Science of the Total Environment 420 (2012) 280–288

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Primary and secondary poisoning by anticoagulant rodenticides of non-target animals in Spain

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form 11 January 2012
Accepted 12 January 2012
Available online 10 February 2012

Keywords:
Wildlife
Pesticide
Anticoagulant rodenticide
Poisoning
Spain

ABSTRACT

Anticoagulant rodenticide (AR) levels were studied in liver of 401 wild and domestic animals found dead in Spain with evidences of AR poisoning, including 2 species of reptiles (n = 2), 42 species of birds (n = 271) and 18 species of mammals (n = 128). Baits (n = 32) were also analyzed to detect the potential use of ARs in their intentional preparation to kill predators. AR residues were detected in 155 (38.7%) of the studied animals and 140 (34.9%) may have died by AR poisoning according to the clinical information, necropsy findings, residue levels and results of other toxicological analysis. Animals considered with sublethal AR exposure had total AR residues (geometric mean with 95% CI) in liver of 0.005 (0.003–0.007) μg/g wet weight (w.w.) and animals diagnosed as dead by AR poisoning had 0.706 (0.473–1.054) μg/g w.w. ARs were detected in 19% of baits illegally prepared to kill predators. In terms of the total incidents studied in our laboratory between 2005 and 2010 (n = 1792 animals), confirmed poisonings represented 40.9% of the cases, and 21.1% of these were due to ARs (8.6% of the total sample). Nocturnal raptors (62%) and carnivorous mammals (38%) were amongst the secondary consumers with highest prevalence of AR exposure, especially to second generation ARs (SGARs). On the other hand, granivorous birds showed the highest prevalence of AR exposure (51%), especially to chlorophacinone in a region treated against a vole population peak in 2007. The presence of hemorrhages was significantly associated with AR levels in liver, but some animals (7.2%) with elevated residue levels (> 0.2 μg/g w.w.) showed no evidence of macroscopic bleeding. The use of accumulative SGARs and the application of baits on surface (i.e. treated grain by spreader machines) should be discontinued in future EU regulations on the use of rodenticides to prevent the poisoning of non-target wildlife species.

1. Introduction

Rodenticides are widely used to control rodent species that inhabit human environments and damage different types of goods. This chemical control is a common practice in farms to prevent rodents spoiling and consuming animal feed and stored grain, damaging buildings and transmitting diseases (Shore et al., 2006; Tosh et al., 2011b). Some rodent species with marked population cycles that may damage large extensions of crops have been controlled by the wide use of rodenticides (Berny et al., 1997; Olea et al., 2009). Rodents can also use and thrive in other human environments such as towns and urban landfills with a consequent risk of transmitting zoonoses (Semenza and Menne, 2009; Vidal et al., 2009). Therefore, treatments with rodenticides are conducted regularly under specific regulations, either as plant protection products or as biocidal products (Berny et al., 2010). Rodenticides are also used to help conserve native species on islands where rodents have been introduced, but adverse effects on non-target species have also been described (Howald et al., 1999; Thorsen et al., 2000; Eason et al., 2002; Spurr et al., 2005). The most widely used rodenticides nowadays are anticoagulants which have an inhibitory action on the enzyme vitamin K epoxide reductase, responsible for recycling the vitamin K necessary for the production and activation of clotting factors II, VII, IX and X (Ishizuka et al., 2008). Two chemical families of commercial anticoagulant rodenticides (ARs), indandiones and coumarines, have this vitamin K-antagonism capacity (Ecobichon, 2001). The development of resistance in rodents to the compounds that were used initially (first generation anticoagulant rodenticides—FGARs) (Ishizuka et al., 2008) led to the introduction in the 1970s of more toxic and bioaccumulative second generation anticoagulant rodenticides (SGARs) (Thomas et al., 2011). Nevertheless, rodents have already developed resistance against some SGARs (Ishizuka et al., 2008; Vein et al., 2011).

The mean rat lethal dose (LD_{50}) from an acute oral exposure to warfarin (the most common FGAR) is 50–100 mg/kg (Stone et al., 1999), but repeated exposures can reduce LD_{50} by two orders of magnitude (1 mg/kg × 5 days; Eason et al., 2002). Acute oral LD_{50} in rats of SGARs ranges from 0.24 mg/kg of brodifacoum to 1.8 mg/kg of

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doi:10.1016/j.scitotenv.2012.01.028

difenacoum (Eason et al., 2002), thus similar lethality may occur with single exposures to SGARs than with repeated exposures (5 days) to FGARs. Moreover, the risk of lethal poisoning by SGARs in non-target vertebrates, especially predators, is greatly enhanced by their hepatic persistence. Mean half-life ($t_{1/2}$) of SGARs in liver of rats ranges from 108 days of difethialone to 220 days of flocoumafen, whereas FGARs showed lower hepatic retention times (warfarin residues detectable for 30–40 days in pig and coumatetralyl $t_{1/2}$ of 55 days in rat) (Eason et al., 2002). In addition to these toxicokinetic features in animals, the persistence of ARs in the baits used in field treatments can be prolonged by certain environmental factors. Bromadiolone $t_{1/2}$ in baits located in galleries of rodents ranged from 3.0 to 5.1 days in autumn and 5.4 to 6.2 days in spring, whereas in the storage cavities used by these rodents it was 4.27 days in autumn and 24.6 days in spring (Sage et al., 2007). Therefore, the environmental stability plus the elevated persistence of bromadiolone in rodents after field controls carry a significant risk of poisoning on predatory species (Giraudoux et al., 2006). After field control operations with bromadiolone against water voles in France, 99.6% of water voles (*Arvicola amphibius*) trapped underground and 41% of common voles (*Microtus arvalis*) trapped above ground contained AR residues in liver, in some cases 135 days after treatment (Sage et al., 2008).

The consequence of the development and extensive use of ARs, especially SGARs, has lead to very important prevalences of AR residues in wildlife. As occurred with other compounds with elevated $t_{1/2}$ in biota (e.g. organochlorine pesticides), ARs have been detected in high percentages of individuals of a wide range of wildlife species around the world (Newton et al., 1990; Shore et al., 1996, 2003; Berny et al., 1997; Howald et al., 1999; Stone et al., 1999; Fournier-Chambrillon et al., 2004; Riley et al., 2007; Walker et al., 2008; Albert et al., 2010; Lemarchand et al., 2010; Elmeros et al., 2011; Murray, 2011; Thomas et al., 2011; Tosh et al., 2011a), especially after the development of sensitive LC–MS analytical techniques (Dowding et al., 2010). In Spain, detection of AR residues in wildlife has been associated with the large-scale treatments against population outbreaks of common voles (Sarabia et al., 2008; Olea et al., 2009; Vidal et al., 2009; Lemus et al., 2011). ARs have been also implicated in the death of 14.9% of mammals (n = 202, mostly pets) analyzed by four Spanish Laboratories of Veterinary Toxicology (Martínez-Haro et al., 2008).

The aim of this study was to describe AR exposure and poisoning in wildlife between 2005 and 2010 obtained as a result of a monitoring program of intentional and accidental poisonings in Spain. This information was related to clinical signs and necropsy findings to give a diagnosis of lethal AR poisoning. Prevalence of exposure and poisoning was compared between different groups of wildlife, and spatial and temporal variations are discussed.

2. Materials and methods

2.1. Sample collection

Between 2005 and 2010, the Laboratory of Toxicology of IREC received 1792 animals suspected to have died intoxicated. These were submitted for toxicological analysis by Wildlife Rehabilitation Centers (WRC), environmental authorities, hunting associations or environmental non-governmental organizations (NGOs). AR analyses were performed in 401 of these animals that were suspected to have died by AR poisoning due to the observation of hemorrhages during the necropsy at WRCs, by the evidence of spatio-temporal associations between AR treatments and wildlife incidents (for samples submitted by hunting associations, environmental authorities and NGOs) or because other poisons had been discarded in previous analyses. This sample included 2 species of reptiles (n = 2), 42 species of birds (n = 271) and 18 species of mammals (n = 128) (see the complete list of species in Supplementary material). Some domestic animals were included because these were submitted as a part of the investigation on the use of poisons in the field. Most of the animals were found dead or moribund in the field. Northern raccoons (*Procyon lotor*) had been trapped and euthanized in Madrid because it is a non-native invasive species. Liver was the sample submitted from most of these animals, but in a few predated carcasses (n = 4) found in the field the muscle was the only tissue available for analysis (and where ARs were detectable). Moreover, 32 baits were submitted to confirm the active ingredient of a commercial formulation or to detect the use of ARs in the preparation of baits intentionally delivered to kill predators. One sample of red fox (*Vulpes vulpes*) feces and one pellet cast of carrion crow (*Corvus corone*) were also analyzed. The geographical area covered by WRCs in this study corresponds to the regions of Asturias, Cantabria, Navarra, Aragon, Catalonia, Madrid and Castilla–La Mancha. Samples from the Basc Country were submitted by environmental authorities. Samples from Castilla y León were submitted by environmental authorities, hunting associations and NGOs after an extensive use of ARs over an area of 375,000 ha against a plague of common vole (*M. arvalis*) occurred in 2007 (Vidal et al., 2009) (Fig. 1).

2.2. Rodenticide analysis

Analyses have been performed upon arrival to the laboratory because the analytical results were necessary for criminal investigations and legal cases related to the intentional poisoning of wildlife in Spain. Several improvements were performed in the analytical methods used during this period between 2005 and 2010, especially as a consequence of the acquisition of a LC–ESI-MS in 2008. Here we describe the definitive LC–ESI-MS method used since October 2008, but before that moment, analyses had been performed by HPLC–DAD as described in Sarabia et al. (2008), Olea et al. (2009) and Vidal et al. (2009) for indandiones or HPLC–FLD for coumarines following Fauconnet et al. (1997).

The extraction procedure for LC–ESI-MS analysis has been modified from Shore et al. (2003). One gram of liver was ground in a mortar with 9 g of anhydrous sodium sulfate (Prolabo, Leuven, Belgium), then the homogenate was transferred to a Teflon-capped 30 mL-glass tube and 20 mL of a mixture of dichloromethane : acetone (70:30) (HiperSolv Cromanorm Gradient grade, Prolabo, Leuven, Belgium) were added, horizontally shaken for 10 min and sonicated for 5 min. The sample was centrifuged at 2000 *g* for 5 min and the supernatant was collected in a conical tube for solvent evaporation in a rotary evaporator. The extraction step was repeated with other 5 mL of the solvent mixture and the supernatant obtained was pooled with the previous one. After solvent evaporation, the dry extract was dissolved in 2 mL of dichloromethane:acetone (70:30). Then, this extract was cleaned-up in a neutral alumina column (SPE ALN 500 mg/3 mL, Upti-clean Interchrom, Montluçon, France). The solid phase extraction (SPE) column was conditioned with 5 mL of dichloromethane and 10 mL of dichloromethane:acetone (70:30). The sample was added to the column and washed with 3 mL of dichloromethane:acetone (70:30). Finally, the anticoagulant rodenticides were eluted with 3 mL of methanol:acetic acid (95:5) (Prolabo, Leuven, Belgium). The solvent was evaporated under N_2 flow and the dry cleaned-up extract was reconstituted in 0.5 mL of methanol and filtered through a 13 mm-filter with a 0.2 μm Nylon membrane (Acrodisk, Pall, NY, USA).

Rodenticide analysis was performed by LC–ESI-MS with an analytical system formed by Agilent 1100 series chromatograph and Agilent 6110 Quadrupole LC/MS with a multimode source (MM). The nitrogen for mass detector was supplied with a high purity nitrogen generator (Whisper 2–50, Ingeniería Analítica, Sant Cugat, Spain). The chromatographic method was developed following Marek and

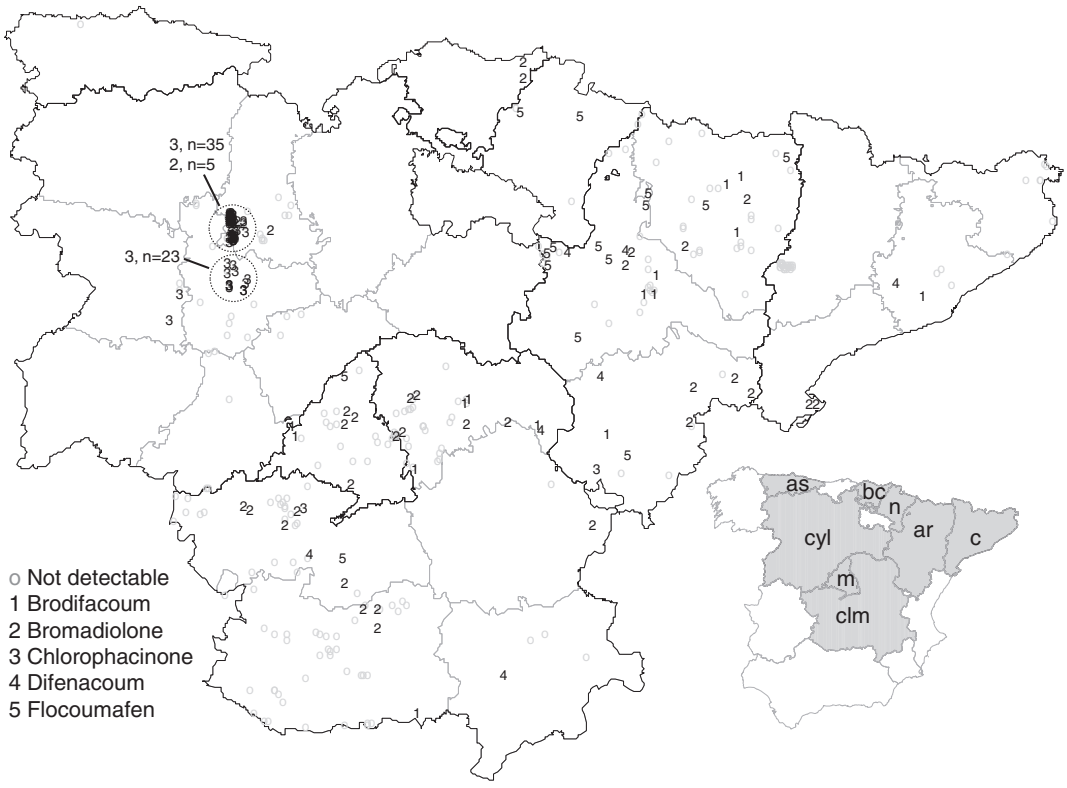

Fig. 1. Distribution of studied cases in eight regions of Spain with the result of the anticoagulant rodenticide analysis. as: Asturias; bc: Basque Country; n: Navarra; ar: Aragón; c: Catalonia; cyl: Castilla y León; m: Madrid, clm: Castilla–La Mancha. Location of some cases was unknown.

Koskinen (2007), with some modifications such as the use of a phenyl–hexyl column (150×2.1 mm, 3 μm) instead of a RX-C8 column. The injection volume was 30 μL. The chromatographic conditions of analysis consisted in a gradient elution of two solvents (A: methanol; B: ammonium acetate 10 mM, pH: 6.8). The initial conditions were 20% A and 80% B, reaching 75% A and 25% B at min 8.75. This was maintained until min 30.62, returning to the initial conditions by min 31.5. Then, column was stabilized with conditions until min 43.75 before the next sample injection. The flow rate was 0.2 mL/min.

ARs were detected using negative ion monitoring with the following MM-ESI source settings. Nebulizer pressure was set at 60 psi, drying gas flow was 4.8 L/min, drying gas temperature was 250 °C, vaporizer temperature was 150 °C, capillary voltage was 2000 V, charging voltage was 1000 V, and fragmentation voltage varied amongst compounds (Table 1). Samples were first run in single ion monitoring (SIM) mode for precursor and/or main ions previously selected by full scan analysis and flow injection analysis sequence (FIAS) of ARs standards (Table 1). If samples contained one of these parent ions at the specific retention times obtained from ARs standards (Table 1), three additional product and/or secondary ions (Table 1) were monitored in a subsequent run. Confirmation was accomplished when the percentage of variation of the relative intensity of the product ion respect to the precursor ion was <30% between samples and standards.

Stock solutions of ARs standards were purchased in methanol at a concentration of 10–100 μg/mL form Dr. Ehrenstorfer (Augsburg, Germany). Calibration curves were performed with concentrations ranging from 0.04 to 2.5 μg/mL of methanol. The recovery of the analytical procedure was calculated with four replicates of chicken liver (1 g) spiked with 1.25 μg of each AR. These spiked samples were processed as described before for liver. Recovery with the extraction method and LC–MS analyses described here was >70% for warfarin, bromadiolone, brodifacoum, difenacoum, flocoumafen and difethialone. Due to the poor recovery obtained with neutral alumina SPE columns for chlorophacinone and diphacinone (<50%), quantification in samples with these residues were done with C18 SPE columns as described by Sarabia et al. (2008), with a recovery of indandiones >60%. Detected AR concentrations were not corrected for recovery rates. Limit of detection (LOD) was calculated with blank samples as 3 times the signal to noise ratio. This LOD was also checked to be the lowest concentration standard allowing unambiguous qualitative analyte detection in liver spiked with ARs. LOD were between 0.001 and 0.002 μg/g for warfarin, bromadiolone, brodifacoum, difenacoum and flocoumafen. LOD were between 0.003 and 0.006 μg/g for chlorophacinone, diphacinone, difethialone and coumatetralyl. AR concentrations were expressed in wet weight of sample.

Table 1
ESI-MS parameters used for anticoagulant rodenticide analysis.

Compound	Fragmentation voltage (V)	Molecular mass (Da)	Monitored ions (Da)		
			1st run[a]	2nd run[b]	
Brodifacoum	200	523.4	521.1	523.1	443.1
Bromadiolone	250	527.4	525.0	527.0	250.1
Chlorophacinone	400	374.8	373.0	201	145.1
Coumatetralyl	150	292.3	291.0	247.0	263.2
Diphacinone	350	340.4	339.0	116.0	167.0
Difenacoum	200	444.5	443.0	444.0	399.2
Diphetialone	200	539.5	539.0	537.0	497.1
Flocoumafen	200	542.5	541.0	542.0	382.1
Warfarin	150	308.3	307.0	250.0	161

[a] Precursor or primary ion.
[b] Product or secondary ions.

2.3. Statistical analysis

Prevalence of liver AR residues was compared between years, taxonomic groups of animals or according to the presence of gross lesions (i.e. hemorrhages) by means chi-square tests. Liver AR levels

were transformed in their natural logarithms to attain a normal distribution. Log-transformed values (only for animals with detectable AR residues) were compared between birds and mammals or between animals with or without signs of hemorrhages with Student's t-tests. The association between AR levels and signs of bleeding was tested at three threshold levels used in the literature as indicative of AR poisoning: presence vs. absence, <0.1 vs. ≥0.1 μg/g, and <0.2 vs. ≥0.2 μg/g of AR residues (Berny et al., 1997; Walker et al., 2008; Thomas et al., 2011). According to the logistic regression obtained by Thomas et al. (2011) to estimate the probability of AR toxicosis, AR poisoning may occur at levels <0.02 μg/g and close to LOD. Significance level was set at $p \leq 0.05$ and statistical analysis was performed with IBM SPSS Statistics v.19.0.0.

3. Results

ARs have been detected in different groups of wildlife between 2005 and 2010, the prevalence being especially high in granivorous birds, nocturnal raptors and carnivorous mammals (Table 2). AR residues were detected in 155 (38.7%) of the studied animals (n = 401) and 140 (34.9%) may have died by AR poisoning according to the clinical information, necropsy findings, residue levels and results of other toxicological analysis. It may be of concern that ARs were detected in illegal baits prepared with eggs, meat or offal to kill predators with concentrated formulations (e.g. bromadiolone 0.25% w/v oily concentrated) or milled commercial baits. ARs were also detected in commercial baits to kill rodents and submitted by regional governments to identify the active ingredients used by farmers (Table 2). Red fox feces and a carrion crow pellet contained 0.013 and 0.011 μg/g of bromadiolone, respectively. The most commonly detected compound in animals was chlorophacinone (19.7% of the total analyzed) followed by bromadiolone (11.0%) and brodifacoum (6.7%) (Fig. 1). The prevalence of ARs differed between years and the highest value (61%) was found in 2007 (Fig. 2) ($\chi^2_5 = 66.2$, $p<0.001$), when a population outbreak of common vole in the region of Castilla y Leon was treated initially with chlorophacinone and later with bromadiolone (Fig. 1). The overall increasing trend in the reported cases was explained by the development and improvement of analytical techniques, especially after 2007. In 2010, when all the analyses were routinely performed by LC–MS in all the animals with suspected exposure to ARs (n = 82), AR residues were detected in 43.9% (n = 36) of the studied animals and 29.2% (n = 24) may have died by AR poisoning (Fig. 2). Animals within the sublethal category (n = 15) had total AR residues levels (geometric mean with 95% IC) in liver of 0.005 (0.003–0.007) μg/g and animals within the lethal category (n = 140) had 0.706 (0.473–1.054) μg/g. In terms of the total incidents studied in our laboratory between 2005 and 2010 (n = 1792 animals), poisonings represented 40.9% (n = 733) of the studied events, and 21.1% (n = 155) of these confirmed poisonings were due to ARs (8.6% of the total sample). The spatial distribution in the studied Spanish

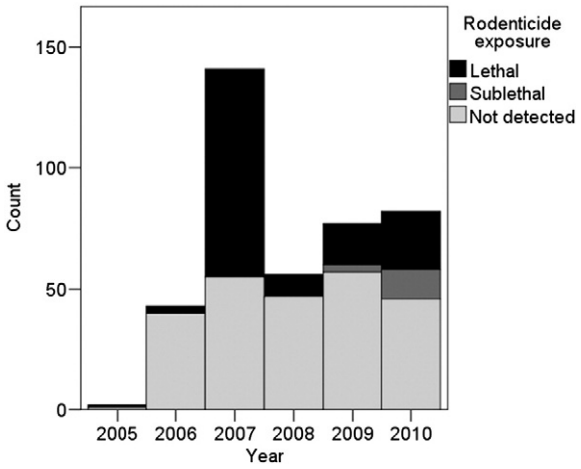

Fig. 2. Studied cases between 2005 and 2010 and interpretation of the anticoagulant rodenticide (AR) levels. Not detected cases were below the limit of detection (LOD). Sublethal cases showed the presence of ARs (>LOD–0.06 μg/g) without signs of coagulopathies. Lethal cases showed the presence of ARs (>LOD–50.1 μg/g) and in most of the cases macroscopic hemorrhages. Prevalence peak observed in 2007 was associated with a population outbreak of common vole (*Microtus arvalis*).

regions showed an aggregation of chlorophacinone and bromadiolone presence in the region of Castilla y León, and a wider spatial distribution of other ARs (Fig. 1). The detected type of ARs differed between primary consumers (granivorous and herbivorous species) and secondary consumers (predators). Chlorophacinone residues were more prevalent amongst primary than secondary consumers ($\chi^2 = 111.5$, $p<0.001$) as a result of widespread surface application of grain formulations of this compound on fields to control common vole plagues. In contrast, SGARs residues were detected more frequently in secondary consumers (bromadiolone: $\chi^2 = 24.6$, $p<0.001$; brodifacoum: $\chi^2 = 13.4$, $p = 0.001$; flocoumafen: $\chi^2 = 9.2$, $p = 0.002$; difenacoum: $\chi^2 = 4.7$, $p = 0.031$) (Fig. 3).

ARs were detected in 21 species of birds, 10 of mammals and 1 reptile (Table 3). Liver levels of ARs tended to be higher in mammals than in birds, although the difference was only significant for bromadiolone ($t_{37.9} = 3.345$, $p = 0.002$; Table 3). The mean detected values were >0.1 μg/g of liver for brodifacoum and chlorophacinone in birds and mammals and for bromadiolone in mammals (Table 3). The percentage of the studied animals with a sum of ARs residues in liver >0.1 μg/g was 26.4%, and similar for birds (28.8%) and mammals (21.1%) ($\chi^2 = 2.27$, $p = 0.13$). These percentages, in terms of animals with detected residues, were 72.2% and 58.7%, respectively, and not significantly different ($\chi^2 = 2.13$, $p = 0.14$).

The proportion of individuals with hemorrhages was greater amongst animals with detectable AR residues (83.1%) than in those

Table 2
Presence of anticoagulant rodenticides in animal groups and baits sampled in Spain.

Sample	Group		n	n^+	$\%^+$
Animals	Reptiles		2	1	50
	Birds	Granivorous	142	72	50.7
		Diurnal raptors	84	23	27.4
		Nocturnal raptors	13	8	61.5
		Other predators	32	5	15.6
	Mammals	Herbivorous	29	8	27.6
		Insectivorous	3	2	66.7
		Carnivores	96	36	37.5
All animals			401	153	38.2
Baits	Illegal baits against predators		26	5	19.2
	Commercial baits against rodents		6	6	100

n = number of individuals analyzed, n^+ = number of individuals with detectable residues, $\%^+$ = percentage of individuals with detectable residues.

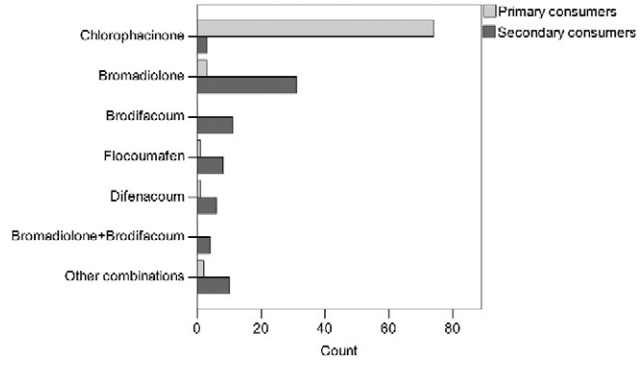

Fig. 3. Number of animals with different anticoagulant rodenticides in liver and grouped as primary and secondary consumers.

Table 3
Species with residues of anticoagulant rodenticides in liver and detected concentrations (μg/g w.w.).

Species	Rodenticide			Brodifacoum		Bromadiolone		Chlorophacinone		Difenacoum		Difethialone		Flocoumafen	
	n	n^+	$\%^+$	n^+	GMean min–max	n^+	GMean min–max	n^+	GMean min–max	n^+	GMean min–max	n^+	Mean	n^+	GMean min–max
Reptiles	2	1	50												
Horseshoe whip snake *Hemorrhois hippocrepis*	1	1	100											1	0.540
Birds	271	108	40	9	0.061	20	0.011[a]	72	3.04	3	0.006			9	0.032
All					0.009–0.830		0.001–0.490		0.004–50.1		0.001–0.056				0.002–0.400
Grey heron *Ardea cinerea*	1	1	100			1	0.010								
Mallard *Anas platyrhynchos*	6	3	50					3	1.21 0.710–2.17						
Red kite *Milvus milvus*	8	7	88	2	0.165 0.129–0.210	4	0.031 0.005–0.490			1	0.001			2	0.146 0.053–0.400
Black kite *Milvus migrans*	5	3	60	1	0.025									2	0.068 0.055–0.084
Bearded vulture *Gypaetus barbatus*	3	1	33			1	0.001								
Eurasian griffon *Gyps fulvus*	23	3	13			1	0.208	1	0.004	1	0.001				
Short toed snake-eagle *Circaetus gallicus*	1	1	100	1	0.009	1	0.010							1	0.002
Northern goshawk *Accipiter gentilis*	2	1	50	1	0.038										
Eurasian buzzard *Buteo buteo*	15	5	33			4	0.008 0.001–0.028	1	0.120						
Spanish imperial eagle *Aquila adalberti*	8	1	13							1	0.008				
Golden eagle *Aquila chrysaetos*	4	1	25											1	0.006
Red-legged partridge *Alectoris rufa*	7	1	14											1	0.143
Lesser black-backed gull *Larus fuscus*	8	3	38			3	0.002 0.002–0.005								
Rock dove *Columba livia*	97	64	66					64	4.15 0.550–55.1						

Eurasian collared-dove *Streptopelia decaocto*	8	1	13			1	0.127								
Great spotted cuckoo *Clamator glandarius*	1	1	100					1	0.006						
Common Barn-owl *Tyto alba*	4	3	75	1	0.028	2	0.008 0.007–0.010								
Eurasian eagle-owl *Bubo bubo*	7	4	57	3	0.116 0.010–0.830	1	0.004							2	0.011 0.003–0.032
Little owl *Athene noctua*	1	1	100							1	0.056				
Calandra lark *Melanocorypha calandra*	7	2	29					2	1.47 1.04–2.09						
Common starling *Sturnus vulgaris*	3	1	33			1	0.015								
Mammals	128	46	36	18	0.151	24	0.104[a]	7	2.11	8	0.029	1	0.926	8	0.064
All					0.005–4.50		0.001–17.9		0.580–9.52		0.004–0.520				0.008–0.353
Feral cat *Felis catus*	4	3	75	2	0.109 0.034–0.350	1	0.052			1	0.070			1	0.072
Common genet *Genetta genetta*	7	2	29	2	0.184 0.016–2.02	2	0.028 0.001–0.350			1	0.012			1	0.060
Domestic dog *Canis familiaris*	11	4	36			3	0.031 0.006–0.308			1	0.004				
Red fox *Vulpes vulpes*	31	12	39	5	0.093 0.005–4.50	8	0.115 0.005–12.3			1	0.078				
Northern racoon *Procyon lotor*	10	2	20			2	2.72 1.09–6.80								
Stone marten *Martes foina*	19	11	58	5	0.144 0.019–0.390	6	0.155 0.007–17.9			3	0.049 0.007–0.520	1	0.926	5	0.045 0.008–0.230
Eurasian otter *Lutra lutra*	3	1	33											1	0.353
Least weasel *Mustela nivalis*	1	1	100	1	2.93										
Iberian hare *Lepus granatensis*	25	8	32	2	0.187 0.130–0.270			7	2.11 0.580–9.52	1	0.015				
European hedgehog *Erinaceus europeaus*	2	2	100	1	0.092	2	0.026 0.013–0.049								

n = number of individuals analyzed, n^+ = number of individuals with detectable residues, $\%^+$ = percentage of individuals with detectable residues, GMean = geometric mean.

[a] Significantly different between birds and mammals ($t_{37.9} = 3.345$, $p = 0.002$).

without detected residues (37.4%; $\chi^2 = 74.3$, $p < 0.001$). The percentage with hemorrhages was slightly higher at threshold levels of >0.1 μg/g (90%) and >0.2 μg/g (92.8%). Amongst the animals with detected AR residues, those with hemorrhages had higher AR levels in liver (n = 123, geometric mean (95%CI) = 0.657 (0.410–1.051) μg/g) than those without hemorrhages (n = 25, geometric mean (95%CI) = 0.067 (0.022–0.197) μg/g; $t_{146} = 3.94$, $p < 0.001$; Fig. 4). Some animals with elevated residue levels showed no evidence of macroscopic bleeding (10% with ≥0.1 μg/g and 7.2% with ≥0.2 μg/g).

4. Discussion

Liver residues of ARs have been detected in a large number of species in Spain, and in most of the cases, ARs exposure were considered to be involved in the death of the animal. Nocturnal raptors (61.5%) and carnivorous mammals (37.5%) were amongst the groups with higher prevalence of secondary AR exposure, especially to SGARs. On the other hand, granivorous birds showed the highest prevalences of primary AR exposure (50.7%), especially to chlorophacinone in a region treated against a vole population peak in 2007. This field application with chlorophacinone treated grain was performed on surface by spreader machines, and this produced the death of granivorous species by chlorophacinone poisoning (Sarabia et al., 2008; Olea et al., 2009). Under this scenario, the risk of poisoning in grazing animals should be considered (Del Piero and Poppenga, 2006).

The prevalence of AR residues and lethal poisoning in wildlife from Spain has been within the range of values detected in other studies elsewhere. Cases of AR poisoning in non-target wildlife in New York state (USA) have been mostly produced by brodifacoum (80%) (Stone et al., 1999). ARs were detected in 49% of 265 raptors in New York state, and it was the cause of death in 9 cases (14.6% of animals with AR residues, 7.2% overall). AR residues were especially frequent in great horned owl (*Bubo virginianus*) (81%, n = 53), and the most frequently detected ARs were brodifacoum (84%) and bromadiolone (22%) (Stone et al., 2003). Similarly, the liver analysis of birds of prey sampled in Massachusetts (n = 161) revealed that 86% had AR residues and 6% were diagnosed as killed by AR poisoning (Murray, 2011). Albert et al. (2010) have reported the presence of AR residues in 70% of the liver of 167 owls of three species in Western Canada. AR levels in owl species from Canada ranged from 0.001 to 1.092 μg/g, and six out of 115 birds (5.2%) with detectable levels were considered as to have died by AR (brodifacoum) poisoning (Albert et al., 2010). Thomas et al. (2011) have also observed in Canada that a minimum of 11% of the sampled great horned owls (n = 196) were at risk of being directly killed by SGARs. We have observed a similar percentage of cases in which the AR could be involved in the death of the animal (8.6% of all submitted animals, and 21.1% of confirmed poisonings) than in these studies from North America. In France, SAGIR network that studies mortality causes in wildlife have observed that AR poisoning accounted for 1–3% of deaths in non-target animals (Berny et al., 1997). SAGIR network probably resembles better the sampling procedure of our study than others, because it is also focused to identify causes of mortality in wildlife. However, wildlife samples were only routinely submitted to our laboratory when veterinarians at WRCs suspected poisoning and this may explain the higher mortality associated with AR exposure in our study.

In Great Britain, 19.2% of 172 tawny owls (*Strix aluco*) contained detectable residues of one or more SGARs, and this percentage remained unchanged between the periods 1990–93 (18.2%) and 2003–05 (20.2%) (Walker et al., 2008). Slightly higher prevalences were found in barn owls from Britain in these two periods (25.5% and 33.0%, respectively) and a much higher value was found in kestrel in the second period (67.1%) (Walker et al., 2008). On the contrary, we have observed higher prevalences of AR residues in nocturnal than in diurnal raptors. The stability of AR exposure levels also contrasts with our results. The increase of AR poisoning found in Spain in 2007 was associated with large scale use of bromadiolone and chlorophacinone in Castilla y Leon region against a plague of common vole in arable land. Common vole is only found on some small islands in Britain and field vole (*Microtus agrestis*), which also has population outbreaks (Burthe et al., 2008), does not affect crops as common vole does in Continental Europe and eat less frequently rodenticide baits than other rodent species (Brakes and Smith, 2005). Therefore, peaks of AR poisoning of non-target species due to the chemical control of vole outbreaks may be more pronounced in Continental Europe than in the British Islands (Jacob and Tkadlec, 2010).

Stone marten in Spain, as occurs with other mustelids in Europe, was one of the mammal species with highest AR prevalences (58%, n = 19). Bromadiolone was detected in liver of 9% (n = 122) of mustelids in France, including the endangered European mink (*Mustela lutreola*), and chlorophacinone was detected in 4% of the same sample (Fournier-Chambrillon et al., 2004). The presence of one or more SGARs was observed in 31% (n = 100) of polecats (*Mustela putorius*) analyzed in Britain and this value has been constant during the 1990s (Shore et al., 2003). More recent studies have found higher prevalences of AR residues in European wild mammals. ARs were detected in 97% (n = 61) of stoats (*Mustela erminea*) and 95% (n = 69) of weasels (*Mustela nivalis*) from Denmark (Elmeros et al., 2011). ARs were detected in liver in 84% (n = 115) of red foxes from Northern Ireland and this elevated prevalence was probably due to the high predation on commensal rodents and non-target wood mouse (*Apodemus sylvaticus*) most likely to take AR baits (Tosh et al., 2011a). The proportion of hedgehogs (*Erinaceus europeaus*) from Britain with LC–MS detectable ARs in their liver was 66.7% (n = 80), and 22.5% contained more than one compound (Dowding et al., 2010). Differences amongst studies may be explained by biases due to the sampling method because many predators and scavengers are protected species and therefore cannot be randomly sampled by hunting. Sampling is then based on found dead animals and the contribution of other causes of mortality can affect the prevalence of AR poisoning. Non-invasive sampling methods such as the analysis of pellets of birds of prey (Newton et al., 1994) or carnivorous feces (Sage et al., 2010) may allow collection of less biased information about exposure rates to ARs in predators and scavengers that may be readily comparable amongst studies. In fact, we have detected bromadiolone in the unique samples of red fox feces and carrion crow pellet analyzed. Another significant bias may result from the different analytical methods used. LOD of the analytical techniques (LC–MS or HPLC–DAD/FLD) differ significantly and prevalence tends to be higher by LC–MS (Dowding et al., 2010). For instance, the increasing trend observed at the end of our study period (Fig. 2) was largely due to greater sampling and analytical efforts. The use of LC–MS in the last two years of the present study has permitted the detection of some sublethal cases of AR exposure, that may remain undetected by other less sensitive techniques.

Detection of AR in liver has been a diagnostic tool for AR poisoning when accompanied by signs of coagulopathies in animals. Berny et al. (1997) considered that AR poisoning was confirmed when animals showed compatible signs and/or lesions and liver AR concentrations ≥0.2 μg/g. Newton et al. (1999) detected AR levels ≥0.1 μg/g in liver of four barn owls (*Tyto alba*) that may have died by AR poisoning, but hemorrhages were seen in only one of these birds with 0.158 μg/g of difenacoum. Walker et al. (2008) detected AR levels ≥0.1 μg/g in liver of 21 tawny owls, but only one bird with 1.2 μg/g of brodifacoum had hemorrhages. Murray (2011) diagnosed AR poisoning in birds of prey clinically and/or on gross postmortem examination, and AR residues in liver (brodifacoum in all cases, n = 6) ranged from 0.012 to 0.269 μg/g. Thomas et al. (2011) have made a probabilistic approach to determine SGARs levels that may be associated with mortality in birds, because sensitivity may vary markedly

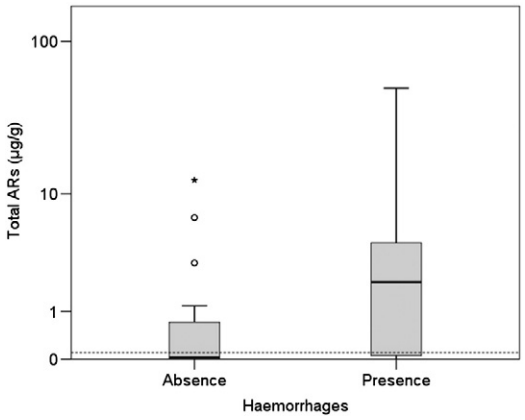

Fig. 4. Box-plot (median, 25–75%, range) of levels of anticoagulant rodenticides (sum of all) in liver of animals with or without macroscopic hemorrhages. Dashed line (—) represents a threshold level of toxic AR exposure (>0.1 µg/g).

amongst individuals and species. Such analysis made with several nocturnal and diurnal raptors from Canada revealed a significant likelihood of AR poisoning below previously suggested liver concentrations of concern (<0.1 µg/g; Thomas et al., 2011). The most sensitive species was great horned owl, for which 5% chance of showing signs of AR toxicosis would occur at liver SGAR levels of 0.02 µg/g (Thomas et al., 2011). However, diagnosis of AR poisoning for every animal found dead is always difficult because the presence of macroscopic hemorrhages is not fully linked to AR residue levels and there are some animals (7.2–10%) with elevated AR levels and no signs of bleeding. Internal bleeding in birds poisoned by ARs can be modest or remain undetected in some cases (Sarabia et al., 2008), and histological examination of tissues may be necessary to detect microscopic hemorrhages in birds (Rattner et al., 2011). Clotting assays such as prothrombin time, Russell's viper venom time, and thrombin clotting time are affected by ARs in birds, and the increase in these in vitro clotting times is indicative of the onset of overt signs of toxicity (Rattner et al., 2010, 2011). Probably, as occurs with other highly toxic pesticides (e.g. organophosphates and carbamates), the use of effect biomarkers should be necessary to reinforce results obtained by chemical analysis and pathological examination.

5. Conclusions

The presence of AR residues in liver of non-target wildlife species has been detected in a large number of species in Spain. This has been a common finding of several recent studies developed with highly sensitive analytical techniques as a result of the wide use of bioaccumulative SGARs in urban and agricultural environments. Moreover, large-scale treatments against rodents that show cyclic population outbreaks may exacerbate the risk of exposure of predatory species to SGARs (Berny and Gaillet, 2008), and may seriously affect populations of granivorous animals when AR treated grain is distributed on surface with spreader machines (Sarabia et al., 2008). Risk of primary poisoning of granivorous animals may be reduced if baits were distributed in places only accessed by the target rodent (i.e. burrows). In the case of rodent controls in farms, AR poisoning in non-target animals may be reduced by ensuring that baiting away from buildings is targeted on areas of high rodent activity, is limited in duration and searches are frequently conducted to remove poisoned animals (Shore et al., 2006). However, according to questionnaires responded by British farmers, the users of ARs almost never searched for and removed poisoned carcasses and many baited for prolonged periods or permanently (Tosh et al., 2011b). The only reptile species with AR residues in the present study was a horseshoe whip snake (*Hemorrhois hippocrepis*) found dead after a flocoumafen treatment for a sea bird colony protection in Chafarinas Islands (Mediterranean Sea). These conservation actions must also consider a wide risk assessment to avoid adverse effects on non-target species (Brooke et al., 2011). The use of accumulative SGARs and the application of baits on surface (i.e. treated grain by spreader machines) should be discontinued in future EU regulations on the use of rodenticides to prevent the poisoning of non-target wildlife species (Mateo, 2010).

Supplementary materials related to this article can be found online at doi:10.1016/j.scitotenv.2012.01.028.

Acknowledgments

This work was funded by the Environmental Agencies of the regional Governments of Aragon, Castilla-La Mancha, Catalonia, Madrid, Asturias, Navarra and Basque country through contracts for toxicological analysis of wildlife incidents. Samples from Castilla y Leon were kindly supplied by the Spanish Hunting Federation, Spanish Greyhound Hunting Federation, Global Nature Foundation and J. Viñuela. We thank all the personnel involved in sampling procedures, from collection of death animals to pathologic examinations.

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