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Second-generation anticoagulant rodenticides in the blood of obligate and facultative European avian scavengers☆

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ABSTRACT

The widespread use of second-generation anticoagulant rodenticides (SGARs) and their high persistence in animal tissues has led to these compounds becoming ubiquitous in rodent-predator-scavenger food webs. Exposure to SGARs has usually been investigated in wildlife species found dead, and despite growing evidence of the potential risk of secondary poisoning of predators and scavengers, the current worldwide exposure of free-living scavenging birds to SGARs remains scarcely investigated. We present the first active monitoring of blood SGAR concentrations and prevalence in the four European obligate (i.e., vultures) and facultative (red and black kites) avian scavengers in NE Spain. We analysed 261 free-living birds and detected SGARs in 39.1% (n = 102) of individuals. Both SGAR prevalence and concentrations (ΣSGARs) were related to the age and foraging behaviour of the species studied. Black kites showed the highest prevalence (100%), followed by red kites (66.7%), Egyptian (64.2%), bearded (20.9%), griffon (16.9%) and cinereous (6.3%) vultures. Overall, both the prevalence and average ΣSGARs were higher in non-nestlings than nestlings, and in species such as kites and Egyptian vultures foraging in anthropic landscapes (e.g., landfill sites and livestock farms) and exploiting small/medium-sized carrions. Brodifacoum was most prevalent (28.8%), followed by difenacoum (16.1%), flocoumafen (12.3%) and bromadiolone (7.3%). In SGAR-positive birds, the ΣSGAR (mean ± SE) was 7.52 ± 0.95 ng mL^{-1}; the highest level detected being 53.50 ng mL^{-1}. The most abundant diastereomer forms were *trans*-bromadiolone and flocoumafen, and *cis*-brodifacoum and difenacoum, showing that lower impact formulations could reduce secondary exposures of non-target species. Our findings suggest that SGARs can bioaccumulate in scavenging birds, showing the potential risk to avian scavenging guilds in Europe and elsewhere. We highlight the need for further studies on the potential adverse effects associated with concentrations of SGARSs in the blood to better interpret active monitoring studies of free-living birds.

1. Introduction

The contamination of food webs with anticoagulant rodenticides (ARs) is currently of major concern to environmental toxicologists and wildlife ecotoxicologists (Rattner et al., 2014; Lohr and Davis, 2018; van den Brink et al., 2018; Ravindran et al., 2022). Rodents comprise the largest mammalian order with >2500 species (Kay and Hoekstra, 2008) and human-rodent conflicts occur worldwide (e.g., by consuming and spoiling crops and stored grain, damaging infrastructure, predating endemic species, and spreading human and livestock diseases; van den Brink et al., 2018) and cost several billion Euros each year (Jacob and Buckle, 2018). The multi-faceted nature of these conflicts requires increasing continuous anthropogenic controls, and the use of ARs has been the most frequent lethal method since the 1950s. However, the rapid development of rodent resistance to the early forms of rodenticides (i.e., first generation ARs or FGARs) has led to the development of more toxic and bioaccumulative second generation ARs (SGARs), also so-called "super-warfarins" (Thomas et al., 2011). The bioaccumulation

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of SGARs after repeated secondary exposure negatively affects the vitamin K-dependent coagulation pathway in birds of prey, leading to bleeding in or around critical organs (e.g., brain, heart, lungs) and causing sublethal impairments or death (Murray, 2018).

Although the use of ARs may be necessary for rodent control, the well-known risks of primary and secondary poisoning of non-target species necessitates debate on the need to require measures to mitigate the risks of ARs use (Buckle and Prescott, 2018; Eisemann et al., 2018; Witmer, 2018; Thornton et al., 2022). Indeed, the Convention on the Conservation of Migratory Species of Wild Animals (the CMS, or Bonn Convention) highlighted secondary exposure to ARs as one of the most important toxicological hazards for migratory birds (CMS, 2014). The high acute toxicity and persistence of ARs in animal tissues, especially of SGARs, has led to their becoming ubiquitous in rodent-predator-scavenger food webs (López-Perea and Mateo, 2018; Pay et al., 2021; Elliott et al., 2022; Cooke et al., 2022). Baits containing ARs may be consumed by a number of non-target primary consumers (e.g., invertebrates, fish, wild birds and mammals), increasing the risk of exposure across the entire food web (Shore and Coeurdassier, 2018; Regnery et al., 2020; Alabau et al., 2020).

Predator species that are highly specialized rodent-feeders, such as snakes, kestrels, owls and mustelids, are at a high risk of SGAR exposure (Lettoof et al., 2020; Roos et al., 2021; Elliott et al., 2022). However, generalist predators and scavengers may be exposed to secondary SGAR poisoning at similar or even higher levels than specialist predators (López-Perea and Mateo, 2018). This could be because clinically poisoned (e.g., sick) animals containing high SGAR concentrations can be easily caught and consumed by predators such as raptors (e.g., eagles and kites) and mammalian carnivores. They could also be eaten by obligate scavengers (i.e., species that depend entirely on carrion, such as vultures) and facultative scavengers (i.e., species that exploit carrion opportunistically, including raptors, corvids and mammalian carnivores) (Hindmarch and Elliott, 2018). In addition, avian scavengers frequently exploit food sources in urban landfill sites (Tauler-Ametller et al., 2017, 2019; Plaza and Lambertucci, 2018; Arévalo et al., 2022; Fernández-Gómez et al., 2022), where SGARs are constantly deployed (Coeurdassier et al., 2018). Despite these risk factors, few studies have investigated the exposure of avian scavengers to SGARs, particularly vulture species (Sánchez-Barbudo et al., 2012; Mateo et al., 2015; Plaza et al., 2019; Rial-Berriel et al., 2021; Moriceau et al., 2022).

The prevalence of wildlife exposure to SGARs has usually been assessed by passive monitoring of animals found dead in the field or admitted to wildlife rehabilitation centres. However, this method is probably biased towards higher prevalence values due to the inherent over-representation of poisoned animals in the samples. Active monitoring (i.e., by sampling live animals in the field) may therefore provide more accurate data on the prevalence of contaminant exposure than passive monitoring (Descalzo et al., 2021). However, active monitoring studies of wild bird exposure to SGARs are much less common. Regarding raptors, Martínez-Padilla et al. (2017) detected bromadiolone in the blood of 16.9% (n = 112) of common kestrel (*Falco tinnunculus*) fledglings with an average (±SE) concentration of 0.25 ± 0.02 ng mL^{-1}, in a region of central Spain during a common vole (*Microtus arvalis*) population outbreak. Badry et al. (2022) analysed ARs in the blood of nestling raptors from Germany and detected ARs in 22.6% (n = 53) of red kites (*Milvus*) and 8.6% (n = 35) of common buzzards (*Buteo*), with the highest median concentration (of brodifacoum at 13 ng mL^{-1}) observed in red kites, evidencing this species' risk of AR exposure. In France, Powolny et al. (2020) detected SGARs in the blood of 30% (n = 47) of red kite nestlings, with a median (and range) of 6.1 ng mL^{-1} (0.2–29.4). It should be noted that, as with other chemicals, blood samples may show lower SGAR concentrations than other tissues in which these compounds tend to bioaccumulate and persist for longer periods (i.e., the liver; see Horak et al., 2018), limiting their detectability in active monitoring studies relying on blood SGAR levels. For example, Murray (2020) detected SGARs in the liver of 100% (n = 43) of red-tailed hawks (*Buteo jamaicensis*) admitted to a wildlife clinic in the north-eastern United States, while only 32.6% showed SGARs in their serum. Another current limitation of blood SGAR levels is the lack of reference toxicity thresholds in blood, contrary, for example, to the established toxicity threshold in the liver (i.e., >0.1 μg g^{-1} in wet weight (Thomas et al., 2011; Rattner et al., 2014).

We carried out an active monitoring program in NE Spain to determine the prevalence and concentration of different SGARs (difenacoum, bromadiolone, brodifacoum and flocoumafen) in the blood of the four European obligate scavengers (griffon *Gyps fulvus*, cinereous *Aegypius monachus*, bearded *Gypaetus barbatus*, and Egyptian, *Neophron percnopterus* vultures) and facultative avian scavengers (black and red kites, *Milvus migrans* and *M. milvus*) with different dietary preferences to test five hypotheses: (1) that a higher proportion of rodents in the diet may increase the exposure to SGARs, because rodents are the target species for these biocides; (2) that non-nestlings bioaccumulate more SGAR residues than nestlings, because of their longer time at risk of exposure; (3) that certain diastereomer (*cis* vs *trans*) forms tend to bioaccumulate more in predator/scavenger species because of their different half-lives in animal tissues; (4) that species foraging in anthropic habitats would suffer greater exposure to these compounds because of the potential association between the exploitation of anthropogenic food resources found on landfill sites and livestock farms; and (5) that the SGAR prevalence observed in living individuals would be lower than that described in animals found dead in other studies in the same geographical area, because SGARs bioaccumulate and persist for longer periods in liver tissue.

2. Material and methods

2.1. Study area

The study was carried out in the Pyrenees (NE Spain) and adjacent regions (Fig. 1). This mountain range covers around 50,000 km^2 and runs from east-west forming a natural boundary between France and Spain (Améztegui et al., 2010). It is characterized by a strong altitudinal gradient ranging from sea level to >3000 m a.s.l. and encompasses a great diversity of vegetation types (Ninot et al., 2007). The area is characterized by extensively and semi-extensively grazed livestock (cattle, sheep and horses) and holds important populations of wild herbivorous ungulates, which provide most of the biomass for the scavenger guild (Colomer et al., 2011; Margalida et al., 2018). The scavenger assemblage (mainly facultative avian species) also exploits carnivorous mammal carcasses (see Fig. 2). The Pyrenees holds breeding populations of the four obligate avian scavengers (Table 1) and a rich community of facultative avian (e.g., raptors and corvids) and mammalian scavenger species (e.g., red fox *Vulpes*, wild boar *Sus scrofa*, *Martes*spp. and badgers *Meles*) (Oliva-Vidal et al., 2022). A network of active supplementary feeding stations (SFS) for avian scavengers is present throughout the Pyrenees (Moreno-Opo et al., 2015) and wildlife, mainly avian species, frequently exploit anthropogenic food resources at the urban open-air landfills that occur throughout the study area (Arizaga et al., 2018; Tauler-Ametller et al., 2018, 2019).

2.2. Studied species

We collected blood samples from 261 free-living obligate and facultative avian scavengers, from 2017 to 2021. Our study included the four European obligate avian scavengers (griffon, cinereous, bearded and Egyptian vultures). As for the facultative avian scavengers, we sampled red and black kites, one golden eagle (*Aquila chrysaetos*) and one Bonelli's eagle (*Aquila fasciata*) (Table S1), the latter being the only one that scavenges only rarely. The Spanish breeding population sizes and overall population trends of these species, and their main biological traits (e.g., breeding behaviour and principle feeding habits) are detailed in Table 1.

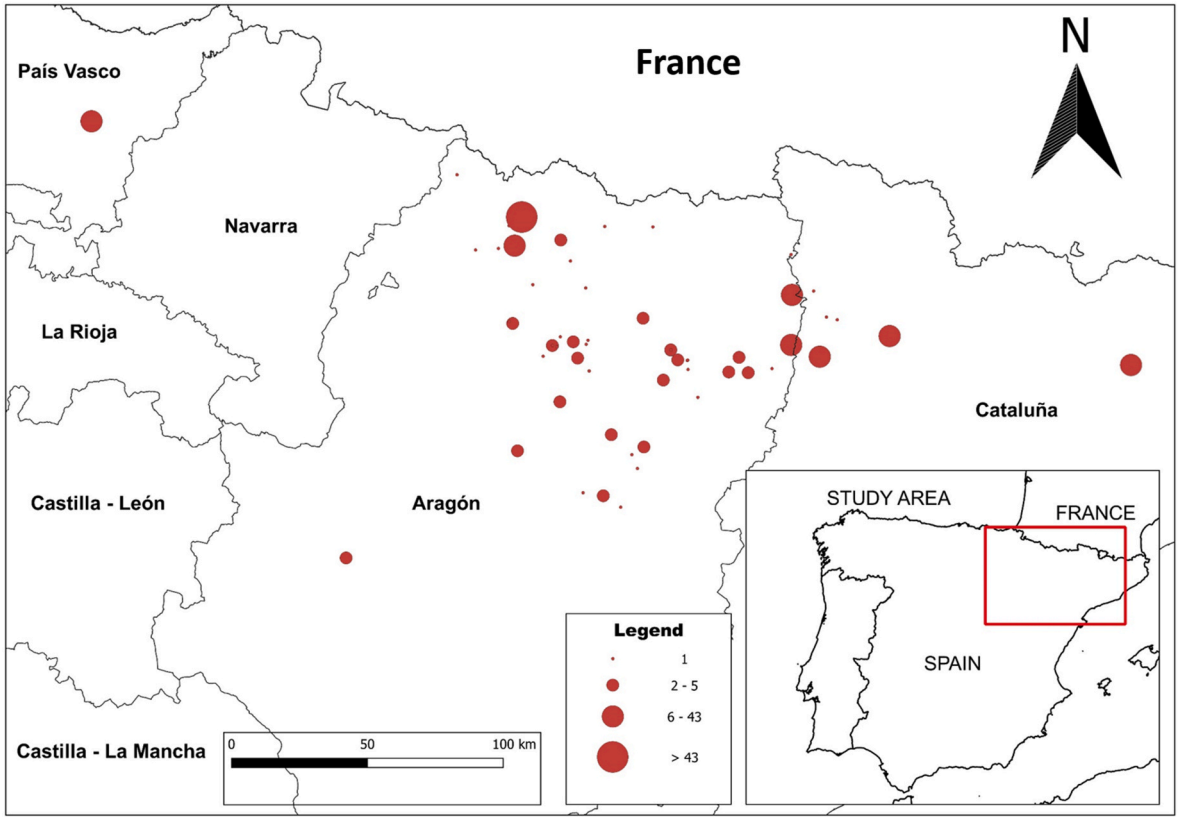

Fig. 1. Map of the study area showing the Pyrenees and adjacent regions (NE Spain) that were sampled from 2017 to 2021. The sampling points are shown according to the number of birds sampled at each location. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

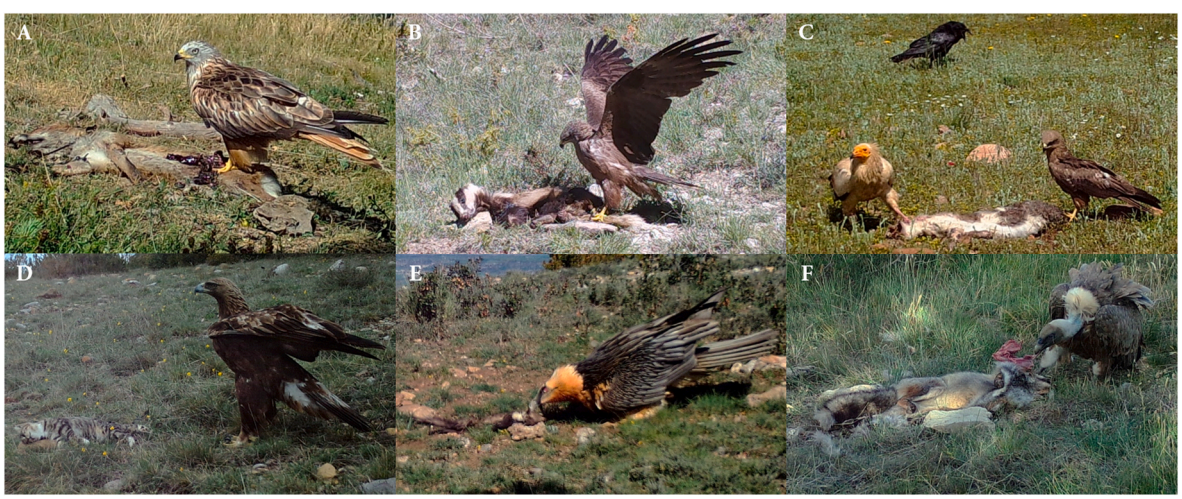

Fig. 2. Main avian scavenger species included in this study consuming carcasses of different mammalian carnivores in the study area. The consumption of carnivore carrion could help explain tertiary SGAR exposure or poisoning routes to scavenging species. A) red kite consuming a red fox; B) black kite consuming a badger; C) Egyptian vulture, black kite and common raven consuming a domestic cat (*Felis catus*); D) golden eagle consuming a domestic cat; E) bearded vulture consuming a stone marten (*Martes foina*); and F) griffon vulture consuming a red fox (see Oliva-Vidal et al., 2022). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

2.3. Sampling

Blood samples were collected when birds were handled to be fitted with satellite transmitters or ringed and marked with patagial tags. Both nestlings and non-nestling individuals (juveniles, subadults and adults, Table S1) were sampled. Each individual's age class was identified according to its moult pattern and plumage characteristics (Forsman, 2016). All individuals were handled by trained and authorized personnel. Specialized climbers visited nests when nestlings were feathered but not yet ready to fly. Non-nestling individuals were captured using a variety of methods (e.g., baited traps) according to the species concerned (Table S2). Whole-blood samples (3–5 mL, and never exceeding 1% of the body weight) were collected from the brachial vein, placed into EDTA or heparinized tubes and stored at −80 °C until

Table 1
Number of breeding pairs, population trend, breeding behaviour, main feeding habits and migratory status in Spain of the four obligate avian scavengers (vultures) and the main facultative avian scavenger species included in this study. The percentage of the European population represented by the Spanish populations of the four European vulture species are shown.

Species	Scavenger group	Breeding pairs	Population trend	Breeding behaviour[8]	Main feeding habits[9, 10, 11]	Migratory status
Griffon vulture	Obligate	30,946[1] [90%]	+	Colonial	Medium-sized and large carrion, garbage	Sedentary (adults) Migrant (juveniles)
Cinereous vulture	Obligate	2,548[2] [> 90%]	+	Colonial and territorial	Medium-sized and large carrion remains (tendons, muscle), small carrion	Sedentary
Bearded vulture	Obligate	140[3] [63%]	+	Territorial	Bone remains of medium-sized and large carrion, small carrions	Sedentary
Egyptian vulture*	Obligate	1,490[4] [32.2%]	–	Territorial	Small pieces of medium-sized and large carrion, small carrions, garbage	Migrant
Red kite	Facultative	1,994[5]	–	Territorial	Small carrion, garbage	Sedentary
Black kite*	Facultative	2,061[6]	+	Territorial	Small carrion, garbage	Migrant
Golden eagle+	Facultative	1,553(7)	+	Territorial	Small and medium-sized prey/carrion	Sedentary

Medium-sized and large carrion items mainly represent domestic and wild ungulate carcasses. Small carrion items represent rodents, birds, invertebrates and small/medium-sized mammals. Garbage refers to food sources found at open-air landfill sites.
(1) Del Moral and Molina (2018a); (2) Del Moral (2017); (3) Margalida and Martínez (2020); (4) Del Moral and Molina (2018b); (5) Cardiel (2006); (6) Palomino (2006); (7) Del Moral (2009); (8) van Overveld et al., (2020); (9) Moreno-Opo et al. (2015); (10) Tauler-Ametlller et al. (2019); (11) Ortiz-Santaliestra et al. (2019).
*Species with a landfill-based diet.
+Apex predator.

analysis. Two drops of blood were used for sexing the individuals using molecular procedures.

2.4. SGAR analysis

SGAR extraction was performed following the method described by Martínez-Padilla et al. (2017) with some modifications. Briefly, whole blood samples were thawed, and 400 μl were placed in a 10 mL-glass tube with 3 mL of dichloromethane:acetone (70:30), 2 g of sodium sulphate and 50 μl of internal standard (brodifacoum-d4 at 0.1 ng μL^{-1}) in methanol. Then, the sample was vortexed (10 min), sonicated (5 min) and centrifuged (1048 rcf, 5 min). The organic phase was transferred to another glass tube. This extraction was repeated again with another 3 mL of dichloromethane:acetone (70:30) and the extract obtained was pooled with the first one. The extract was then evaporated to dryness under a stream of N_2, resuspended in 200 μL of methanol, filtered through a 0.2 μm mesh nylon filter, and collected in a chromatography vial for analysis using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). The analytical equipment consisted of a liquid chromatograph (Agilent UHPLC Series 1290 Infinity II) coupled to a triple quadrupole mass spectrometer (Agilent 6470 LC/TQ). The chromatographic separation was performed using a reverse-phase column (Agilent InfinityLab Poroshell 120 EC–C18, 2.1 × 150 mm, 2.7 μm) in an oven at 40 °C. The injection volume was 10 μL. The mobile phase comprised a gradient elution of two solvents (A: ammonium acetate 10 mM, pH: 6.03; B: methanol). The initial conditions were 50% A and 50% B, reaching 100% B at min 13. This was maintained until min 14, returned to the initial conditions at min 15, and then left to stabilize until min 20 before the next sample injection. The flow rate was 0.250 mL min^{-1}. Ionization was performed using an electrospray ionization source (ESI) in negative mode with an Agilent Jet Stream. The conditions were as follows: gas temperature 300 °C; gas flow 8 L min^{-1}; nebulizer at 40 psi; sheath gas temperature 300 °C; sheath gas flow 11 L min^{-1}; capillary at 4000 V; and nozzle voltage/charging 1750 V. Fragment ion spectra were obtained in a dynamic multiple reaction monitoring (dMRM) scan. The fragmentor voltage, collision energy and ion fragments for quantification or qualification were optimised for every compound (Table S3). Up to four dMRM transitions were performed for each SGAR and identifications were considered positive when all the ratios between qualifier and quantifier ions in the sample differed by less than 20% compared with the standard ratios. *Cis* and *trans* diastereomers of the SGARs (with indistinguishable mass spectra) were identified according to the elution order of each form given by Fourel et al. (2017a). Data collection and processing were performed using the Masshunter™ Work-station from Agilent Technologies.

Matrix-matched calibrations were performed using partridge whole-blood extracts at concentrations of 6.25–250 ng mL^{-1} in a final volume of 200 μL, including 25 ng mL^{-1} of internal standard. Procedural blanks were analysed for each sample batch. Precision and accuracy were calculated using partridge whole-blood fortified with 31.25, 62.5 and 125 ng mL^{-1} of SGAR standards. The recovery percentage ranged between 89.0 and 95.8% and the relative standard deviation (RSD) was between 3.3 and 9.9%. The limits of quantification based on the minimum detected concentrations followed the identification criteria described above and ranged between 0.04 and 0.5 ng mL^{-1} with a response to noise ratio greater than 10 (Table S3).

2.5. Statistical analysis

We first calculated the blood concentration (ng mL^{-1}) of each SGAR (brodifacoum, bromadiolone, difenacoum and flocoumafen) in SGAR-positive birds (n = 102 individuals). Then, to ascertain whether avian species, age class (nestling or non-nestling) or sex (female or male) affected the prevalence and concentrations of SGARs, we fitted generalized linear models (GLMs) where 'SGARs presence' (positive or negative), 'ΣSGARs concentration' (i.e., sum of the concentrations of all the compounds detected in each bird) and 'ΣSGARs concentration > LOQ' (limit of quantification) were the response variables; and 'avian species', 'age class' and 'sex' were categorical predictors. Given that we did not sample individuals of all age classes for all species (see Table S1), we lumped the age data into two categories, 'nestling' and 'non-nestling' in all models. We used binomial error distributions and log link functions for SGARs prevalence, and Gaussian error distributions and identity link functions for ΣSGARs concentration and ΣSGARs concentration > LOQ. To improve normality assumptions, both ΣSGARs concentration and ΣSGARs concentration > LOQ were log-transformed. When we found significant differences among avian species, we used a post hoc Tukey's HSD test for pairwise comparisons using the *multcomp* package with the Bonferroni correction. At the intraspecific level, we reported 'SGARs presence', 'ΣSGARs concentration' and 'ΣSGARs concentration > LOQ' for all age class categories (nestling, juvenile, subadult and adult). Given sample size limitations for some age class categories (see Table S1), we did not model SGARs prevalence and concentrations at the species level. We used the Kruskal-Wallis test and post hoc pairwise

comparisons using the pairwise Wilcoxon rank sum test with Bonferroni adjustment to test for intraspecific differences in ΣSGAR concentrations among age classes for species with both sample sizes ≥ seven individuals and with individuals of at least three age classes (griffon vultures, bearded vultures, Egyptian vultures and red kites; Table S1). To explore differences in the proportions of the most abundant *cis* or *trans* diastereomers of each SGAR, we performed GLMs where 'proportion of *cis* forms' or 'proportion of *trans* forms' were the response variables, and 'avian species', 'age class' and 'sex' were categorical predictors. We used Gaussian error distributions and identity link functions, and only birds with residues of each SGAR were considered. Analyses were performed using R version 3.6.1 (R Development Core Team, 2019).

3. Results

SGARs were detected in 102 (39.1%) of the 261 avian scavengers analysed, including every species studied except Bonelli's eagle (Table 2). The highest prevalence of SGARs was observed in black kites (100%), followed by red kites (66.7%), and Egyptian (64.2%), bearded (20.9%), griffon (16.9%) and cinereous vultures (6.3%) (Table 2). SGARs prevalence differed among species with sample sizes ≥7 ($p < 0.001$), with higher values in black kites, red kites and Egyptian vultures than in the other species (Tables 2 and 3). SGARs prevalence was lower in nestlings compared with individuals of other age classes ($p = 0.003$), whereas sex had no influence (Table 3). Regarding the specific SGARs, brodifacoum showed the highest overall prevalence in the birds studied (28.8%), followed by difenacoum (16.1%), flocoumafen (12.3%) and bromadiolone (7.3%). We found that 18.4% (n = 48) of all birds analysed contained residues of multiple (>1) SGARs, representing 47.1% (48/102) of the SGAR-positive birds. Of all SGAR-positive birds, 30.4% contained residues of two SGARs, 15.7% of three SGARs, and four different SGARs were detected in one individual (Fig. S1).

Considering concentrations in SGAR-positive birds (n = 102) only, ΣSGARs showed a mean ± SE value of 7.52 ± 0.95 ng mL^{-1}, with a maximum level of 53.50 ng mL^{-1}. Considering all individuals analysed (n = 261), the mean total ΣSGARs was 2.94 ± 7.03 ng mL^{-1}. Brodifacoum was the compound found with the highest levels in birds with any SGARs present (5.95 ng mL^{-1}), followed by bromadiolone (0.72 ng mL^{-1}), difenacoum (0.72 ng mL^{-1}) and flocoumafen (0.13 ng mL^{-1}) (Fig. 3). The maximum blood concentration of brodifacoum (51.66 ng mL^{-1}) was found in a golden eagle without evident signs of toxicosis; the 27-year-old female eagle had been equipped with a GPS transmitter since the time of sampling and died by electrocution two years later without showing any uncharacteristic behaviours.

In all of the species studied with sample sizes ≥7 individuals, the mean individual concentration of ΣSGARs considering all individuals (n = 259) was significantly higher in black kites, red kites and Egyptian vultures than in bearded, cinereous and griffon vultures ($p < 0.001$; Fig. 4, Table 3). ΣSGARs concentration was lower in nestlings than in non-nestlings ($p < 0.001$; Table 3), particularly in Egyptian vultures and red kites (Fig. 5). The mean concentration of ΣSGARs differed between species ($p < 0.001$) in individuals with SGARs concentration > LOQ, with higher concentrations in red kite, black kite and Egyptian vultures than in bearded and griffon vultures, and showed no significant differences related to age class (nestling vs. non-nestling) or sex (Tables 2 and 3).

The proportions of SGAR diastereomers did not differ between species, sexes or age classes. The *trans* diastereomer was the most frequent form of bromadiolone and flocoumafen, whereas the *cis* diastereomer was the most frequent form of brodifacoum and difenacoum. The highest difference between diastereomer forms was found for bromadiolone, in which the *cis* form was almost absent (Fig. 6).

4. Discussion

Our active monitoring indicated that medium-sized facultative avian

Table 2
Prevalence (%) and concentration (ng mL^{-1})[1] of second-generation anticoagulant rodenticides (SGARs) in the blood of free-living obligate and facultative avian scavengers sampled in the Pyrenees and adjacent regions (NE Spain).

	SGARs				Brodifacoum			Bromadiolone			Difenacoum			Flocoumafen		
	N	N+	%	Mean ± SE [min-max]	N+	%	Mean ± SE [min-max]	N+	%	Mean ± SE [min-max]	N+	%	Mean ± SE [min-max]	N+	%	Mean ± SE [min-max]
Obligate																
Egyptian v.	67	43	64.18^{A}	6.74 ± 1.1^{A} [0.13–28.87]	39	58.21	6.54 ± 1.11 [0.37–27.15]	8	11.94	1.48 ± 0.25 [0.85–2.95]	18	26.87	0.85 ± 0.18 [0.13–2.84]	16	23.88	0.47 ± 0.08 [0.12–1.39]
Bearded v.	67	14	20.90^{B}	2.29 ± 0.5^{B} [0.1–6.53]	12	17.91	2.48 ± 0.53 [0.17–6.53]	1	1.49	0.50	1	1.49	0.15	5	7.46	0.35 [0.04–1.31]
Griffon v.	65	11	16.92^{B}	2.23 ± 1.85^{B} [0.04–20.73]	3	4.62	7.15 ± 6.53 [0.53–20.21]	ND	–	–	4	6.15	0.19 ± 0.08 [0.1–0.43]	7	10.77	0.33 ± 0.13 [0.04–0.90]
Cinereous v.	16	1	6.25^{B}	0.17	ND	–	–	ND	–	–	1	6.25	–	ND	–	–
Facultative																
Black kite	8	8	100^{A}	7.61 ± 2.20^{A} [0.20–16.71]	7	87.50	7.60 ± 2.28 [0.14–16.71]	ND	–	–	5	62.50	1.44 ± 0.85 [0.07–4.77]	1	12.50	0.50
Red kite	36	24	66.67^{A}	12.75 ± 2.29^{A} [0.49–45.95]	13	36.11	15.08 ± 3.62 [0.49–45.95]	10	27.78	6.08 ± 2.20 [0.58–18.44]	12	33.33	4.02 ± 1.56 [0.23–17.38]	3	8.33	0.29 ± 0.02 [0.26–0.32]
Golden e.	1	1	100	53.50	1	–	51.66	ND	–	–	1	–	1.84	ND	–	–
Bonelli's e.	1	0	–	–	ND	–	–	ND	–	–	ND	–	–	ND	–	–

[1]Concentrations in birds with values > LOQ.
[2]Percentages with different letters are significantly different.

Table 3
Generalized linear models (GLMs) used to assess the effects of 'avian species' (red kite, black kite, Egyptian, bearded, griffon and cinereous vultures), 'age class' (nestling or non-nestling) and gender (female or male) on SGARs prevalence, ΣSGARs concentration (i.e., sum of concentrations of all compounds detected in each bird) and ΣSGARs concentration > LOQ (i.e., considering only individuals with SGARs levels > LOQ). The coefficients and standard errors (SE) of all models are shown. Significant p-values (<0.05) are highlighted in bold.

Response variable	Explanatory variable	Coefficient	SE	p-value
SGARs prevalence	Black kite	15.073	–	0.986
	Egyptian vulture	−0.206	0.453	0.649
	Bearded vulture	−0.716	0.542	< **0.001**
	Griffon vulture	−2.867	0.552	< **0.001**
	Cinereous vulture	−2.962	1.105	**0.007**
	Age class ~ non-nestling	1.1966	0.408	**0.003**
	Sex ~ male	−0.302	0.319	0.344
ΣSGARs concentration	Black kite	0.678	0.837	0.418
	Egyptian vulture	−0.596	0.430	0.167
	Bearded vulture	−3.645	0.459	< **0.001**
	Griffon vulture	−3.905	0.458	< **0.001**
	Cinereous vulture	−3.251	0.642	< **0.001**
	Age class ~ non-nestling	1.220	0.337	< **0.001**
	Sex ~ male	−0.274	0.268	0.308
ΣSGARs concentration > LOQ	Black kite	−0.482	0.558	0.390
	Egyptian vulture	−0.530	0.337	0.119
	Bearded vulture	−1.735	0.449	< **0.001**
	Griffon vulture	−3.136	0.514	< **0.001**
	Age class ~ non-nestling	0.095	0.309	0.758
	Sex ~ male	0.396	0.283	0.164

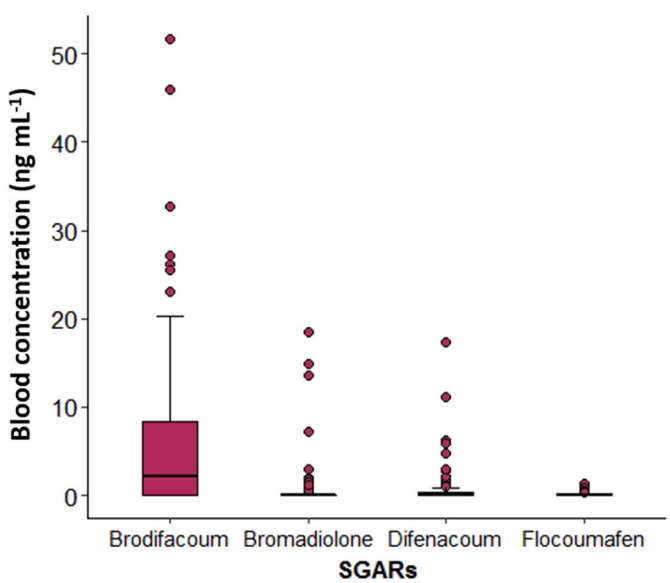

Fig. 3. Boxplots showing the blood concentration (ng mL^{-1}) of each second-generation anticoagulant rodenticide (SGAR) analysed in the avian scavenger species sampled in the Pyrenees and adjacent regions (NE Spain). Only SGAR-positive birds are shown. Boxes encompass the 25–75th quartiles from the median (thick line); vertical lines represent the maximum and minimum values, excluding outliers, shown as circles and defined as values further than 1.5 times the interquartile range.

scavengers, such as red and black kites, and Egyptian vultures (an obligate scavenger) were the species most exposed to SGARs, with a prevalence >64% in red kites and Egyptian vultures, and up to 100% in black kites. Prevalence values were lower for the larger obligate avian scavengers (bearded, griffon and cinereous vultures) but still ranged between 6.25% and 20.9%. The average blood SGAR concentrations

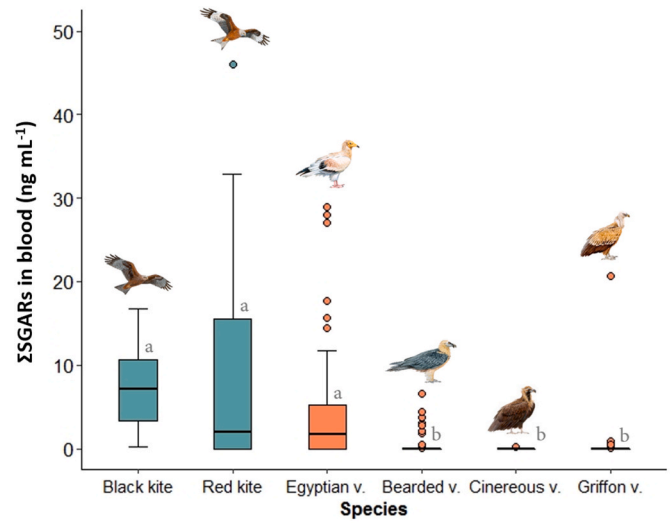

Fig. 4. Boxplots representing the blood concentration of the sum of second-generation anticoagulant rodenticides (ΣSGARs) in obligate and facultative avian scavengers with sample sizes ≥7 individuals sampled in the Pyrenees and adjacent regions (NE Spain). Boxes encompass the 25–75th quartiles from the median (thick line); vertical lines represent the maximum and minimum values, excluding outliers, shown as circles and defined as values further than 1.5 times the interquartile range. Different letters show significant differences between species as indicated by post hoc Tukey's tests after Bonferroni correction of ΣSGARs concentration generalized linear model (GLM; see Table 3). Facultative (green boxes) and obligate (orange boxes) avian scavenger species are shown separately. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

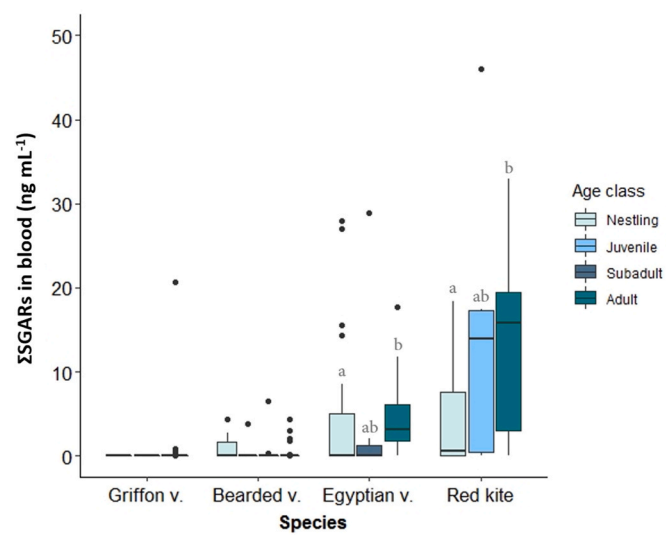

Fig. 5. Boxplots representing differences in blood concentrations of the sum of second-generation anticoagulant rodenticides (ΣSGARs) in the obligate and facultative avian scavengers studied in the Pyrenees and adjacent regions (NE Spain) according to age class (i.e., nestling, juvenile, subadult and adult; also see Table S4). Boxes encompass the 25–75th quartiles from the median (thick line); vertical lines represent the maximum and minimum values, excluding outliers, shown as circles and defined as values further than 1.5 times the interquartile range. Different letters show significant intraspecific differences between age classes as indicated by the Kruskal-Wallis test with Bonferroni adjustment (see Methods).

were similar in kites and Egyptian vultures (ranging between ~7 and 13 ng mL^{-1}) while among large vultures blood levels were lower (<2.5 ng mL^{-1}). The highest concentration was detected in the only golden eagle

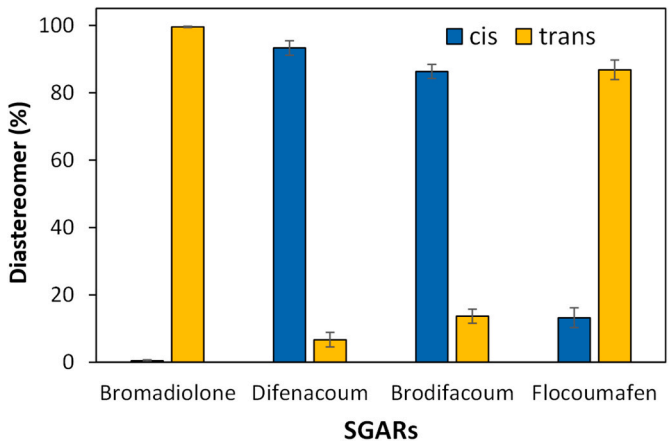

Fig. 6. Proportion (mean ± SE) of *cis* and *trans* diastereomer forms of each SGAR detected in the obligate and facultative avian scavengers studied in the Pyrenees and adjacent regions (NE Spain).

(i.e., an apex predator) we sampled, being 53.50 ng mL^{-1}. These results showed that bioaccumulation of SGARs presents a risk not only to top predators, but also to the European avian scavenger guild as a whole.

Exposure of avian scavengers to SGARs has previously been investigated mainly by analysing the livers of animals found dead, but such passive monitoring method may introduce significant bias in the observed concentration and prevalence values. For example, Sánchez-Barbudo et al. (2012) detected ARs in 13% of griffon vultures throughout Spain (n = 23) and López-Perea et al. (2019) found that 19% of griffon vultures were affected (n = 42) in NE Spain. In griffon vultures in France, Berny et al. (2015) detected ARs in 4.2% of the liver samples examined (n = 119) and Moriceau et al. (2022) in 32.2% of livers (n = 90). Regarding bearded vultures, López-Perea et al. (2019) detected ARs in 22.2% of the birds examined (n = 9) in NE Spain. In France, Berny et al. (2015) did not detect residues in bearded vultures (n = 8), but Moriceau et al. (2022) found AR residues in 33.3% of birds (n = 9). Concerning cinereous vultures, Moriceau et al. (2022) detected ARs in 76.5% of the birds examined (n = 17). For Egyptian vultures, Berny et al. (2015) detected ARs in 22.2% of the individuals examined (n = 9) while López-Perea et al. (2019) found residues in two out of three birds in NE Spain. In the Canary Islands (Spain), Rial-Berriel et al. (2021) detected ARs in 29.9% of the Egyptian vultures analysed (n = 67).

Considering the number of birds studied by Berny et al. (2015) and López-Perea et al. (2019), in areas which overlap or are close to our study area, we estimate an overall prevalence of AR occurrence of 8.1% (13/161) for griffon, 11.8% (2/17) for bearded and 33.3% (4/12) for Egyptian vultures. The values estimated from birds found dead were half of those we obtained by analysing living individuals (i.e., griffon vulture: 16.9%, n = 65; bearded vulture: 20.9%, n = 67; and Egyptian vulture: 64.2%, n = 67), highlighting the need for further studies on the relationship between AR levels in blood and liver tissues. Assuming similar limits of detection in the studies mentioned, we observed that, contrary to our expectations, the prevalence of ARs discovered in birds found dead may underestimate those we observed in living individuals. This fact could be due to significant bias in the sampling method. Most birds found dead could have died from other non-natural causes, such as those related to anthropogenic infrastructures (e.g., trauma or electrocution due to collision with energy infrastructures; Pérez-García et al., 2022) and their carcasses could be more easily detected at the site of death (González et al., 2008). In contrast, clinically poisoned birds tend to move less and remain in safer places (e.g., roosting sites) before dying in the field, making them much harder to find (Peshev et al., 2022). Indeed, there are significant differences in the reported causes of mortality of dead vultures discovered by chance (e.g., 75% relating to shootings and collisions with power lines) compared with dead radio-tagged vultures (e.g., 86% relating to intentional and unintentional poisoning), demonstrating a methodological sampling bias (Margalida et al., 2008). Thus, assessment of threats to avian scavengers by analysing untagged individuals found dead may lead to important biases in the accurate calculation of epidemiological parameters (Franson et al., 1996; González et al., 2008).

Regarding New World vultures, Hosea (2000) found AR residues in one of the two turkey vultures (*Cathartes aura*) examined between 1997 and 2010. McMillin (2012) reported death due to AR poisoning in four turkey vultures in California (USA). Stone et al. (2003) detected AR residues in the liver of both of two turkey vultures in New York state (USA), while Kelly et al. (2014) detected ARs in the liver of seven out of 23 (30.4%) turkey vultures in California (USA). Turkey vultures therefore show a prevalence of exposure to ARs similar to that of Egyptian vultures in Europe. The dietary habits of these opportunistic avian scavengers are similar, feeding on substantial quantities of garbage and the carrion of wild and domestic mammals, ranging from small rodents to small/medium-sized mammals, large ungulates, birds, reptiles, amphibians and invertebrates (Margalida et al., 2012; Hill et al., 2022).

Exposure to ARs in facultative avian scavengers such as kites or large eagles has been more frequently studied. Regarding kites, Badry et al. (2021) detected ARs in the liver of 80.5% of the birds (n = 41) in Germany. In England, SGAR poisoning was diagnosed as the cause of death in 17.3% of reintroduced red kites found dead (n = 110) (Molenaar et al., 2017). In Scotland, 70% of red kites found dead (n = 114) contained ARs in their liver and in 10% of the individuals AR poisoning was the cause of death (Hughes et al., 2013). In France, Coeurdassier et al. (2014) sampled in an area where bromadiolone has been intensively used to control water voles (*Arvicola terrestris*) and poisoning by this compound was confirmed (or highly suspected) in all red kites found dead (n = 28). More recently, Moriceau et al. (2022) detected AR residues in the liver of 100% of red kites (n = 16) and in one black kite (n = 1) found dead in France. In Spain, ARs have been detected by López-Perea et al. (2019) in the liver of red kites (77%, n = 13) and black kites (33%, n = 6) and Sánchez-Barbudo et al. (2012) found a similar situation (red kites, 88%, n = 8; black kites, 60%, n = 5). In Asia, Hong et al. (2018) detected SGARs in the liver of 42.9% of black kites (n = 7) in Taiwan.

Regarding large eagles, Badry et al. (2021) detected ARs in 38.3% of white-tailed sea eagles (*Haliaeetus albicilla*) (n = 60) in Germany. Sell et al. (2022) detected SGARs in 100% of white-tailed sea eagles found dead with suspected poisoning (n = 40) in Poland. In France, Moriceau et al. (2022) detected ARs in the liver of 100% of golden eagles found dead (n = 7). In Norway, Langford et al. (2013) detected SGARs in the liver of 68.8% of golden eagles (n = 16). In Spain, López-Perea et al. (2019) found no ARs in the liver of golden eagles (n = 5) but Sánchez-Barbudo et al. (2012) detected SGARs in one individual (25%, n = 4). In the USA, Viner et al. (2022) detected ARs in the liver of 38.7% of golden eagles (n = 62) found dead under power lines or wind turbines, with no significant differences in AR prevalence between these infrastructures, and Niedringhaus et al. (2021) detected ARs in the liver of 83% of bald eagles (*Haliaeetus leucocephalus*) (n = 96) and 77% of golden eagles (n = 17). AR poisoning was also previously detected in bald and golden eagles by Stone et al. (1999). In Australia, Pay et al. (2021) detected AR residues in 74% of Tasmanian wedge-tailed eagles (*Aquila audax fleayi*) found dead (n = 50). We detected SGARs in the only golden eagle we analysed, but every previous study of large eagles has indicated significant exposure levels varying from 25% to 100%, depending on the sampling method or location.

Our findings evidence that facultative avian scavengers (black and red kites) showed the highest prevalence of SGARs (100% and 66.7%, respectively) although Egyptian vultures (an obligate scavenger) also showed a high SGAR prevalence (64.2%). Moreover, we found that both kites and Egyptian vultures showed a high prevalence of individuals containing multiple different SGARs (four compounds were identified in a single Egyptian vulture). These exposures could relate to the foraging

and dietary habits of these species, which comprise the carrion of small and medium-sized mammals, such as rodents, as well as carnivores (Oliva-Vidal et al., 2022) and large quantities of garbage and food items gleaned from landfill sites and livestock farms (Margalida et al., 2012; Tauler-Ametller et al., 2017, 2018; Arévalo et al., 2022; Fernández-Gómez et al., 2022). In the specific case of the red kite, a diet based on small rodents is more likely to result in bioaccumulation of ARs (Coeurdassier et al., 2014) although this species is well-known for frequently exploiting garbage dumps, slaughterhouses and agricultural areas (Seoane et al., 2003; García-Macía et al., 2022). The widespread use of ARs at landfills and farms could result in a high number of contaminated rodents around these areas, which could be consumed by predators and scavengers foraging in such anthropogenic landscapes. Thus, species which exploit anthropogenic habitats are very likely to suffer greater exposure to SGARs through secondary or even tertiary pathways due to their consumption of contaminated rodents or their predators (López-Perea and Mateo, 2018).

Large obligate avian scavengers (griffon, cinereous and bearded vultures) showed lower prevalence and concentration values than Egyptian vultures and kites. However, our results showed that the endangered bearded vulture suffered the highest prevalence (20.9%) among the large vultures. The consumption of small carrion items (e.g., birds, rodents and small/medium-sized mammals), which represent 14% of their diet (Margalida et al., 2009), could explain the vulnerability of this species to ARs and the prevalence that we found. Their greater dietary plasticity could also explain the high AR exposure observed in Egyptian vultures, since small to medium-sized vertebrate carrion items are frequent in their diet during the breeding season (Margalida et al., 2012). Direct and indirect poisoning is the most widespread non-natural mortality factor for Egyptian vultures and other avian scavengers in Spain (Hernández and Margalida, 2009; Mateo et al., 2015) and our findings demonstrate the critical vulnerability of these threatened species to ARs.

We found that both SGAR prevalence and average concentration were related to the age of an individual. Overall, our results showed that nestlings exhibited lower prevalence and concentration values than non-nestling individuals. Badry et al. (2022) detected ARs in the blood of 22.6% of red kite nestlings in Germany, and Powolny et al. (2020) in the blood of 30% of red kite nestlings in France, much lower prevalence values than we found in red kite nestlings (55.0%, 11/20), juveniles (75%, 6/8) and adults (87.5%, 7/8). Several factors may explain the age class differences we found. There may be dietary differences in the trophic spectrum between chicks and adults. In some species, variations in the diet during the breeding period are related to specific energetic requirements or food quality. However, the high bioaccumulative capacity of SGARs could help explain this age-related exposure, resulting in lower concentrations in nestlings than in adults because of the shorter time nestlings have spent at risk. SGARs have long half-lives in the liver of an exposed animal (e.g., 91.7–307.4 days), which could explain the higher concentrations we observed in adults compared with nestlings (Shore and Coeurdassier, 2018). As for differences in SGAR diastereomer concentrations, our results are in line with other studies in which the prevalence in birds of prey of the *trans* forms of bromadiolone and flocoumafen and the *cis* forms of brodifacoum and difenacoum were greatest because of their higher persistence in animal tissues (Fourel et al., 2017b, 2021). This is a relevant finding and highlights the need for developing SGAR formulations with a lower impact on non-target wildlife (Damin-Permink et al., 2016). Currently, commercial SGAR formulations have varying proportions of *cis*:*trans* forms, ranging between 35:65 and 55:45 (Alabau et al., 2020), and the proportions observed in free-living avian scavengers clearly show that the different diastereomer forms bioaccumulate differently across the food chain.

From a conservation and management point of view, our results suggest that scavenging birds foraging in anthropogenic landscapes (e.g., landfill sites and livestock farms) are more vulnerable to SGAR exposure, as reported in other raptor and scavenger species (Lopez-Perea et al., 2019; Badry et al., 2021) and for other pollutants in Egyptian vultures (Ortiz-Santaliestra et al., 2019). The exploitation of predictable food resources at landfills has increased coinciding with the changes in the Spanish sanitary regulations between 2006 and 2011, which have resulted in a sudden reduction in food availability by restricting the availability of livestock carcasses in the field, causing changes in the foraging behaviour and dietary habits of avian scavengers, particularly griffon vultures (Donázar et al., 2010; Fernandez-Gómez et al., 2022). As with other chemical pollutants, the risk of avian scavenger exposure to SGARs could be mitigated by securing food availability using managed SFSs where livestock and game animal carcasses can be provided regularly under strict controls to prevent wildlife exposure to toxic compounds (e.g., veterinary pharmaceutical residues or lead).

Our study highlights the problems associated with widespread use of ARs to control commensal rodents, which can affect both facultative avian scavengers, such as kites and eagles, and obligate avian scavengers that frequently include rodents and small/medium-sized carrion items in their diet (e.g., bearded and Egyptian vultures). Oliva-Vidal et al. (2022) found that some facultative avian scavengers, such as golden eagles and red kites, exploit carnivorous mammal carcasses more frequently than herbivore carcasses, although other avian and mammalian scavengers can also consume carnivore carrion (Fig. 2), so increasing the risk of secondary or tertiary AR exposure routes across the scavenger guild. The use of bromadiolone against field voles (*Arvicola terrestris*), coypu (*Myocastor coypu*) and musk rat (*Ondathra zibethicus*) in France has been associated with AR exposure and poisoning of both predators and scavengers (Berny et al., 1997; Fournier-Chambrillon et al., 2004; Coeurdassier et al., 2014). A similar scenario could occur in other countries and affect facultative and obligate avian scavengers due to the current underestimation of the potential problems. Future research to reduce biases in the study of SGAR exposure could focus on sampling individuals tagged with GPS transmitters in order to provide temporal and spatial information to identify conflict areas that should be monitored by managers and policy-makers to reduce the negative impact of SGARs on scavenger populations.

5. Conclusions

We performed the first ever active monitoring of SGAR concentrations and prevalence in the blood of the four European obligate (vultures) and facultative (red and black kites) avian scavengers in the Pyrenees and adjacent areas (NE Spain). SGARs were detected in 39.1% of the birds sampled, with variations in prevalence and concentrations among species that could be explained by differences in their foraging and trophic behaviours. Red and black kites and Egyptian vultures, which mainly feed on small/medium-sized carrion items (e.g., rodents and mammals, including carnivores) and frequently forage in anthropic areas (e.g., landfill sites and livestock farms), showed the highest prevalence of SGARs (64%–100%). In contrast, large vulture species mainly exploiting medium-sized and large domestic and wild ungulate carcasses, such as griffon, cinereous and bearded vultures, showed the lowest prevalence (<21%), the highest being found in bearded vultures. By analysing the blood of free-living vultures, we found prevalence values higher than those previously described from the analysis of liver samples from individuals found dead in the same area, which may point to methodological sampling biases. Both prevalence and ΣSGAR concentrations showed differences between age classes, being higher in adult individuals than in nestlings, which could be explained by their longer exposure to ARs leading to greater levels of bioaccumulation. The most abundant SGAR was brodifacoum, followed by difenacoum, flocoumafen and bromadiolone, and in all of these compounds one diastereomer form was clearly more bioaccumulated than the other. These results highlight the need to monitor and regulate the use of anthropogenic compounds such as SGARs. The current exposure levels in scavenging birds could have negative impacts on conservation efforts,

particularly for the most endangered European avian scavengers. Further studies on the potential adverse effects associated with blood SGAR levels are necessary to better interpret the concentrations found in the blood of free-living birds and to better understand their potential population effects.

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Credit author statement

Pilar Oliva-Vidal: Fieldwork & Sample collection, Conceptualization, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization; **José Ma Martínez**: Fieldwork & Sample collection, Investigation; **Inés Sánchez- Barbudo**: Validation, Investigation; **Pablo R. Camarero**: Validation, Investigation; **Antoni Margalida**: Fieldwork & Sample collection, Conceptualization, Resources, Methodology, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition; **Ma Àngels Colomer**: Resources, Methodology, Writing – review & editing, Funding acquisition; **Rafael Mateo**: Conceptualization, Resources, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120385.

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