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Potential threat to Eurasian griffon vultures in Spain from veterinary use of the drug diclofenac

Rhys E. Green[1,2†], José A. Donázar[3], José A. Sánchez-Zapata[4] and Antoni Margalida[5,6*†]

[1]*Conservation Science Group, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK;* [2]*RSPB Centre for Conservation Science, Royal Society for the Protection of Birds, The Lodge, Sandy SG19 2DL, UK;* [3]*Department of Conservation Biology, Estación Biológica de Doñana – CSIC, Avenida de Américo Vespucio s/n, Seville E-41092, Spain;* [4]*Department of Applied Biology, Universidad Miguel Hernández, Ctra Beniel km 3·2, Alicante E-03312, Spain;* [5]*Faculty of Life Sciences and Engineering, University of Lleida, Lleida E-25003, Spain; and* [6]*Division of Conservation Biology, Institute of Ecology and Evolution, University of Bern, Bern 3012, Switzerland*

Summary

1. Spain holds > 95% of the European breeding population of the Eurasian griffon vulture *Gyps fulvus*. Vultures provide important ecosystem services in carcass removal and influence emissions of greenhouse gases. Despite the known toxicity of the non-steroidal anti-inflammatory drug diclofenac to this species and other *Gyps* vultures, in March 2013 the *Agencia Española de Medicamentos y Productos Sanitarios* (AEMPS) approved the use of two medicines containing diclofenac for veterinary use in horses, pigs and cattle in Spain.

2. To assess the potential impact of medicated ungulate carcasses on Eurasian griffon vulture populations in Spain, we first used information on the metabolism and elimination of diclofenac from medicated cattle and pigs to calculate residue levels in relation to time elapsed between dosing and death. Secondly, probabilities of the death of a vulture per meal were calculated based upon experimental studies of diclofenac toxicity. Finally, annual numbers of vulture deaths expected to be caused by diclofenac were obtained by multiplying the death rates per meal by the estimated numbers of vulture meals taken from expected numbers of medicated carcasses suggested by AEMPS.

3. Assuming that vultures feed on carcasses that were treated with diclofenac 8 h before the animal's death, the annual number of vulture deaths caused by diclofenac was estimated at 715–6389, depending upon the estimate of numbers of medicated carcasses assumed and the version of the dose–response model used. Using a density-independent simulation model of a vulture population, the expected rate of decline of the Spanish population of Eurasian griffon vultures caused by these deaths is 0·9–7·7% per year. A density-dependent simulation model also indicated substantial population-level effects. Formal estimates of precision and sensitivity analyses of effects of unmeasured variables highlight the uncertainty of estimates using currently available data.

4. *Synthesis and applications*. Due to the possibility of causing an important impact on vulture populations, our findings justify a precautionary ban on the veterinary use of diclofenac in Spain and encourage the use of meloxicam, a vulture-safe alternative drug. A programme of monitoring of non-steroidal anti-inflammatory drug contamination of ungulate carcasses available to vultures and of moribund and dead obligate and facultative avian scavengers would be needed to be confident that a damaging level of contamination is not present.

Key-words: avian scavengers, diclofenac, ecosystem services, Eurasian griffon vulture *Gyps fulvus*, population dynamics, simulation model, veterinary drugs

*Correspondence author. E-mail: antoni.margalida@iee.unibe.ch
†These authors contributed equally to this work.

Introduction

Gyps vultures are obligate scavengers that feed on carcasses of ungulates, including wild and domesticated species. By doing this, they modify ecosystem processes that influence human and animal health (Markandya *et al.* 2008) and greenhouse gas emissions (Morales-Reyes *et al.* 2015). They are characterized by a long life span (annual adult survival >0·9) and low fecundity (a maximum of one fledgling per pair per year, Del Hoyo, Elliott & Sargatal 1994). These demographic traits make their population trends very sensitive to additional mortality of adults caused by non-natural factors, as was illustrated by the recent rapid collapse of Asian vulture populations. Populations of three vulture species (oriental white-backed vulture *Gyps bengalensis*, Indian vulture *G. indicus* and slender-billed vulture *G. tenuirostris*) were reduced to near extinction in the Indian subcontinent within a decade (Prakash *et al.* 2003). The principal cause of these declines was the ingestion of tissues from carcasses of domesticated ungulates treated with the veterinary non-steroidal anti-inflammatory drug (NSAID) diclofenac shortly before death (Oaks *et al.* 2004; Green *et al.* 2006). Although only *Gyps* species are known from experiments to be affected by diclofenac, populations of other vulture species in the region have declined substantially. The Egyptian vulture *Neophron percnopterus* population in India declined by 80% between the early 1990s and 2003 and that of the red-headed vulture *Sarcogyps calvus* declined by 91% in the same period (Cuthbert *et al.* 2007). There is also circumstantial evidence of nephrotoxicity of diclofenac to the steppe eagle *Aquila nipalensis* (Sharma *et al.* 2014). Hence, it is possible that obligate and facultative scavenging birds other than *Gyps* vultures are affected by diclofenac as well as by other nephrotoxic NSAIDs, such as ketoprofen (Naidoo *et al.* 2010) and possibly also by flunixin (Zorrilla *et al.* 2015) and nimesulide (Cuthbert *et al.* 2016). It has been suggested that NSAIDs that are nephrotoxic to a particular bird species are those with long elimination times from the plasma in that species (Hutchinson *et al.* 2014).

Experimental dosing of captive vultures with diclofenac at levels corresponding to likely exposure from feeding on tissues from ungulates treated with a standard veterinary dose resulted in visceral gout followed by death at 28–56 h post-exposure (Oaks *et al.* 2004; Swan *et al.* 2006a; Naidoo *et al.* 2009). The toxicity of diclofenac, accompanied by kidney failure and visceral gout, has been demonstrated experimentally in oriental white-backed, African white-backed *G. africanus*, Cape griffon *G. coprotheres* and Eurasian griffon *G. fulvus* vultures. Diclofenac is highly toxic to *G. bengalensis* with a median lethal dose (LD_{50}) of 0·10–0·23 mg kg^{-1} vulture body weight (Swan *et al.* 2006a). The LD_{50} has not been determined for the other *Gyps* species. However, when doses of diclofenac expected from the results of Oaks *et al.* (2004) to kill 87% of *G. bengalensis* were administered to two *G. africanus* and three *G. fulvus*, all five birds died within two days of dosing and were found to have extensive visceral gout at necropsy (Swan *et al.* 2006a). Hence, it is likely that the toxicity of diclofenac to these other *Gyps* species is similar to that in *G. bengalensis*.

Following reports of the scientific evidence on the causes of the vulture population crash, the Government of India introduced a ban on the manufacture, importation and sale of veterinary diclofenac products in 2006, with similar measures being taken in Pakistan and Nepal soon afterwards and in Bangladesh in 2010 (Balmford 2013).

Despite these experiences in Asia, the Governments of Spain, Italy, Estonia, Latvia and the Czech Republic have recently approved the veterinary use of diclofenac. Spain is particularly important for the global conservation of avian scavengers because it holds >95% of the European vulture population, 97% of the world population of facultative scavengers such as the Spanish imperial eagle *Aquila adalberti* and internationally important numbers of red kites *Milvus milvus* (Del Hoyo, Elliott & Sargatal 1994). Although livestock husbandry systems and veterinary care in Spain differ markedly from those of Asian countries, there is currently insufficient information on these differences to be certain that the risk of animals treated with diclofenac in Spain becoming available to avian scavengers is negligibly low. Previous simulation modelling of *Gyps* vulture populations has shown that a very low prevalence of ungulate carcasses with a lethal level of diclofenac is sufficient to cause rapid population declines. Contamination of just 0·3–0·7% of ungulate carcasses with a lethal level of diclofenac was shown to be sufficient to cause the population of the oriental white-backed vulture *Gyps bengalensis* to decline at a rate of about 50% per year, as observed in India and Pakistan (Green *et al.* 2004).

Current sanitary legislation in Spain (Royal Decree 1632/2011) allows that dead livestock from intensive and extensive rearing can be available to avian scavengers in the field and/or feeding stations (Margalida *et al.* 2012). The risk of vultures ingesting carrion containing diclofenac is acknowledged by regulatory authorities to exist, but the possible number of vulture deaths has only been assessed in a report not subject to peer review (AEMPS & MAGRAMA 2014). This assessment put the number of Eurasian griffon vultures that might die per year in Spain as a result of the nephrotoxicity of diclofenac at 15–39 birds per year. The range of values given reflects minimum and maximum assumptions about how many carcasses of domestic ungulates medicated with diclofenac might be accessible to feeding vultures per year.

In this study, we used previously published information on the concentrations of diclofenac in ungulate tissues in relation to time elapsed since treatment, the toxicity of diclofenac to vultures and the numbers of medicated carcasses available to vultures proposed by AEMPS and MAGRAMA (2014) to estimate expected annual numbers

of vulture deaths. We then used estimates of national vulture population size and a simulation model of vulture demography and foraging ecology previously developed by Green *et al*. (2004), to estimate the potential effect of veterinary use of diclofenac on the vulture population trend.

Materials and methods

STUDY AREA AND VULTURE ECOLOGY

Our study concerns Spain, which holds more than 95% of the European breeding population of vultures. The study focuses on the Eurasian griffon vulture because it is the most numerous and the only one of these vulture species in which the toxicity of diclofenac has been established by experiments (Swan *et al*. 2006a). For the present study, we considered that the main species consuming carrion potentially contaminated by diclofenac is the Eurasian griffon vulture, because this species was seen in 84–100% of carrion consumption events by vultures in feeding stations (Cortés-Avizanda *et al*. 2012; Moreno-Opo *et al*. 2015).

The diet of vultures in Spain consists mainly of carcasses of domestic ungulates (cattle *Bos taurus*, sheep *Ovis aries* and pigs *Sus scrofa domesticus*) and, to a lesser extent, wild ungulates (roe deer *Capreolus capreolus*, wild boar *Sus scrofa*, red deer *Cervus elaphus*, Pyrenean chamois *Rupicapra pyrenaica*) (Donázar 1993; Margalida, Colomer & Sanuy 2011). For the Eurasian griffon vulture, carrion from domestic livestock, mainly cows, pigs and sheep, constitutes the main food (Donázar 1993). In addition, food is provided by deaths of free-ranging ungulates in extensive grazing management systems. Spain has a tradition of artificial feeding of avian scavengers at feeding stations or 'muladares' (see review in Donázar, Margalida & Campión 2009), at which carcasses of both intensively and extensively managed livestock are provided to avian scavengers.

A Eurasian griffon vulture can ingest an average of 1·2 kg in each meal (Donázar 1993). On average, vultures locate carrion and begin to feed 6 min after death or deposition of the carcass at a feeding station (range 0–480 min) and 60 min (range 0–1800 min) for carcasses placed at unpredictable times at random locations (Cortés-Avizanda *et al*. 2012).

CALCULATION OF THE PROBABILITY OF A VULTURE DYING AS A RESULT OF A MEAL TAKEN FROM A CARCASS MEDICATED WITH DICLOFENAC

Green *et al*. (2006) estimated the mean concentration of diclofenac in the tissues of cattle *Bos taurus* and *B. indicus* in relation to the time elapsed between the administration of the last dose of diclofenac and the death of the treated animal. The length of this interval is the main factor affecting the concentration of diclofenac in the carcass of a treated animal after death. In addition, experiments have shown that the time elapsed between the death of the ungulate and the consumption of its carcass by vultures has only a small effect on diclofenac concentration (Taggart *et al*. 2006). The data used for the analyses for European cattle *B. taurus* came from experiments conducted to establish maximum residue limits for diclofenac (EMEA 2004).

Green *et al*. (2006) fitted two-phase piecewise exponential regression models of diclofenac concentration in relation to the time elapsed between treatment and slaughter to the data for different tissues of *B. taurus*. We used a similar procedure to obtain an equivalent relationship between the mean diclofenac concentration averaged over all of the tissues of the animal likely to be eaten by vultures in relation to the time elapsed between treatment and slaughter for the domestic pig. The mean concentrations of diclofenac for various tissues, measured at 3, 12 and 24 h after diclofenac treatment, were obtained from EMEA (2004). An exponential regression model was fitted, but with only one phase because concentration was measured at too few times for fitting a piecewise model. Because estimates were only made for treatment–death intervals up to 50 h, the lack of a two-phase model is unlikely to have much effect on the estimates. Concentrations were then averaged over tissues likely to be eaten by vultures in relation to time after treatment using the same procedure as that of Green *et al*. (2006).

We used this relationship, in combination with the dose–response curves for vulture mortality from Swan *et al*. (2006a), to obtain the relationship between the proportion of vultures killed by diclofenac and the time elapsed between treatment of an ungulate and its death. Although the method used was similar to that used for *Gyps bengalensis* by Green *et al*. (2006), the dose of diclofenac per unit of vulture body weight had to be calculated separately for *Gyps fulvus*. We assumed that a Eurasian griffon vulture meal weighs 1·2 kg and that the mean body weight W of the vulture is 7·4 kg (Donázar 1993). The dose in mg kg^{-1} relative to vulture body weight for a vulture feeding on mixed tissues from a contaminated ungulate was taken to be the product of 1·2 and the mean concentration of diclofenac in mixed edible tissues (in mg kg^{-1}) divided by 7·4. The expected proportion of vultures killed was calculated from the dose–response functions given by Swan *et al*. (2006a). We used each of the two versions of the dose–response function given by Swan *et al*. (2006a), depending upon whether the datum from an outlier (Vulture 11) in the experiments of Oaks *et al*. (2004) was included or not.

CALCULATION OF THE ANNUAL NUMBER OF VULTURE MEALS TAKEN FROM UNGULATE CARCASSES MEDICATED WITH DICLOFENAC

The minimum and maximum numbers of carcasses of domestic ungulates of each species medicated with diclofenac and assumed to be available to vultures annually were obtained from AEMPS & MAGRAMA (2014), using *Tabla 1* of their *Anejo III*. The numbers of carcasses were rounded to integers in AEMPS & MAGRAMA (2014), but we give the calculated values to two decimal places so as not to propagate the rounding errors into later calculations. The first two columns of our Table 1 show these values. We converted the minimum and maximum proposed annual numbers of ungulate carcasses medicated and consumed by vultures to total weights, summed across all medicated carcasses, using typical mean weights of ungulates (Table 1). Not all of the total weight of a carcass is eaten by scavengers. A study of the proportion of the carcass weight of wild ungulates of various species completely consumed by avian and mammal scavengers in Poland found that 81·4% was eaten for the European bison *Bos bonasus*, 80·5% for red deer and 79·4% for wild boar (Selva 2004). In South Africa, the proportion consumed ranges from 90·6% for medium-sized ungulates to 79·1% for large ungulates and megaherbivores (Moleón *et al*. 2015). We assumed that these relatively constant proportions (around 80%) can also be applied

Table 1. Estimated minimum and maximum annual numbers of ungulate carcasses medicated with diclofenac and available to vultures based upon the results in AEMPS & MAGRAMA (2014) [columns (A) and (B)]. Columns to the right of these use species-specific ungulate weights (column C) to calculate the total weight of medicated carcasses (D, E) and the numbers of vulture meals (F, G) according to the arithmetic specified by the numerical labels in the column headings. The numbers of meals were multiplied by the proportions of vultures killed by eating mixed tissues from an animal that died 8 h after the treatment, assuming a dose–response model fitted with (columns H, I) and without (J, K) the datum from the outlier Vulture 11 (v11) to give estimates of annual numbers of vulture deaths caused by diclofenac. For the purposes of the calculation, the mean concentration of diclofenac in the tissues of horses was assumed to be the same as that for European cattle. Numbers in parentheses in the vulture deaths columns are 95% confidence intervals based upon bootstrap analyses that only take into account the uncertainties shown in Fig. 1 in (a) in the relationship of diclofenac concentration in ungulate tissue with time between dosing and death, and (b) the relationship of the proportion of vultures killed with diclofenac concentration in the vulture's meal. The terms 'intensive' and 'extensive' refer to intensively and extensively managed livestock

Animal type	Carcasses treated with diclofenac & accessible to vultures (minimum) (A)	Carcasses treated with diclofenac accessible to vultures (maximum) (B)	Weight of carcass per animal (kg) (C)	Minimum total weight of medicated carcasses accessible to vultures (kg) (D = A × C)	Maximum total weight of medicated carcasses accessible to vultures (kg) (E = B × C)	Minimum medicated vulture meals per year (80% cadaver wt eaten) (F = D × 0·8/1·2)	Maximum medicated vulture meals per year (80% cadaver wt eaten) (G = E × 0·8/1·2)	Vulture deaths per year: Minimum meals and dose response with v11 (H = F × proportion killed from Fig. 1)	Vulture deaths per year: Maximum meals and dose response with v11 (I = G × proportion killed from Fig. 1)	Vulture deaths per year: Minimum meals and dose response without v11 (J = F × proportion killed from Fig. 1)	Vulture deaths per year: Maximum meals and dose response without v11 (K = G × proportion killed from Fig. 1)
Cow (intensive)	1·25	3·18	425	530	1350	354	900	213 (91–304)	542 (232–774)	122 (4–226)	310 (10–574)
Cow (extensive)	2·37	4·75	330	783	1567	522	1044	314 (135–449)	629 (269–897)	180 (6–333)	360 (12–667)
Pig (intensive)	55·61	92·15	200	11 122	18 430	7415	12 287	2781 (898–5249)	4609 (1489–8699)	364 (8–1094)	603 (14–1813)
Pig (extensive)	11·53	23·06	100	1153	2306	769	1538	288 (93–544)	577 (186–1089)	38 (1–113)	76 (2–227)
Horse	0·10	0·17	500	50	83	33	55	20 (9–29))	33 (14–48)	12 (0–21)	19 (1–35)
All categories								3617 (1452–6187)	6389 (2914–11047)	715 (139–2120)	1368 (274–3781)

to domesticated ungulates fed upon by vultures, and therefore take the proportion of the weight of carcasses of domesticated cattle, pigs and horses *Equus caballus* available to vultures. Thus, we multiplied the total weight of all medicated carcasses by 0·8 to give the total weight of tissue from medicated carcasses eaten by vultures per year (Table 1). Then, we divided this total weight eaten by vultures by the mean weight of a vulture meal (1·2 kg) to give the annual number of vulture meals obtained from medicated carcasses.

CALCULATION OF THE ANNUAL NUMBER OF VULTURE DEATHS ATTRIBUTABLE TO DICLOFENAC

We first calculated expected numbers of vultures killed annually by diclofenac by following AEMPS & MAGRAMA (2014) in assuming that ungulate carcasses eaten by vultures were from animals medicated 8 h before they died. The proportions of vultures killed at 8 h after diclofenac treatment for a given ungulate species were multiplied by the numbers of meals from medicated ungulates to estimate the annual number of vulture deaths. We assumed that the relationship between the concentration of diclofenac in the tissues of horses and the time elapsed between drug administration and death was the same as that for cattle. It should be noted that our results are not sensitive to this assumption because of the small number of medicated carcasses of horses.

We stress that we used the value of 8 h between dosing and death only to make our calculations comparable to those of AEMPS & MAGRAMA (2014). The duration of the period between dosing and death has not been estimated for animals available as vulture food in Spain and was only assumed by AEMPS & MAGRAMA (2014). To illustrate the sensitivity of our results to this uncertainty, we repeated the calculations for a range of times between medication and ungulate death between 0 and 50 h. We took 50 h as the upper limit because diclofenac concentrations in cattle and pigs treated with the drug have fallen to very low levels unlikely to cause vulture mortality by that time (Green *et al*. 2006).

CALCULATION OF ANNUAL PER CAPITA MORTALITY RATES CAUSED BY DICLOFENAC

We converted the numbers of vulture deaths per year to annual per capita death rates D by dividing them by the estimated total number of Eurasian griffon vultures in Spain. We took the number of breeding pairs of Eurasian griffon vultures in Spain to be 25 075 (50 150 individuals), which is the mid-point of the range (24 609–25 541 pairs) obtained from a census in 2008–2009 (del Moral 2009). We used a density-independent Leslie matrix model with six age categories (juveniles, immatures aged 1–4 years and adults >4 years old), equal sex ratio, stable total full-grown population size (free-flying immatures and adults combined) and stable age structure to estimate the number of free-flying individuals (all age classes combined) per breeding pair. For this model, we required values for the mean annual breeding productivity K (0·62 fledglings per pair per year from del Moral 2009), mean age at first reproduction B (5 years, Cramp & Simmons 1980) and mean annual survival rate of adults in the absence of diclofenac S_0. Using mark–resighting data, Martínez-Abraín *et al*. (2012) estimated the annual survival of adult griffon vultures in the province of Castellón, Spain, to be 0·939 ± 0·026 (±1 SE) during periods when survival was not adversely affected by the removal of food supplies and collisions with wind turbines. Green *et al*. (2004) considered that a plausible range of values for S_0 in *Gyps* vultures, based upon studies of this and other large-bodied avian scavengers, was S_0 = 0·90–0·97 (see also Sarrazin *et al*. 1994; Grande *et al*. 2009; Margalida, Colomer & Oro 2014). We therefore selected S_0 = 0·95 as a reasonable value for our analysis. Given these values of K, B and S_0, and assuming that annual survival of immature age classes was constant with respect to age, the mean annual survival rate of immature vultures S_i was calculated as $(2(1 - S_0)/K)^{1/B} = 0{\cdot}694$. Using these parameter values and assumptions, the Leslie matrix model gives a total population size of all age classes of 86 594 individuals.

CALCULATION OF MORTALITY RATES CAUSED BY DICLOFENAC PER VULTURE MEAL

If a typical vulture takes a meal at mean intervals of F days, of which a proportion of meals C contains enough diclofenac to kill the bird, the expected annual probability of dying from diclofenac poisoning is $D = 1 - (1 - C)^{(365{\cdot}25/F)}$ (Green *et al*. 2004). By rearranging this expression, the annual per capita death rates D can be converted to mean proportions of vultures killed per meal C as $C = 1\text{-}\exp(\log_e(1\text{-}D)F/365{\cdot}25)$.

The mean interval between vulture meals F has not been estimated directly from field data for any *Gyps* vulture species, but vulture experts cited by Green *et al*. (2004) ranged it between 2–4 days for *Gyps* vultures. It was assumed that the mean wet weight of tissue in a Eurasian griffon vulture meal is 1·2 kg (Donázar 1993). We therefore estimated F by dividing mean meal weight by the daily food requirement of this species. We calculated the daily food requirement from the mean body weight W of Eurasian griffon vulture which we took to be 7·4 kg (Donázar 1993). We used the method of Mundy *et al*. (1992) to estimate the daily energy requirements in kJ of a vulture of this size as $668{\cdot}4W^{0{\cdot}622}$. Assuming that the tissue consumed has an energetic content of 6000 kJ kg^{-1} (wet weight) and that the assimilation efficiency of vultures is 0·86, this gives a daily food requirement of 0·45 kg per day. Dividing the meal weight (1·2 kg) by the daily food requirement gives an estimate of 2·67 days for the mean interval F between meals, which is close to the mid-point of expert opinion on F reported by Green *et al*. (2004).

CALCULATION OF RATES OF VULTURE POPULATION DECLINE CAUSED BY DICLOFENAC

The mean probabilities of death per meal C caused by diclofenac, calculated as described above, are equivalent to the proportions of carcasses with lethal levels of diclofenac C in the density-independent population model of the impact of diclofenac on vulture population trend of Green *et al*. (2004). We therefore used the model of Green *et al*. (2004) with the mean death rates per meal calculated above to obtain expected rates of vulture population decline.

The population model of Green *et al*. (2004) requires values for mean age at first reproduction B, mean interval between vulture meals F and mean annual survival rate of adults in the absence of diclofenac S_0. These are the same parameters as those required for the Leslie matrix model and the calculations described previously, and we used the same estimates of B, F and S_0. The model also requires a value for the duration of parental

care during reproduction M. We used an estimate $M = 187$ days derived from studies of *Gyps fulvus* in the Pyrenees (Leconte & Som 1996).

We assessed the potential effects of density dependence on our conclusions using the model of density-dependent reproduction of Eurasian griffon vultures in Spain proposed by García-Ripollés & López-López (2011). Density dependence would be expected to lead to some compensatory enhancement of demographic rates induced by the additional mortality caused by diclofenac poisoning and this would reduce its effect on population growth rate. We used the function relating to breeding productivity, relative to that at carrying capacity, to population size, applying the equation used in *Vortex* (Miller & Lacy 2005): $P(N) = \left(P(0) - (P(0) - P(K)) \cdot \left(\frac{N}{K}\right)^1\right)$, where $P(N)$ is the percentage of females that breed at population size N, $P(K)$ the percentage of breeding when the population is at carrying capacity (K) and $P(0)$ the percentage of breeding when the population is close to zero (see García-Ripollés & López-López 2011; Margalida *et al.* 2015). We used the parameter values of the Iberico Massif subpopulation (García-Ripollés & López-López 2011). We then used the population model and parameter values described above to estimate the effect of additional mortality caused by diclofenac and adapted it by making reproduction dependent upon population size. We first checked that the simulated population without additional mortality stabilized at the carrying capacity defined by the parameter K. We then started the population at its carrying capacity and imposed additional per capita mortality rates due to diclofenac derived from mean proportions of vultures killed per meal C as described above. We simulated the population size for 2000 years.

ASSESSMENT OF UNCERTAINTY OF MODEL OUTPUTS AND THEIR SENSITIVITY TO ASSUMED PARAMETER VALUES

Our calculations require values to be estimated or assumed for many parameters, but only for some of them was it possible for us to make formal estimates of uncertainty. However, we were able to quantify the uncertainty in parameter values for two key processes in our model: with the relationship of the concentration of diclofenac in cattle tissues and the probit dose–response model of the proportion of vultures killed in relation to diclofenac dose.

We quantified uncertainty associated with the relationship of the concentration of diclofenac in cattle tissues to the time between dosing and death, using the experimental data for diclofenac concentrations in five tissues (intestine, kidney, liver, muscle and fat) in Figure 1 of Green *et al.* (2006). For a given tissue type, we calculated the residual difference between each observed value of the logarithm of diclofenac concentration and value expected from the tissue-specific maximum-likelihood regression models presented in Table 1 of Green *et al.* (2006). We then resampled these residuals for a given tissue at random, with replacement, and added them to the values of the logarithm of diclofenac concentration expected for each observation from the regression model for that tissue. The same type of regression model was then fitted to this bootstrap sample to give a bootstrapped set of regression parameter estimates. This resampling and parameter estimation was performed 10 000 times and repeated for each of the five tissues. The model parameters for each bootstrapped replicate were used to calculate the expected mean diclofenac concentration value for each tissue at a given time between dosing and death. Following Green *et al.* (2006), a weighted mean of the expected values was calculated across the five tissues for each bootstrap replicate using proportion by weight of the edible cadaver contributed by each tissue as weights. This resulted in 10 000 bootstrap replicate estimates of the mean concentration of diclofenac in mixed edible tissues from the whole cadaver. The analysis was repeated at hourly intervals of the time between dosing and death up to 50 h.

We also quantified uncertainty associated with the two-parameter probit dose–response model of the proportion of vultures killed in relation to diclofenac dose of Swan *et al.* (2006a) using the bootstrap method described in Green *et al.* (2007). We then calculated, for each bootstrap replicate, the dose of diclofenac per unit vulture body weight as the expected concentration in the tissues of the whole cadaver multiplied by meal size (1·2 kg) and divided by mean vulture body weight (7·4 kg) and used this with bootstrap values of the dose–response model parameters to calculate a bootstrap value of the expected proportion of vultures killed per meal. This was done for each of the 10 000 sets of bootstrap replicates and for hourly intervals of the interval between dosing and death up to 50 h. For each hour, we took the bounds of the central 9500 bootstrap values as the 95% confidence limits of the expected proportion of vultures killed per meal.

We used the bootstrap distributions of the proportion of vultures killed per meal multiplied by the number of meals from medicated cadavers and the estimated total number of vultures in Spain to define 95% confidence intervals for expected annual numbers of vulture deaths caused by diclofenac. We do not have access to the data for individual animals from the study of diclofenac in the tissues of pigs in relation to time between dosing and death, so we assumed that the sampling variation in logit-transformed death rate per meal was the same for pigs as that determined by the bootstrap method described above for cattle.

For some other parameters for which we did not have formal estimates of uncertainty, we conducted a sensitivity assessment in which we increased and decreased the selected parameter value by 10% whilst keeping all other parameters at their usual values. We calculated the change in the estimated annual number of vulture deaths for a 10% change in the selected parameter by averaging the absolute percentage change in the number of deaths produced by a 10% increase and a 10% decrease. In all cases, these two values were very similar.

Results

PROBABILITY OF A VULTURE DYING AS A RESULT OF A MEAL TAKEN FROM A CARCASS MEDICATED WITH DICLOFENAC

Whether feeding on cattle or pigs, relationships between the expected proportion of Eurasian griffon vultures killed per meal in relation to the time elapsed between diclofenac treatment of an ungulate and its death show that more than half of the vultures feeding on a carcass were expected to die if the animal died immediately after diclofenac treatment, but this proportion declined rapidly with increasing time since treatment (Fig. 1).

The results indicate that the proportion of vultures killed by feeding on the carcass of a contaminated cow

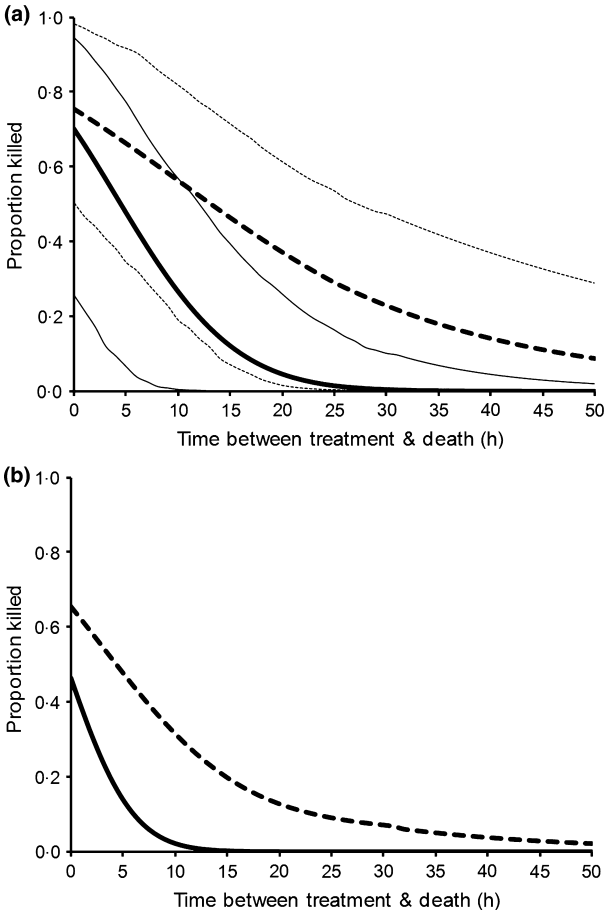

Fig. 1. Proportion of Eurasian griffon vultures *Gyps fulvus* expected to be killed upon eating a meal of 1·2 kg (wet weight) of mixed tissues from carcasses of (a) European cattle *Bos taurus* and (b) pigs *Sus scrofa domesticus* medicated with diclofenac in relation to the time (hours) between the administration of the last dose and the death of the animal. The thick lines show the results for a dose–response model fitted to data including (dashed line) and excluding (solid line) an outlier (Vulture 11). The thin lines in (a) represent 95% confidence limits.

8 h after the treatment would be 0·602 for the dose–response model that included Vulture 11 and 0·345 for the model that excluded it (Fig. 1). The equivalent proportions of vultures killed by feeding on a contaminated pig 8 h after the treatment would be 0·375 for the dose–response model that included Vulture 11 and 0·049 for the model that excluded it (Fig. 1). It is evident that the proportion of vultures killed was strongly affected by the time between dosing and death of the treated ungulate. In addition, the confidence limits for the proportions of birds killed at a given dosing–death interval are wide.

EXPECTED ANNUAL NUMBER OF VULTURE DEATHS ATTRIBUTABLE TO DICLOFENAC POISONING

If the dose–response model fitted including the outlier Vulture 11 was used, the expected annual number of vulture deaths was 3617–6389, the range being for the minimum and maximum number of medicated carcasses

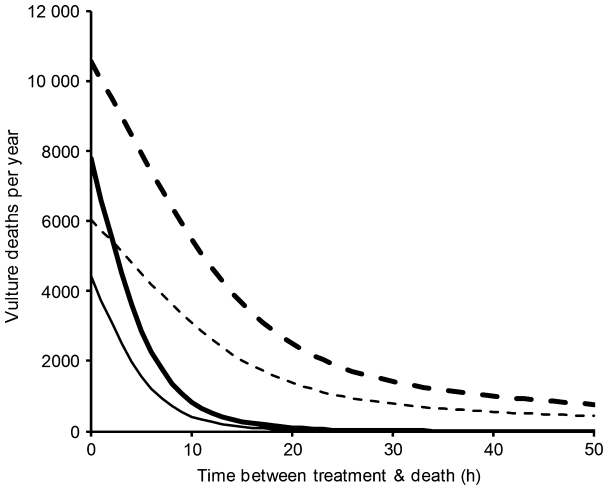

Fig. 2. Expected annual number of deaths of Eurasian griffon vultures *Gyps fulvus* in Spain in relation to the time between the medication of an ungulate and its death. Results are shown for the minimum (thin lines) and maximum (thick lines) numbers of medicated carcasses assumed by AEMPS & MAGRAMA (2014) and for a dose–response model fitted to data including (dashed line) and excluding (solid line) an outlier (Vulture 11).

assumed (Table 1). If the outlier was excluded when fitting the dose–response model, the expected number of deaths was 715–1367. As was the case for the proportions of vultures killed at a given dosing–death interval, the confidence intervals for the numbers of deaths were wide and the expected number of deaths was strongly affected by the time between medication and ungulate death (Fig. 2).

ANNUAL PER CAPITA MORTALITY RATES CAUSED BY DICLOFENAC POISONING

Dividing annual numbers of expected vulture deaths from Table 1 by the estimated population size (86 594 individuals) gives per capita annual death rates due to diclofenac poisoning. If the dose–response model fitted including the outlier Vulture 11 was used, the rates were 0·0417–0·0738, with the range being for the minimum and maximum assumed numbers of medicated carcasses. If Vulture 11 was excluded when fitting the dose–response model, the per capita rate was 0·0083–0·0158.

RATES OF VULTURE POPULATION DECLINE CAUSED BY DICLOFENAC POISONING

Using these values of annual per capita death rates and $F = 2{\cdot}67$ gave mean probabilities of death per meal C caused by diclofenac of 0·000312–0·000560 if the dose–response model fitted including the outlier Vulture 11 was used and rates of 0·000061–0·000116 if the outlier was excluded.

For these values of C, the density-independent population model of Green *et al.* (2004) gave expected rates of

population decline if the dose–response model fitted including the outlier Vulture 11 was used of 4·3–7·7% per year. If the outlier was excluded when fitting the dose–response model, the expected rate of population decline was 0·9–1·6% per year.

ASSESSMENT OF UNCERTAINTY OF MODEL OUTPUTS AND THEIR SENSITIVITY TO ASSUMED PARAMETER VALUES

As we reported above, the precision of our estimates of the proportion of vultures killed per meal is quite low. This also applies to the estimates of numbers and proportions of vultures killed per year, which are derived from the death rate per meal from a medicated carcass. This means that the values we give could be considerably lower or higher than our best estimates.

There was a strong effect on our estimates of the inclusion or exclusion of the outlier Vulture 11 in the fitting of the dose–response model. For reasons given by Swan *et al.* (2006a), it is unclear whether this outlier should be included or not, so uncertainty from this source must be accepted.

No estimates are available of the mean and distribution of the interval between dosing of an ungulate with diclofenac and its death for those animals whose carcasses are eaten by vultures. The duration of this interval has a strong effect on the estimates of death rate per meal and annual numbers of vultures killed. Our sensitivity calculation showed that a 10% increase in the interval between dosing of an ungulate with diclofenac and its death for those animals whose carcasses are eaten by vultures (from the 8-h assumed value) decreased the estimate of the annual number of vultures killed by diclofenac by 6·5% when the dose–response relationship including Vulture 11 was used and by 32·4% when the model excluding Vulture 11 was used.

The value of annual survival used to calculate the size of the total vulture population from the count of breeding pairs had a modest effect on the estimated number of vulture deaths caused by diclofenac. In our sensitivity assessment for this parameter, we increased and decreased survival by 0·005 (10% of mortality rather than survival) because differences as large as 10% in the true value of adult survival are not plausible. Increasing adult survival by this amount from the assumed value of 0·95 decreased the estimate of the number of vultures killed annually by diclofenac by 1·2%.

The value of annual breeding productivity used to calculate the size of the total vulture population from the count of breeding pairs had a slightly larger effect on the estimated number of vulture deaths caused by diclofenac. In our sensitivity assessment for this parameter, an increase of 10% compared with the assumed value of 0·62 increased the estimate of the number of vultures killed annually by diclofenac by 3·5%.

We did not perform sensitivity assessments for some parameters because the consequences of errors are obvious. The number of diclofenac-contaminated vulture meals would obviously increase by 10% for a 10% increase in any of the following: the number of medicated carcasses, the proportion of carcasses accessible to vultures, the average weight of an ungulate carcass and the mean proportion of the carcass eaten by vultures.

Effects of errors in some other parameter values tend to cancel themselves out. For example, an increase in the average weight of a vulture meal would decrease the number of meals taken annually from medicated carcasses, but it would increase the dose of diclofenac per unit vulture body weight and the death rate per meal. The extent to which these effects cancel out would depend upon the interval between dosing and death of the ungulate which is unknown.

ASSESSMENT OF EFFECTS ON POPULATION OUTCOMES OF INCLUDING DENSITY-DEPENDENT REPRODUCTION IN THE POPULATION MODEL

We evaluated the population outcomes for the ranges of the mean probabilities of death per meal C caused by diclofenac, described above, using the population model with density-dependent reproduction (see Materials and methods). The simulated population declined to extinction in model runs for all values of C calculated when the dose–response model fitted including the outlier Vulture 11 was used. When we used the dose–response model fitted excluding the outlier Vulture 11, the simulated population declined to extinction if C was >0·000065. The population persisted with C at the lowest value we calculated under this scenario (0·000061), but stabilized at a level 39% lower than carrying capacity.

Discussion

The expected numbers of vulture deaths per year from our calculations (715–6389) are much larger than the numbers of vulture deaths (15–39 deaths per year) calculated by AEMPS & MAGRAMA (2014). This large difference is because the AEMPS and MAGRAMA study assumed that vultures only died from diclofenac poisoning if a threshold dose of diclofenac per unit vulture body weight was exceeded. Diclofenac doses even slightly below this level were assumed not to kill any vultures. The available information on the toxicity of diclofenac to *Gyps* vultures shows that the simplified procedure used for the calculation of numbers of vulture deaths is likely to lead to a substantial underestimation of vulture mortality. The dose–response curves derived from the experimental study of Oaks *et al.* (2004) by Swan *et al.* (2006a) indicate that the lethal dose of diclofenac is variable among individuals and has an S-shaped form, rather than being a step function. For this reason, diclofenac dose levels substantially lower than the LD_{50}, or other thresholds used by AEMPS & MAGRAMA (2014), kill a proportion of vultures. Studies of the probability of the density distribution of

concentrations of diclofenac in the tissues of domestic ungulates available to vultures in the field in India showed a broad, left-skewed distribution (Green *et al*. 2007; Cuthbert *et al*. 2011). Hence, there is a high proportion of ungulate carcasses with diclofenac concentrations well below the LD_{50} for vultures that are nonetheless capable of causing the deaths of many vultures in India. This point is illustrated graphically in Figure 4 of Cuthbert *et al*. (2011).

Our results suggest that the potential impact of diclofenac on Eurasian griffon vulture populations could be substantial. The estimated numbers of deaths lead to expected population decline rates in a density-independent population model of 4·3–7·7% per year or 0·9–1·6% per year, depending upon the dose–response model used. These values are lower than the annual rate observed in the Indian subcontinent (~50% per year, Green *et al*. 2004), but some of them are rapid compared with some rates of decline of European bird species that are of significant concern to conservationists. It can be argued that accurate models of the population impact of additional mortality should include density dependence. Available information on population processes in *Gyps* vultures and other large birds of prey indicates that density dependence largely affects fecundity rather than survival (Fernández, Azkona & Donázar 1998; Newton 1998). Using the particular form of density dependence of reproduction (see Materials and methods), we found that the substantial effects of additional mortality potentially caused by diclofenac remained after density dependence was taken into account. This result is not unexpected, given that population growth rate in long-lived birds like vultures and other large raptors is highly sensitive to changes in adult survival and, in general, less sensitive to changes in fecundity and immature survival (Grande *et al*. 2009; Margalida *et al*. 2015). However, the strength and form of density dependence is not well quantified for vultures. For these reasons, we argue that the use of a density-independent model is appropriate and precautionary.

Our estimates are highly sensitive to the time assumed to elapse between the administration of diclofenac and the death of the treated ungulate, which has not been estimated in Spain. AEMPS & MAGRAMA (2014) assumed a value of 8 h for this interval, but it is not clear that this value is well substantiated. The numbers of vulture deaths would be much higher if the time interval was shorter and much lower if it was longer. In reality, the interval has not been estimated and is likely to differ substantially among ungulate carcasses and to be dependent on the local circumstances of veterinary treatment. Hence, we do not argue that we have estimated the effects of diclofenac on vulture populations in Spain with any degree of certainty. Rather, we have estimated what they could potentially be for a plausible set of assumptions about unmeasured variables, the most influential of which is the time elapsed between the administration of diclofenac and the death of the treated ungulate. Measurements of diclofenac residues in ungulate tissues collected from feeding stations in Spain provide the only practical way to estimate this key variable.

Our results are also sensitive to which version is used for the relationship between diclofenac dose and the probability of death of a vulture ingesting contaminated tissues. The relationship between the proportion of vultures killed and the dose of diclofenac per unit of vulture body weight has only been measured for *Gyps bengalensis* and markedly different versions of the dose–response model were obtained, depending upon whether or not the datum from an outlier was included (Swan *et al*. 2006a). Despite a detailed consideration, Swan *et al*. (2006a) were unable to conclude that the outlying datum for Vulture 11 should be included or excluded. For this reason, we recommend that the uncertainty attributable to this source is unavoidable.

The dose–response relationship has not been measured for the Eurasian griffon vulture, but an experimental study indicates that this species is as susceptible to the drug as *Gyps bengalensis* (Swan *et al*. 2006a). Hence, it is prudent to assume that low doses of diclofenac will be toxic similar to Eurasian griffon vultures in Spain. It may also be that diclofenac affects other vulture species, such as bearded *Gypaetus barbatus*, Egyptian *Neophron percnopterus* and cinereous vultures *Aegypius monachus*, and facultative avian scavengers, such as the red kite, Spanish imperial eagle, golden eagle *Aquila chrysaetos* and black kite *Milvus migrans*. Circumstantial evidence suggests that the steppe eagle which is in the same family (Accipitridae) as vultures, kites and other eagles, may be susceptible to diclofenac (Sharma *et al*. 2014).

We relied upon the estimates of the numbers of potentially available diclofenac-medicated ungulate carcasses provided by AEMPS & MAGRAMA (2014), but these are estimations and, from the information given in the report, we were unable to assess how well founded they are. It can be argued that regulatory controls on veterinary treatments and carcass disposal in Spain will be strict enough to prevent carcasses contaminated with diclofenac being fed upon by vultures. However, this cannot be regarded as certain, given a recent observation of a Eurasian griffon vulture found dead in Spain with visceral gout associated with high levels of flunixin, another veterinary NSAID, in its liver and kidneys (Zorrilla *et al*. 2015). This observation indicates that at least one carcass contaminated with a veterinary drug has been accessible to vultures in Spain, in spite of the regulations. If this occurs more widely, there might be a real risk from veterinary NSAIDs to Spanish avian scavenger populations (Zorrilla *et al*. 2015). Recent changes in sanitary policies (Margalida *et al*. 2012) will allow a greater availability of domestic ungulate carcasses to vultures at feeding stations and some of the ungulate carcasses involved will come from intensive husbandry systems. Hence, the potential risk of vulture intoxication should not be disregarded. However, it is also the case that domestic ungulate car-

casses are an important source of food for vultures and are required for the maintenance of their populations and the ecological processes they provide (DeVault, Rhodes & Shivik 2003; Wilson & Wolkovich 2011; Moreno-Opo & Margalida 2013). Vultures provide important ecosystem services, and vulture declines would have far-reaching consequences for the ecosystems they share with humans (Margalida & Colomer 2012; Ogada, Keesing & Virani 2012; Moleón *et al*. 2014). For example, the scavenging services provided by vultures in Spain may save tens of thousands of tonnes of greenhouse gas emissions and up to $50 million (Morales-Reyes *et al*. 2015).

The presence of pharmaceuticals in the environment and evidence of their having significant effects on the physiology and demography of wild species are increasing (Arnold *et al*. 2014). The example of diclofenac and vultures shows that the current system of environmental risk assessment and regulation of veterinary pharmaceuticals does not take a sufficiently quantitative approach to risk assessment (Margalida *et al*. 2014). Measures to reduce veterinary use of diclofenac in India were made easier by the experimental demonstration that the off-patent NSAID meloxicam did not cause death or hyperuricemia in African or Asian *Gyps* vultures and other scavenging birds at levels found in the tissues of ungulates given a standard veterinary dose and therefore represented a suitable alternative vulture-safe drug (Swan *et al*. 2006b; Swarup *et al*. 2007). Over a period of about four years after the ban analysis of the tissues from dead cattle showed that diclofenac was at least partially replaced by meloxicam (Cuthbert *et al*. 2014). Such a ban on veterinary use of diclofenac and encouragement of the use of meloxicam would now be appropriate as a precautionary measure in Spain. Given the uncertainty about whether regulations will prevent diclofenac and other NSAIDs from being present at toxic levels in the food of vultures, it will be necessary to monitor ungulate carcasses for the presence and concentration of diclofenac because illegal veterinary use can occur, even if there is a ban. Illegal veterinary use of diclofenac after the ban was widespread in India, though it has decreased over time (Cuthbert *et al*. 2014). Because a very low prevalence of carcasses with lethal levels of diclofenac can cause rapid vulture population declines (Green *et al*. 2004), annual monitoring of diclofenac contamination in large numbers of ungulate carcasses will be necessary, focusing on carcasses of pigs and cattle provided in feeding stations and those from intensive rearing. Vultures and facultative scavenging birds received in raptor recovery centres should be used as sentinels to detect possible cases of NSAID ingestion and toxicity. The responsible authorities in Spain should immediately design and implement monitoring programmes.

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Data accessibility

All data used in the modelling were taken from the published sources cited in the Materials and methods.

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