



Wildlife contamination with fluoroquinolones from livestock: Widespread occurrence of enrofloxacin and marbofloxacin in vultures



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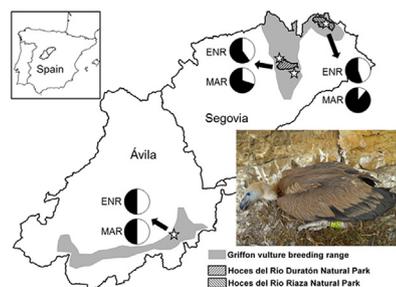
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HIGHLIGHTS

- We evaluated the presence of fluoroquinolones in nestling vultures.
- A high proportion of individuals showed fluoroquinolone residues at variable concentrations.
- These and other drugs were potentially ingested throughout nestling development.
- This is the first study showing wildlife contamination with fluoroquinolones from livestock carrion.
- The chronic ingestion of quinolones can have negative health consequences for nestlings.

GRAPHICAL ABSTRACT



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ABSTRACT

There is much recent interest in the presence and impact of veterinary pharmaceuticals in wildlife. Livestock carcasses are often disposed of in supplementary feeding stations for avian scavengers, as a management and conservation tool for these species worldwide. In feeding stations, vultures and other scavengers can consume carcasses almost immediately after disposal, which implies the potential ingestion of veterinary pharmaceuticals as a non-targeted consequence of supplementary feeding. Using UPLC-MS/MS and HPLC-TOF, we evaluated the presence and concentration of fluoroquinolone residues in plasma of nestling vultures feeding on domestic livestock carrion. Three different fluoroquinolones (marbofloxacin, enrofloxacin and its metabolite ciprofloxacin) and a non-targeted β -lactam (nafcillin) were detected in vulture plasma. The high proportion of individuals (92%) with fluoroquinolone residues at variable concentrations (up to $\sim 20 \mu\text{g L}^{-1}$ of enrofloxacin and $\sim 150 \mu\text{g L}^{-1}$ of marbofloxacin) sampled in several geographically distant colonies and on different dates suggests that these and other drugs were potentially ingested throughout nestling development. Contamination with veterinary fluoroquinolones and other pharmaceuticals should be considered as an unintended but alarming consequence of food management in threatened wildlife.

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1. Introduction

Veterinary medicines are widely used in livestock operations worldwide (Boxall et al., 2003; Sarmah et al., 2006; Woodward, 2009). In an attempt to prevent pharmaceuticals in food for human consumption, sanitary legislations generally establish farm and slaughterhouse withdrawal periods without medication, as well as controls of legal residue limits in livestock meat and its products (Woodward, 2009; Toldrá and Reig, 2012). These waiting periods and residue surveillance do not apply to animal by-products not intended for human consumption. Thus, entire carcasses and meat remains such as viscera and offal from stabled and intensive livestock operations are often disposed of in supplementary feeding stations for avian scavengers, as a management and conservation tool for these species worldwide (Gilbert et al., 2007; Donázar et al., 2009; Fielding et al., 2014). Carrion used for this purpose is often that of sick livestock, medicated just before death and disposed of in feeding stations a short time afterwards. Avian scavengers can consume carcasses almost immediately after disposal (Cortés-Avizanda et al., 2012), which implies the likely and potentially frequent ingestion of veterinary pharmaceuticals and harmful pathogens harbouring multi-resistance to these drugs (Marín et al., 2014; Mora et al., 2014; Blanco, 2015), as a non-target consequence of supplementary feeding.

The decline to near extinction of vultures in Asia due to the ingestion of the non-steroidal anti-inflammatory diclofenac from livestock carcasses is the most patent example of the catastrophic consequences of veterinary pharmaceuticals for wildlife (Watson et al., 2004; Gilbert et al., 2007). A large variety of drugs, including antibiotics, antiparasitics, antifungals, analgesics, hormones, etc. are used on a routine basis in curative and preventive treatments in factory farming and other less productive systems (Sarmah et al., 2006; Woodward, 2009). However, apart from unintended poisoning by euthanasic drugs (Friend and Franson, 1999; O'Rourke, 2002), there is a general lack of knowledge about the occurrence and impact of poisoning and subtle intoxication of scavengers with the wide array of veterinary drugs habitually administered to livestock. Therefore, assessing the eventual ingestion of these pharmaceuticals and their effects on the health of scavengers is crucial given its implications in public health and wildlife conservation. Because Spain is the main stronghold for vultures in Europe, an intense debate has been aimed at reconciling sanitary and environmental policies, including the risk of ingestion of veterinary pharmaceuticals from livestock carcasses consumed by wild scavengers in the field or at supplementary feeding stations (Donázar et al., 2009; Margalida et al., 2014).

The animal husbandry industry used different families of antibiotics depending on their availability and suitability for the treatment of bacterial infections, but also for growth promotion (now banned in the European Union) and performance enhancement. From the 1980's to today, the farming industry has widely used fluoroquinolones as the selected antimicrobials in food-producing animals (Brown, 1996; Martínez et al., 2006). Fluoroquinolones are synthetic antimicrobials with a broad spectrum of action against Gram-negative, and some Gram-positive, bacteria, as well as against mycoplasma (Andriole, 2005; Sharma et al., 2009). Thus, these drugs have a wide use for treatment of many different infections in all livestock and pet species (EMEA, 2006; Martínez et al., 2006). Enrofloxacin was the first fluoroquinolone approved for exclusive use in the farm industry, especially for the treatment of swine, cattle, poultry and rabbits, depending on each country's specific regulation (Anderson et al., 2003; EMEA, 2006; Woodward, 2009). Other fluoroquinolones have been approved and are used in human

medicine, especially ciprofloxacin, but not in livestock farming, in order to preserve their efficacy and avoid bacterial resistance in animals destined for human consumption (EMEA, 2006; Martínez et al., 2006; Dalhoff, 2012). In addition, the occurrence of antibiotic residues in the environment has been recorded as due to contamination by urban and livestock effluents and waste (Kummerer, 2010; Van Doorslaer et al., 2014; Vasquez et al., 2014; Gothwal and Shashidhar, 2015), with concerning consequences for the emergence of antibiotic-resistant pathogens (Levy, 2002; McEwen and Fedorka-Cray, 2002; Martínez et al., 2006). Pollution with fluoroquinolones is also concerning because of their high persistency in environment, with consequences on bio-accumulation and toxicity (e.g., Boxall et al., 2003; Sturini et al., 2009, 2012; Vasconcelos et al., 2009). Therefore, the presence of fluoroquinolones (hereafter quinolones) as biologically active compounds in wildlife warrants specific research evaluating their potential impact on biodiversity, as well as on public health through the role of wild animals as reservoirs and dispersers of bacterial antibiotic resistance.

The determination of quinolones in livestock plasma and tissue has been conducted using high-performance liquid chromatography coupled with fluorescence and mass spectrometry detection (Hernandez-Arteseros et al., 2002; Barbosa et al., 2009; Hermo et al., 2011; Jiménez-Díaz et al., 2013). In this study, we used ultra high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QqQ) and high-performance liquid chromatography time-of-flight mass spectrometry (HPLC-ToF) to determine the presence and concentration of quinolones in the plasma of nestling vultures feeding regularly on carcasses of intensively-reared livestock. We assessed the extent of contamination with quinolones by sampling nestling vultures of different ages and sexes on different dates in several breeding colonies in central Spain. To our knowledge, this is the first study evaluating the contamination with fluoroquinolones through ingestion with medicated livestock in vultures.

2. Material and methods

2.1. Study area and species

The study was conducted in the distribution range of the Eurasian griffon vulture (*Gyps fulvus*) in the Central Mountains and associated canyons of the Castilian Highlands, in Avila and Segovia provinces, central Spain. The area includes a complex of cliffs, canyons and pinewoods where a large population of griffon vultures and other avian scavengers nest (Martínez et al., 1997; Fargallo et al., 1998). We selected four sampling sites corresponding to different vulture eyries, including the main breeding colonies within or near the Natural Parks of Hoces del Río Riaza and Hoces del Río Duratón, in the Segovia province, as well as another colony in the south of Ávila province (Fig. 1).

The griffon vulture is a large (~8–10 kg) obligate top scavenger showing high sociality at carcasses, and breeding and roosting sites. This species inhabits hilly and open areas throughout the Palearctic region, where it acts as a key scavenger foraging on wild ungulates and domestic livestock. Breeding griffon vultures are year-round residents in the study area; laying begins in late December and young fledge from June–August (Martínez et al., 1997, 1998). Females lay one egg per clutch and both sexes are responsible for incubation and feeding of the nestling until independence >100 days after hatching. In the study area, griffon vultures and other scavengers are highly dependent upon livestock carrion found at carcass dumps provided by stabled livestock operations, mostly of swine and poultry (Blanco, 2014).

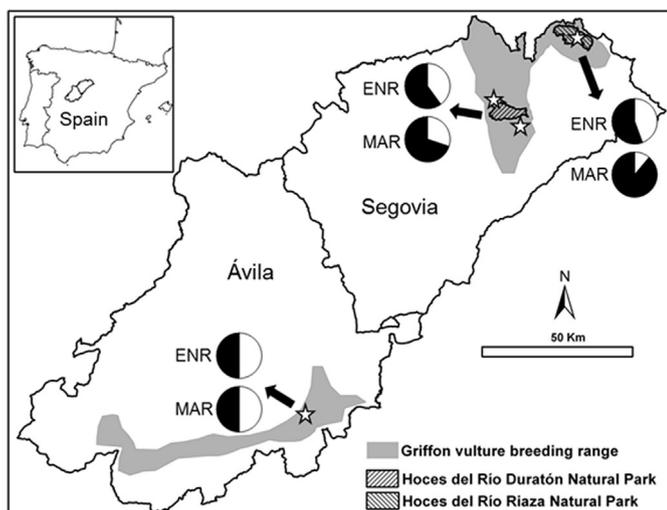


Fig. 1. Map of the study area showing the location of the sampled breeding colonies within the distribution range of griffion vultures in Segovia and Ávila provinces, central Spain. The areas covered by the Natural Parks of Hoces del Río Riaza and Hoces del Río Duratón, in the Segovia province, are shown. Sampling eyries are represented as white stars. The circles represent the proportion (%) of individuals with (black areas) or without (white areas) plasma residues of enrofloxacin (ENR) and marbofloxacin (MAR).

2.2. Fieldwork

During the breeding season of 2013, a sample of vulture nests was intensively monitored as part of a long-term population monitoring program conducted in the study area (Blanco et al., 1997; Martínez et al., 1997). Vulture nests were accessed by climbing and nestlings ($n = 25$) were sampled at about 50–80 days of age within or near the Natural Parks of Hoces del Río Riaza ($n = 9$) and Hoces del Río Duratón ($n = 10$), in the Segovia province, and in a colony in Ávila province ($n = 6$).

A blood sample (3–5 mL) was taken from the brachial vein, transferred to vials containing heparin and kept chilled until arrival at the laboratory within 5 h after collection. On the day of collection, blood samples were centrifuged at 13,000 g for 10 min to obtain plasma, which was frozen at $-20\text{ }^{\circ}\text{C}$ until analysis. A drop of blood was used for sexing the individuals through molecular procedures (Wink et al., 1998).

2.3. Reagents, standards and stock solutions

Quinolones were purchased from various pharmaceutical firms: ciprofloxacin (Ipsen Pharma, Barcelona, Spain), sarafloxacin, difloxacin, enrofloxacin (Cenavisa, Reus, Spain), danofloxacin (Pfizer, Karlsruhe, Germany), marbofloxacin (Sigma, St. Louis, MO, USA), and pipemidic acid (Prodesfarma, Barcelona, Spain). Pipemidic acid was used as an internal standard. Structures of the studied quinolones are shown in Fig. S1.

All reagents were of analytical grade. Merck (Darmstadt, Germany) supplied formic (HCOOH) and trifluoroacetic (TFA) acids and Fluka, the acetic acid (Sigma, St. Louis, MO, USA). Acetonitrile (MeCN) was purchased from VWR (Radnor, PA, USA) and methanol (MeOH) from Panreac (Castellar del Vallès, Spain). Ultrapure water generated by the Milli-Q system (Millipore, Billerica, MA, USA) was used.

Individual quinolone stock solutions of marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin and difloxacin were prepared at concentrations of $250\text{ }\mu\text{g mL}^{-1}$. All solutions were prepared in acetic acid 50 mM. An individual quinolone stock solution of pipemidic acid at a concentration of $100\text{ }\mu\text{g mL}^{-1}$ was

prepared in 50 mM acetic acid: MeCN (80:20; v/v). Individual stock solutions of each quinolone were prepared for the optimization of ionization potentials at the MS in a mixture of MeOH with 0.1% HCOOH. All solutions were stored at $4\text{ }^{\circ}\text{C}$.

2.4. Instrumentation, analytical conditions and procedures

Chromatographic separation of the quinolones was performed on a Symmetry C8 5 mm (2.1 mm i.d. x 50 mm) from Waters (Milford, MA, USA).

Chromatographic analysis by UHPLC was performed using an Acquity-Ultra Performance LC from Waters (Milford, MA, USA) equipped with an autosampler. API 3000 triple-quadrupole mass spectrometer (QqQ) (PE Sciex, Framingham, MA, USA) with a turbo ionspray source was used in positive mode. The system was controlled by the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA) and the Acquity Console to control the UHPLC.

A HP Agilent Technologies HPLC 1100 system was also used. This system was equipped with an autosampler and coupled to a 6220 ToF LC/MS mass spectrometer with an electrospray ionization source (ESI) from Agilent Technologies (Santa Clara, CA, USA). The system was controlled by Mass Hunter workstation software.

Solid phase extraction (SPE) was carried out using a Supelco vacuum 24-cartridge manifold (Bellefonte, PA, USA) connected to a Supelco vacuum tank. The SPE cartridges used in this study were Strata X ($1\text{ cm}^3/30\text{ mg}$; Phenomenex, Torrance, USA). The $0.45\text{ }\mu\text{m}$ pore size nylon filter membranes (Sharlab, Barcelona, Spain) were used to filter the extracts before injection into the chromatographic system. A Vacuum system miVac Duo Concentrator from GeneVAC (Ipswich, UK) was used to evaporate samples. A Mikro 220R centrifuge from Hettich Zentrifugen (Hettich Zentrifugen, Germany) was also used.

The method used to treat samples was developed previously to analyse quinolones in pig and cow plasma samples (Hermo et al., 2011). Briefly, a sample (0.5 mL) of plasma was placed in a 1.5 mL polypropylene Eppendorf tube. An appropriate volume of I.S. was added. When the final extracts were analysed by LC, the concentration of IS was $100\text{ }\mu\text{g L}^{-1}$. For the protein precipitation, prior to carrying out SPE extraction, 1 mL MeCN was added and mixed for 1 min using a vortex. The samples were then centrifuged at 12,000 rpm for 5 min. Supernatant was transferred to a 15 mL polypropylene tube and 10 mL of Milli-Q water were added to decrease any high concentrations of MeCN.

Strata X, based on a poly(benzylpiperidone) polymer, was used to extract and preconcentrate analytes. 1 mL MeOH was used to precondition the cartridge, 10 mL plasma sample were added and 2 mL Milli-Q water and 2 mL 1% TFA: MeCN (25:75, v/v) were used to wash and elute antibiotics, respectively. Extraction solution was evaporated to dryness using a vacuum concentrator and the residue was dissolved in $200\text{ }\mu\text{L}$ of Milli-Q water.

2.5. Chromatographic and mass spectrometric conditions

For HPLC and UHPLC, optimization of the chromatographic separation was carried out. Gradient programs featuring a mobile phase that combined solvent A (MeOH) and solvent B aqueous solution of 0.1% HCOOH were used. The chromatographic gradient used in the separation of the studied quinolones is shown in Table S1. The flow rate was 0.3 mL min^{-1} and the injection volume was $10\text{ }\mu\text{L}$.

For UHPLC-QqQ, a turbo ion-spray source in positive mode was used, since the amino group present in most quinolones is easily protonated in acidic media. Ionization potential (IP), declustering potential (DP), focussing potential (FP), entrance potential (EP) and collision energy (CE) were optimized directly injecting each

compound individually at an automatic flow rate of $10 \mu\text{L min}^{-1}$. Optimized parameters are shown in Table S2. 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} .

MS–MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions, in the collision cell of the triple quadrupole mass spectrometer. They were then mass-analysed using the second analyser of the instrument. In all experiments, CAD gas (N_2) of 4 (arbitrary units) was used. Multiple reaction monitoring (MRM) mode was chosen for the experiments in MS–MS. Two transitions were followed for each quinolone. The specific transitions and collision energies used to quantify and confirm the quinolones in plasma samples are shown in Table S2.

The optimum parameters of ToF in positive mode were as follows: capillary voltage, 4000 V; drying gas (N_2) temperature, 300°C ; drying gas (N_2) flow rate, 9 mL min^{-1} ; nebulizer gas (N_2), 40 psi; fragmentor voltage, 150 V; skimmer voltage, 60 V; and OCT I RF voltage, 250 V. The ToF-MS mass resolving power was approximately 10,000 full width at half-maximum (FWHM) at m/z 922. Spectra were acquired over the m/z 150–650 range. Data store was in profile and centroid modes. The exact mass for the quinolones studied in this work are shown in Table S3.

Because complex matrices require extensive sample preparation procedures in order to quantitatively extract the antibiotics, avoiding matrix interferences, the observation of signal suppression or signal enhance (matrix effect) for fluoroquinolones in MS was studied. To evaluate the presence or absence of matrix effect, calibration curves prepared in matrix and subjected to the sample treatment were compared with calibration curves obtained when the plasma was substituted with water. In order to establish calibration curves with UHPLC-QqQ and HPLC-ToF, six concentration levels of spiked blank plasma ($5\text{--}200 \mu\text{g L}^{-1}$) were prepared in duplicate and injected into the separation system. As internal standard, a compound that is very similar, but not identical to the chemical species of interest in the samples should be selected. When a chromatographic system is used, a similar retention time is also required. In this work, pipemidic acid was selected as internal standard, because all these requirements are met. Pipemidic acid at $100 \mu\text{g L}^{-1}$ was added at all points of the calibration curves. The occurrence of pipemidic acid has been reported in aqueous environment (e.g. Zhang et al., 2014), but this compound was not observed in blank nor plasma samples analysed. A mixture of plasma from Magellanic penguins (*Spheniscus magellanicus*) from Argentinean Patagonia and Red-billed coughts (*Pyrhhorax pyrhororax*) from Spain were used as blank plasma. In these sampling areas, these species feed primarily on pelagic schooling fishes and to a lesser extent on squid (Forero et al., 2002) and hypogeal invertebrates (Sánchez-Alonso et al., 1996), respectively, and thus were not exposed to quinolones, which was confirmed in this study. Results were presented as the signal of quinolone/IS ratio vs. the quinolone/IS concentration ratio. The same concentration levels were prepared substituting blank plasma with water.

The comparison of detection methods of quinolones in plasma required a previous optimization of liquid chromatography conditions. The ionization efficiency of the analyte may be affected when analytes co-elute with compounds originating from the matrix and then enter the ion source simultaneously causing enhanced or suppressed signals. Thus, chromatographic gradient conditions were adjusted to keep chromatographic run times as short as possible and to achieve nearly complete separation of all analytes. The separation of the antibiotics in the system UHPLC-QqQ, obtaining an adequate separation in less than 5 min, is shown in Fig. S2.

All compounds presented good linearity in the concentration

range studied (from 5 to $200 \mu\text{g L}^{-1}$) with a correlation coefficient higher than 0.98 for both matrices (water and plasma). Table S4 shows the calibration curves obtained by UHPLC-QqQ.

The influence of matrix in the sensitivity was evaluated by comparison of slopes of standard calibration curves in biological matrices after sample preparation with those established in water (Macarov et al., 2012). As can be observed in Table S4, all substances studied show different slopes in plasma and water. The results of the statistical analysis show statistically significant differences between the calibration curves of the quinolones prepared in water from those prepared in plasma, because the F-values (10.59) > F-critical (6.60). The behaviour of the quinolones depends on the analysed matrix.

3. Results

We found circulating quinolone residues in 23 of 25 nestling vultures (92.0%), from the analysis with UHPLC-QqQ considering the presence of ciprofloxacin (Table 1; the presence of ciprofloxacin was not determined by HPLC-ToF). Quantifiable residues of enrofloxacin and marbofloxacin were found in the same proportion of individuals by HPLC-ToF and UPLC-QqQ (Table 1). The chromatographic peak corresponding to enrofloxacin and marbofloxacin were also found in other samples (24.0% and 36.0% respectively, $n = 25$) but their signals were below the calibration curves and could not be quantified. 20.0% of individuals ($n = 25$) showed both enrofloxacin and marbofloxacin at quantifiable concentrations. Ciprofloxacin, the metabolite of enrofloxacin, was also found in 32.0% of individuals ($n = 25$) at concentrations too low to be quantified (Table 1).

When there was sufficient plasma volume, the samples were analysed in duplicate by UHPLC-QqQ (low resolution) and HPLC-ToF (high resolution). This allowed us to confirm that both detection methods yielded repeatable quinolone concentrations between duplicate samples from the same individuals (UHPLC-QqQ: Pearson $r = 0.95$, $p < 0.001$, $n = 7$, $r = 0.96$, $p = 0.002$, $n = 6$; HPLC-ToF: $r = 0.83$, $p = 0.020$, $n = 7$, $r = 0.922$, $p = 0.009$, $n = 6$, for enrofloxacin and marbofloxacin, respectively). The concentration of quinolones found in each individual, considering average values when there was more than one determination per sample (see above), was repeatable between detection techniques ($r = 0.92$, $p < 0.001$, $n = 8$ and $r = 0.99$, $p < 0.0001$, $n = 8$, for enrofloxacin and marbofloxacin, respectively, Table 1). As an example, the chromatogram obtained for a sample (MHX) positive for the three quinolones by UPLC-QqQ is shown in Fig. 2; chromatograms of standards of these quinolones and chromatograms with the transitions of identification have been included for comparison and confirmation. The sample contained a high concentration ($\sim 140 \mu\text{g L}^{-1}$) of marbofloxacin and $\sim 11 \mu\text{g L}^{-1}$ of enrofloxacin. The concentration of marbofloxacin was significantly higher than that of enrofloxacin as determined by HPLC-ToF (using mean values for each quinolone when more than one sample was analysed per individual, see above), both considering average concentration (t-test, $t = 3.70$, $p = 0.002$, $n = 8$ and 9) and concentrations corresponding to individuals with the simultaneous presence of both quinolones in plasma (paired t-test, $t = 3.00$, $p = 0.04$, $n = 5$, similar results were found for concentrations obtained with UPLC-QqQ, results not shown).

The proportion of individuals with circulating quinolones, including those with quantifiable and non-quantifiable levels, was similar between vulture breeding colonies for enrofloxacin and marbofloxacin (Fisher exact test, $p = 1.00$ and $p = 0.33$, respectively, Fig. 1). When quinolone concentrations were below the limit of detection, we considered half of this value ($5.0 \mu\text{g L}^{-1}/2$) to include a higher sample size in the analysis for differences among colonies.

Table 1
Results of the presence and concentration ($\mu\text{g L}^{-1}$) of quinolones detected by different HPLC techniques in plasma of nestling griffon vultures ($n = 25$) from central Spain.

	Enrofloxacin	Ciprofloxacin	Marbofloxacin	Total quinolones
UHPLC-QqQ				
Positive samples (%)	14 (56.0)	8 (32.0)	18 (72.0)	23 (92.0) ^b
Quantifiable samples (%)	8 (32.0)	0 (0.0)	9 (36.0)	12 (48.0) ^b
Mean concentration (SD)	11.4 (3.1)	—	62.1 (35.2)	54.2 (40.9) ^{b,c}
HPLC-ToF				
Positive samples (%)	14 (56.0)	— ^a	18 (72.0)	— ^d
Quantifiable samples (%)	8 (32.0)	—	9 (36.0)	12 (48.0) ^b
Mean concentration (SD)	13.4 (3.0)	—	62.1 (37.0)	55.5 (42.8) ^{b,c}

^a Not determined by HPLC-ToF.

^b Note that several individuals showed the simultaneous presence of several quinolones.

^c The sum of enrofloxacin and marbofloxacin concentrations.

^d Not calculated because the presence of ciprofloxacin was not determined by HPLC-ToF.

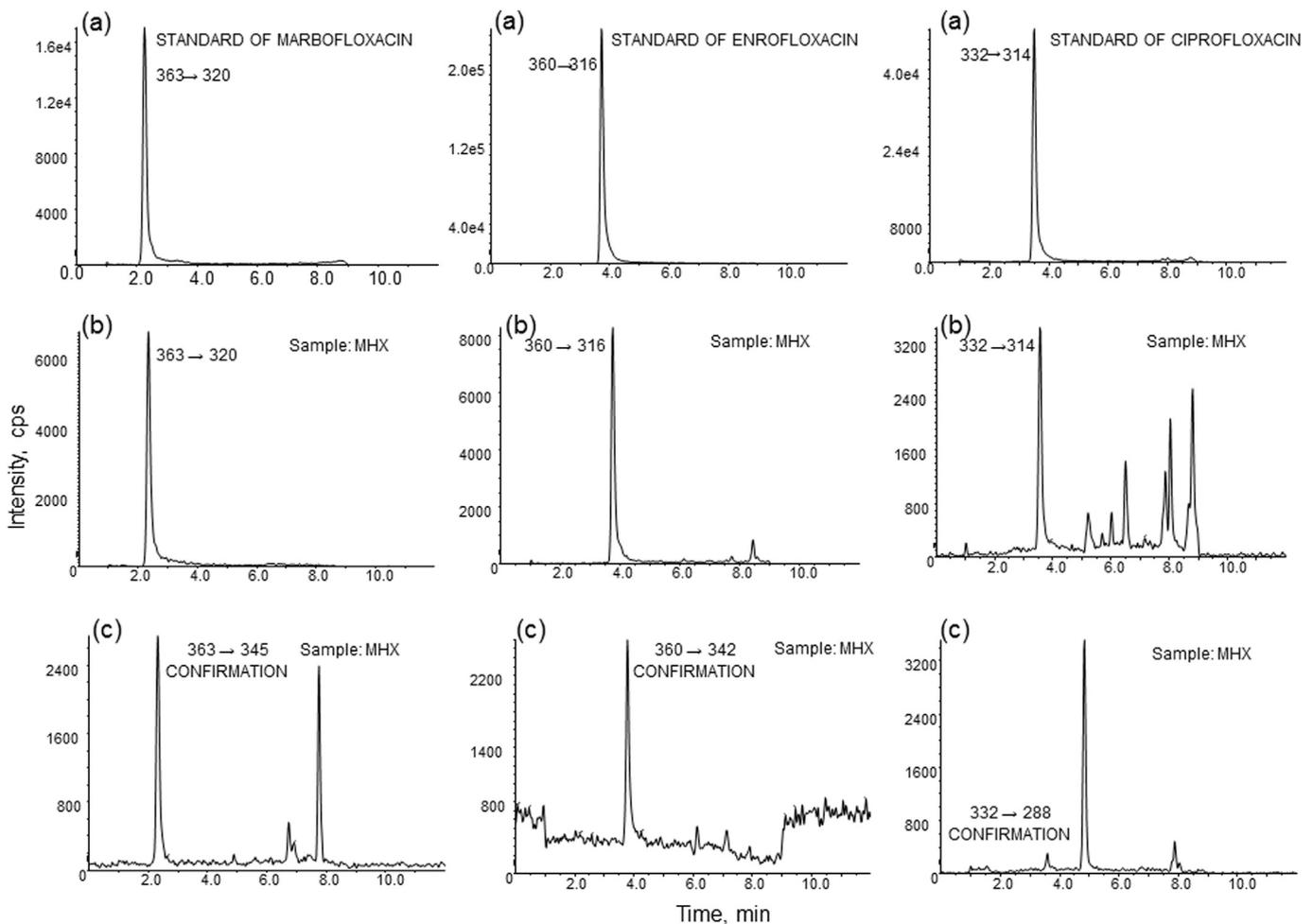


Fig. 2. UHPLC-QqQ chromatograms of (a) standard mixture of marbofloxacin, enrofloxacin and ciprofloxacin at a concentration of $200 \mu\text{g L}^{-1}$. The quantification transitions of marbofloxacin (363–320), enrofloxacin (360–316) and ciprofloxacin (332–314) are shown. (b) Chromatograms corresponding to vulture plasma (sample with reference MHX) positive for marbofloxacin, enrofloxacin and ciprofloxacin respectively is shown by their quantification transitions. (c) Confirmation transition for marbofloxacin, enrofloxacin and ciprofloxacin in vulture plasma (sample: MHX) is also shown.

These analyses showed that the concentration of enrofloxacin differed between colonies (one-way ANOVA, $F_{2,13} = 5.67$, $p = 0.020$, $R^2 = 0.51$), being lower in Ávila, where only non-quantifiable concentrations were found, than in the Natural Park of Hoces del Río Riaza (DMS post-hoc test, $p = 0.007$, Fig. 3); the concentration of marbofloxacin was similar among colonies ($F_{2,17} = 0.31$, $p = 0.74$, $R^2 = 0.04$, Fig. 3). No significant difference was found in the proportion of individuals with quinolones (enrofloxacin and

marbofloxacin) or their concentrations according to nestling sex (Fisher exact test and t-test, respectively, all $p > 0.05$).

The determination by UHPLC-QqQ only allows the analysis of target substances, while their analysis by HPLC-ToF was also used to identify non-target substances because spectra were acquired in full scan mode over the m/z 150–650 range. When samples of plasma were analysed by HPLC-ToF, a peak of m/z 415.1512 was observed in six of the analysed samples. A peak of m/z 415.1321 was

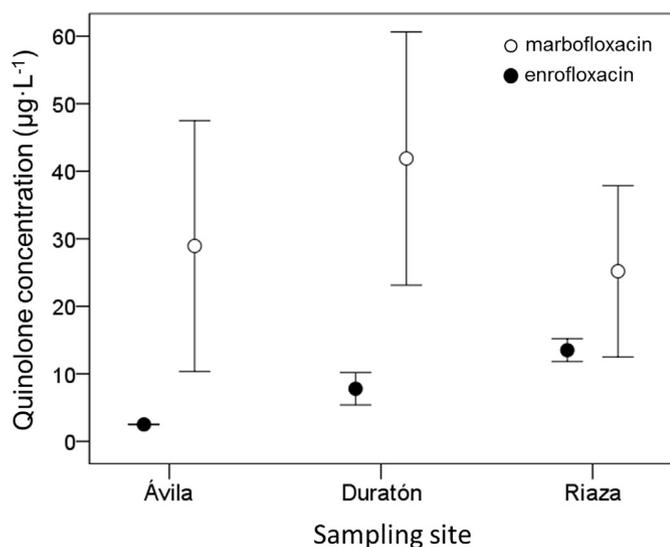


Fig. 3. Average \pm SD plasma concentration of enrofloxacin and marbofloxacin in nestling griffon vultures sampled in several breeding colonies in central Spain (see Fig. 1 for reference) as determined by HPLC-ToF.

also observed in one sample. The maximum discrepancy acceptable between experimental and accurate mass of unknown compounds was 5 ppm. The high resolution ToF system ensures that both masses belong to different compounds. Taking into account that working in positive mode the molecules must be protonated, the peak with m/z 415.1321 corresponding to $[M+H]^+$ fit with the accurate mass of nafcillin (415.1322), an antibiotic belonging to the family of β -lactams, with an assignment error of 0.2 ppm; previously a standard of nafcillin was injected in the LC-MS system and the same retention time and mass was obtained. The peak with m/z 415.1512 giving an assignment error of 46 ppm could not be identified.

4. Discussion

There is much recent interest in the presence and impact of veterinary pharmaceuticals in wildlife (Arnold et al., 2014). However, there is scarce information on contamination with antibiotics in wild birds (Li et al., 2012). In this study, we demonstrate the presence of three different fluoroquinolones in nestling plasma of a key avian scavenger using different HPLC techniques.

Most (92%) nestling griffon vultures sampled in central Spain showed at least one type of quinolone in plasma, and often the simultaneous quantifiable presence of enrofloxacin and marbofloxacin, as well as non-quantifiable residues of ciprofloxacin, the metabolite of enrofloxacin. We found similar proportions of individuals with each quinolone and slight differences in the concentration of enrofloxacin among breeding colonies from different geographical areas. This suggests that enrofloxacin and marbofloxacin were the quinolones of choice for livestock treatment across the study area, and that these drugs frequently pass to vultures through the livestock carcasses on which they feed. The detection of enrofloxacin in vulture plasma was not unexpected given that it has been extensively used in multiple treatments of different animal species since its introduction in livestock operations, especially in intensive stabled facilities (EMEA, 2006; Woodward, 2009; Moreno, 2014). The presence of marbofloxacin in even a higher proportion of individuals than enrofloxacin suggests that this quinolone is currently used, probably extensively, in livestock operations in central Spain in single treatments or in combination with enrofloxacin (EMEA, 2006; Moreno, 2014).

Although in this study we specifically focused on quinolones, we also found residues of the β -lactam nafcillin in a single individual. The adequate sampling and analytical procedures for the detection of β -lactams and other antibiotics require specific protocols because their chemical structures make them unstable and easily degradable (Okerman et al., 2007; Berendsen et al., 2009; Pérez-Parada et al., 2011). Therefore, the presence of nafcillin in a single sample can be considered indicative of the potential presence in vultures of this β -lactam and other antibiotics not specifically tested for.

Given that vultures can consume carcasses almost immediately after disposal in refuse dumps and feeding stations, the combination of antibiotics present in plasma found in this study may represent a sample of the potential mixture of pharmaceuticals likely present in vultures and other avian scavengers. Carcasses generally disposed of in supplementary feeding stations often correspond to sick livestock that died during disease and other treatments. Thus, the carcasses of these livestock can promote the ingestion of veterinary pharmaceuticals used to combat infection (e.g. antimicrobials, antiparasitics, antifungals) and their effects (e.g. analgesics, anti-inflammatories) while simultaneously promoting the transmission of livestock pathogens to wild scavengers (Marin et al., 2014; Blanco, 2015). Taken together, the fact that several quinolones were found in a high proportion of individuals of variable age since hatching in several geographically distant colonies sampled on different dates suggests that these and other drugs can be potentially ingested throughout the development stage of nestlings. This is supported by the presence of residues of ciprofloxacin in several individuals, both with or without its precursor enrofloxacin. This antibiotic is commonly used in livestock farming but its use has not been approved in human medicine, while the contrary is true for ciprofloxacin, in order to preserve efficacy in their respective uses while avoiding crossed bacterial resistances (Martinez et al., 2006; Dalhoff, 2012). Therefore, unless ciprofloxacin use in livestock was occurring illegally, their residues in vultures likely correspond to the metabolization from enrofloxacin in live livestock before their carcasses were available to vultures, or in the vultures themselves.

Levels of circulating quinolones were variable among vulture individuals, with concentrations up $\sim 20 \mu\text{g L}^{-1}$ of enrofloxacin and $\sim 150 \mu\text{g L}^{-1}$ of marbofloxacin. Average concentrations of marbofloxacin were consistently higher than those of enrofloxacin across sampling sites, which suggest a differential frequency of use and concentration in livestock treatment, or the different metabolism and excretion of these drugs by vultures. Of concern is the fact that a proportion of samples showed more than one quinolone at quantifiable concentrations, potentially promoting drug–drug interactions that may increase their concomitant adverse effects of non-targeted hosts (Martínez et al., 2006; Backhaus, 2014). These drugs may promote similar or different antimicrobial activity, activate different physiologic pathways and induce different or similar detrimental secondary effects on target individuals of each livestock species (Martinez et al., 2006; Backhaus, 2014). Adverse side-effects are known and generally reduced by other veterinary treatments under controlled conditions of drug administration against target pathogens, therapeutic dosage and veterinary control in livestock exploitations (Martínez et al., 2006; Cunningham et al., 2010). In the case of vultures, both the concentration and frequency of ingestion of each quinolone and other drugs present in the carcasses can be variable depending on the rate of nestling feeding and the kind of food provided by their parents. Therefore, the ingestion of quinolones can be chronic or discontinuous in frequency and pulsed in terms of concentrations coinciding with the consumption by vultures of medicated carcasses.

This unintended quinolone ingestion without any regular

patterns regarding frequency and concentration in vultures can potentially cause similar adverse side-effects than in livestock, especially poultry and pet birds (Flammer, 2006; Maslanka and Jaroszewski, 2009). Direct adverse effects and side-effects from antibiotic misuse or overuse are diverse, including the alteration of normal microbiota and overgrowth of pathogenic bacteria and fungi, gastrointestinal disturbances, hypersensitivity reactions, cartilage disorders, etc. (Patterson, 1991; Robinson et al., 2005; Cunha, 2001; Montagnac et al., 2005; Martinez et al., 2006; Granowitz and Brown, 2008), but there is scarce information on the detrimental effects of long-term contamination with variable concentrations of antibiotics and other pharmaceuticals in wildlife, including those derived from drug–drug interactions (Backhaus, 2014; Vasquez et al., 2014). These potential effects can especially jeopardize growing nestlings because their developing organs and metabolism are more vulnerable to external threats such as contamination (Markman et al., 2011; Morrissey et al., 2014). In fact, it has been argued that the adverse effects of quinolones are exacerbated during organismal development, especially causing alterations in joint cartilages, bone growth and tendons (Peters et al., 2002; Stahlmann, 2003; Chide and Orisakwe, 2007; Woodward, 2009). In addition to potential detrimental effects on wild animals, contamination with antibiotics can promote novel bacteria–antibiotic resistance interactions in natural environments (Allen et al., 2010; Berendonk et al., 2015), especially in bacterial species exclusive to or more common in particular wild host species (Keesing et al., 2010; da Costa et al., 2013). This bacterial resistance can be host-amplified, carried and spread in the environment due to the often long-distance and migratory movements of wild birds, which represent a major concern for the emergence of bacterial-resistant disease worldwide (Mølbak, 2004; Tsiodras et al., 2008; Bonnedahl and Jarhult, 2014).

In conclusion, a high proportion of nestling griffon vultures from central Spain showed fluoroquinolone residues at variable concentrations in plasma. To our knowledge, this is the first study showing wildlife contamination with fluoroquinolones through consumption of medicated livestock carcasses. Fluoroquinolones and other drugs were potentially ingested throughout nestling development, suggesting a chronic contamination with potential negative health consequences. The disposal of diseased and subsequently medicated livestock carcasses in feeding stations can imply the concerning spillover of veterinary pharmaceuticals and pathogens as a non-target consequence of feeding management of threatened wildlife.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://>

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