Journal of Applied Ecology 2006
43, 949–956

PRIORITY CONTRIBUTION

Collapse of Asian vulture populations: risk of mortality from residues of the veterinary drug diclofenac in carcasses of treated cattle

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Summary

1. The populations of three species of South Asian vultures (*Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*) have declined rapidly within the last decade and all are now critically endangered. Veterinary use of the non-steroidal anti-inflammatory drug diclofenac appears to be a major cause of the declines. Vultures are likely to be exposed to the drug when they feed on carcasses of livestock that were treated with diclofenac before death.
2. We measured the concentration of diclofenac in the tissues of treated Indian humped and European cattle (*Bos indicus* and *Bos taurus*) in relation to the interval between dosing and death. We used a dose–response model to assess the risk posed to wild vultures if they feed on carcasses of treated livestock.
3. Diclofenac concentrations in fat, intestine, kidney and liver were considerably higher than those in muscle, but concentrations in the first four tissues initially depleted more rapidly (half-life 6–8 h) with time since the last injection of the drug, compared with muscle (half-life 15 h). Depletion rates became much slower in all tissues 25–98 h after the last injection.
4. Diclofenac concentration, averaged across the carcass, was enough to cause appreciable mortality (> 10% of birds per meal) if oriental white-backed vultures *G. bengalensis* were to take a large meal from the carcass of an animal that was given its last dose of the drug within a day or two before death. Vultures that feed selectively on tissues with high concentrations of the drug, such as kidney, liver and intestine, would be exposed to a higher risk and for longer after dosing.
5. *Synthesis and applications.* The tissues of cattle treated with diclofenac are a hazard to wild vultures that feed on an animal that dies within a few days after treatment. Intestine, kidney and liver have the highest diclofenac concentrations, but the concentration averaged across all the edible tissues of the carcass is also hazardous. Withdrawal of diclofenac from veterinary use on animals whose carcasses may become available to scavenging vultures is recommended. In *ex situ* and *in situ* conservation projects, vultures should be fed on carcasses of animals that are known not to have been treated with diclofenac in the week before death.

Key-words: bird population decline, dose–response relationship, endangered species, non-steroidal anti-inflammatory drug, pharmacokinetics, renal failure, visceral gout

Journal of Applied Ecology (2006) **43**, 949–956
doi: 10.1111/j.1365-2664.2006.01225.x

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Introduction

Three species of vultures endemic to South Asia, the oriental white-backed vulture *Gyps bengalensis* (Gmelin), long-billed vulture *Gyps indicus* (Scopoli) and slender-billed vulture *Gyps tenuirostris* Gray, are at high risk of global extinction and are listed as critically endangered because of rapid population declines within the last decade in the Indian subcontinent (Prakash *et al*. 2003; Green *et al*. 2004; IUCN 2004). There is strong evidence that veterinary use of the non-steroidal anti-inflammatory drug (NSAID) diclofenac is a major cause of the population declines (Oaks *et al*. 2004; Green *et al*. 2004; Shultz *et al*. 2004). Diclofenac is widely available as a veterinary drug in the Indian subcontinent, where it is used to treat inflammation, fever and pain associated with disease and injury in domestic livestock. Vultures are believed to be exposed to the drug when they feed on carcasses of livestock that were treated with diclofenac before death. Following experimental exposure to tissues of ungulates treated with a veterinary dose of diclofenac, *G. bengalensis* died within a few days from kidney failure. Extensive visceral gout was evident at post-mortem examination (Oaks *et al*. 2004). Rapid death associated with visceral gout has also been observed in two other vulture species, African white-backed vulture *Gyps africanus* Salvadori and Eurasian griffon vulture *Gyps fulvus* (Hablitzl), treated experimentally with diclofenac (Swan *et al*. 2006a). Hence, susceptibility to diclofenac poisoning seems to be widespread in the genus, so *G. indicus* and *G. tenuirostris* are also likely to be susceptible. Visceral gout and diclofenac residues in tissues have also been found in most carcasses of wild *Gyps* vultures from across India, Pakistan and Nepal examined since the decline began (Oaks *et al*. 2004; Shultz *et al*. 2004). The proportion of carcasses of *G. bengalensis* and *G. indicus* found dead in the wild with signs of diclofenac poisoning is consistent with this being the main, and possibly the only, cause of the vulture decline (Green *et al*. 2004).

No detailed studies have been made previously of the exposure of vultures to diclofenac and the risk of death posed by each exposure. In this paper, we report on the concentrations of diclofenac in the tissues of experimentally treated cattle and assess the risk to wild vultures if they were to feed on them, in relation to the time between treatment and the death of the treated animal. For this we used data from three experiments. One was carried out to estimate the exposure of wild vultures to diclofenac administered to cattle using the standard daily dose of the drug recommended for veterinary use in India (experiment 1). The other experiments (experiments 2 and 3) were undertaken by a pharmaceutical manufacturing company to establish maximum residue limits, as required by the European Agency for the Evaluation of Medicinal Products (EMEA, London, UK).

Methods

EXPERIMENT 1

Ten female Indian humped cattle *Bos indicus* L., 1–7 years old (mean 3·8 years) with an average mass of 202 kg (range 30–300 kg), were housed on a farm in Kerala, India. After keeping the animals for at least 2 weeks to ensure that they were free of any previous NSAID they might have received, each was given one injection of a diclofenac sodium formulation (25000 mg L^{-1}; 3-D Vet, Intas, India) into the neck muscle at a dose of 1 mg kg^{-1} live weight. At 21 h (20·7–22·5 h), 46 h (45·8–46·8 h), 71 h (71·0–71·3 h), 167 h (166·4–168·8 h) and 334 h (333·1–334·3 h) after the injection, respectively, two animals were slaughtered. Samples of tissue weighing 25–30 g were taken from intestine, kidney and liver and from intercostal or gluteal muscle from the opposite side of the body from the injection site. Samples were frozen at −20 °C.

EXPERIMENT 2

Sixteen young European cattle *Bos taurus* L. (eight males and eight females), 0·6–2·0 years old (mean 1·1 years) and weighing 140–280 kg (mean 187·5 kg), were housed at a facility in France. Each animal received an injectable proprietary veterinary formulation of diclofenac sodium (50 000 mg L^{-1}) injected into the neck muscle at a dose of 2·5 mg kg^{-1} live weight on each of 6 consecutive days. At 2–4 h, 12 h, 24 h and 144 h after the last injection, respectively, four animals were slaughtered by exsanguination after stunning. Samples of tissue weighing 2–5 g were taken from perirenal fat, kidney, liver and triceps muscle. A summary of this experiment has been reported previously in item 18 of EMEA (2004).

EXPERIMENT 3

Eight mature dairy cows *B. taurus*, aged 2·5–10·0 years old (mean 5·3 years) and weighing 555–715 kg (mean 638·8 kg), were housed at a facility in France. Each animal received an injectable proprietary veterinary formulation of diclofenac sodium (50 000 mg L^{-1}) injected into the neck muscle at a dose of 2·5 mg kg^{-1} live weight on each of 6 consecutive days. Four animals were slaughtered by exsanguination after stunning at 96 h, and another four at 176 h, after the last injection. Samples of perirenal fat (10 g), kidney (20 g), liver (20 g) and triceps muscle (20 g) were taken. A summary of this experiment has been reported previously in item 18 of EMEA (2004).

DICLOFENAC ANALYSES

Diclofenac concentrations in tissues were measured using high-performance liquid chromatography (HPLC) methods calibrated against a known standard concentration of the drug. In experiment 1, diclofenac was extracted from 0·5 g of tissue by homogenization with

2 mL of HPLC-grade acetonitrile, which was then centrifuged at 1000 *g* for 5 min. Analysis of diclofenac tissue concentrations was undertaken using a validated HPLC method with electrospray ionization mass spectrometry (ESI/MS) detection, following the methods of Oaks *et al*. (2004). The Agilent 1100 series instrument (1946D) (Agilent Technologies UK, Cheadle Royal Business Park, Stockport, SK8 3GR, UK) was calibrated using six diclofenac sodium salt (Sigma-Aldrich; D6899, New Road, Gillingham, Dorset, SP8 4XT, UK) standards ranging from 0·005 to 1 mg L^{-1}. The calibration was linear across this range, with an r^2 value of > 0·999. The limit of quantification (LOQ) was 0·01 mg kg^{-1}.

In experiments 2 and 3, diclofenac was extracted using a multistep process. For fat, aliquots of 0·5–1·0 g of tissue were homogenized in 8 mL of dichloromethane. For kidney, liver and muscle, aliquots of 0·5–1·0 g of tissue were homogenized in 4 mL of ethanol. Quantitative determination of diclofenac was performed using a Hewlett Packard HP 1050 series system. Chromatography was carried out on a column packed with a C18 stationary phase. The mobile phase was a mixture of acetonitrile/sodium acetate 0·1 M/methanol (2/38/60; v/v) and was used at a flow rate of 0·7 mL min^{-1}. A coulometric detection method was used. The LOQ was 0·005 mg kg^{-1} in experiments 2 and 3 in all tissues except fat in experiment 3, for which the LOQ was 0·01 mg kg^{-1}.

MODELLING RELATIONSHIPS BETWEEN THE DICLOFENAC CONCENTRATION IN TISSUE AND TIME SINCE LAST INJECTION

Inspection of the data indicated that the concentration of diclofenac in tissue declined approximately exponentially after the last injection, but that the rate of decline slowed abruptly after a certain time, which we called the transition time (Fig. 1). We decided to model the diclofenac concentration d as a function of time since the last injection t using the piece-wise relationship $d = \exp(a_{ij} + b_{i.1}t)$ for $t < t_i^*$, and $d = \exp(a_{ij} + b_{i.1}t_i^* + b_{i.2}(t - t_i^*))$ for $t > t_i^*$, where a_{ij} is a constant specific to the ith tissue and jth experiment, $b_{i.1}$ is the exponential rate of decline of diclofenac concentration in the ith tissue between the last injection and transition time t_i^*, after which the decline rate changes to $b_{i.2}$. Deviations from expected values were assumed to be log-normally distributed with residual variance s_i^2 specific to each tissue.

There were insufficient data to estimate decline rates and transition times for a given tissue separately for each of three experiments, so only the a parameters were treated as specific to both tissue and experiment. The b and t^* parameters were assumed to differ among tissues, but to be the same for a given tissue in each experiment. In practice, this gave a reasonable fit to the data (Fig. 1).

Parameter values were estimated by a quasi-Newton maximum-likelihood (M-L) method using the NONLIN module of SYSTAT 5.03. A M-L procedure was preferred to ordinary linear least-squares regression after log transformation of d because some observed concentrations were below the LOQ. If least-squares linear regression had been used, these observations would have had to be assigned an arbitrary concentration lower than the LOQ so that logarithms could be taken. The choice of this value would affect the resulting parameter estimates. Omitting the < LOQ observations would also be unsatisfactory as it would bias the parameter estimates. Therefore, the presence of observations < LOQ was handled in the M-L procedure by incorporating into the model left-censoring of d at the LOQ value appropriate to a given experiment and tissue (Kalbfleisch 1979).

As a measure of the goodness-of-fit of the models, we calculated expected values from them and then obtained the Pearson correlation coefficient r between observed and expected diclofenac concentrations for each tissue. Data for which the observed value was < LOQ were excluded from this calculation.

COMPOSITION OF CARCASSES OF CATTLE AND BUFFALOES

We wished to calculate the concentration of diclofenac averaged over all parts of the carcass of a cow or domesticated water buffalo *Bubalus bubalis* (L.) available to vultures as food. To do this, we needed to know the proportion of the edible mass of the entire carcass that is made up of each tissue. The composition of typical carcasses of *Bos taurus*, *Bos indicus* and *Bubalus bubalis* was determined from the scientific literature. Ideally we would have determined composition separately for each species from samples of animals representative of those available dead as food for vultures in India. In practice, the information available did not allow this and it was necessary to assume that the same mean values could be used for the three species.

Initially we expressed the mass of each body component as a percentage of the animal's live weight. The mass of the liver is 1·3–1·5% of live weight for *Bos taurus* (Budras & Habel 2003) and 1·5% for *Bos indicus* (Kumar Ghosh 1998). We used 1·5% as the typical value. The mass of the kidneys is 0·25% of live weight in *Bos taurus* according to Budras & Habel (2003), and 0·55% in *Bos indicus* Kumar Ghosh (1998). Using the relative mass of the kidneys and liver from table 14 of Grubh (1974) and the value for liver given above, we estimated that the mass of the kidneys is 0·41% of live weight in *Bubalus bubalis*. The mean of these three estimates is 0·40%.

The mass of all internal organs (offal) of *Bos taurus* is given as 14% of live weight by the Competition Commission (1985). After subtracting the estimates of the mass of the liver and kidneys given above, this leaves 12·1% of live weight comprised by the other internal organs.

The percentage of muscle present in carcasses dressed for meat preparation (i.e. with the head, feet, hide, blood, alimentary tract and contents removed) was taken to be 33% of live weight for *Bos taurus* (Callow 1962) and 37·1% for *Bubalus bubalis* (Charles & Johnson 1972), giving a mean of 35·0%. We estimated the mean mass of edible tissue, assumed to be mostly muscle, on heads of

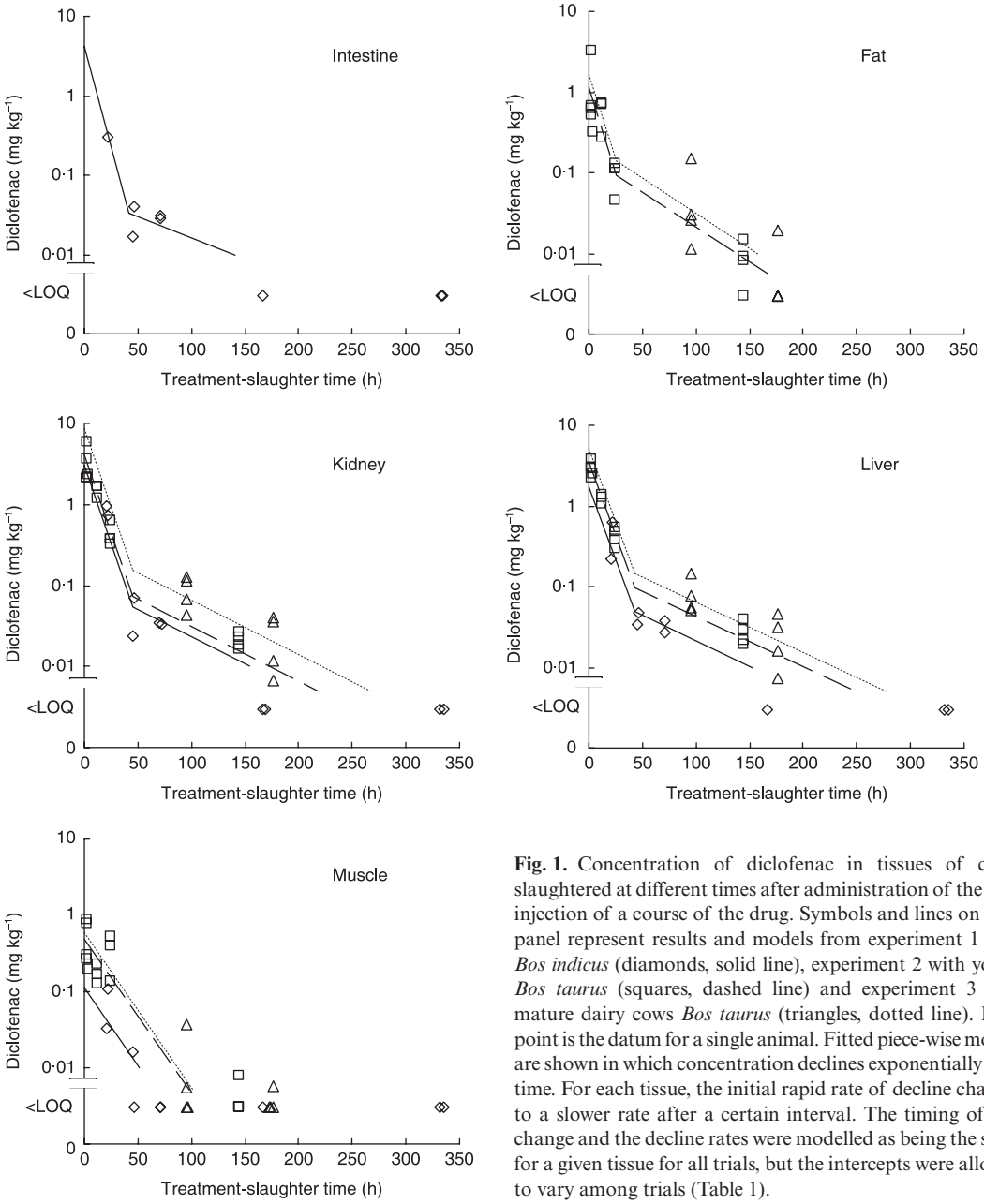

Fig. 1. Concentration of diclofenac in tissues of cattle slaughtered at different times after administration of the final injection of a course of the drug. Symbols and lines on each panel represent results and models from experiment 1 with *Bos indicus* (diamonds, solid line), experiment 2 with young *Bos taurus* (squares, dashed line) and experiment 3 with mature dairy cows *Bos taurus* (triangles, dotted line). Each point is the datum for a single animal. Fitted piece-wise models are shown in which concentration declines exponentially with time. For each tissue, the initial rapid rate of decline changes to a slower rate after a certain interval. The timing of this change and the decline rates were modelled as being the same for a given tissue for all trials, but the intercepts were allowed to vary among trials (Table 1).

Bos indicus and *Bubalus bubalis* from data in table 14 of Grubh (1974) as 2·0% of live weight. Combining these two estimates gave total muscle mass as 37·1% of live weight.

The percentage of fat present in carcasses dressed for meat preparation, averaged across *Bos taurus*, *Bos indicus* and *Bubalus bubalis*, is 6·4% of live weight (Charles & Johnson 1972). For *Bos taurus*, the mass of fat, other than that in the dressed carcass, is 8% of live weight (Competition Commission 1985), giving a total fat content of 14·4% of live weight. The remaining body components, the hide and the blood, were taken to be 7% and 3% of live weight, respectively (Competition Commission 1985).

We took the total mass of edible tissue available to vultures on a typical carcass to be the sum of these components. The hide of cattle and buffaloes is often removed for leather production soon after death, so we assumed arbitrarily that the hide was only available to vultures on half of carcasses. After conversion to percentages of total edible tissue, the masses of the different body components are: liver 2·1%, kidneys 0·6%, alimentary tract and other offal 16·8%, muscle 51·5%, fat 20·0%, hide 4·9% and blood 4·2%. We took the diclofenac concentration measured in samples of intestine to apply to the whole alimentary tract and to offal other than liver and kidney, and the concentration in samples of muscle to apply also to hide and blood.

CALCULATING THE DICLOFENAC CONCENTRATION AVERAGED OVER THE ENTIRE CARCASS

The average diclofenac concentration in the edible parts of the entire carcass was calculated separately for

Table 1. Estimates of the parameters of piece-wise models relating tissue concentrations of diclofenac in mg kg^{-1} wet weight to the time in hours since the last injection. Details of the models are given in the Methods. a_{ij} is an intercept specific to the ith tissue and jth experiment, b_{i1} is the exponential rate of decline of diclofenac concentration in the ith tissue between the last injection and the transition time t_i* in hours, b_{i2} is the rate of decline after the transition time and s_i is the residual standard deviation. Half-life, in hours, was calculated as $\log_e(0{\cdot}5)/b$ for the periods before and after the transition. Values of a_{ij} in italics are for experiment–tissue combinations for which observations were not available and were calculated using the mean of a values for kidney, liver and muscle (see Methods). Also shown is the Pearson correlation coefficient r between observed and modelled concentrations

	Parameter estimates				
Parameter	Fat	Intestine	Kidney	Liver	Muscle
a_{i1}	*–0·7077*	1·4120	1·1095	0·5490	–2·2125
a_{i2}	0·1192	*2·2389*	1·4048	1·2441	–0·7222
a_{i3}	0·5202	–	2·1658	1·6468	–0·5331
b_{i1}	–0·09916	–0·11538	–0·08775	–0·08347	–0·04684
b_{i2}	–0·01982	–0·01208	–0·01554	–0·01435	–0·01021
t_i*	25·19	41·76	45·83	42·73	98·34
s_i	0·6760	0·3256	0·4551	0·3858	0·7326
Half-life$_1$	6·99	6·01	7·90	8·30	14·80
Half-life$_2$	34·97	57·37	44·60	48·30	67·89
r	0·646	0·997	0·895	0·979	0·698

experiments 1 and 2. We considered it inappropriate to perform this calculation for experiment 3 because the first animals were slaughtered after the transition time. For each experiment and each hour after the last injection, we multiplied the diclofenac concentration for each tissue, obtained from the fitted model, by the proportion of the edible mass of the entire carcass that is made up of each tissue (see above). We then summed these products to obtain the weighted mean diclofenac concentration for the entire carcass. We did not have measurements of diclofenac concentration for fat in experiment 1, or for intestine in experiment 2, so experiment-specific a values were not available to use in the calculations for these tissue experiment combinations. To overcome this, we calculated the means, for each of the two experiments, of the a values for the three tissues, kidney, liver and muscle, that were measured in both. To estimate the a value for fat in experiment 1, we took the a value for fat in experiment 2, added the mean for the three tissues from experiment 1 and subtracted the mean from experiment 2. To estimate the a value for intestine in experiment 2, we took the a value for intestine in experiment 1, added the mean for the three tissues from experiment 2 and subtracted the mean from experiment 1.

TOXICITY TO *G. BENGALENSIS* OF TISSUES FROM DICLOFENAC-TREATED CATTLE

Following Swan *et al*. (2006b), we assumed that a wild vulture would ingest 1·023 kg of various tissues from the carcass of a treated animal in a short time. This quantity of food would sustain a free-living *G. bengalensis* for 3 days. We assumed that the average concentration of diclofenac in the food was the same as the average for all edible parts of the carcass, obtained from the fitted model, as described above. This concentration was multiplied by 1·023 and the product was divided by 4·75 (the average mass in kg of *G. bengalensis*; del Hoyo, Elliott & Sargatal 1994) to give the ingested dose in mg kg^{-1} vulture body weight. We then used dose–response models fitted by probit analysis by Swan *et al*. (2006a) to experimental data for captive *G. bengalensis* from Oaks *et al*. (2004), which estimated the relationship between the rate of diclofenac-induced mortality and the dose of diclofenac ingested. We used two versions of the model, which were fitted with and without data from an outlier, a vulture that died with visceral gout after apparently receiving a very low dose of diclofenac (Swan *et al*. 2006a).

Results

RELATIONSHIP OF TISSUE CONCENTRATION OF DICLOFENAC TO TIME SINCE THE LAST INJECTION

Diclofenac concentration declined rapidly (half-life 6–8 h) in fat, intestine kidney and liver up to the transition time (25–46 h), after which it declined more slowly (half-life 35–57 h; Fig. 1 and Table 1). The concentration in muscle declined more slowly than in other tissues (half-life 15 h) and the transition occurred later (98 h). The models fitted the data reasonably well for all five tissues (Fig. 1 and Table 1). There was a non-significant tendency (Pearson $r_3 = 0{\cdot}687$, $P = 0{\cdot}20$) for those tissues with a long half-life in the period before the transition to also have a relatively long half-life after the transition.

In all three experiments, diclofenac concentrations in kidney and liver were considerably higher than those in muscle (Fig. 1). Concentrations in intestine approached those in kidney and liver and were higher than those in muscle. Concentrations in fat were intermediate. For a given tissue, diclofenac concentrations tended to be higher in experiments 2 and 3 than in experiment 1 (Fig. 1). Hence the estimated average diclofenac concentration in the entire carcass was considerably

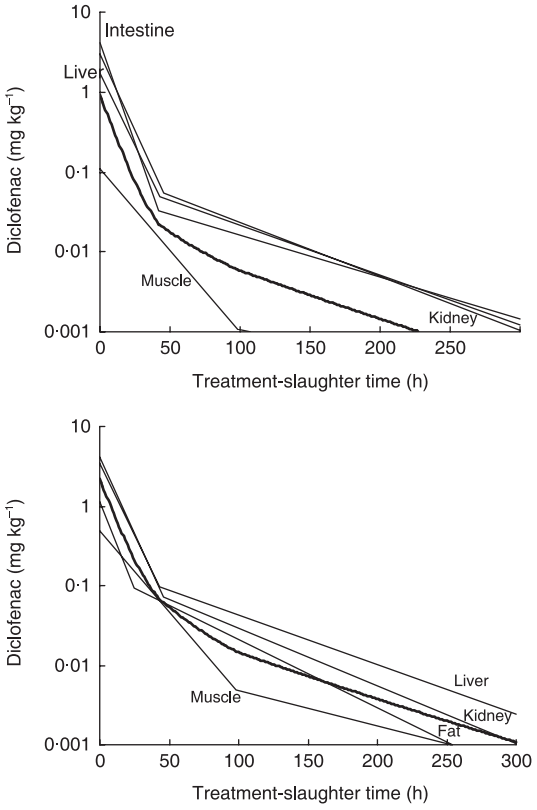

Fig. 2. Concentration of diclofenac in various cattle tissues in relation to the time between administration of the final injection of a course of the drug and slaughter. Models are the same as those presented in Fig. 1. The thin lines on each panel represent results for different tissues. The thick line shows the estimated diclofenac concentration averaged over all parts of a carcass that are available to vultures (see text). The upper panel shows results from experiment 1 with *Bos indicus* and the lower panel shows results from experiment 2 with young *Bos taurus*.

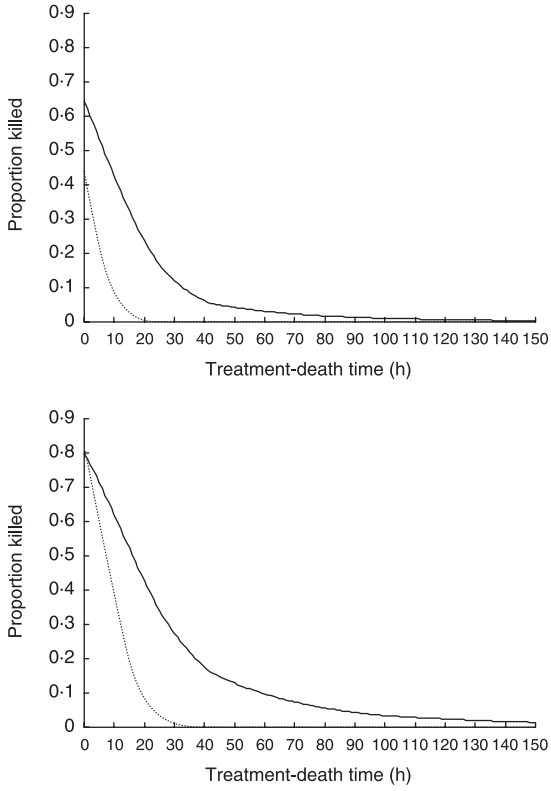

Fig. 3. Proportion of *Gyps bengalensis* estimated to be killed by diclofenac in relation to time between administration of the final injection of a course of the drug and the death of the treated cow (see text). Results are shown separately for a dose–response model fitted to all data from the experiments of Oaks *et al*. (2004) (solid line) and after excluding an outlier (dotted line). The upper panel shows results from experiment 1 with *Bos indicus* and the lower panel shows results from experiment 2 with young *Bos taurus*.

higher at all times after the last injection for experiment 2 than experiment 1 (Fig. 2).

TOXICITY TO *G. BENGALENSIS* OF TISSUES FROM DICLOFENAC-TREATED CATTLE

The average diclofenac concentrations in tissues of the cattle experimentally treated with the drug were sufficient to cause appreciable mortality in vultures that fed on the carcass of an animal that died within 1–4 days of the last treatment (Fig. 3). Vultures that ingested the average concentration across all tissues would receive a sufficient dose to kill > 10% of birds if a treated cow had died within 33 h (experiment 1) or 59 h (experiment 2) of the last injection. These results are for the dose–response model that includes the datum from an outlier (see the Methods). If the version of the model that excludes this datum is used, then the equivalent times are 9 h (experiment 1) and 19 h (experiment 2).

Vultures that fed exclusively on tissues with higher than average concentrations of diclofenac would be at risk over a much longer interval after treatment. For example, if birds were to feed only on liver they would receive a sufficient dose to kill > 10% of them if the treated cow had died within 56 h (experiment 1) or 105 h (experiment 2) of the last injection (model including the outlier). If the dose–response model that excludes the outlying datum is used, the equivalent times are 19 h (experiment 1) and 28 h (experiment 2).

Discussion

Our study demonstrates that diclofenac concentrations in the tissues of treated cattle decline rapidly with time after the last injection. However, enough diclofenac remains to cause appreciable mortality (> 10%) if birds were to take a large meal from the carcass of an animal that was given its last dose of the drug within a few days of death, although the relationship of mortality to the time since injection was affected by the version of the dose–response model used. Lower mortality rates were calculated if a vulture that died with visceral gout after apparently ingesting a very small dose of diclofenac was excluded when fitting the model. However, for reasons given by Swan *et al*. (2006a), it is unclear whether the results for this bird should be included in the calculation or not. Hence, we present calculations based upon both versions.

The treatment regimes used in our experiments are likely to be broadly comparable with practice in the Indian subcontinent. In India, the recommended veterinary course of diclofenac for cattle and buffaloes is 1·0 mg kg^{-1} on each of 3 consecutive days. This is the dose we used in experiment 1, although only one injection was administered rather than three. Given the short half-life of diclofenac in cattle tissue, giving a course of three injections at 24-h intervals would probably have elevated tissue concentrations by a modest amount. In Pakistan, the recommended course is 2·5 mg kg^{-1} on each of 3 consecutive days. This daily dose level was used in experiments 2 and 3, although injections were given for 6 rather than 3 days. It seems likely that larger doses of diclofenac than recommended are sometimes used in practice in India. The highest concentrations of diclofenac found in liver from livestock carcasses sampled in the field in India were up to three times the maximum we found in liver in these experiments (M. Taggart, manuscript in preparation).

The average concentration of diclofenac in the entire edible carcass is likely to be affected by the number of doses and particularly the daily dose level. The concentration was higher in experiments 2 and 3, in which 2·5 mg kg^{-1} was used, than in experiment 1, in which the dose was 1·0 mg kg^{-1}. In experiment 2, the ratio of the diclofenac concentration in the entire carcass, calculated over the first 24 h after the last injection, was 2·58 times that for experiment 1; a very similar ratio to that of the dose levels used. The estimated concentration of diclofenac in the entire edible carcass immediately after the last injection (calculated as for the intercepts in Fig. 2) was about two-thirds of the amount of diclofenac injected into the cattle per kilogram of edible tissue in both experiments (experiment 1, post-injection carcass concentration 0·91 mg kg^{-1} cf. 1·39 mg kg^{-1} drug injected; experiment 2, carcass concentration 2·19 mg kg^{-1} cf. 3·47 mg kg^{-1} drug injected, given that 72% of the live weight consists of edible tissues). However, it should be noted that several other things differed between the experiments, such as the species of cattle, the number of injections given and the laboratory where the diclofenac assays were performed, so the difference in tissue concentration cannot be attributed unambiguously to the difference in the amount given per dose.

The concentrations of diclofenac we measured in tissues of cattle in our experiments are broadly similar to results reported by Oaks *et al*. (2004) for a *Bubalus bubalis* that was slaughtered 4 h after receiving the last of three daily injections of diclofenac at 2·5 mg kg^{-1}. Diclofenac concentrations in the kidney, liver and muscle of this animal were 5·7, 1·5 and 0·76 mg kg^{-1}, respectively, compared with values of 2·87, 2·48 and 0·40 mg kg^{-1} for the same tissues of young *Bos taurus*, estimated 4 h after the last treatment from the results of experiment 2. When expressed as normal standard deviates using the residual standard deviation from the model, the values for *Bubalus bubalis* are +1·51, −1·31 and +0·87 relative to the modelled values for young *Bos taurus*. Hence the differences are well within the expected range. However, the values observed for the *Bubalus bubalis* are high relative to the modelled values for *Bos indicus* from experiment 1 (2·14, 1·24 and 0·09 mg kg^{-1}, or +2·16, +0·49 and +2·90 as standard normal deviates). The better agreement of the *Bubalus bubalis* data with the expected values from experiment 2 than experiment 1 may be because the *Bubalus bubalis* and the *Bos taurus* in experiment 2 both received the same daily dose of diclofenac (2·5 mg kg^{-1}), whereas the *Bos indicus* in experiment 1 received a much lower dose (1·0 mg kg^{-1}).

Our findings are of practical value for efforts to prevent the extinction of critically endangered vulture species in two main ways. First, they extend the experimental results of Oaks *et al*. (2004) by measuring the period after treatment for which contamination of cattle tissues causes vulture mortality. This new information is being used to develop a more realistic version of the model, described by Green *et al*. (2004), of the relationship between the prevalence of diclofenac in ungulate carcasses and vulture population trends. From this, it should be possible to ascertain more accurately, from field surveys of diclofenac residues in cattle and buffalo carcasses, whether a sufficient proportion is contaminated with diclofenac to account for the observed vulture population declines. Better means of interpreting such surveys will be essential for monitoring the effectiveness of future action taken to remove diclofenac from the food supply of vultures, such as government measures to restrict veterinary use of diclofenac and encourage its replacement by meloxicam (Swan *et al*. 2006b). Secondly, the findings are useful for the husbandry of captive vultures being held for captive breeding and future reintroduction projects, and the supplementary feeding of wild vultures at 'vulture restaurants', which is intended to divert them from feeding on contaminated tissue. Our results indicate that these birds are likely to be safe from diclofenac poisoning if they are fed on carcasses of cattle that are known not to have been treated with diclofenac within about a week before death.

Acknowledgements

We gratefully acknowledge Fatro S.p.A. (Italy) for permission to use data from the experiments they conducted to establish maximum residue limits for diclofenac (experiments 2 and 3) and thank Beata Truszkowska for providing the raw data and details of the experimental methods. We thank Vibhu Prakash and Susanne Shultz for help in planning and setting up experiment 1. We are grateful to Ian Newton, Simon Thirgood and two anonymous referees for useful criticisms of a previous version. The research is part of a programme co-funded by the UK government's Darwin Initiative for the Survival of Species, the Royal Society for the Protection of Birds and the Zoological Society of London.

References

Budras, K.-D. & Habel, R.E. (2003) *Bovine Anatomy: An Illustrated Text*. Schlütersche, Hanover, Germany.

Callow, E.H. (1962) The relationship between the weight of a tissue in a single joint and the total weight of the tissue in a side of beef. *Animal Production*, **4**, 37–46.

Charles, D.D. & Johnson, E.R. (1972) Carcass composition of the water buffalo (*Bubalus bubalis*). *Australian Journal of Agricultural Research*, **23**, 905–911.

Competition Commission (1985) *Animal Waste. A Report on the Supply of Animal Waste in Great Britain.* Competition Commission, London, UK.

EMEA (2004) *The European Agency for the Evaluation of Medicinal Products. Committee for Veterinary Medicinal Products, Diclofenac Summary Report.* EMEA/MRL/885/03-FINAL. EMEA, London, UK.

Green, R.E., Newton, I., Shultz, S., Cunningham, A.A., Gilbert, M., Pain, D.J. & Prakash, V. (2004) Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. *Journal of Applied Ecology*, **41**, 793–800.

Grubh, R.B. (1974) *The birds of Gir Forest (the ecology and behaviour of vultures in Gir Forest)*. PhD Thesis. University of Bombay, Bombay, India.

del Hoyo, J., Elliott, A. & Sargatal, J. (1994) *Handbook of the Birds of the World. 2. New World Vultures to Guineafowl.* Lynx Edicions, Barcelona, Spain.

IUCN (2004) IUCN Red list of Threatened Species. http://www.iucnredlist.org, accessed 5 December 2005. IUCN, Gland Switzerland.

Kalbfleisch, J.G. (1979) *Probability and Statistical Inference*, Vol. II. Springer-Verlag, New York, NY.

Kumar Ghosh, R. (1998) *Primary Veterinary Anatomy*, 2nd edn. Current Books International, Calcutta, India.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivasprasad, H.L., Ahmed, S., Chaudry, M.J.I., Arshad, M., Mahmood, S., Ali, A. & Khan, A.A. (2004) Diclofenac residues as the cause of vulture population declines in Pakistan. *Nature*, **427**, 630–633.

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 bengalensis* and long-billed *Gyps indicus* vulture populations. *Biological Conservation*, **109**, 381–390.

Shultz, S., Baral, H.S., Charman, S., Cunningham, A.A., Das, D., Ghalsasi, G.R., Goudar, M.S., Green, R.E., Jones, A., Nighot, P., Pain, D.J. & Prakash, V. (2004) Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proceedings of the Royal Society of London B*, (Supplement) **271**, S458–S460. DOI 10.1098/Rsbl.2004.0223.

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. *Biology Letters*, **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 2006. *Public Library of Science Biology*, **4**, 396–402.

Received 18 May 2006; final copy received 21 June 2006
Editor: Simon Thirgood