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Food safety in scavenger conservation: Diet-associated exposure to livestock pharmaceuticals and opportunist mycoses in threatened Cinereous and Egyptian vultures

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ABSTRACT

Pharmaceuticals from veterinary treatments may enter terrestrial food webs when medicated livestock are available to wildlife in supplementary feeding stations aimed at the conservation of endangered scavengers. Here, we hypothesized that the exposure risk to livestock fluoroquinolones, as indicators of pharmaceutical burden in food, is related to the variable reliance of scavengers on domestic versus wild animal carcasses. Since the misuse of broad-spectrum antibiotics is a major predisposing factor for opportunistic mycoses, we evaluated disease signs potentially associated with diet-dependent drug exposure in nestlings of two threatened vultures. A greater occurrence (100%, n=14) and concentration of fluoroquinolones (mean ± SD=73.0 ± 27.5 μg L^{-1}, range=33.2–132.7), mostly enrofloxacin, were found in Cinereous vultures, *Aegypius monachus*, due to their greater dependence on livestock carcasses than Egyptian vultures, *Neophron percnopterus* (fluoroquinolones occurrence: 44%, n=16, concentration: 37.9 ± 16.6 μg L^{-1}, range=11.5–55.9), which rely much more on carcasses of wild animals (42% of remains vs. 23% in the cinereous vulture). The chaotic, chronic and pulsed ingestion of these drugs throughout nestling development is proposed as one of the most plausible explanations for the high occurrence and intensity of oral *Candida*-like lesions in nestling vultures. The high occurrence of fluoroquinolone residues and disease hindered the probing of a cause-effect relationship between both factors in individual vultures. This relationship could be evaluated through a population-based approach by sampling vultures not exposed to these drugs. The high dependence of vultures on domestic animals today compared to past decades and the growing intensification of livestock farming, imply an expected increase in the impact of pharmaceuticals on scavenger populations. This requires further evaluation due to potential consequences in biodiversity conservation and environmental health. We encourage the prioritization of efforts to promote the use of less medicated free-ranging livestock carcasses left in the countryside, rather than stabled stocks made available in vulture restaurants. Additionally, attention should be paid to the population recovery of wild species that dominated scavenger diets in the past.

1. Introduction

Environmental contamination with pharmaceuticals is a major public health concern (Boxall, 2010; Arnold et al., 2014). The occurrence of anti-inflammatories, antibiotics, analgesics, hormones, etc. has been repeatedly recorded in aquatic environments as a consequence of their continuous release from urban sewage systems and livestock operations (Daughton and Ternes, 1999, Kummerer, 2010; Gothwal and Shashidhar, 2015). Pharmaceuticals, generally designed to be highly specific and potent in their respective therapeutic effects, may be biologically active and toxic to non-target organisms even at very low concentrations (Christen et al., 2010; Corcoran et al., 2010; Brausch et al., 2012). These compounds are prone to bioaccumulation in long-term or chronic exposure conditions and bio-magnification in food chains (Boxall et al., 2010; Lazarus et al., 2015). As a consequence, multiple adverse effects of their residues have been reported in aquatic wildlife (Robinson et al., 2005; Christen et al., 2010; Brausch et al., 2012; Fabbri and Franzellitti, 2016). In terrestrial

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ecosystems, wildlife exposure to pharmaceuticals may arise as a consequence of the use of contaminated effluents into irrigation of agricultural areas or by direct manuring with litter and excreta of medicated livestock (Sharma et al., 2009; Daughton and Ruhoy, 2009; Boxall, 2010). Pharmaceuticals from veterinary treatments may also enter terrestrial food webs when excreta and carcasses of medicated livestock become available for coprophagous and scavenger wildlife (e.g. Floate et al., 2005; Richards et al., 2014; Hammer et al., 2016).

Attempts to reconcile sanitary and environmental policies have addressed the exposure to veterinary pharmaceuticals from livestock carcasses in the management and conservation of avian scavengers in Spain (Donázar et al., 2009; Margalida et al., 2014). Spain is the major stronghold for obligate and facultative scavengers in the Western Palearctic. Several species that were very abundant in the past, especially the Egyptian vulture (*Neophron percnopterus*) and the Red kite (*Milvus milvus*), have suffered severe declines (Blanco and Montoya, 2004; Donázar, 2004; Carrete et al., 2007; Blanco, 2014). In addition, decreases in population growth and breeding success in other species, such as the Eurasian griffon vulture (*Gyps fulvus*) and the Cinereous vulture (*Aegypius monachus*), have been recorded in Spain over the past few decades (Camiña, 2004, Donázar et al., 2002, 2009, authors' unpublished data). The reduction and changes in the nature of food supplies available for scavengers has been repeatedly highlighted as one of the main causes of these declines and the reduction in breeding success (Donázar, 2004; Sánchez, 2004). However, despite great interest and concern in the distribution, accessibility and availability of food for avian scavengers due to the impact of sanitary regulations (Tella, 2001; Donázar et al., 2009), there exists little information on the role of the different livestock species from stabled and extensive farming schemes in scavenger diets (Donázar et al., 2010; Blanco, 2014), contamination with pharmaceuticals (Mateo et al., 2015; Blanco et al., 2016) and exposure to pathogens (Marín et al., 2014; Blanco, 2015).

Livestock carcasses used to feed avian scavengers may include sick and medicated livestock, consumed by scavengers a short time after disposal in carcass dumps and supplementary feeding stations, colloquially termed "vulture restaurants" (Cortés-Avizanda et al., 2016). Therefore, the exposure to livestock pharmaceuticals and pathogens that are potentially harmful to scavengers is likely an unintentional consequence of conservation management of these species (Cortés-Avizanda et al., 2016). This exposure may have direct adverse effects on scavenger populations, exemplified by the crash of vulture populations in Asia due to the anti-inflammatory diclofenac (Watson et al., 2004; Gilbert et al., 2007). Information is also available on pathogen transmission from livestock to wildlife (Friend and Franson, 1999; Thomas et al., 2007). However, the likely indirect negative side-effects of pharmaceuticals via the overgrowth of disease-causing opportunistic pathogens remain largely unexplored.

A previous study recorded lesions caused by *Candida*-like yeast in the oral cavity of griffon vultures in central Spain (López-Rull et al., 2015), where most nestlings of this species showed circulating residues of several fluoroquinolones (Blanco et al., 2016). Opportunistic fungal pathogens may cause infection when a disruption of the host's natural barriers or an impairment of the host's immune system occurs (Lin et al., 2005; Pfaller and Diekema, 2007; Hebecker et al., 2014). The prolonged use of broad-spectrum antibiotics can cause these effects directly or indirectly by altering the host's normal microbiota composition (Patterson,1991; Lin et al., 2005; MacCallum, 2010; Dethlefsen and Relman, 2010; Cho et al., 2012; Keeney et al., 2014). Gut dysbiosis and other microbial imbalances may be especially detrimental in nestling vultures subjected to long-term or chronic exposure to antibiotics and other pharmaceuticals (Blanco et al., 2016), exacerbated by an immature immune system (López-Rull et al., 2015). However, no information is available on exposure to antibiotics in vulture species that include a proportion of wild animals in their diets, thereby being less dependent on livestock carcasses. This lack of information precludes the proper evaluation of the factors determining exposure to pharmaceuticals from different livestock species due to the variable availability and elimination methods of their carcasses, and its implications on the conservation of their populations.

In this study, we evaluated the exposure to livestock pharmaceuticals in nestling cinereous and Egyptian vultures, two threatened scavenger species exploiting carcasses of domestic livestock and wild animals. Egyptian vultures exploit carcasses of a wider variety of wild animals than cinereous vultures, which today rely much more heavily on livestock carrion than in the past (Hiraldo, 1976; Donázar, 1993). Therefore, a greater dependence on domestic versus wild animals in the cinereous vulture was predicted to be reflected in a higher exposure to pharmaceuticals than in Egyptian vultures. This prediction was tested by using fluoroquinolones as indicators of the overall exposure to livestock pharmaceuticals, as these antibiotics are often used in combination with other drugs in multiple treatments (Andriole, 2005; Sharma et al., 2009) and because they are highly stable compared with other pharmaceuticals (Martinez et al., 2006; Okerman et al., 2007). Because the prolonged use of broad-spectrum antibiotics is a major predisposing factor for opportunistic mycoses (Cunha, 2001; Lin et al., 2005; Pfaller and Diekema, 2007), we evaluated signs of disease caused by *Candida*-like yeast. Specifically, we hypothesized potential cause-effect relationships between diet-associated exposure to livestock antibiotics and occurrence of disease. We critically assessed whether the evaluation of these relationships may be obscured by variations in exposure and the concentration of fluoroquinolones, and their effects on disease signs, due to chronic contamination with these drugs throughout the nestling development period (Blanco et al., 2016). We discuss whether the interactions between diet, exposure to pharmaceuticals and disease can be modulated by species-specific life-history traits such as size, metabolism and foraging strategies. Finally, we reviewed information on the occurrence and concentration of pharmaceuticals in avian scavengers, to call attention to the increasing impact of these drugs on scavenger populations and environmental health due to supplementary feeding with medicated livestock.

2. Material and methods

2.1. Study area and species

The study was conducted in central Spain (provinces of Avila and Segovia). This area holds numerically important populations of vultures that are highly dependent on livestock carrion at vulture restaurants and other carcass dumps (see details of the study area and vulture populations in Donázar et al., 2002; Blanco, 2014). In addition to the griffon vulture, which shows a large and increasing population (Martínez et al., 1997; authors' unpublished data), about two hundred pairs of cinereous vultures and about 30 pairs of Egyptian vultures nest in the study area (Donázar et al., 2002; authors' unpublished data).

The cinereous vulture is the largest avian scavenger in the Palearctic (8–12 kg). It is a year-round resident in the study area, where it nests in pines and forages across large open areas around the colonies (Fargallo et al., 1998; Donázar et al., 2002). It searches for carcasses of livestock and wild prey, especially lagomorphs, that may be opportunistically predated when injured or sick (Hiraldo, 1976). Exploited livestock carcasses are mainly pigs and poultry predominantly present in vulture restaurants in the study area (Blanco, 2014). This species is categorized globally as "Near Threatened" and included in category SPEC 1 in Europe, due to the declining population trend over the last century (BirdLife International, 2016). In Spain, the stronghold for the species in Europe, major threats include illegal persecution through poisoning, habitat alteration and reduction of food supplies, especially of wild prey and free-ranging livestock (Sánchez, 2004).

The Egyptian vulture is a small (~1.8 kg) cliff-nesting vulture living

in open, arid and rugged landscapes in the Mediterranean, Africa and Asia. Mainland populations in Europe are migratory, with wintering areas in Africa. It is listed as globally endangered due to severe declines experienced throughout its range (BirdLife International, 2016). The Spanish population represents the main stronghold in Europe, despite the fact that it has suffered a strong decline due to illegal persecution through poisoning, habitat alteration and reduction of food supplies (Donázar, 2004; Carrete et al., 2007). The primary food sources for this species are carcasses of livestock and small and medium-sized wild animals and, to a lesser extent, livestock faeces (Donázar, 1993).

2.2. Nestling sampling

During the breeding season of 2007–2015, a sample of nestling cinereous vultures (n=14 nestlings from different nests, one nestling per nest) and Egyptian vultures (n=16 nestlings from 11 breeding events: 6 nests with one nestling and 5 nests with two nestlings) were accessed by climbing to the nests. The nestlings were sampled at ages of about 50–80 days depending on the species, when they were feathered at the final stage in the nest. Fledglings with a risk of leaving the nests were avoided. A sample of blood (3–5 mL) was taken from the brachial wing vein. The blood samples were transferred to vials containing heparin and kept chilled until arrival at the laboratory within 5 h after collection. On the day of collection, blood was centrifuged at 13,000g for 10 min to obtain plasma, which was frozen at −20 °C until analysis.

2.3. Nestling diet

During the breeding season of 2003–2015, food remains found in and below the nests of the two study species were collected at the time of nestling ringing. Prey remains were identified macroscopically and quantified assuming the smallest possible number of individuals (Blanco, 1997). Pellets found in the nests were not considered to avoid including the diet of breeding adults.

To assess broad differences between species in the consumption of livestock carrion, food remains were grouped into two categories: (a) wild animals obtained by scavenging and occasional predation (e.g. wild birds, mammals, reptiles) and (b) livestock carcasses (mainly pigs, cows, sheep, poultry and domestic rabbits) intentionally discarded for elimination by scavenger species in carcass dumps, vulture restaurants and around farms (Blanco, 2014). Carcasses of each livestock species can be sources of pharmaceuticals for vultures, with a presumably higher content in livestock from intensive and stabled farming using fluoroquinolones and other pharmaceuticals on a regular basis versus free-ranging livestock, which are generally much less medicated (Page and Gautier, 2012; Moreno, 2014). Therefore, food remains from livestock were classified as coming from intensive stock farms (swine, poultry and domestic rabbits) or primarily from free-ranging farms (sheep, goats, cows and horses) in the study area. Remains from marine fish and domestic refuse were assumed to have been obtained in rubbish dumps (Blanco, 1997). By using food remains, diet was likely biased in favour of the most durable prey remains and did not reflect the importance of arthropods and skinned carcasses and viscera of livestock and large game species (Blanco, 1997; Sánchez-Zapata et al., 2010). The biomass of each food item was not estimated because of the difficulty in obtaining accurate measurements from scavenged food, particularly from livestock remains.

2.4. Determination of fluorquinolone residues in plasma

The presence and concentration of fluoroquinolones (ciprofloxacin, sarafloxacin, difloxacin, enrofloxacin, danofloxacin and marbofloxacin) were determined in nestling vulture plasma by ultra high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QqQ), as described previously (Blanco et al., 2016). Briefly, the sample treatment included a protein precipitation prior to carrying out the solid phase extraction (Hermo et al., 2011). For UHPLC, optimization of the chromatographic separation was carried out. Gradient programs featuring a mobile phase that combined solvent A (MeCN with 0.1% HCOOH) and solvent B aqueous solution of 0.1% HCOOH were used. The chromatographic gradient used in the separation of the studied fluoroquinolones was as follows: from 0 to 2 min, the mobile phase contained 7% of MeCN; from 2 to 4 min, the percentage of MeCN linearly increased to 10%; from 4 to 6 min, it increased to 30%. Finally, the MeCN percentage decreased to 7% from 6 to 7 min. The flow rate was 0.3 mL min^{-1} and the injection volume was 10 μL.

Optimized parameters used were described elsewhere (Blanco et al., 2016) except for levofloxacin with a declustering potential of 45 V, focusing potential of 175 V and entrance potential of 11 V. The quantification transition for levofloxacin was 362→318 (28 V). Other parameters were capillary voltage 4500 V, and nebulizer gas (N_2) 10 (arbitrary units). Drying gas (N_2) was heated to 400 °C and introduced at a flow rate of 4500 mL min^{-1}.

To establish calibration curves, six concentration levels of spiked blank plasma (5–200 μg L^{-1}) were prepared in duplicate and injected into the separation system. Levofloxacin was selected as an internal standard (IS) because it met all the suitable requirements (very similar compound to analytes but not identical to the chemical species of interest and a similar retention time). Levofloxacin at 100 μg L^{-1} was added at all points of the calibration curves and vulture plasma samples. Results were presented as a ratio of signal of quinolone/IS vs. the quinolone/IS concentration ratio. Plasma from an organic chicken farm in Spain was used as a blank.

The separation of the fluoroquinolones in the UHPLC-QqQ system was achieved in less than 5 min. All compounds presented good linearity in the concentration range studied (from 5 to 200 μg L^{-1}) with a correlation coefficient (r) higher than 0.990 for all fluoroquinolones studied. The calibration curves obtained by UHPLC-QqQ were: enrofloxacin, y=3.22 x+0.038 (r=0.998); ciprofloxacin, y=0.34 x +0.0032 (r=0.996); difloxacin, y=0.98 x+0.027 (r=0.994); marbofloxacin, y=0.12 x+0.017 (r=0.990); danofloxacin, y=2.08 x−0.018 (r=0.997) and sarafloxacin, y=0.36 x+0.0036 (r=0.997).

2.5. Presence of lesions in the oral cavity

The presence and number of gross lesions compatible with *Candida*-like yeast infection were determined by inspecting the oral cavity of nestlings. These lesions, previously recorded in vultures from central Spain (López-Rull et al., 2015), appeared as milky prominent nodules of variable size, with a solid consistency and circular to elliptic form that often extended in larger and ulcerated plaque-like areas on the tongue and other parts of the oral cavity (Fig. 1). By gently scraping on the lesions with sterile swaps, abundant yeast cells and hyphae compatible with infection by *Candida*-like yeast were observed directly by microscope at the laboratory. At the same time, we discarded the presence of *Trichomomas* (Phylum Metamonada) flagellates, *Capillaria* (Phylum Nematoda) worms and other organisms as the causative agent of the lesions. Although these parasites can cause lesions in the oral cavity of birds, these lesions show a characteristic appearance that differs from that of lesions caused by yeast (Friend and Franson, 1999; Velasco 2000; Carter et al., 2008). In addition, multiple yeast species, especially of the *Candida* genus, have been identified to cause these lesions (A. Pitarch, C. Gil, G. Blanco unpublished data). Although yeast and other fungi can be normal inhabitants of the upper alimentary tract of birds, the presence of lesions implies an ongoing infection by these opportunistic pathogens (Ritchie et al., 1994, Friend and Franson, 1999). Therefore, the number of lesions was considered to reflect the intensity of infection..

Fig. 1. Appearance of lesions caused by *Candida*-like yeast in the oral cavity of vulture nestlings in central Spain. The picture shows the lesions in a nestling cinereous vulture.

2.6. Statistical analysis

Proportions of food items within the considered food categories were compared between vulture species using Fisher's exact tests, with *p* values adjusted with the Bonferroni correction for multiple comparisons, and an odds ratio and 95% confidence intervals (CI) as a measure of effect size. Continuous data, including the concentration of fluoroquinolones and the number of lesions, were compared between the two vulture species by means of Student's t or Mann-Whitney *U* tests, as appropriate. Relationships between the concentration of enrofloxacin and ciprofloxacin in particular samples corresponding to individual nestlings was conducted by paired *t*-tests, considering half of the more diluted level of the calibration curve (5.0 μg $L^{-1/2}$) when the ciprofloxacin concentration was below this value, to include a higher sample size in the analysis. The relationships between vulture species, the presence of fluoroquinolone residues and *Candida*-like lesions were evaluated by conducting log-linear analysis. The test was run hierarchically, beginning with the three-order interaction and proceeding backwards until all two-order interactions retained by the model reached significance ($p < 0.05$). The occurrence and number of yeast-like lesions were evaluated by using General Linear Models (GLM) with binomial and Poisson error respectively, by including the presence or absence of fluoroquinolone residues (fixed factor, pooling all compounds), their concentrations (covariate, summing the levels of all compounds within particular nestlings) and vulture species (fixed factor) as explanatory variables. The occurrence and concentration of fluoroquinolones were included as explanatory variables in different models to avoid collinearity. Statistical analyses were performed using SPSS software.

3. Results

3.1. Nestling diet

A total of 1374 food remains were collected, 181 in cinereous vulture nests (*n*=60 breeding attempts; mean ± SD remains per nest=3.0 ± 3.2, range=0–13, considering the smallest possible number of individual animals consumed, see methods), and 1193 in Egyptian vulture nests (*n*=88 breeding attempts; 13.6 ± 10.7 remains per nest, range=0–51). Nestling cinereous vultures feed primarily on pork from carcasses dumped in vulture restaurants and farm surroundings, and on wild lagomorphs (Table 1), mostly European rabbit (*Oryctolagus cuniculus*) but also Iberian hare (*Lepus granatensis*). Nestling Egyptian vultures mainly feed on scavenged pigs, wild birds (35 species) and lagomorphs (Table 1). The proportions of food items within the considered categories differed between vulture species only for wild birds, as no wild bird remains were found in cinereous vulture nests (Table 1), and for large free-ranging livestock (cattle and horses), which was significantly less consumed by nestling Egyptian than cinereous vultures (Table 1).

Overall, the proportion of the three major groups of food items differed between vulture species (χ^2_2=24.29, $p < 0.0001$, Fig. 2), mainly because the proportion of carrion from livestock versus wild animals was lower in the diet of nestling cinereous vultures than Egyptian vultures (Fisher exact test, $p < 0.0001$, Odds ratio =0.42, 95% CI=0.28–0.61). Remains from stabled and free-ranging livestock were found in a similar proportion in the two vulture species (Fisher exact test, *p*=0.28, Odds ratio=1.25, 95% CI=0.81–1.92, excluding remains from unidentified livestock species and rubbish dumps), with stabled livestock dominating in the diet of both species (Fig. 2)..

3.2. Presence and concentration of fluoroquinolones in plasma

Enrofloxacin and ciprofloxacin were found in cinereous vultures,

Table 1
Number and percentage of food items in each wild and domestic animal category from remains found in nests of cinereous and Egyptian vultures sampled in central Spain (2003–2015). Boldface *p* values indicate significance after the Bonferroni adjustment for multiple comparisons. Odds ratio was considered to show important changes in the frequency of each prey group between species when the value 1 was not within the 95% CI. *n* represents the number of analysed food items.

Food item	Cinereous vulture	Egyptian vulture		
	n (%)	*n* (%)	Fisher's exact test, *p*	Odd ratio (95% CI)
Wild animals				
Reptiles	2 (1.1)	64 (5.4)	0.008	5.07 (1.21–30.23)
Lagomorphs	27 (14.9)	137 (11.5)	0.18	0.74 (0.46–1.18)
Other mammals	12 (6.6)	70 (5.9)	0.74	0.88 (0.45–1.75)
Birds	0 (0.0)	213 (17.9)	**< 0.0001**	∞[b] (8.18-∞)
Fishes	0 (0.0)	11 (0.9)	0.377	∞ (0.32-∞)
Domestic animals				
Sheep and goats	31 (17.1)	155 (13.0)	0.131	0.72 (0.47–1.13)
Cattle and horses	8 (4.4)	11 (0.9)	**0.002**	0.20 (0.07–0.56)
Pigs	75 (41.4)	444 (37.2)	0.29	0.84 (0.60–1.17)
Domestic rabbits	5 (2.8)	13 (1.1)	0.076	0.39 (0.13–1.26)
Poultry	17 (9.4)	60 (5.0)	0.024	0.51 (0.28–0.93)
Others[a]	3 (1.7)	15 (1.3)	0.72	0.76 (0.20–3.32)
Total	181	1193		

[a] Included unidentified livestock remains and food remains obtained in rubbish dumps, including marine fish and domestic refuse.
[b] ∞ indicates that the odds ratio and its 95% CI tends toward infinite.

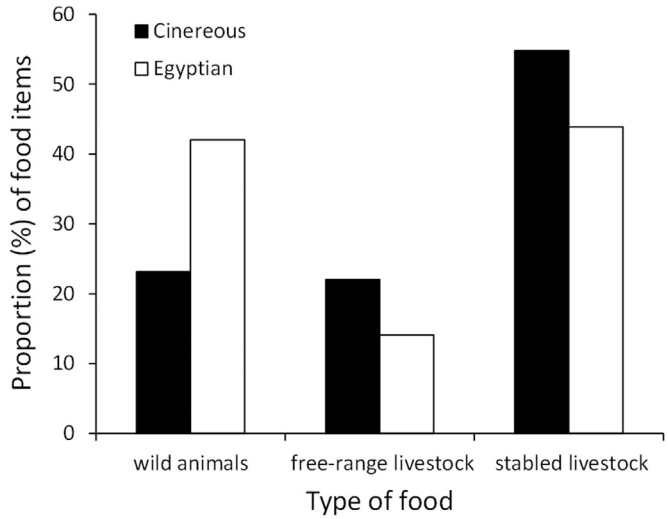

Fig. 2. Percentage of remains of carrion from wild animals, free-range and stabled livestock as food for nestling Cinereous and Egyptian vultures in central Spain.

while enrofloxacin but not ciprofloxacin was found in Egyptian vultures (Table 2). Marbofloxacin was only detected in a single Egyptian vulture (Table 2). The number and proportion of samples with quantifiable and unquantifiable residues of each fluoroquinolone, as well as those with no detectable residues are shown in Table 2. The proportion of individuals with enrofloxacin (including those with quantifiable and unquantifiable levels) was higher in cinereous than Egyptian vultures (Fisher exact test, p=0.0003, Table 2). Overall, residues of fluoroquinolones (pooling all types of compounds) were found in all sampled cinereous vultures and in a lower proportion of Egyptian vultures (Fisher exact test, p=0.0009, Fig. 3a).

The concentration of enrofloxacin was higher in cinereous than Egyptian vultures (t-test, t=2.28, df=17, p=0.035, Table 2; ciprofloxacin was not found in Egyptian vultures). The comparison of total levels of fluoroquinolones (pooling the simultaneous presence of the different fluoroquinolones in individual nestlings) showed the same trend (Fig. 3b, t=2.88, df=18, p=0.010). Previously published values of occurrence and concentrations of fluorquinolones found in nestling griffon vultures from the same area (Blanco et al., 2016) are also shown for comparison purposes (Fig. 3a, b).

An example of chromatograms obtained by UPLC-QqQ from plasma samples positive for enrofloxacin and ciprofloxacin is shown for a cinereous vulture (Fig. 4a). The concentration of enrofloxacin was always higher than that of ciprofloxacin in cinereous vultures (paired t-test, t=15.24, df=11, $p < 0.0001$), and within-individual values were positively related (Fig. 4b, Pearson linear regression, r=0.92, $p < 0.0001$, n=12). In both analyses, half of the lowest level of the calibration curve was considered for four positive samples with unquantifiable concentrations of ciprofloxacin; when these four samples were excluded the results were similar (paired t-test, t=12.96, df=7, $p < 0.0001$ and r=0.91, p=0.002, n=8)..

3.3. Yeast-like lesions in the oral cavity and relationship to fluoroquinolone residues

Gross lesions compatible with yeast-like infections were recorded in the oral cavity of 85.7% of nestling cinereous vultures (n=14) and 62.5% Egyptian vultures (n=16) sampled for the determination of circulating residues of fluoroquinolones (Fig. 5). The proportion of nestlings with lesions did not differ between vulture species (Fisher exact test, p=0.23). The number of lesions did not differ between cinereous (median=1.5, range=1–6, n=12) and Egyptian (median=2, range=1–5, n=10) vultures (Mann-Whitney U test, z=0.21, p=0.84)..

A log-linear analysis taking into account the relationships between vulture species, presence of fluoroquinolone residues and lesions did not show a significant three-way interaction (χ^2=0.002, p=0.97, df =1). Only the interaction between vulture species and the presence of fluoroquinolone residues was significant (χ^2=14.72, $p < 0.0001$, df=1) indicating a higher proportion of cinereous than Egyptian vultures with fluoroquinolone residues (see above, Fig. 3a). The interaction of species x lesions and residues x lesions were not significant (both $p > 0.14$; the fit of the model was adequate, goodness-of-fit χ^2=2.57, p=0.46, df =3).

The occurrence of lesions was not associated with the qualitative presence of fluoroquinolones (GLM, binomial error, logit link function, Wald χ^2=0.42, p=0.52, df=1) or their concentrations (summing all compounds found in particular nestlings with residues, Wald χ^2=0.27, p=0.61, df=1, respectively) after controlling for the effect of species (both $p > 0.44$). The number of lesions was not associated with the occurrence of fluoroquinolone residues (GLM, Poisson error, log link function, Wald χ^2=1.49, p=0.22, df=1) or their concentrations (excluding nestlings without residues, GLM, Poisson error, log link function, Wald χ^2=1.43, p=0.23, df=1) after controlling for the effect of species (both $p > 0.22$).

4. Discussion

There is widespread concern over the exposure to pharmaceuticals, and its associated adverse effects, in wildlife (Arnold et al., 2014). However, little data on these topics are available for terrestrial environments. In this study, we found that all (100%) cinereous vultures and about 44% of the Egyptian vultures sampled in central Spain showed fluoroquinolone residues in plasma, mostly enrofloxacin. These synthetic broad-spectrum antibiotics are frequently used by the animal husbandry industry, especially in factory farming, for multiple treatments (Andriole, 2005; EMEA, 2006; Woodward, 2009). Therefore, as occurs with other vulture species in Spain (e.g. Mateo et al., 2015; Blanco et al., 2016), cinereous and Egyptian vultures are exposed to pharmaceuticals through the available livestock carcasses, which in the case of stabled livestock are invariably disposed in vulture restaurants and other feeding stations around farms in the study area (Blanco, 2014).

Carcasses disposed for vultures are often those of sick livestock that died after a variable treatment period with pharmaceuticals to combat infection (e.g. antibiotics, parasiticides, antifungals) and their effects (e.g. analgesics, anti-inflammatories). As a consequence, exposure to pharmaceuticals should be directly related to the extent of the exploitation of medicated livestock by scavengers. We confirmed this prediction by showing a greater occurrence and concentration of fluoroquinolones in nestling cinereous vultures, due to their greater dependence on livestock carcasses for food than Egyptian vultures,

Table 2
Results of the occurrence and mean ± SD concentration (μg L^{-1}) of fluoroquinolones detected through UHPLC-QqQ in plasma of nestling cinereous and Egyptian vultures in central Spain. Mean concentrations were calculated considering those samples with quantifiable residues.

	Residues of fluoroquinolones in plasma		
	Enrofloxacin	Ciprofloxacin	Marbofloxacin
Cinereous vulture (n=14)			
Quantifiable (%)	14 (100)	8 (57.1)	0 (0.0)
Not quantifiable (%)	0 (0.0)	4 (28.6)	0 (0.0)
Not detected (%)	0 (0.0)	2 (14.3)	14 (100)
Mean concentration ± SD	64.1 ± 19.0	15.5 ± 5.8	–
Egyptian vulture (n=16)			
Quantifiable (%)	5 (31.3)	0 (0.0)	1 (6.3)
Not quantifiable (%)	1 (6.2)	0 (0.0)	0 (0.0)
Not detected (%)	10 (62.5)	16 (100)	15 (93.7)
Mean concentration ± SD	43.2 ± 11.7	–	11.5

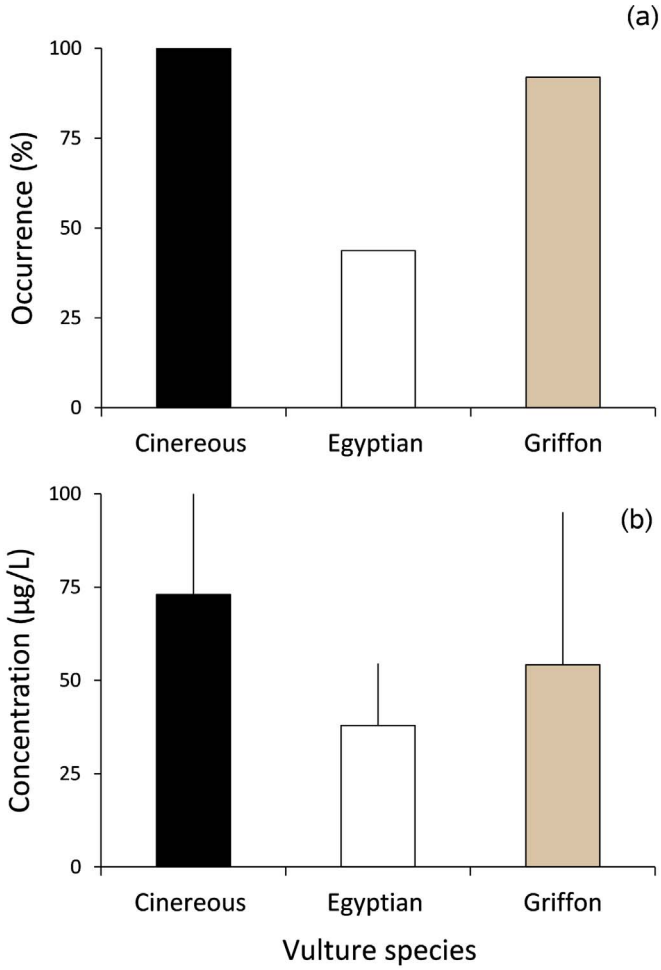

Fig. 3. (a) Frequency (%) of fluoroquinolone occurrence (considering quantifiable and unquantifiable residues) and (b) average ± SD plasma concentration (µg L^{-1}) of fluoroquinolones (summing the levels of different fluoroquinolones simultaneously present in particular individuals) in nestling Cinereous and Egyptian vultures sampled in central Spain. Mean concentrations were calculated considering those samples with quantifiable residues. Data from nestling griffon vultures from the same area (brown bars), determined by using the same analytic methodology (Blanco et al., 2016), are shown for comparison purposes.

which rely much more on carcasses of wild animals (Donázar, 1993). Regarding livestock, both vulture species feed primarily on pork, the most predictable, available and abundant food providing the most biomass disposed in vulture restaurants and farm surroundings in the study area (Blanco, 2014). Swine is reared under intensive conditions including heavy medication with fluoroquinolones and other drugs (Salichs et al., 2012; Moreno, 2014). Carcasses of other stabled and intensively reared livestock also disposed in vulture restaurants included poultry and domestic rabbits (Blanco, 2014), which are generally heavily medicated (Hofacre, 2006; Goetting et al., 2011) and thereby may also be a source of fluoroquinolones for vultures. The proportion of free-ranging sheep and goats, which are generally only mildly medicated, did not differ in the diets of the two vulture species. Cattle and horses show variable rearing, housing and medication conditions depending on stock age, season and particular exploitation operations (Page and Gautier, 2012). Thus, they may contribute to the greater exposures to fluoroquinolones in the cinereous vulture because this species consumes these livestock more frequently than Egyptian vultures. In addition, while the cinereous vulture can feed on livestock organs such as liver with high antibiotic concentrations, the Egyptian vulture generally feeds on small pieces of skin and raw muscle (Donázar 1993), where antibiotics may be less concentrated. Regarding wild animals, the main difference was the consumption of

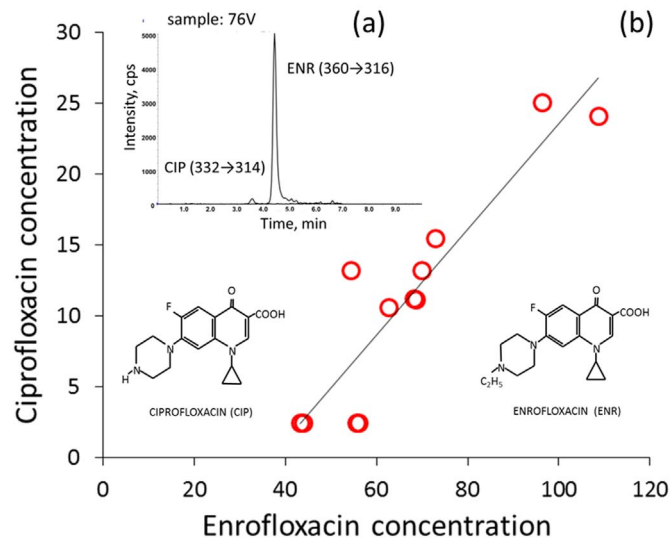

Fig. 4. (a) An example of UHPLC-QqQ chromatogram corresponding to plasma of a cinereous vulture nestling (sample reference: 76 V) positive for enrofloxacin (ENR) and ciprofloxacin (CIP). The quantification transitions of enrofloxacin (360→316) and ciprofloxacin (332→314) are shown. (b) Relationship between concentrations (µg L^{-1}) of enrofloxacin and ciprofloxacin in plasma of nestling Cinereous vultures from central Spain. Least squares regression line is shown. The structures of enrofloxacin and its metabolite ciprofloxacin were also shown.

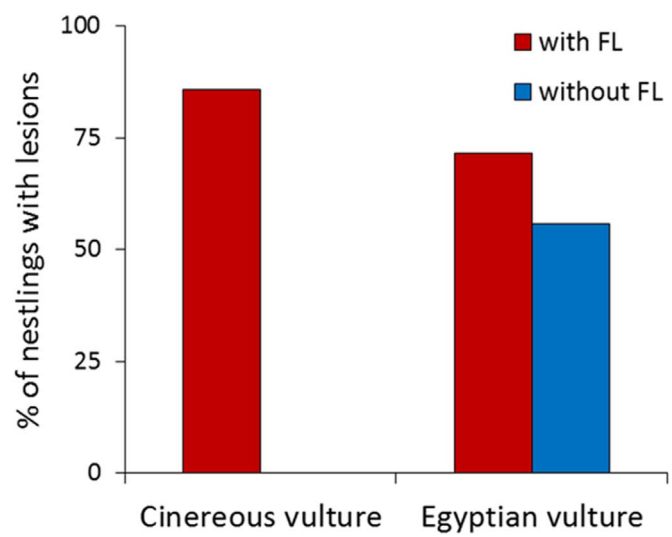

Fig. 5. Percentage of nestlings with *Candida*-like lesions in the oral cavity according to the presence of fluoroquinolone (FL) residues (pooling the presence of all compounds) in plasma of Cinereous and Egyptian vultures from central Spain.

birds by the Egyptian vulture but not by the cinereous vulture. Overall, both vulture species rely comparatively more on stabled livestock in the study area than in other regions (Donázar, 1993; Hidalgo et al., 2005; Costillo et al., 2007, Margalida et al., 2012). This likely determines a high exposure of vultures to pharmaceuticals in central Spain compared to other population nuclei, which should be tested in future studies.

The high occurrence of fluoroquinolones in both vulture species suggests a chronic ingestion of these drugs throughout nestling development, as has been shown for the griffon vulture in the same region (Blanco et al., 2016). This was supported by the fact that the nestlings were sampled across the study area, at different ages and on different dates, given their variable breeding phenology and length of the nestling stage, which is longest in the larger cinereous vulture (Hiraldo, 1983; Donázar, 1993). Despite their almost total dependence on domestic livestock carcasses in the study area (Arroyo and Garza,

1995; Acha et al., 1998; Blanco, 2014), griffon vultures showed an intermediate occurrence and concentration of fluoroquinolones compared to cinereous and Egyptian vultures (Fig. 2a and b, Blanco et al., 2016). In the griffon vulture, the occurrence of marbofloxacin (Blanco et al., 2016) was higher than in Egyptian vultures, and this drug was not found in cinereous vultures. This indicates a high exposure to this drug in griffon vultures owing to the exploitation of a great variety of domestic animals over a wide range of livestock operations across their large foraging range (Arroyo and Garza, 1995; Donázar et al., 2010; authors' unpublished data). However, despite including a proportion of carcasses of wild animals in their diet, the highest occurrence and concentration of fluoroquinolones in the largest and heaviest cinereous vulture suggests a potential role for size-related allometries and scaling relationships regarding food processing (Blanco et al., 2014) and drug metabolization and excretion (Garcia-Montijano et al., 2011; Lashev and Haritova, 2012). This was supported by the fact that the residues of ciprofloxacin, the metabolite of enrofloxacin, were found at quantifiable concentrations in a proportion of cinereous vultures, but were not found in Egyptian vultures with enrofloxacin residues, and only were found at unquantifiable concentrations in griffon vultures (Blanco et al., 2016). In addition, the concentration of enrofloxacin was always higher and positively related to that of ciprofloxacin in particular nestling cinereous vultures. This suggests that slow, size-related, metabolism in this species can promote a long dose-dependent permanence of circulating enrofloxacin and its metabolization to ciprofloxacin.

As recorded in griffon vultures from the study area (López-Rull et al., 2015), gross lesions compatible with yeast-like infections were found in the oral cavity of a large proportion of nestling cinereous and Egyptian vultures. The prolonged use and misuse of broad-spectrum antibiotics are major predisposing factors for opportunistic mycoses (Cunha, 2001; Lin et al., 2005; Pfaller and Diekema, 2007). Therefore, a cause-effect relationship between diet-associated exposure to livestock fluoroquinolones and occurrence of disease is proposed as one of the most plausible explanations for the large prevalence and intensity of yeast-like infections in vultures, although other potential concurrent source of infection such as opportunistic bacteria can also contribute to the pathogenicity of the lesions, which requires further research. However, owing to the high prevalence of circulating fluoroquinolones and clinical mycoses in both vulture species, a cause-effect relationship cannot be demonstrated by testing the association of the two factors in individual vultures. Thus, despite the fact that all cinereous vultures showed circulating fluoroquinolones, "only" about 86% of these nestlings showed detectable yeast-like lesions in the oral cavity. This may be because a proportion of the nestlings did not develop clinical manifestations of infection due to fluoroquinolones or, alternatively, some birds may be infected at lower areas of their digestive tract hidden from the observer, as recorded during necropsy of vultures conducted in rehabilitation centers (pers. observ.). In the Egyptian vulture, 76% of nestlings with fluoroquinolones showed lesions, while 56% of nestlings without fluoroquinolones showed lesions. This suggests that although the ingestion of fluoroquinolones can be chronic throughout nestling development, the discontinuous exposure to and pulsed concentration of these drugs coinciding with the consumption of medicated carcasses can promote infections persisting beyond fluoroquinolone metabolization and excretion. In this sense, the similar occurrence and intensity of infection in both vulture species despite the higher proportion of cinereous compared to Egyptian vultures with fluoroquinolone residues is noteworthy. The expected size-related slower metabolism of cinereous compared to Egyptian vultures (Blanco et al., 2014) may explain these results through the likely longer period of fluoroquinolone metabolization and excretion (Lashev and Haritova, 2012). Thus, the impact of these drugs on yeast infections in the cinereous vulture would be greater. However, this requires further research.

To properly demonstrate a within-individual cause-effect relationship between exposure to fluoroquinolones and disease is logistically challenging, but could be evaluated through a population-based approach by sampling vulture populations not exposed to these drugs. The hypothesis that the yeast infections are directly triggered by the chronic and chaotic conditions surrounding antibiotic ingestion predicts that vulture populations feeding on carcasses without antibiotics (e.g. those from non-medicated wild animals) should not show clinical candidiasis, or should show it in a much lower prevalence that found in this study. However, no European vulture population relies exclusively on non-domestic animals (Cortés-Avizanda et al., 2016), which hampers the testing this hypothesis in the wild under completely controlled conditions regarding exposure to antibiotics and other drugs from livestock carcasses. However, this question can be approached by assessing partial differences in diet among populations, or by means of an interspecific comparison approach including facultative scavengers. Experimental food-borne exposure in captivity simulating the chronic and chaotic ingestion of antibiotics in the wild is expected to dramatically alter the microbiota in the digestive tract, thus increasing the susceptibility to fungal and other infections by opportunistic pathogens (Levy, 2002, Keeney et al., 2014). In fact, antibiotics are often used to break colonization resistance in microbial-mediated disease research with animal models (Keeney et al., 2014). However, there are clear ethical implications of these experiments in vultures and other threatened wildlife. The expected detrimental impact on experimental subjects seems unnecessary given the extensive evidence that mycotic infection is one of the more common side effects of exposure to antibiotics, even under adequate therapeutic treatment against targeted bacterial infections (Cunha, 2001; Lin et al., 2005).

Whatever the likely conditional and context-dependent outcome of fluoroquinolone-disease interactions, the concomitant impact on health of chaotic antibiotic ingestion and mycoses is likely more detrimental than if these threats act independently. This relies on the fact that the weakened status of individuals with a mycotic infection can be worsened if they are exposed to antibiotics, due to multiple side effects of these drugs under non-therapeutic conditions (Levy, 2002; Keeney et al., 2014). Fluoroquinolones can have adverse effects exacerbated during organismal development by causing alterations in joints, bones and tendons (Peters et al., 2002; Chide and Orisakwe, 2007; Maslanka and Jaroszewski, 2009) and by depressing the immune system (Walker, 2000; Keeney et al., 2014). These effects can be especially detrimental in developing nestlings, as occurs with other contaminants (Blanco et al., 2004; Markman et al., 2011; Morrissey et al., 2014). Importantly, chronic contamination with antibiotics may promote the alteration of the normal microbiota and the overgrowth of pathogenic fungi, especially *Candida* yeasts, which are among the more frequent direct adverse effects from antibiotic misuse or overuse (Patterson, 1991; Cunha, 2001; Montagnac et al., 2005; Martinez et al., 2006; Granowitz and Brown, 2008; Caliendo and Bull, 2011). Thus, by eliminating or disturbing competing bacteria of the normal flora, the ingestion of antibiotics can be directly implied in the acquisition and overgrowth of opportunist yeast able to cause invasive candidiasis (Lin et al., 2005; Pfaller and Diekema, 2007; MacCallum, 2010), as well as of pathogenic bacteria proliferating due to the imbalance of competing and protective microbiota (Levy, 2002; Dethlefsen and Relman, 2010; Cho et al., 2012; Keeney et al., 2014). Antibiotics can also indirectly promote invasive candidiasis by depressing the still immature ability of the immune system in developing nestlings to fight against infection (Fellah et al., 2008; López-Rull et al., 2015). Therefore, both direct and indirect effects of fluoroquinolones can act together to cause candidiasis or to increase the invasiveness and effects of a prior infection in vultures. In addition to the potential detrimental effects on vultures, contamination with antibiotics can promote bacteria resistance to these drugs, which represents a major concern for the emergence of resistant disease worldwide.

The present study further confirms the concerning exposure of avian scavengers to livestock pharmaceuticals, expanding our knowl-

edge to two endangered vultures that include wild animals in their diet. As cited above, the presence of several fluoroquinolones was previously recorded in nestling griffon vultures from central Spain (Blanco et al., 2016). Enrofloxacin was also found in adult griffon vultures largely dependent on stabled livestock carcasses in northern Spain (Casas-Díaz et al., 2016). Other antibiotics have been found in this species in south-eastern and central Spain, including trimethoprim, sulfadiazine, tetracycline, oxytetracycline, chlortetracycline, erythromycin, nalidixic acid and nafcillin (García-Fernández et al., 2013; Blanco et al., 2016). Topical antiparasitics (diazinon and permethrin) were found in bearded vultures (*Gypaetus barbatus*) and livestock carcasses used in supplementary feeding of this threatened species in the Pyrenees (Mateo et al., 2015). In addition, a griffon vulture was found exposed and presumably killed by flunixin (Zorrilla et al., 2015), a non-steroidal anti-inflammatory used in livestock farming.

5. Conclusions and recommendations

Fluoroquinolones, as indicators of general exposure to pharmaceuticals with detrimental effects on health, are especially concerning in the cinereous vulture, although further research is required to assess the impact on nestling survival and fitness, and their consequences on population dynamics. This is especially important since the increasing population trends of this species over the last decades (BirdLife International, 2016) may mistakenly downplay the impact of fluoroquinolones on health without sufficient knowledge of the metapopulation structure and functioning. The less pronounced exposure to pharmaceuticals in Egyptian vultures due to diet could change if their dependence on livestock carcasses further increases in the future as a consequence of the growth of stabled stock and the elimination of their carcasses in vulture restaurants (Cortés-Avizanda et al., 2016). This effect may be augmented due to the general biodiversity loss in the Mediterranean (Cuttelod et al., 2009) in parallel with the population reductions of many of the wild species that constituted a primary part of the diet of these vultures, especially lagomorphs and medium-sized birds and reptiles of open habitats (Pleguezuelos et al., 2002; Virgós et al., 2007; Escandell, 2015). Given the delicate conservation status of Egyptian vulture, the impact of fluoroquinolones requires priority attention for its consequences on fledgling survival during the critical migration to Africa.

In sum, the currently strong dependence of cinereous and Egyptian vultures on domestic, rather than wild, animals compared with past decades (Garzón, 1973; Hiraldo, 1976; Donázar, 1993), and the growing intensification of livestock farming implies an expected increase in the impact of pharmaceuticals on scavenger populations. This requires further evaluation regarding consequences on biodiversity conservation and environmental health. Special attention should be paid to the concerning exposure to pharmaceuticals due to the increasing use of stabled livestock in supplementary feeding programs, and its consequences contrary to their primary aim of the conservation of endangered species (Cortés-Avizanda et al., 2016). We encourage the prioritization of efforts to promote the use of free-range livestock carcasses in the countryside by scavengers. Additionally, effort should be made to encourage the population recovery of wild species that once dominated the diets of vultures.

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