



Veterinary diclofenac threatens Africa's endangered vulture species

V. Naidoo^{a,*}, K. Wolter^b, R. Cuthbert^c, N. Duncan^a

^a Departmental of Paraclinical Sciences, Section of Pharmacology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, Gauteng 0110, South Africa

^b Vulture Programme of the Rhino and Lion Non-profit Organisation, Krugersdorp, South Africa

^c Royal Society for the Protection of Birds (RSPB), The Lodge, Sandy, United Kingdom

ARTICLE INFO

Article history:

Received 3 October 2008

Available online 1 February 2009

Keywords:

Diclofenac

Gyps vultures

Cape Griffon Vulture

Toxicity

Gout

Asian vulture crisis

Gyps coprotheres

ABSTRACT

Veterinary diclofenac has been responsible for the devastation of three species of *Gyps* vulture on the Indian subcontinent, and it is now regarded as one of the worst environmental contaminants in the recent past. While measures have been taken to control the manufacture of veterinary diclofenac in South Asia, the promotion of diclofenac on the African continent poses a risk to vultures in this region. In Southern Africa, the species of greatest conservation concern is the Cape Griffon Vulture (*Gyps coprotheres*), as only 2900 breeding pairs remain in the wild. The objective of this study was to test if this species is toxicologically sensitive to diclofenac. In a single dose-toxicity study, two adult Cape Griffon Vultures with severe injuries, that were considered to have a very poor prognostic outcome, were dosed intravenously with diclofenac at 0.8 mg/kg. The changes in the clinical pathology were compared to the normal reference range established for 24 healthy Cape Griffon Vultures. Both birds died within 48 h of dosing. The clinical signs, clinical pathology, gross pathology and histopathological finding were typical for diclofenac toxicity. It would appear that the sensitivity of the Cape Griffon is similar to that of their Asian counterparts and the African White-backed Vulture (*Gyps africanus*). Diclofenac is almost certainly toxic to all *Gyps* vultures species and strong efforts must be taken to ensure that veterinary diclofenac products are not licensed or introduced to the African continent.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

In the past fifteen years, diclofenac has been responsible for the devastating decline in populations of three resident *Gyps* vulture species on the Asian subcontinent (Prakash et al., 2003). The crash in vulture numbers is a consequence of their consumption of carcasses contaminated with residues of diclofenac following the veterinary treatment of cattle, prior to their death (Oaks et al., 2004). While diclofenac was considered safe in cattle, the drug proved to be one of the most toxic compounds to vultures with an LD₅₀ in the range of 0.098–0.225 mg/kg (Swan et al., 2006a). While this exposure was accidental via contamination of the food chain, it highlighted the potential impact of veterinary medicines on susceptible species and delicate ecosystems. The loss of tens of millions of vultures in South Asia has had major repercussions on the ecology of the region, as the principal species of scavenger are now absent across large areas of the subcontinent. This has had potential serious consequences upon environmental and human health, including an increase in feral dog numbers and increased risk of diseases such as rabies, TB and brucellosis. Markandya et al. (2008) estimates the human health costs in India

from increasing dogs and rabies to be around US\$1.5 billion per year. With the devastation seen on the Asian subcontinent, it has become important to determine if other vulture species could also be susceptible, especially since pharmaceutical companies are now marketing diclofenac in other countries in Africa, where *Gyps* vultures are endemic (BirdLife International, 2007b).

In Southern Africa seven of the nine vulture species, of which three are *Gyps* vultures, endemic to the region are listed on the IUCN red list as being threatened. Of these, the Cape Griffon Vulture (*Gyps coprotheres*) is most threatened and considered globally vulnerable, as only 2900 breeding pairs are still found in the wild (Birdlife International, 2007a). While the low number of this species is already of major concern, the phylogenetic relationship of this species to other species within the *Gyps* genus is of great concern, as diclofenac toxicity has been established across the group, including in the African White-backed Vulture (AWBV) (*G. africanus*) (van Wyk et al., 2001; Swan et al., 2006a). In their study, Swan et al. (2006a) demonstrated that the African White-backed Vulture was at least as sensitive to diclofenac as the Oriental White-backed Vulture (*G. bengalensis*), in all aspects from clinical signs of toxicity, clinical pathology changes, necropsy findings and histopathological lesions.

Further concern for the species comes from studies of the foraging behaviour of the species as radio-tracking and satellite

* Corresponding author. Fax: +2712 529 8304.

E-mail address: vinny.naidoo@up.ac.za (V. Naidoo).

telemetry studies indicate Cape Griffon Vultures have an extremely wide foraging range with birds routinely crossing borders in the Southern African region (Bamford et al., 2007). If the Cape Griffon Vulture is sensitive to the effects of diclofenac as the African White-backed Vulture, the regional registration of veterinary diclofenac in any Southern African country could pose a major risk to the survival of the species. As the first step towards advocating a ban on the marketing authorisation of diclofenac in the region, the aim of this study was to determine the toxicological sensitivity of the Cape Griffon Vulture to diclofenac toxicity.

2. Materials and methods

2.1. Animals and dosing

Two adult Cape Griffon Vultures were intravenously dosed with diclofenac (Adco-diclofenac, Adcock Ingram South Africa) at 0.8 mg/kg vulture body weight, the same dose as utilised in previous toxicity studies based upon the dose-toxicity curve for the Oriental White-backed Vulture (Swan et al., 2006a). With a dose of 0.8 mg/kg bw, testing of two Cape Griffon Vultures is sufficient to demonstrate that this species is as least as sensitive to diclofenac as the Oriental White-backed Vulture. The route of exposure (intravenous versus oral) was, however, different than that used by Swan et al., 2006a, as the pharmacokinetic of the diclofenac was also evaluated in this study. The birds were obtained after rehabilitation efforts had failed i.e., humane euthanasia was considered the only option by the treating veterinarian (Vulture Programme of the Rhino and Lion Non-Profit Organisation). Due to the scarcity of birds where rehabilitation efforts had failed it took approximately 6 months between dosing of the two individual birds. Control animals were not available for sham dosing or necropsy (c.f. Swan et al., 2006a) due to the endangered nature of the species. To overcome this and to allow for the interpretation of the clinical pathology results, 24 adult healthy Cape Griffon Vultures housed at the DeWildt Cheetah and Wildlife Trust (DeWildt, South Africa) were sampled to establish normal reference intervals for selected parameters for the species.

2.2. Clinical pathology

Samples were collected in serum tubes for clinical chemistry analysis prior to dosing, after 2, 24 and 36 h. The electrolytes Sodium (Na^+), Potassium (K^+), Calcium (Ca^{2+}) were measured with a blood gas analyser (Rapidlab 34E Chiron diagnostics, Bayer SA). Uric acid (UA), alanine aminotransferase (ALT), creatine kinase (CK) and albumin (Alb) were measured with a Nexet Chemistry Analyser (Alfa Wasserman, Bayer SA). For the interpretation of the results from the two treated birds, the normal species reference ranges (mean \pm 2SD) was established for the listed serum chemistry parameters ($n = 24$) using a Kolmogorov–Smirnov test and Lilliefors table to assess the normality of distribution (SPSS15, SPSS Inc.) (Marco et al., 2000). When normality was demonstrated on natural log (Ln) transformed data, confidence intervals were determined as backed transformed mean ($\text{Ln}(X) \pm 2\text{SD}(\text{Ln}(X))$), where X represents the clinical pathological parameter being evaluated (Bland and Altman, 1996).

2.3. Pathology and histopathology

Tissue samples collected for residue analysis (liver, kidney, fat and muscle) were frozen or preserved in buffered formalin for histopathology (liver, kidney, heart, spleen, lungs). Samples collected in formalin were trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin using standard methods. All lesions seen at necropsy were recorded.

2.4. Tissue concentrations

Kidney, liver and muscle collected from the vultures were analysed for their concentration of the diclofenac parent molecule in the tissues. Organs were stored at -30°C until analysis. Half a gram of thawed tissue was minced, using a scalpel blade, and subsequently extracted with 2 ml acetonitrile, using a multitube vortex for 10 min (Naidoo et al., 2007). The mixed samples were centrifuged at 4500g for 10 min, the supernatant was decanted, dried under a steady stream of nitrogen in a water bath at 50°C and stored at -25°C until analysis by high performance liquid chromatography (HPLC) as described below.

2.5. Pharmacokinetic parameters

The limited samples (0, 0.5, 1, 2, 24 and 36 h) collected from the two birds were analysed for their diclofenac concentration (Naidoo et al., 2007). In brief, plasma samples (1.5 ml) were mixed with diethyl ether (3 ml), 0.3 M potassium dihydrogen phosphate (3 ml) and vortexed. The organic layer was subsequently separated in an ice bath (methanol/solid carbon dioxide), evaporated to dryness and dissolved in 400 μl mobile phase. Samples were analysed on a Beckman System Gold HPLC consisting of an autosampler module 507, programmable solvent module 126, diode array detector module (DAD) 168, and 32 Karat™ software package, was used (Beckman Instruments, Fullerton, California, USA). Separation was achieved with a Synergi Max-RP column (80A, 150×4.6 mm, 4; Phenomenex, Torrance, California, USA). The mobile phase consisted of 0.05 M sodium dihydrogen phosphate ($\text{pH} = 4.85$ to 4.89): CH_3CN , 42.5:57.5. 100 μl of the reconstituted samples were injected onto the HPLC column at 1 ml/min in an isocratic run. Detection of diclofenac and flunixin (internal standard) was carried out at 275 nm. The total runtime per sample was 8 min with retention times as follows: flunixin at 3.9 min, diclofenac at 4.9 min. Control values showed a mean% CV of 0.156% accuracy of 98% and regression coefficients greater than 0.99 for the analytical run. The LLQ was established at 0.1 $\mu\text{g}/\text{ml}$ and the LOD at 0.05 $\mu\text{g}/\text{ml}$, with a linear relationship between concentration and peak area being demonstrated for the total concentration range between 0.1 and 20 $\mu\text{g}/\text{ml}$.

Pharmacokinetic parameters were determined in Pharmacokinetics 4.4 (Thermo Electron Corporation) using non-compartmental modelling. The elimination rate constant (λ) and the elimination half-life ($T_{1/2}$) were calculated from the terminal phase. The concentration at 0 h (C_p0) was extrapolated by linear regression. The area under the plasma concentration vs. time curve (AUC) was obtained by the linear trapezoidal rule, up until the last measurable concentration (C_{last}), with extrapolation to infinity (AUC_{inf}) using the elimination rate constant (C_{last}/λ). Total body clearance (Cl), volume of distribution (Vd), and the mean residence time (MRT) were calculated using standard formulae.

3. Results

Both dosed birds died following treatment within the 48 h period. Clinical signs of depression viz. loss of appetite, failure to drink and dehydration and drooping neck became evident 24 h after exposure. The animals subsequently became more depressed until they became semi-comatose just before dying. The actual times of death was not recorded as the birds died during the early hours of the morning, with bird 1 dying within 48 h and bird 2 within 36 h of dosing. The specific changes in plasma chemistry are listed in Table 1. The 36 h sample was not collected in the second Cape vulture as the bird died before the sampling point. When compared to the normal values generated from healthy animals (Table 2 and

Table 1

Clinical pathology parameters and plasma diclofenac concentration evaluated in the two dosed birds.

Parameter	Bird 1				Bird 2		
	0 h	2 h	24 h	36 h	0 h	5 h	26 h
Alb (g/l)	13.2	12.7	12.3	15.5	15.3	15.2	12.3
ALT (U/l)	24	26	90	295	31	21	43
UA (umol/l)	0.23	0.5	4.92	4.92	0.46	0.42	0.93
Na ⁺ (mmol/l)	143	142	140	139	145	144	136
K ⁺ (mmol/l)	2.99	2.64	4.79	6.53	2.72	4.52	3.38
Ca ²⁺ (mmol/l)	0.56	0.73	0.83	0.83	0.96	1.04	0.93

Alb, Albumin; ALT, Alanine aminotransferase; UA, Uric acid; Na⁺, Sodium; K⁺, Potassium; Ca²⁺, Calcium.

Table 2

Normal parameter established for adult Cape Griffon Vultures (n = 24).

Parameter	Unit	Min	Max	Mean	SD	LL	UL
UA	mMol/L	0.18	0.67	0.31	0.36	0.15	0.65
Alt ^a	U/L	3.00	180.00	36.72	0.74	31.20	60.10
Alb	g/L	10.70	18.50	12.80	0.15	9.46	17.31
CK	U/L	172.00	1485.00	441.81	0.52	156.22	1249.51
Na ⁺	mMol/L	134.00	150.00	142.76	0.02	136.36	149.45
K ⁺	mMol/L	0.92	7.00	3.26	0.39	1.49	7.15
Ca ²⁺ ^b	mMol/L	0.46	1.16	0.90	0.23	0.44	1.35

UA, Uric acid; ALT, Alanine aminotransferase; Alb, Albumin; CK, Creatine kinase; Na⁺, Sodium; K⁺, Potassium; Ca²⁺, Calcium.

^a Normality could not be determined so values are presented as 95% confidence intervals.

^b Normality was demonstrated on non-transformed data.

Fig. 1) only UA was above the recommended interval from 24 h in both birds. All other parameters were normal except ALT which was raised at 36 h time point for bird 1 (Table 2 and Fig. 1).

At necropsy, lesions in the birds were characterised by wide-spread urate deposits on the air sacs, heart and liver, while the kidneys were swollen, pale and granular. On histopathology, numerous tophi were seen within the lung tissue, spleen and the liver. The liver lesion was characterised by amorphous urate starting to form spicules with resultant physical damage to nucleated erythrocytes and adjacent hepatocytes. The renal architecture was disrupted by numerous tophi with many of the tubular cells showing signs of injury and necrosis. As for the liver, the tubular

Table 3

Pharmacokinetic parameters and tissue concentrations obtained by non-compartmental modelling.

Pharmacokinetic parameters	Cape Griffon Vulture				African White-backed Vulture ^a			
	Bird 1	Bird 2	Mean	SD	Bird 1	Bird 2	Mean	SD
AUC _{last} (μg/mL h)	116.46	44.11	80.28	51.16	23.46	100.43	61.95	54.43
AUC _{inf} (μg/mL h)	135.72	54.26	94.99	57.6	31.60	132.72	82.16	71.50
λ (1/h)	0.05	0.06	0.06	0	0.06	0.03	0.05	0.02
AUMC _{last} (μg/mL (h) ²)	1393.98	238.42	816.2	817.1	247.94	1923.57	1085.76	1184.85
T _{1/2} (h)	12.94	11.53	12.24	0.99	11.05	22.50	16.78	8.09
MRT (h)	18.03	12.18	15.11	4.13	18.14	34.07	26.10	11.26
Cl (L/h)	0.006	0.015	0.01	0.006	0.03	0.01	0.02	0.01
Vd (L/kg)	0.11	0.25	0.18	0.1	0.40	0.20	0.30	0.15
Cp ₀ (μg/mL)	7.41	4.61	6.01	1.98				
<i>Organ concentrations</i>								
Kidney (mg/kg)	0.04	0.39	0.215	0.25	0.13	0.50	0.31	0.26
Muscle (mg/kg)	0.25	0.15	0.2	0.07	0.03	0.13	0.08	0.07
Liver (mg/kg)	0.25	0.15	0.2	0.07	0.11	0.53	0.32	0.30

AUC_{last}, Area under curve to the last sampled point; AUC_{inf}, Area under curve extrapolated to infinity; λ, Slope of the terminal curve; AUMC_{last}, Area under the moment curve to the last sampled point; T_{1/2}, Half-life of elimination; MRT, Mean residence time; Cl, Clearance; Vd, Volume of distribution; Cp₀, Extrapolate plasma concentration to time 0 h.

^a AWBV results calculated from results presented by Naidoo et al. (2007).

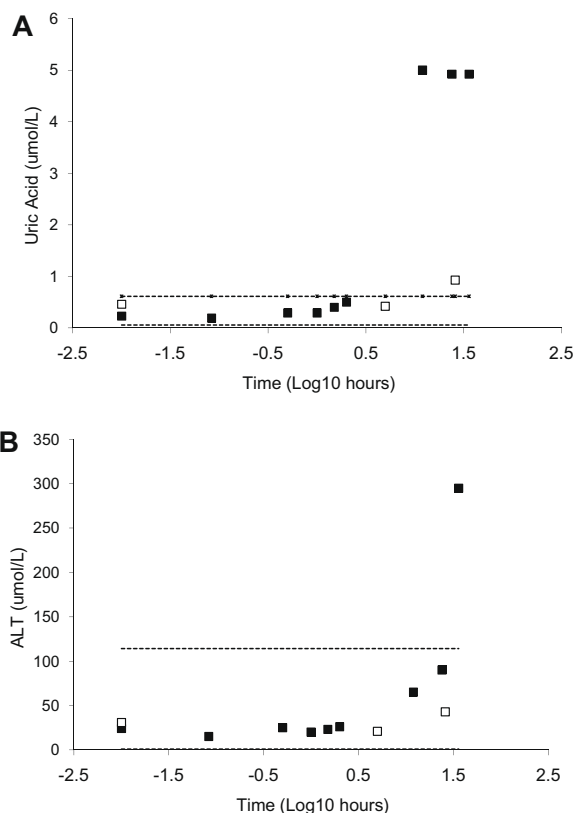


Fig. 1. Change in plasma uric acid (A) and ALT (B) from pre-dosing levels to last sample point. (Solid block = Vulture 1, Open block = Vulture 2). The broken lines represent the species upper and lower limits that were determined from 24 healthy Cape Griffon Vultures.

lumen was filled with spicule and globular urates with adjacent cell injury.

The pharmacokinetics from the limited profile in the two birds is listed in Table 3 and the average plasma profile in Fig. 2. In both animals, the drug was characterised by slow clearance and a small volume of distribution. The drug had a half-life of elimination of 12 h, which was similar in both birds. At death, diclofenac was detectable in the kidney, liver and muscle with an average concentration of 0.2 mg/kg being detectable in all tissues (Table 3).

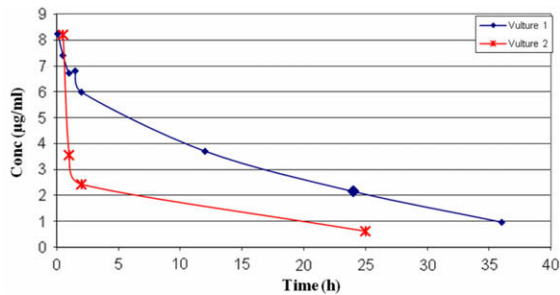


Fig. 2. Diclofenac plasma vs. time profile for the two dose Cape Griffon Vultures following dosing at 0.8 mg/kg intravenously.

4. Discussion

In comparison to the African White-backed Vulture, which were exposed to diclofenac at 0.8 mg/kg orally, the clinical signs of toxicity, time to depression and death, abnormal uric acid, necropsy and histopathological finding were almost identical (Swan et al., 2006a) and correspond closely to findings of diclofenac toxicity in Oriental White-backed Vultures (Oaks et al., 2004). Pharmacokinetic parameters were also similar, with both species characterised by a half-life above 10 h, low rate of clearance, small volume of distribution and similar tissue concentrations at death. The Area under Curve for African White-backed Vulture was marginally lower than the Cape Griffon Vulture (76% and 86% for AUC_{last} and AUC_{inf} , respectively), and most likely resulted from the different routes of administration namely African White-backed Vultures were dosed orally while the Capes were dosed intravenously. While these are different species, the similarities with regards to the other pharmacokinetic parameters tends to suggest that a degree of presystemic elimination is most likely occurring following oral administration. The latter is in agreement with the pharmacokinetics of the NSAIDs in mammalian species, in which a degree presystemic elimination results following oral exposure in the absence of food (Willis et al., 1979; Peris-Ribera et al., 1991). The increase in uric acid was also characteristic for diclofenac toxicity and this parameter was the best early indicator of toxicity. While ALT was increased in vulture 1, the absence of this increase in vulture 2 is most likely representative of the inability to obtain a 36 h sample. Merit for this argument was seen with the severe hepatic injury evident on histopathology.

This study therefore demonstrates that the Cape Griffon Vulture is as sensitive to diclofenac as the *Gyps* vulture species that have been devastated on the Indian subcontinent. This study also provides further information on the species specific toxicity of diclofenac in birds, as to date diclofenac has proven to be toxic in four species of *Gyps* vultures and the domestic chicken (*Gallus gallus*) (Oaks et al., 2004; Swan et al., 2006a; Naidoo et al., 2007; this study), while no cases of mortality (even at very high doses) have been reported for the Turkey Vulture (*Cathartes aura*) or Pied Crow (*Corvus albus*) (Rattner et al., 2008; Naidoo unpublished information). This adds support to our previous supposition, that toxicity is related to species specific metabolism (Naidoo and Swan, 2008).

In South Africa, the Cape Griffon Vulture is almost wholly dependent on “vulture restaurants” for their source of food. Consequently, the species is very susceptible to the accidental entry of diclofenac into their food chain. The latter is important as at

vulture restaurants, farmers and/or conservationists place out dead livestock or trophy kills to provide a food supply for vulture colonies. Unfortunately, since the process is often reliant on the donation of dead livestock from commercial farms, it is possible that these animals could have been treated with NSAIDs prior to death. This therefore highlights the importance of regulatory control of diclofenac and other NSAIDs in Southern Africa. This may be best achieved by the regional and hopefully world-wide ban on the manufacture and/or sale of veterinary diclofenac in support of the vulture safe NSAID meloxicam (Swan et al., 2006b).

Acknowledgments

This study was approved by the Animal Use and Care Committee of the University of Pretoria. The birds evaluated for the normal values were obtained from the DeWildt Cheetah and Wildlife Trust. This study was approved by Gauteng Nature Conservation (GDACE) and North West Nature Conservation. The study was funded in part by the National Research Foundation of South Africa and the University of Pretoria.

References

- Bamford, A.J., Diekmann, M., Monadjem, A., Mendelsohn, J., 2007. Ranging behaviour of Cape Vultures *Gyps coprotheres* from an endangered population in Namibia. *Bird Conserv. Int.* 17, 331–339.
- BirdLife International, 2007a. Vulture-killing drug now on sale in Africa. Available from: http://www.birdlife.org/news/news/2007/10/africa_diclofenac.html.
- BirdLife International, 2007b. The 2006 IUCN Red list of threatened species.
- Bland, J.M., Altman, D.G., 1996. Transformation, mean and confidence intervals. *Brit. Med. J.* 312, 1079.
- Marco, I., Martinez, F., Pastor, J., Lavin, S., 2000. Hematologic and serum chemistry values of the captive European wildcat. *J. Wildl. Dis.* 36, 445–449.
- Markandya, A., Taylora, T., Longic, A., Murtyd, M.N., Murtyd, S., Dhavalad, K., 2008. Counting the cost of vulture decline — An appraisal of the human health and other benefits of vultures in India. *Ecol. Econ.* 67, 194–204.
- Naidoo, V., Duncan, N., Bekker, L., Swan, G., 2007. Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in *Gyps* vultures. *Environ. Toxicol. Pharmacol.* 24, 260–266.
- Naidoo, V., Swan, G.E., 2008. Diclofenac toxicity in *Gyps* vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* [Epub ahead of print].
- Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., 2004. Diclofenac residues as the cause of vulture population declines in Pakistan. *Nature* 427, 630–633.
- Peris-Ribera, J.E., Torres-Molina, F., Garcia-Carbonell, M.C., Aristorena, J.C., Pladelfina, J.M., 1991. Pharmacokinetics and bioavailability of diclofenac in the rat. *J. Pharmacokinet. Pharmacodyn.* 19, 647–665.
- Prakash, V., Pain, D.J., Cunningham, A.A., Donald, P.F., Prakash, N., Verma, A., Gargi, R., Sivakumar, S., Rahmani, A.R., 2003. Catastrophic collapse of Indian white-backed *Gyps begalensis* and long-billed *Gyps indicus* vulture populations. *Biol. Conserv.* 109, 381–390.
- Rattner, B.A., Whitehead, M.A., Gasper, G., Meteyer, C.U., Link, W.A., Taggart, M.A., Meharg, A.A., Pattee, O.H., Pain, D.J., 2008. Apparent tolerance of turkey vultures, *Cathartes aura*, to the non-steroidal anti-inflammatory drug diclofenac. *Environ. Toxicol. Chem.* 27, 2341–2345.
- Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A., Duncan, N., Meharg, A.A., Oaks, J.L., Parry-Jones, J., Shultz, S., Taggart, M.A., Verdoorn, G., Wolter, K., 2006a. Toxicity of diclofenac to *Gyps* vultures. *Biol. Lett.* 2, 279–282.
- Swan, G., Naidoo, V., Cuthbert, R., Green, R.E., Pain, D.J., Swarup, D., Prakash, V., Taggart, M., Bekker, L., Das, D., Diekmann, J., Diekmann, M., Killian, E., Meharg, A., Patra, R.C., Saini, M., Wolter, K., 2006b. Removing the threat of diclofenac to critically endangered Asian vultures. *PLoS Bio.* 4, e66.
- van Wyk, E., van der Bank, H., Verdoorn, G.H., 2001. Allozyme variation in four populations of African whitebacked vultures (*Gyps africanus*) and phylogenetic relationships between four vulture species from southern Africa. *Biochem. Syst. Ecol.* 29, 485–512.
- Willis, J.V., Kendall, M.J., Flinn, R.M., Thornhill, D.P., Welling, P.G., 1979. The pharmacokinetics of diclofenac sodium following intravenous and oral administration. *Eur. J. Clin. Pharmacol.* 16, 405–410.