Science of the Total Environment 738 (2020) 139828

Contents lists available at ScienceDirect

Science of the Total Environment

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

Accumulation of diastereomers of anticoagulant rodenticides in wild boar from suburban areas: Implications for human consumers

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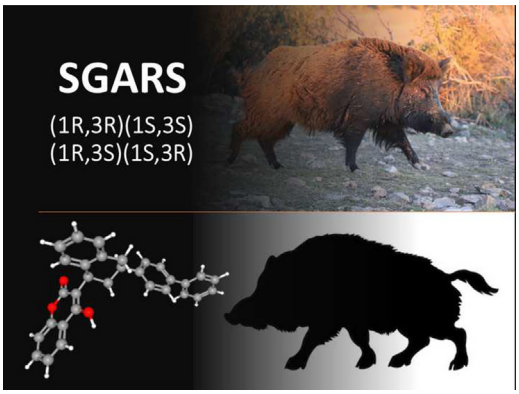
HIGHLIGHTS

- Wild boar around Barcelona city were frequently exposed to SGARs.
- Liver accumulates higher concentrations of SGARs than muscle.
- The most polar diastereomers of SGARs were more abundant in boar samples.
- Game liver consumption should be considered in the risk assessments of SGARs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 February 2020
Received in revised form 27 May 2020
Accepted 28 May 2020
Available online 30 May 2020

Editor: Damia Barcelo

Keywords:
Biocides
Pesticides
Diastereoisomers
Urban wildlife
Food safety
Game meat

ABSTRACT

We studied the prevalence of anticoagulant rodenticides (ARs) in liver and muscle tissues of wild boar captured in the urban area of Barcelona, the suburban area of Collserola Natural Park and the rural area of Santa Quiteria, next to Cabañeros National Park, in Spain. The objective was to assess the influence of both urbanisation and wild boar (*Sus scrofa*) trophic opportunism on the accumulation of these compounds. We have also evaluated the risk for human consumers of this game meat. Wild boars from Barcelona city showed the highest prevalence of ARs detection (60.8%), followed by the adjoining suburban area of Collserola N.P. (40%) and the rural distant area of Santa Quiteria (7.7%). Liver bioaccumulated ARs (45.2%) more frequently than muscle (11.9%). A significant proportion (13.7%) of wild boar captured in Barcelona city exceeded 200 ng/g of total ARs in liver, a threshold for adverse effects on blood clotting. For difenacoum, there was a predominance of *cis* isomer, while for brodifacoum and bromadiolone *cis* and *trans* isomers appeared in a similar proportion. According to the scarce available information on ARs toxicity in humans, the risk of acute poisoning from game meat consumption seems to be low. However, repeated exposure through liver consumption should be considered in further risk assessments because of the high concentration detected in some samples (up to 0.68 mg/kg).

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https://doi.org/10.1016/j.scitotenv.2020.139828
0048-9697/

1. Introduction

Rodent infestations cause substantial property damage and pose health risks in both urban settings and rural farmland, and different methods, including poisoning, have been used to reduce their negative impacts (Van den Brink et al., 2018; Jacob and Buckle, 2018). Anticoagulant rodenticides (ARs) are currently the most common pesticides and biocides used to control rodents by predisposing them to fatal haemorrhage (Rattner et al., 2014). However, the long-term persistence of ARs in animal tissues, especially in liver, makes them bioaccumulative, with the consequent transfer along the food chain (Erickson and Urban, 2004; Ruiz-Suárez et al., 2016) and the risk to cause secondary poisoning of predators (Dowding et al., 2010; Sánchez-Barbudo et al., 2012).

Commonly used ARs nowadays, such as brodifacoum, bromadiolone, difenacoum, flocoumafen and difethialone, are classified as second-generation ARs (SGARs). These types of rodenticides were introduced in the 1970s following widespread development of rodent resistance to first-generation ARs (FGARs) such as warfarin, chlorophacinone and diphacinone. SGARs are much more acutely toxic than the FGARs, generally providing a lethal dose after a single feeding, and tend to be considerably more persistent in animal tissues and have higher affinity for liver tissue (Rattner et al., 2014). SGARs have two asymmetric carbons in their chemical structure conferring them the property of having two diastereomers and thus four stereoisomers. Therefore, commercial SGARs are mixtures of two diastereoisomeric forms (1*R*,3*R*)(1*S*,3*S*)-isomers and (1*R*,3*S*)(1*S*,3*R*)-isomers in different proportions. These pairs of diastereomers have different chemical and physical properties and likely have different pharmacokinetic properties and biological activities (Fourel et al., 2017a). There is usually one diastereoisomeric form with a shorter half-life than the other one, so the risk of secondary poisoning in predators may differ between isomers (Lefebvre et al., 2017).

In a toxic-epidemiological context, we can identify two different scenarios to explain how wild animals can become exposed to rodenticides: (1) animals foraging in and around urban or agricultural areas where the use of ARs against commensal rodents is continuous because of the permanent presence of rodents attracted to anthropogenic food resources, and (2) animals from farmland areas where temporary outbreaks of rodent population (i.e. voles) lead to the intensive use of ARs during these specific periods (Hindmarch and Elliott, 2018; López-Perea and Mateo, 2018). A positive relationship between human population density and the presence of SGARs residues in wildlife has been observed, probably because of the intensive use of these pesticides against commensal rodents (López-Perea et al., 2015, 2019). The exposure to ARs in wildlife can be also associated with intensive livestock farming, where these biocides are commonly used (Alterio and Moller, 2000; Morzillo and Mertig, 2011; Hughes et al., 2013; López-Perea et al., 2019). Since secondary exposure through the ingestion of poisoned rodents is feasible, the risk of population effects on non-target species should be considered in these scenarios of high prey availability (i.e. rodents) to avoid the creation of ecological traps with the use of SGARs (López-Perea et al., 2019; Fernandez-de-Simon et al., 2019).

Game animals are at risk of exposure to SGARs, either by the direct ingestion of anticoagulant baits or by the consumption of carcasses of animals poisoned by these rodenticides (Eason et al., 1999). Omnivorous species such as wild boar (*Sus scrofa*) can be exposed by these two potential routes. Previous studies have described the accumulation of brodifacoum in wild boar, ranging from 0.007 to 1.7 mg/kg in liver and 0.01 to 0.07 mg/kg in muscle; in red deer (*Cervus elaphus*), with up to 0.02 mg/kg in muscle and 0.03 mg/kg in liver; and in goat (*Capra hircus*), with up to 0.01 mg/kg in liver (Eason et al., 1999, 2001). All this information indicates a risk of exposure to SGARs for game meat consumers, although the observed levels up to now are well below those that are likely to be lethal or to affect blood clotting in humans after acute exposures (Eason et al., 2001). However, game animals around urban areas can be more intensively exposed to SGARs and this may increase the risk of exposure in game meat consumers. Therefore, the information about the potential exposure to SGARs in animals that can be consumed by humans can be relevant for the regulation on use of this group of pesticides/biocides (Eason et al., 1999; López-Perea and Mateo, 2018), for the regulation of hunting and game meat consumption as well as to modulate consumers' habits (Conejero et al., 2019).

The aim of this study is to describe ARs exposure, and especially SGARs, in urban, suburban and rural areas in Spain where rodenticide baits are frequently used and to evaluate the potential adverse effects that such exposure may have on wild boars and on the consumers of their meat. The presence of *cis* and *trans* diastereomers of SGARs was also investigated to explore differences in the accumulation of both isomers respect to the proportions observed in commercial AR baits.

2. Materials and methods

2.1. Sample collection and study areas

Liver and muscle samples (≥10 g each) were obtained from 84 wild boars either captured (n = 51) or hunted (n = 33) for management purposes. Fifty-one were urban wild boars captured to manage or prevent real or potentially dangerous situations in Barcelona city (Fig. 1). They were dart-anesthetized (xylazine, tiletamine and zolazepam; 3 mg/kg each) and euthanized (T-61®; 1.2 mL/10 kg) by a veterinarian. Another twenty non-urban wild boars were harvested by local hunters during the regular hunting season in the adjoining Collserola Natural Park, a Mediterranean woodland formed mainly by helm oak *Quercus ilex* with some small crop fields between forested and urbanized areas of single-family houses (Fig. 1). Both Barcelona city and Collserola N.P. are part of the highly populated Metropolitan Area of Barcelona (~5030 inhabitants/km^2 on average) and host a growing population of urban and suburban wild boars (González-Crespo et al., 2018). For comparison, the remaining 13 non-urban hunted wild boars were sampled in a distant rural area of central Spain (Santa Quiteria, in the surroundings of Cabañeros National Park, Ciudad Real; Fig. 1), in order to have a reference wild boar population from a sparsely populated area (4.84 inhabitants/km^2). The habitat of this reference area is formed by hills covered by a Mediterranean woodland in which helm oak is the predominant tree species, surrounded by farmland areas. See Table 1 for further details on the sex and age of the sampled wild boar. Some potential bias may exist in the sample of wild boars from Barcelona city as they were dart-anesthetized and euthanized by veterinarians because these animals were invading urban areas. In any case, the sample is representative of the wild boar invading the city of Barcelona because most of the detected animals are sacrificed. Wild boars from Collserola N.P. and Santa Quiteria were killed by hunters in similar driven hunts, so no bias between these two areas is expected by sampling method. No wild boar was specifically hunted or captured for this study and all procedures were performed under the regulation of the competent public administrations and in compliance with current guidelines for ethical use of animals in research, following European (2010/63/EU) and Spanish (R.D. 53/2013) legislation.

Samples (n = 22) of commercial formulations (baits) of SGARs commercially available in Spain were analysed to know the proportion of *trans*:*cis* isomers. These formulations (12 of bromadiolone, 4 of brodifacoum, 3 of difenacoum and 3 of flocoumafen) had been received for forensic analysis in our laboratory (IREC, Ciudad Real) between 2009 and 2019.

2.2. Rodenticide analysis

The analysis of ten ARs (brodifacoum, difenacoum, bromadiolone, flocoumafen, difethialone, coumachlor, coumatetralyl, chlorophacinone, diphacinone and warfarin) was performed following the method described by López-Perea et al. (2015). Firstly, 1 g of tissue or bait was

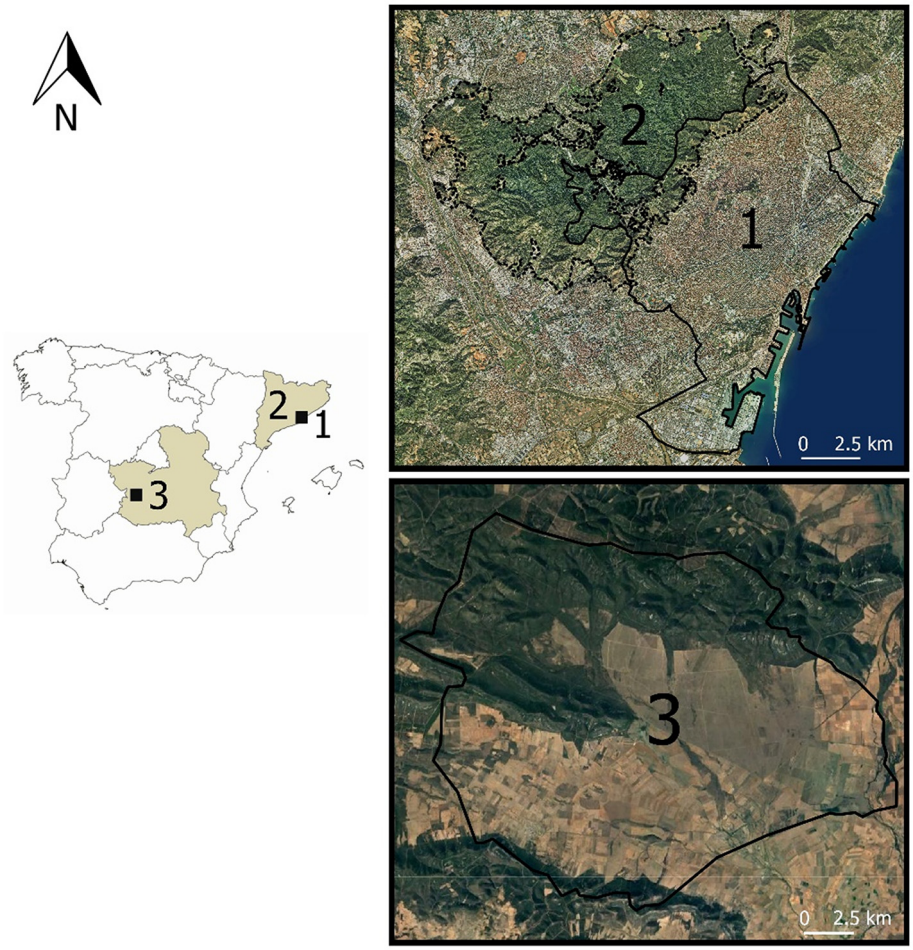

Fig. 1. Sampling areas in Spain. Shaded areas show the region of Catalonia with sampling areas 1-Barcelona and 2-Collserola Natural Park in Barcelona province and the region of Castilla-La Mancha with sampling area 3-Santa Quiteria in Ciudad Real province.

homogenized in a mortar with 10 g of anhydrous sodium sulfate (Prolabo, Leuven, Belgium). The result was transferred to a Teflon-capped 30 mL-glass tube and 20 mL of a mixture of dichloromethane:acetone (70:30) (HiperSolv Chromanorm Gradient grade, Prolabo, Leuven, Belgium) were added, horizontally shaken for 10 min and sonicated for 5 min. The extract was filtered through a Whatman paper filter and collected in a conical tube for solvent evaporation in a rotary evaporator. The extraction was repeated with 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 solid phase extraction (SPE) column filled with neutral alumina (SPE ALN 500 mg/3 mL, Upti-clean Interchrom, Montluçon, France). The 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 (25:75). Finally, the ARs 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 liquid chromatography coupled to mass spectrometry with electrospray ionization source (LC–ESI–MS). The analytical system was formed by an Agilent 1100 series chromatograph and an Agilent 6110 Quadrupole LC/MS with a multimode source (MM). The N_2 for the ionization source was supplied with a high purity generator (Genius 1024, Peak). For the chromatography we used an Eclipse column XDB-C18 (4.6 × 12.5 mm, 5 μm). The injection volume was 30 μL. The chromatographic conditions of analysis consisted in a gradient elution of two solvents (A: ammonium acetate 10 mM, pH: 6.03; B: methanol). The initial conditions were 35% A and 65% B, reaching 15% A and 85% B at min 5. This was maintained until min 10, returning to the initial conditions by min 12. Then, column was stabilized with the initial conditions until min 15 before the next sample injection. The flow rate was 1.2 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 fragmentor value varied among compounds (Table S1). Four ions previously selected for each compound by means of analysis of complete scanning and flow injection analysis of sequences (FIAS) of AR standards were monitored in SIM mode (Table S1; Fig. S1). Method set-up and calibrations were performed with standards purchased from Fluka Pestinorm (brodifacoum, difenacoum, bromadiolone,

Table 1
Wild boars grouped by gender and age by catch area in the urban and suburban areas of Barcelona. The wild boars from Santa Quiteria (rural area) were not aged.

Age (years)	Barcelona (urban area)		Collserola N.P. (suburban area)	
	N of males	N of females	N of males	N of females
<1	14	3	3	2
1–2	4	13	3	1
3–6	2	12	1	4
≥7 years	0	3	2	4

difethialone, coumachlor chlorophacinone, and warfarin), Sigma Pestinorm (coumatetralyl) and Dr. Ehrenstorfer Reference Materials (flocoumafen and diphacinone) (Fig. S2). The limits of detection (LOD) of the studied ARs varied between 1 and 6 ng/g and recovery values for the detected ARs were > 70% (López-Perea et al., 2015). Details of the validation of this method for diastereomer analysis are shown in the supplementary material. The recovery of the diastereomers of the four SGARs in pig liver spiked with 0.5 μg/g (n = 4) were all >73.9%, with RSD values <5.6% (see Table S2 and Fig. S3).

2.3. Statistical analysis

The occurrence (% of positives) of each SGAR and the sum of them were compared among the three sampling areas (Barcelona, Collserola N.P. and Santa Quiteria) by means of χ^2 tests. The percentage of wild boars with residues >200 ng/g of the total of SGARs in liver was calculated and compared among areas by means of χ^2 tests, because this threshold has been considered as indicative of elevated exposure and potentially toxic in animals (Berny et al., 1997; Newton et al., 1999; Walker et al., 2008). Because of the high number of values below the limit of detection we used non-parametric tests. The total tissue concentration of ARs both in all the samples and only in the positive samples was compared among areas with a Kruskal-Wallis test. We can hypothesize that in addition to a higher occurrence of ARs in wild boar in more anthropized habitats, we can also find higher concentrations in the tissues of the exposed animals in these areas. We studied the relationship between age and total AR concentration or *trans*:*cis* ratios with the Spearman correlation. We can hypothesize that older wild boars may accumulate higher ARs concentrations in tissues and tend to accumulate one of the two isomers (the most persistent for each SGAR). Moreover, we used the Mann-Whitney test to explore differences by sex in total AR concentrations and *trans*:*cis* ratios. An effect can be also expected by sex because of differences in the metabolism of SGARs. The isomer ratio was also compared between positive animal samples and baits with the Mann-Whitney test. The relationship in SGARs concentrations in liver and muscle were studied with Spearman's correlations (r_s) for each compound. The relationship between the occurrence of SGARs in wild boar liver and the number of commercial formulations registered in Spain (Ministerio de Sanidad, 2020) was studied with a Spearman's correlation. The number of registered formulations was taken as a surrogate of the use of each SGAR in the absence of official data of SGARs use in Spain. The level of significance of the statistical tests was set at $p < 0.05$. All the analyses were carried out with IBM SPSS Statistics v.24.

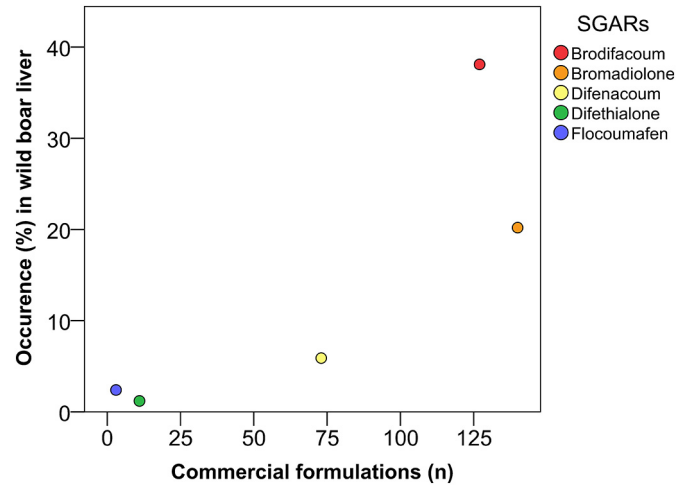

Fig. 2. Relationship between number of registered formulations of SGARs in Spain and the occurrence (%) of each SGAR in liver of wild boar.

3. Results

Considering both liver and muscle samples, the wild boar from Barcelona city showed the highest prevalence of ARs detection (60.8%), followed by those from the suburban area of Collserola N.P. (40%) and the rural area of Santa Quiteria (7.7%).

3.1. ARs residues in liver

SGARs residues were detected in the liver of 38 (45.2%) wild boars (Table 2). A single SGAR was found in 21 (25.0%) samples, two SGARs were found in 15 (17.8%) and three SGARs were found in two (2.4%) wild boars. The SGARs detected were brodifacoum (38.1%), bromadiolone (20.2%), difenacoum (5.9%), flocoumafen (2.4%), and difethialone (1.2%). The occurrence of the different SGARs in wild boar livers was marginally correlated with the number of commercial formulations available in Spain ($r_s = 0.8$, one-tailed $p = 0.052$; Fig. 2). SGARs were detected more frequently in the urban area of Barcelona (56.9%) than in Santa Quiteria (7.7%; $\chi^2 = 10.4$, $p = 0.006$; Table 2). The percentage of wild boars with residues >200 ng/g was 9.5%, without statistically significant differences among Barcelona (13.7%), Collserola N.P. (5%) and Santa Quiteria (0%). The concentrations for the total SGARs detected in wild boar ranged between non-detected values to 678.8 ng/g,

Table 2
Prevalence and concentrations (ng/g) of anticoagulant rodenticides (ARs) detected in liver of wild boars in three areas from Spain.

ARs	Barcelona (urban area) n = 51			Collserola N.P. (suburban area) n = 20			Santa Quiteria (rural area) n = 13		
	n+ (%)	Mean (SE)	Median min-max	n+ (%)	Mean (SE)	Median min-max	n+ (%)	Mean (SE)	Median min-max
Bromadiolone	13 (25.5)	47.5 (20.6)	17.0 3.7–274.4	4 (20)	62.0 (50.2)	15.0 5.5–212.4	0 (0)	n.d.	n.d.
Difenacoum	5 (9.8)	53.8 (28.3)	24.2 3.7–153.2	0 (0)	n.d.	n.d.	0 (0)	n.d.	n.d.
Flocoumafen	2 (3.9)	7.0 (3.4)	7.0 3.6–10.3	0 (0)	n.d.	n.d.	0 (0)	n.d.	n.d.
Brodifacoum	24 (47.1)	109.7 (37.2)	25.9 1.6–678.8	7 (35)	19.7 (5.9)	14.9 5.4–50.4	1 (7.7)	8.7	8.7
Difethialone	1 (1.9)	5.9	5.9	0 (0)	n.d.	n.d.	0 (0)	n.d.	n.d.
Total of ARs	29 (56.9)[A]	122.0 (32.1)	54.5 1.7–678.8	8 (40)[AB]	48.2 (24.4)	24.5 5.4–212.4	1 (7.7)[B]	8.7	8.7
n > 200 ppb (%)	7 (13.7)			1 (5.0)			0 (0)		

[A,B]Percentages of SGARs occurrence sharing a letter are not significantly different among areas. n.d.: not detected.

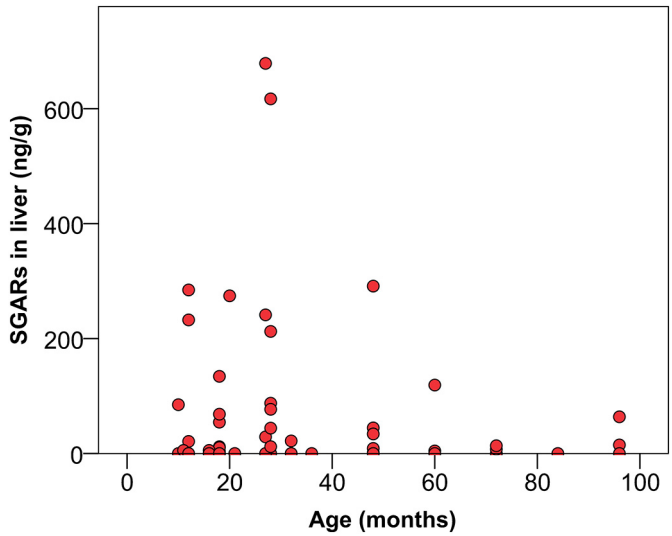

Fig. 3. Relationship between age and sum of SGAR concentrations in liver of wild boar. These variables were not significantly correlated ($r_s = -0.196$, $p = 0.303$), but all the oldest animals (>48 months) showed <200 ng/g of SGARs in liver.

with differences among areas ($\chi^2 = 10.9$, $p = 0.004$). In the positive individuals, the average concentration was 122.0 ng/g and no differences were found among areas in total ARs levels (Table 2). The liver SGAR concentrations were not statistically affected by sex. Regarding the effect of age, none of the wild boars older than -four years showed SGAR residues >200 ng/g in liver (Fig. 3). However, this difference (0/13 vs. 8/53) was not significant ($\chi^2 = 2.23$, $p = 0.135$). SGAR concentration was not significantly correlated with age (Fig. 3).

3.2. ARs residues in muscle

ARs residues were detected in 10 (11.9%) muscle samples. The three detected compounds were two SGARs, difenacoum (2.4%) and brodifacoum (4.8%); and one FGAR, diphacinone (4.8%). In contrast with liver, only one AR was detected in each positive muscle sample. Only the wild boar from Barcelona showed AR residues in muscle (Table 3). Differences in the occurrence of ARs were detected among the study areas, being significantly higher in the urban area of Barcelona (19.6%) than in Santa Quiteria and in Collserola N.P. (0%; $\chi^2 = 7.3$, $p = 0.025$; Table 3). The concentrations for the total ARs detected in the muscle tissue of wild boar ranged between non-detected values to 162.7 ng/g, with significant differences among areas ($\chi^2 = 7.2$, $p = 0.027$). The average value of total ARs in the positive wild boars was 34.5 ng/g. No significant relationship was found between total AR concentration and sex or age.

Table 3
Prevalence and concentrations (ng/g) of anticoagulant rodenticides (ARs) detected in muscle of wild boars from the urban area of Barcelona (n = 51). None of the wild boars from Collserola N.P. (n = 20) and Santa Quiteria (n = 13) presented ARs in muscle.

ARs	n+ (%)	Mean (SE)	Median min-max
Difenacoum	2 (3.9)	2.8 (0.9)	2.8 1.9–3.7
Brodifacoum	4 (7.8)	11.8 (6.1)	7.7 2.9–29.0
Diphacinone	4 (7.8)	73.1 (72.5)	42.4 n.d.-162.7
Total of ARs	10 (19.6)	34.5 (17.1)	18.5 1.9–162.7

n.d.: not detected.

3.3. Cis and trans isomers of SGARs in wild boar tissues and commercial formulations

Cis and *trans* isomers of the different SGARs were detected in liver (Fig. 4) and muscle. We observed an average predominance of the first-eluting SGAR isomer (more polar) in the reverse-phase HPLC analysis. According to Fourel et al. (2017a) this is the *trans* isomer in the case of bromadiolone and flocoumafen and the *cis* isomer in the case of brodifacoum and difenacoum. Despite being more abundant *trans* than *cis* isomer of bromadiolone in baits (64:36), this proportion was more balanced in wild boar livers (54:46). In the case of brodifacoum, *trans*:*cis* isomer proportions were similar in baits and wild boar livers (46:54 vs. 45:55). In the other two SGARs with lower prevalence, the first-eluting isomers (*trans*-flocoumafen and *cis*-difenacoum) were slightly predominant in baits (52:48 and 44:56, respectively) and this was more evident in liver samples (70:30 and 18:82, respectively; Fig. 4). All these ratios were not significantly different between baits and livers. In muscle, *trans* and *cis* isomers of brodifacoum were in similar proportion (47:53) and *cis* isomer was the only one detected for difenacoum (>98.8%). The high proportion of *cis*-difenacoum in muscle might not be representative because only two samples were positive and with very low concentrations. None of the isomer ratios differed statistically between wild boar tissues or were affected by age or sex.

3.4. Relationship between liver and muscle ARs concentrations

For the two SGARs detected in both liver and muscle (i.e. brodifacoum and difenacoum), the concentrations were significantly correlated between tissues only for brodifacoum, possibly because of its higher occurrence ($r_s = 0.417$, $p < 0.001$, $n = 84$; Fig. 5). Fig. 5 shows that brodifacoum was only detected in the muscle of those wild boars with >150 ng/g of this SGAR in liver.

4. Discussion

The comparative study of ARs residues in liver and muscle tissues of wild boar from different areas of Spain revealed differences in the occurrence and concentration of these compounds depending on the anthropization of the habitat used by the animals.

4.1. SGARs exposure and habitat anthropization

The results show a significant relationship between SGARs occurrence in wildlife and the density of human population. These results are probably due to the extensive use of SGARs against commensal rodents in highly populated sites, which can lead to long-term chronic accumulation of SGARs in wild boars. In urban areas, anticoagulant residues have been detected in wastewater, originating from the surface run-off from baits, the use of ARs in sewers, and the excretion of anticoagulants used therapeutically in humans (Shore and Coeurdassier, 2018). However, since wild boar presence in the urban areas of Barcelona is regular and driven by food resources (Castillo-Contreras et al., 2018), the high exposure detected in wild boar from the urban and suburban areas of Barcelona can be due to the direct ingestion of rodenticide baits and the consumption of carcasses of poisoned rodents or their predators (e.g. cats). In the case of Santa Quiteria, the human density and the degree of habitat anthropization is much lower than in Barcelona and Collserola N.P.; despite this, one of the 13 wild boars from Santa Quiteria also showed brodifacoum residues. This is not unexpected at all, since cattle farms are present in this area and rodenticides are commonly used in and around farm buildings. In a previous study, López-Perea et al. (2019) found a significant association between cattle farms and ARs occurrence in wildlife in the Aragon region (NE Spain). Other authors have observed this association with other types of livestock production (i.e. pig farming in Germany, Geduhn

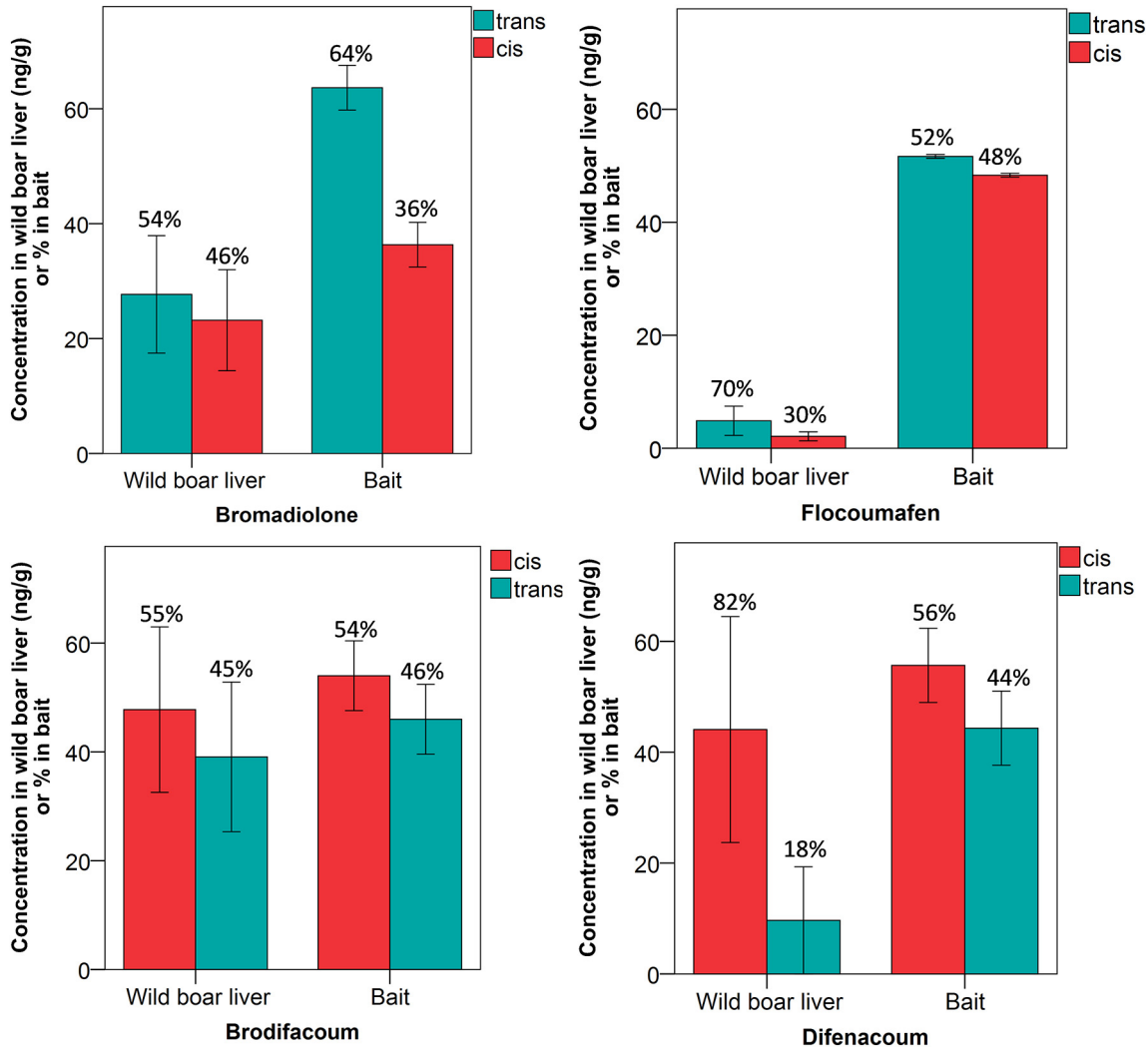

Fig. 4. Concentrations (ng/g) and proportions (%) of SGAR diastereomers in liver of wild boar from the study areas (positive animals) compared with the proportions of diastereomers in SGAR formulations (baits) commercially available in Spain.

et al., 2015). Moreover, ARs are used in farmland environments during rodent population peaks at large spatial scales with consequences for non-target and predatory species (Olea et al., 2009; Jacob and Tradlec, 2010; Luque-Larena et al., 2013), but this was not the case of Santa Quiteria area. In summary, the differences between urban and rural environments can affect the probability of exposure and the accumulation of ARs in non-target species, including those that can feed on rodenticide baits or exploit poisoned rodents as a food resource (Tosh et al., 2011).

4.2. Cis and trans isomers of SGARs

This study shows a similar *trans*:*cis* ratio in baits and animal tissues, with a predominance of the polar isomers (eluting first in HPLC) in both types of samples. These were *trans* isomers of bromadiolone and flocoumafen, and *cis* isomers of brodifacoum and difenacoum. This predominance of the polar isomers was more evident for difenacoum and flocoumafen in liver, which agrees with previous studies showing an accumulation in of *cis* and *trans* isomers of these two SGARs, respectively (Damin-Pernik et al., 2016; Damin-Pernik et al., 2017). In the case of bromadiolone, there is a more balanced proportion of isomers in liver in comparison with baits, which had more *trans*-bromadiolone. Considering that *trans*-bromadiolone has a longer half-life than *cis*-bromadiolone in rats (Lattard and Benoit, 2018), this finding may be unexpected. In the case of brodifacoum, we have not detected a significant accumulation of the *cis* form as could expected by its longer half-life in rats respect to its *trans* form (121 vs. 69 h; Lattard and Benoit, 2018). Interspecific differences in diastereomer metabolism may explain these inconsistencies with previous studies. Differences in the AR enantiomers binding to serum albumin have been observed between rats and other species (Brown et al., 1979; Lagercrantz et al., 1981). Toxicokinetics of SGAR isomers are not known in species other than rats, so experimental studies in pig may be necessary to investigate interspecific differences in SGAR diastereomers metabolism.

Metabolism, distribution, and excretion of ARs are affected by a series of factors. Hepatic metabolism of ARs exhibits a biphasic profile. Initial metabolism is rapid, often clearing a large percentage of the compound within the first days following exposure. In the terminal phase of hepatic metabolism, clearance of anticoagulant compounds occurs more slowly, leading to the low residual levels of SGARs often found for long periods of time post-exposure (Watt et al., 2005). The toxicokinetic properties of diastereomers are very different in rodents, with one of them more rapidly cleared than the other one (Damin-Pernik et al., 2016; Damin-Pernik et al., 2017; Fourel et al., 2017a, 2017b). Therefore, the manufacture of baits with the less persistent isomer could reduce the bioaccumulation of SGARs in the organism of the rodents and the risk for their predators, while maintaining the inhibition of the vitamin K epoxide reductase (VKOR) (Damin-Pernik et al., 2016; Lattard and Benoit, 2018).

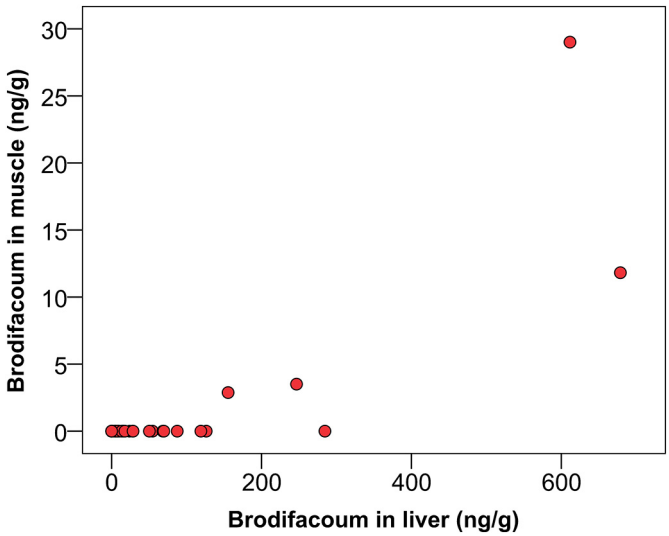

Fig. 5. Relationship between the concentration (ng/g) of brodifacoum in liver and muscle of wild boars.

4.3. Individual traits and SGAR accumulation

Spatial activity pattern in animal species is determined by mobility, diet, age and sex; hence, all these factors can be considered as potential drivers of mammal exposure to ARs. On one side, we would expect an increase with age in ARs concentrations because of a bioaccumulation process, but on the other side the animals highly exposed to ARs may result lethally poisoned or be more susceptible to traumatisms, so their lifespan could become shortened (Fournier-Chambrillon et al., 2004; López-Perea et al., 2015). We have not detected any age or sex effect on liver ARs concentrations, but we have observed that wild boars with an ARs concentration of >200 ng/g did not exceed 48 months of age (Fig. 2). This could be explained by a higher exposure to ARs in younger animals. However, considering the bioaccumulation capacity of these chemicals (Frankova et al., 2019), the differential mortality between animals exposed and non-exposed to ARs may be another feasible explanation.

Often, ARs concentration in liver is not enough to kill the animals, but some sub-lethal effects can impair the fitness of individuals. The main effect of ARs is a reduction in blood clotting capacity (i.e. increase in thrombin time, prothrombin time and activated partial thromboplastin time) (Brakes and Smith, 2005). Other effects attributed to sublethal AR exposure are reduced body condition, susceptibility to disease, reduced resilience or tolerance to extreme weather and sensitivity to other toxicants (Rattner et al., 2014). Sub-lethal adverse effects mediated by ARs may occur not only at the individual level. Several studies reveal adverse effects on the breeding success of predators and population dynamics (Naim et al., 2011; Proulx and MacKenzie, 2012; Jacquot et al., 2013; Coeurdassier et al., 2014) and the maternal transfer of ARs to progeny (Fisher, 2009; Gabriel et al., 2012).

4.4. ARs residues in game animals and implications in food safety

The occurrence of ARs in wild boar tissues is relevant because this is a game species largely hunted and consumed in Europe. Muscle is more commonly consumed than liver, and our results can be used to recommend avoiding offal consumption in areas with intensive SGAR use. While in liver 45.2% of samples were positive, in muscle these were only 11.9%. These results also show that the target organ and the best for monitoring ARs is the liver, even though these biocides can be also found at lower level and frequency in muscle and other tissues or even in non-invasive samples (Newton et al., 1994; Berny et al., 2010; Albert et al., 2010; Rattner et al., 2020). The proportion of AR in liver has been estimated to be about 25% of total body burden (Giraudoux et al., 2006). We also observed combinations of ARs accumulated in the liver (20.2%) of wild boars more frequently that in muscle (0%), which is another sign that liver can accumulate residues from subsequent exposures during long periods of time (López-Perea et al., 2015). In the liver, the main combination was bromadiolone + brodifacoum, which has been described as the most frequent in other animal species (Walker et al., 2008). All this information must be considered in terms of food safety for game meat consumers.

The risk of secondary poisoning in humans has been evaluated in previous studies, but some gaps persist and hamper proper assessment. Eisemann and Swift (2006) showed that the exposure scenarios of risk are not likely to occur in a single day; however, the risk of such exposure should not be discounted as ARs have much lower effective doses at repeated exposures and because some SGARs are highly bioaccumulative. These exposure scenarios were based on maximum diphacinone residues in pig muscle (0.25 mg/kg) and pig liver (3.07 mg/kg), and the conclusion was that a person of 55 kg of body weight would need to eat 28.48 kg of pig muscle and 2.33 kg of pig liver to ingest a dose of diphacinone enough to show adverse effects on blood clotting (Eisemann and Swift, 2006). Clinical assays have established the therapeutic dose of diphacinone in humans at 5–63 mg per day (Willis et al., 1953). Here we observed a maximum brodifacoum level of 0.68 mg/kg in liver. If humans were as sensitive as dogs to brodifacoum (LD_{50}: 0.25 mg/kg, ChemIDplus, 2020), and according to a human equivalent dose calculation based on body surface area respect to the dog (Nair and Jacob, 2016), the LD_{50} in humans could be around 0.14 mg/kg. This means that a 60 kg person would need to eat approximately 12.4 kg of liver containing 0.68 mg/kg of brodifacoum to achieve an acute lethal dose. As a boar liver has an average weight of 1.36 kg (Babicz et al., 2018), it is highly unlikely to reach a lethal exposure level in humans with a single intake, and because game liver is not commonly eaten in Spain. However, since it is unknown the effect of repeated exposures to SGARs, people already on antithrombotic therapy may be at special risk (Eason et al., 1999). Moreover, wild boar liver with high SGAR concentration may represent a risk for hunting dogs fed regularly with game offal.

5. Conclusions

Wild boars are frequently exposed to ARs in urban and suburban areas, which should be considered in terms of game meat safety. We found ARs in 54.9% of liver and/or muscle samples of the wild boar from the metropolitan area of Barcelona (including Collserola N.P.), with bromadiolone and brodifacoum displaying the highest concentrations. This occurrence of ARs is positively associated with human population density and habitat anthropization. In fact, we found the highest occurrence and the highest concentrations of SGARs in the wild boars captured in Barcelona city. Despite SGARs concentrations do not appear to be affected by age, further studies would be needed to clarify a potential relationship with increased wild boar mortality. The proportion of SGAR diastereomers in wild boar liver was similar than in the commercial baits. This contrasts with studies in rodents, in which one of the two diastereomers was more accumulated. This information could be useful for the study of the persistence of SGARs in different wildlife species and for the selection of those less persistent diastereomers for the manufacture of these biocides.

CRediT authorship contribution statement

Enrique Alabau: Formal analysis, Investigation, Methodology, Writing - original draft. **Gregorio Mentaberre:** Resources, Investigation, Funding acquisition, Writing - review & editing. **Pablo R. Camarero:** Resources, Methodology, Writing - review & editing. **Raquel Castillo-**

Contreras: Resources, Investigation, Writing - review & editing. **Inés S. Sánchez-Barbudo:** Investigation, Methodology. **Carles Conejero:** Resources, Investigation, Writing - review & editing. **María S. Fernández-Bocharán:** Investigation, Methodology. **Jorge R. López-Olvera:** Resources, Investigation, Funding acquisition, Writing - review & editing. **Rafael Mateo:** Conceptualization, Formal analysis, Funding acquisition, Writing - original draft.

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.

Acknowledgements

We want to express our gratitude to the Ajuntament de Barcelona, Generalitat de Catalunya, Agents Rurals and Guàrdia Urbana de Barcelona for collecting and providing part of the raw data used to develop this study. Sample collection was funded by the contracts 13/051, 15/0174 16/0243 between the Universitat Autònoma de Barcelona and the Municipality of Barcelona. University of Castilla-La Mancha funded the analysis of samples with project CGT13-0183. Pablo R. Camarero benefited from a contract with reference PTA2017-14583-I financed by the Spanish Ministry of Economy and Innovation and CSIC. Raquel Castillo-Contreras benefitted from a PhD grant 2016FI_B 00425 financed by Generalitat de Catalunya (Secretaria d'Universitats i Recerca, Departament d'Economia i Coneixement) and the European Social Fund (ESF).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139828.

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