Electronic Appendix

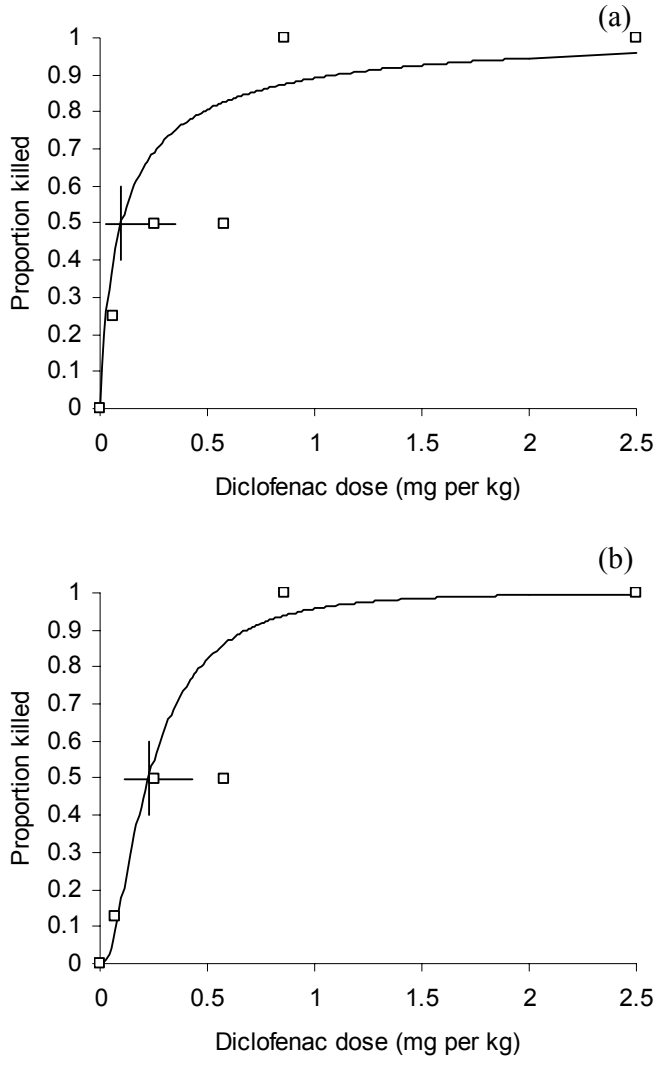
Median lethal dose for *Gyps bengalensis*

Data on the toxicity of diclofenac to *Gyps bengalensis* were obtained from experiments undertaken by the Peregrine Fund and their co-workers (text and Table 2 of Oaks *et al.* 2004). Vultures were fed over a period of a few days on tissues from goats or buffaloes that had been treated with a standard veterinary course of single daily doses of diclofenac, the last being administered a few hours before slaughter. Diclofenac concentrations were determined from samples of ungulate tissues, and the dosage (mg kg^{-1} vulture body weight) was estimated from this concentration and the weight of tissue that each vulture ingested. There were four experimental groups fed in this way and one group of controls (Oaks *et al.* Table 2). In addition, data were also included on two vultures given oral doses of 2.5 mg kg^{-1} vulture body weight and two vultures given oral doses of 0.25 mg kg^{-1} vulture body weight. The estimate of m obtained from birds given tissue from treated livestock was reasonably similar to the estimate from the birds receiving the oral dose (m = -1.056 cf. m = -0.6021 respectively: back transformed LD_{50} 0.088 mg kg^{-1} cf. 0.250 mg kg^{-1} respectively). Observed and expected mortality rates were compared graphically (Figure A1) and by calculating expected mortality rates for each experimental group under the fitted model (Table A1). The data were too sparse for a meaningful goodness-of-fit test, but the model does not appear to fit the grouped data particularly well. Inspection of individual doses revealed that this is primarily because of an outlier (Vulture 11 in Table 2 of Oaks *et al.* (2004)); a bird that received the lowest dose of diclofenac (0.007 mg kg^{-1}), but died of gout. Although the death of this bird from such an apparently low dose and the high concentration of diclofenac in kidney tissue taken from it at post-mortem (Figure A3) might suggest that it actually ingested more diclofenac than was estimated, the low uric acid concentration in plasma taken 24 h after treatment (Figure 2) is consistent with a low dose. Hence, it is not clear whether this low dose estimated for this individual is correct or not. For this reason, we have estimated LD_{50} with and without this datum.

Table A1. Outcome of experiments on *Gyps bengalensis* in which diclofenac was administered to vultures orally or by feeding them on diclofenac-treated ungulate tissue. The modelled percentage mortality was calculated as the mean for each experimental group of the expected individual probabilities of death from the fitted probit model. Two versions of this analysis are shown, with (1) and without (2) the inclusion of an outlier (see methods).

Experimental group	Range of doses mg kg^{-1}	Mean dose	Number of birds	Number died	Percent died	Modelled percent died (1)	Modelled percent died (2)
Untreated control	0	0	6	0	0	0	0
Fed tissue of treated ungulate	0.005 to 0.3	0.066	8	2	25	33	11
Fed tissue of treated ungulate	0.5 to 0.6	0.575	2	1	50	83	86
Fed tissue of treated ungulate	0.8 to 1.0	0.863	10	10	100	87	94
Dosed orally	0.25	0.25	2	1	50	69	55
Dosed orally	2.5	2.5	2	2	100	100	100

Figure A1. Fitted models of the median lethal dose from experiments on *Gyps bengalensis* in which diclofenac was administered to vultures orally or by feeding them on tissues from ungulates treated with the drug shortly before slaughter (Oaks *et al.* 2004). Squares represent the proportion of vultures that died in each experimental group in relation to the mean dose, in mg of diclofenac ingested per kg of vulture body weight, for individuals in that group. The curve shows the probability of death in relation to dose for the fitted probit model. The median lethal dose is indicated by the vertical line and its 95% confidence interval by the horizontal line. Fitted models are shown separately for (a) data for all birds and (b) with one outlier (Gb11) removed (see results).



Toxicity Testing

Injured non-releasable birds were selected for the trials if they were destined for euthanasia, could not be rehabilitated and could not be used for captive breeding purposes (Table A2). The vultures to be tested were habituated to captivity and eating regularly. Birds were fasted for 2-3 days prior to treatment to ensure that their crops were empty and that they would not easily regurgitate when dosed. Meat and food given to the vultures was of known origin, and free of anti-inflammatory or other drugs of any kind. Vultures were fed 4 hours after treatment and thereafter daily until death or termination of the study. For the study in South Africa, birds were transported to the University of Pretoria Biomedical Research Centre (UPBRC) three days prior to the start of the experiment. The vultures were housed individually in stainless steel cages in a fully environmentally controlled room. Diclofenac sodium 2.5% (Valfen, Merinal India; batch W2E 3010, Exo. Nov. 2005) was administered by oral gavage to the vultures at a dose of 0.8 mg kg^{-1}. One ml of diclofenac was diluted in 24 ml sterilized water to achieve a concentration of 1 mg ml^{-1}, and the volume of dilution administered to each vulture corresponded to a diclofenac dose of 0.8 mg kg^{-1}. Control birds were sham-treated with 2.5 ml of sterilized water by oral gavage.

Table A2. Description of African white-backed (AW) and Eurasian griffon (EG) vultures used in the toxicity trials

No	Treatment	Age	Sex	Condition and history
AW1	Diclofenac	Adult	Female	Non-releasable; amputated wing due to power line injury; General good condition. Held in captivity for over 5 years. Not used for breeding purposes.
AW2	Control	Juvenile	Male	Broken right wing and possible fractured leg/hip due to power line injury. In pain. Placed in captivity 3 weeks prior to study and destined for euthanasia.
AW3	Control	Adult	Male	Non-releasable. Amputated wing due to power-line injury. Bird in good condition. Been in captivity for 5 years.
AW4	Diclofenac	Adult	Female	Non-releasable. Amputated wing due to power line injury. Bird in good condition. Been in captivity for 5 years.
EG1	Diclofenac	Juvenile	Male	Non-releasable; chronic and complicated bumblefoot on right leg, due to a chronic luxation of tibiotarsal joint and shortening of left leg. Held in captivity for 2 years.
EG2	Diclofenac	Juvenile	Male	Non-releasable bird due to chronic ankylosis in left carpus, destined for euthanasia.
EG3	Diclofenac	Juvenile	Female	Non-releasable bird kept in rehabilitation centre for 2 years. Amputated wing (mid shaft humerus).
EG4	Control	Juvenile	Female	Bred and housed at Jerez Zoo
EG5	Control	Adult	Male	Non-releasable bird due to elbow joint ankylosis. In captivity for 2 years
EG6	Control	Unknown	Unknown	Admitted into rehabilitation centre 1 week prior to study, and released into wild on completion of trials

Quantification of blood parameters and statistical testing

Blood was taken from control and experimental *G. africanus* to quantify uric acid, albumin, creatine kinase (CK), and serum alanine transferase (ALT). Uric acid acid concentration was measured using ACE™ Uric Acid Reagent, albumin concentration using the NExT™ Albumin reagent, ALT activity using the Alfa Wasserman ALT, and CK using the Alfa Wasserman CK Reagent e ACE™ clinical chemistry system(Alfa Wassermann, Bayer Health). The analyses were performed by means of the ACE™ and NExT™ Clinical Chemistry Systems (Alfa Wassermann, Bayer Health Care, SA). Blood samples from *G. fulvus* were submitted to a commercial veterinary diagnostic laboratory (Greendale Veterinary Diagnostics Ltd., Woking, Surrey, U.K.) for biochemical analysis.

Statistical testing of the effects of diclofenac treatment on blood parameters of *G. africanus* was performed in SYSTAT 5.01, by fitting a repeated measures model in the MGLH module with log-transformed plasma concentration as the dependent variable, with sampling time and the sampling time*treatment interaction as the effects. The significance of the interaction term was tested. A test for variation in the kidney concentration vs. dose relationship among vulture species and routes of diclofenac administration (by feeding tissues from treated livestock or by gavage of drug) was performed by analysis of covariance with diclofenac concentration as the dependent variable and dose as the independent variable. The slope of this relationship was modelled as being constant, but the elevation of the regression was allowed to vary among species and administration routes.

Post-mortem

A full necropsy was undertaken within seven hours after death on all vultures that died. External features examined for abnormalities included skin, feathers, preen glands, muscle mass, eyes, nostrils, ears, wings, feet, cloaca, vent and examination for ectoparasites. Gross internal observations were made on pectoral muscles, thoracic and abdominal air-sacs, heart and major blood vessels, thyroid and adrenal glands, brain, liver, kidneys, spleen, lungs, trachea, intestinal tract, reproductive organs and tibeotarsal, metatarsal and digital joints. Post-mortem examinations for *G. africanus* and *G. fulvus* were undertaken by Professor N. Duncan and Dr. Miguel Quevedo, respectively. The extent and distribution of gout on internal organs varied among individuals (Table A3). There was also evidence for abnormal enlargement of the kidneys, spleen and liver, and these organs were pale and mottled in colour. In all five diclofenac-treated birds gout was found covering the thoracic and abdominal air sacs, and the pericardium and myocardium surface of the heart (Figure A2). The distribution and extent of visceral gout are in agreement with the experimental results for *G. bengalensis* (Figure 1 of Oaks *et al.* 2004) and findings of gout in wild birds.

Table A3. Results of gross internal post-mortem examinations undertaken on Eurasian griffon and African white-backed vultures treated with diclofenac and one control bird (AW2), indicating no abnormalities (NA), the presence of gout on the surface of organs (Gout), abnormal enlargement (+) of organs, and organs pale and mottled in appearance (†)

	Eurasian griffon vulture			African white-backed vulture		
	EG1	EG2	EG3	AW1	AW4	AW2
Pectoral muscles	NA	NA	Gout	NA	NA	NA
Air-sacs	Gout	Gout	Gout	Gout	Gout	NA
Heart*	Gout	Gout	Gout	Gout	Gout	NA
Major blood vessels	NA	NA	NA	NA	NA	NA
Thyroid & adrenal glands	NA	NA	NA	NA	NA	NA
Brain	NA	NA	NA	NA	NA	NA
Liver	NA	Gout	Gout +	†	†	NA
Kidney	†	+ †	Gout + †	Gout + †	Gout + †	NA
Spleen	NA	+	+	†	†	NA
Lungs	NA	NA	Gout	NA	NA	NA
Trachea	NA	NA	NA	NA	NA	NA
Intestinal tract	NA	Gout	Gout	Gout	Gout	NA
Reproductive organs	NA	NA	NA	NA	NA	NA
Joints	NA	NA	Gout	NA	NA	NA

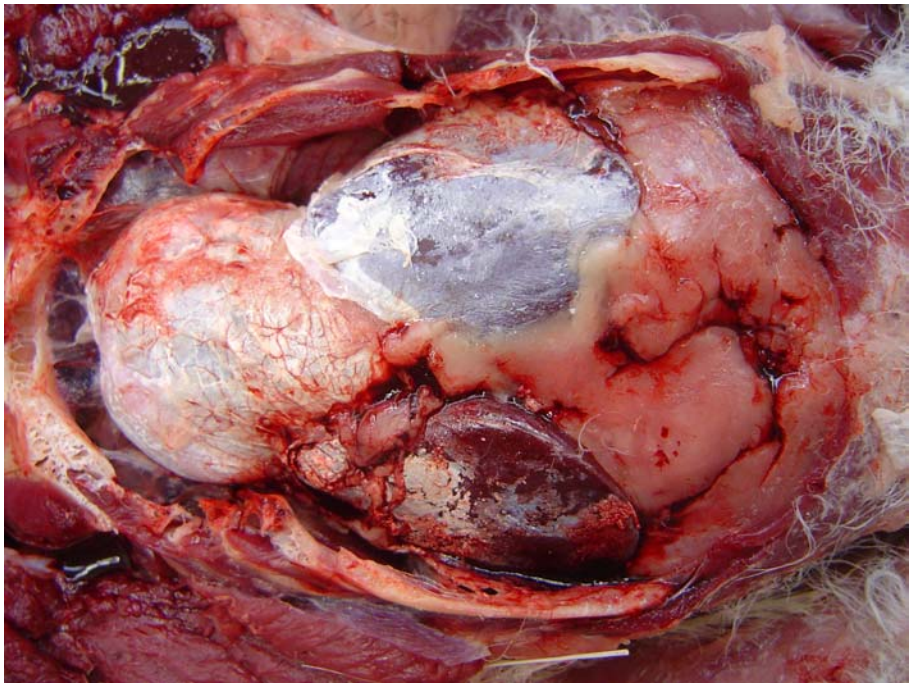


Figure A2. Abdominal cavity of vulture AW4 (*G. africanus*) at necropsy showing visceral gout on the liver surface following experimental dosing with diclofenac.

Concentrations of diclofenac in kidney samples of *G. bengalensis* taken at post-mortem were obtained from Oaks *et al.* (2004) and were measured for diclofenac concentrations in *G. africanus* and *G. fulvus* at the University of Aberdeen using a validated HPLC-MS-MS method following the methods of Oaks *et al.* (2004) and Shultz *et al.* (2004). The relationship between kidney diclofenac concentration and dose is shown in fig. A3.

Histopathology

For *G. africanus*, samples of spleen, kidney, trachea, lung, heart, liver, pancreas, bursa, brain, peripheral nerve, crop, proventriculus, ventriculus, duodenum, ileum, colon, cloaca, and skeletal muscle collected in 10% formalin and 100% ethanol for processing for light microscopic examination. After fixation the tissue were routinely processed, sectioned at 4 microns and stained with Haematoxylin and Eosin for

examination. Histological examinations of both diclofenac-treated *G. africanus* revealed significant lesions in the kidneys, spleen and liver only, with extensive uric acid crystal formation within the kidneys and liver. The changes within the kidneys were widespread and severe, and were characterized mainly by necrosis of the lining cells of the proximal convoluted tubules (characterized by eosinophilia, pyknosis, karryorhexis and desquamation). A lesser number of tubules showed marked dilatation. The glomeruli and distal convoluted tubules appeared unaffected, and within the medullary cones the medullary loops were randomly dilated. Changes within the spleen and liver were similar, with multifocal areas of necrosis characterized by fibrin and uric acid crystal depostition. Similar histological examinations were performed on tissues from *G. fulvus.* At post-mortem all diclofenac-treated *G. fulvus* showed extensive visceral gout, and one bird showed articular gout. Extensive uric acid crystal deposition was found in the liver, spleen thoracic and abdominal air sacs, pericardium and myocardium. Kidneys of *G. fulvus* were pale and mottled in colour. These results *from G. africanus* and *G. fulvus* are similar to those of Oaks *et al.* 2004 for diclofenac-treated *G. bengalensis* (Oaks *et al.* 2004; Supplementary Information).

Figure A3. Concentration of diclofenac at post-mortem in vultures that died after experimental treatment with diclofenac in relation to the dose administered. Open diamonds represent *G. bengalensis* fed on tissue from diclofenac-treated livestock in the experiments of Oaks *et al.* (2004), open squares *G. bengalensis* that were given oral doses of the drug directly, filled squares and grey squares *G. africanus* and *G. fulvus* respectively that were given diclofenac directly by gavage. The *G. bengalensis* that died with gout after receiving a very low dose of diclofenac (see text) is labelled Gb11.