2, plasma concentrations of uric acid were not affected by diclofenac administration ($p > 0.05$). Unlike *Gyps* vultures [6], uric acid concentrations in turkey vultures were not linearly related to diclofenac dose at 12 h ($p > 0.50$) or 24 h ($p > 0.10$) postadministration. For comparative purposes, concentrations of plasma uric acid in both turkey vultures and *Gyps* vultures are presented in Figure 1. A slight elevation ($p < 0.05$) in uric acid levels were noted in all (sham- and diclofenac-dosed) turkey vultures after 12 h (evening, 8:00 to 10:00 PM), perhaps reflecting a postprandial or circadian fluctuation, and concentrations were modestly depressed in all birds after 24 and 48 h. Plasma alanine aminotransferase activity was neither affected by dose of diclofenac nor sampling time ($p > 0.05$).

Of the other analytes, the most pronounced effects of diclofenac included depressed plasma potassium levels at 12 and 24 h postadministration ($p < 0.05$; $n = 4$ birds that received 8 and 25 mg/kg combined; mean ± standard deviation of 3.78 ± 0.15 at 12 h and 3.57 ± 0.10 mmol/L at 24 h vs 3.98 ± 0.10 at time 0, $n = 16$), which is in contrast to hyperkalemia noted in diclofenac-intoxicated *Gyps* species and chickens [8]. In addition, plasma lactic dehydrogenase activity was seeming elevated by higher doses of diclofenac at 24 h ($p < 0.05$; $n = 9$ birds that received 0.8, 2.5, 8, and 25 mg/kg; 957 ± 278 IU/L at 24 h versus 786 ± 243 at time 0, $n = 16$). Dose-

related effects were detected for plasma sodium at 12 and 24 h, blood urea nitrogen at 24 and 48 h, carbon dioxide at 48 h, creatinine at 48 h, amylase on day 7, and lipase at 48 h and on day 7, but values were within the range observed at other sampling times, overlapped the range of values reported for other birds of prey (Andean condor, *Vultur gryphus* [28]; Eurasian vulture and Egyptian vulture, *Neophron pernopterus* [29]) and fluctuations were seemingly modest from a physiological and toxicological standpoint. Hematocrit, plasma aspartate aminotransferase, and creatine phosphokinase activities and calcium, phosphorus, carbon dioxide, glucose, blood urea nitrogen, cholesterol, triglycerides, and total protein concentrations were not affected by diclofenac, but some changes were noted among sampling times. Notably, plasma calcium, creatinine, cholesterol, and triglyceride concentrations were slightly elevated at the 12 h sample collection (evening), possibly reflecting recent feeding or a diurnal rhythm. Plasma gamma glutamyl transferase activity was detectable in only 59% of the samples.

In conclusion, turkey vultures seem to be remarkably tolerant to diclofenac compared to *Gyps* vultures [1,3,6]. This presumably reflects basic physiological differences due to taxonomic distinctions between turkey vultures and *Gyps* species.
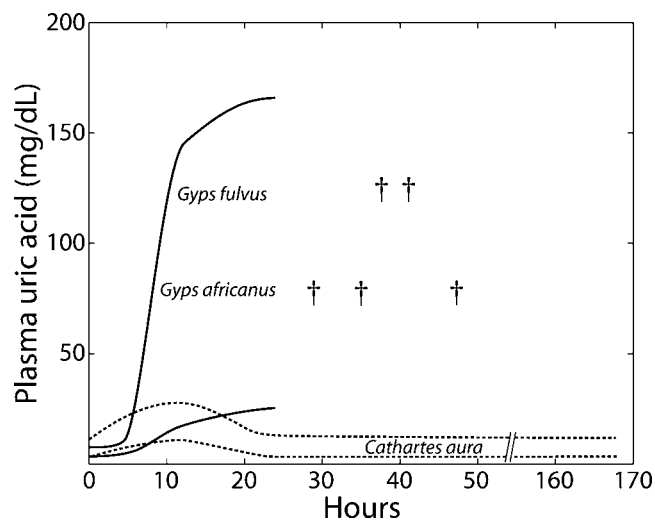

Fig. 1. Plasma uric acid concentration (solid lines indicate range of observed values) in *Gyps* vultures ($n = 2$ *Gyps fulvus* and $n = 3$ *Gyps africanus*) orally dosed with 0.80 mg diclofenac/kg body weight; † = approximate time of death (adapted from Swan et al. [6]). Plasma uric acid concentration (dashed lines indicate range of observed values) in *Cathartes aura* orally dosed with 0.80, 2.5, 8, and 25 mg diclofenac/kg body weight ($n = 2$ birds from trial 1 plus four redosed birds used in trial 2); no mortality occurred.

If in fact turkey vultures are representative of New World vultures, the toxicity and risk of diclofenac to these scavenging birds would be predicted to be minimal. However, differences in sensitivity among avian species seem to be the hallmark of the cyclooxygenase-2 inhibitors, making such interspecific predictions tenuous. Notably, the cyclooxygenase-2 inhibitors carprofen and flunxin have been observed to evoke renal disease and gout in rather diverse species of birds (Harris's hawk, *Parabuteo unicinctus*; northern saw-whet owl, *Aegolius acadicus*; Maribou stork, *Leptoptilos crumeniferus*) [11]. Mortality events in any species need to be investigated, and seemingly unusual exposure routes to toxicants deserve consideration. Use of alternative therapeutic NSAIDs (e.g., meloxicam) that evoke effects through other mechanisms of action has been advocated in regions where species at risk are known to exist [9,11], and despite the tolerance of turkey vultures to diclofenac, additional research in scavenging birds would seem warranted.

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