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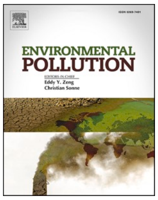

# Widespread use of anticoagulant rodenticides in agricultural and urban environments. A menace to the viability of the endangered Bonelli's eagle *(Aquila fasciata)* populations[☆]

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## ABSTRACT

Anticoagulant rodenticides (ARs) are one of the most toxic groups of compounds currently used worldwide for rodent pest control. Toxic baits are often, directly or indirectly, ingested by non-target animals, resulting in secondary poisoning and frequently affecting apex predators. Their presence in many species of raptors is quite common, particularly scavenger species, with some of these acting as sentinels for the presence of these substances in the environment. However, there is less data on the presence of ARs in Bonelli's eagle, one of the most endangered eagle species in Spain and which is experiencing a negative population trend in Europe. This medium-sized eagle feeds predominantly on live species, and rarely consumes carrion.

In this study, 17 carcasses of Bonelli's eagles from the Eastern Spain were necropsied. Both first and second generation ARs in their livers were analyzed by HPLC-MS-TOF revealing that all the eagles studied had been exposed to at least 5 ARs, out of a total of 10 ARs analyzed, with 7 being the highest number of ARs detected in a sample. Second generation ARs were the most prevalent, particularly bromadiolone and brodifacoum, with the highest concentrations in 94% of the cases. More than a third of the eagles presented a liver concentration of greater than 200 ng/g ARs, suggesting AR poisoning.

The elevated presence of these compounds in Bonelli's eagles could be a new cause of mortality for this species or could explain other causes of death, such as the increased mortality in power lines, and should be taken into account for their conservation. At the same time, the presence of these compounds in the environment also represents a risk to public health, as the most frequent species in the diet of Bonelli's eagle (rabbits and partridges) are also hunted and consumed by hunters and their families.

## 1. Introduction

Exposure to contaminants has been described in many non-target wildlife species and is known to be among the most life-threatening factors affecting fauna. Among these contaminants, exposure to anticoagulant rodenticides (AR) has been well documented in numerous species (Dowding et al., 2010; Elmeros et al., 2011), particularly raptors (García-Fernández et al., 2024; Herring et al., 2017; Niedringhaus et al., 2021; Ruiz-Suárez et al., 2014; Stone et al., 2003; Thomas et al., 2011). This generally occurs as secondary exposure due to the ingestion of previously exposed prey species, including rodents and other small mammals (Niedringhaus et al., 2021; Rattner et al., 2014b; Rattner and Harvey, 2021; Stone et al., 2003; Thomas et al., 2011; van den Brink et al., 2018). These compounds have been widely used worldwide since the middle of the 20th century to control rodents in urban settings, as well as in agricultural environments and habitat conservation or

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restoration settings (Rattner et al., 2014b). Anticoagulant rodenticides disrupt vitamin K-dependent clotting factors in the liver, potentially causing life-threatening hemorrhages in predatory bird species (Bachmann and Sullivan, 1983; Rattner et al., 2014b). These birds are particularly vulnerable to AR poisoning due to their higher susceptibility to these compounds (Thomas et al., 2011), their greater propensity for injuries resulting in hemorrhages during routine activities (Rattner et al., 2014b), and the frequent consumption of rodents targeted using ARs. In some raptor species, mortality caused by AR poisoning may have population-level impacts (Thomas et al., 2011).

ARs are classified into first generation and second-generation anticoagulant rodenticides (FGARs and SGARs, respectively) based on their chemical structure and when they were first synthesized. FGARs were first used for rodent control in the 1940s (Rattner et al., 2014b) and due to their widespread and continuous use, rodent populations were increasingly developing genetic resistance. This prompted the design of new chemical formulations, leading to the appearance of SGARs on the market in the 1970s (Buckle et al., 1994; Elliott et al., 2016). SGARs are persistent (Fisher et al., 2003), bio-accumulative (Vein et al., 2013) toxic compounds (Vandenbroucke et al., 2008), and are currently the most widely used products for controlling rodent populations.

Exposure to anticoagulant rodenticides can be primary (Geduhn et al., 2014; Tosh et al., 2012) (usually through the direct consumption of toxic baits) or secondary (Rattner et al., 2014b) (e.g., the consumption of animals that have been previously exposed to these compounds). The presence of several AR residues in the liver reflects repeated exposure that could have adverse effects (Rattner and Harvey, 2021), with their consequent entry into food webs (Hindmarch and Elliott, 2018). The acute toxicity of SGARs is greater than that of FGARs, requiring only a single bait ingestion to be lethal for the target species (Pelfrène, 2010). Moreover, SGARs are also more persistent in vertebrate livers (Eason et al., 2002; Fisher et al., 2003). Consequently, raptors feeding on animals poisoned with SGARs may have a greater probability of bioaccumulation and secondary poisoning compared to raptors exposed to FGARs (Herring et al., 2017; Mendenhall and Pank, 1980).

The Bonelli's eagle (*Aquila fasciata),* [Vieillot, 1822] is a medium-sized raptor currently classified as "Least Concern" out of Spain (BirdLife International, 2024) although in Spain it is considered "Vulnerable" (Real Decreto 139/2011; Viada, 2021) due to the rapid reduction of important breeding areas (Real, 2004). The habitat of this species ranges across southern Europe from the Iberian Peninsula, to northern Africa, the Near and Middle East and India to southern China (BirdLife International, 2024; del Hoyo et al., 1994). In Spain, this species usually occupies Mediterranean habitats near to the coast with evergreen forests and areas with steep cliffs where it finds adequate nesting sites. This species experienced a decline in the mid-1980s on the Iberian Peninsula (Real and Mañosa, 1997). The diet of this eagle depends on the availability of prey (Cheylan, 1977; Simeón and Wilhelm, 1988), this being mainly rabbits, red-legged partridges, and pigeons, but it may occasionally consume lizards and small mammals (Moleón et al., 2012; Ontiveros et al., 2005). It principally feeds on live species, with rare data on carrion consumption (BirdLife International, 2024; Cheylan, 1977; Ferguson-Lees and Christie, 2001).

In this work, we evaluated the exposure to anticoagulant rodenticides in Bonelli's eagles after necropsy of the carcasses and analysis of the livers of 17 specimens found in the Valencian Community, where this raptor species has been newly listed as critically endangered. Simultaneously, we tried to determine if there was an explanation or relationship between the use of different habitats and the concentrations of these compounds in our study area, focusing our analysis on agricultural and urban land-uses as possible factors that could explain the frequency and intensity of the exposure.

## 2. Materials and methods

### 2.1. Study area

The study was conducted in the Valencian Community (VC), in southeastern Spain, with an area of 23,255 $km^2$ and a population of 5,270,802 inhabitants (INE, 2023). Valencia, the capital city of the VC, is the third largest city in Spain, with a metropolitan population of 1.5 million (INE, 2023). Besides Valencia, Castellón de la Plana and Alicante, the first level administrative capitals of this region, are the other two main towns. At the second administrative division, the Valencian Community is territorially divided into *comarcas*, hereinafter referred to as districts. These are a group of municipalities historically characterized by shared land usage, often focused on specific industries, agricultural practices, or both (Pyke and Sengenberger, 1992), see Fig. 1.

Inland areas often exhibit reliance on traditional agricultural practices, emphasizing rainfed agriculture and extensive livestock rearing, while coastal districts are more inclined toward industrial activities and tourism as well as intensive agricultural production, leveraging their proximity to the sea. This region has exhibited particularly intense urban growth in recent years (Carrasco and Puebla, 2014); new residential settlements have spread along the coast, reflecting the expansion of the second home housing market (Mantecón, 2010; Ribes et al., 2011). Recent urban developments have defined a new metropolitan corridor where the main urban centers give way to a sparse countryside of houses, industrial parks and highways expanding into agricultural lands (Prytherch and Boira Maiques, 2009). Suburbanization has affected not only major cities but also medium-sized towns and peri-urban areas, making the difference even more marked between inland regions and those close to the coast (Carrasco and Puebla, 2014).

At a coarse scale, animal production represents approximately 20–25% of agricultural activity with 4973 farms intensively producing more than a livestock unit (pigs, poultry, cattle, sheep, goats, or horses). Approximately, 44% of the land area is used for agricultural purposes, whereas approximately 56% of the surface area of the VC is forested. The inland zone is characterized by pasture, forest, scrub, rainfed agriculture and olive and wine production, land abandonment and terraces, whereas coastal localities are intensively farmed and have a higher density of intensive holdings. In terms of population, inland regions tend to have lower densities while the coast concentrates higher population densities and the largest cities in the area (>50,000 inhabitants), especially in the southern part, such as Valencia, Alicante and Elche (INE, 2023), see Fig. 1.

### 2.2. Focal species in the study area

Bonelli's Eagle (*Aquila fasciata*) is one of the rarest birds of prey in Europe, where it has suffered a significant decline in recent decades. This raptor typically inhabits coastal regions and mid-altitude mountain areas throughout the Iberian Peninsula, which holds 60% of the European population (Del Moral and Molina, 2018). Previous analyses have highlighted this decline with high levels of adult and pre-adult mortalities (Hernández-Matías et al., 2010; Real and Mañosa, 1997; Soutullo et al., 2008), mainly caused by electrocution, collision and direct persecution (Chevallier et al., 2015; Real et al., 2001). The loss of suitable habitat resulting from changes in land-use including urban development, infrastructure projects and a decline in traditional-extensive farming may also have had an impact (Balbontín, 2005; Carrete et al., 2005; Martínez et al., 2008). Bonelli's Eagles prefer open areas for nesting and hunting potential prey (Carrascal and Seoane, 2009; Carrete et al., 2002; López-López et al., 2006; Ontiveros et al., 2005) It is a raptor species with large home ranges and movements (Martínez-Miranzo et al., 2016; Morollón et al., 2022a, 2022b; Pérez-García et al., 2013) and is an active hunter that principally preys on live animals; there is little data about its feeding on carrion (Cheylan, 1977; Ferguson-Lees and Christie, 2001). Its principal prey are rabbits, pigeons and

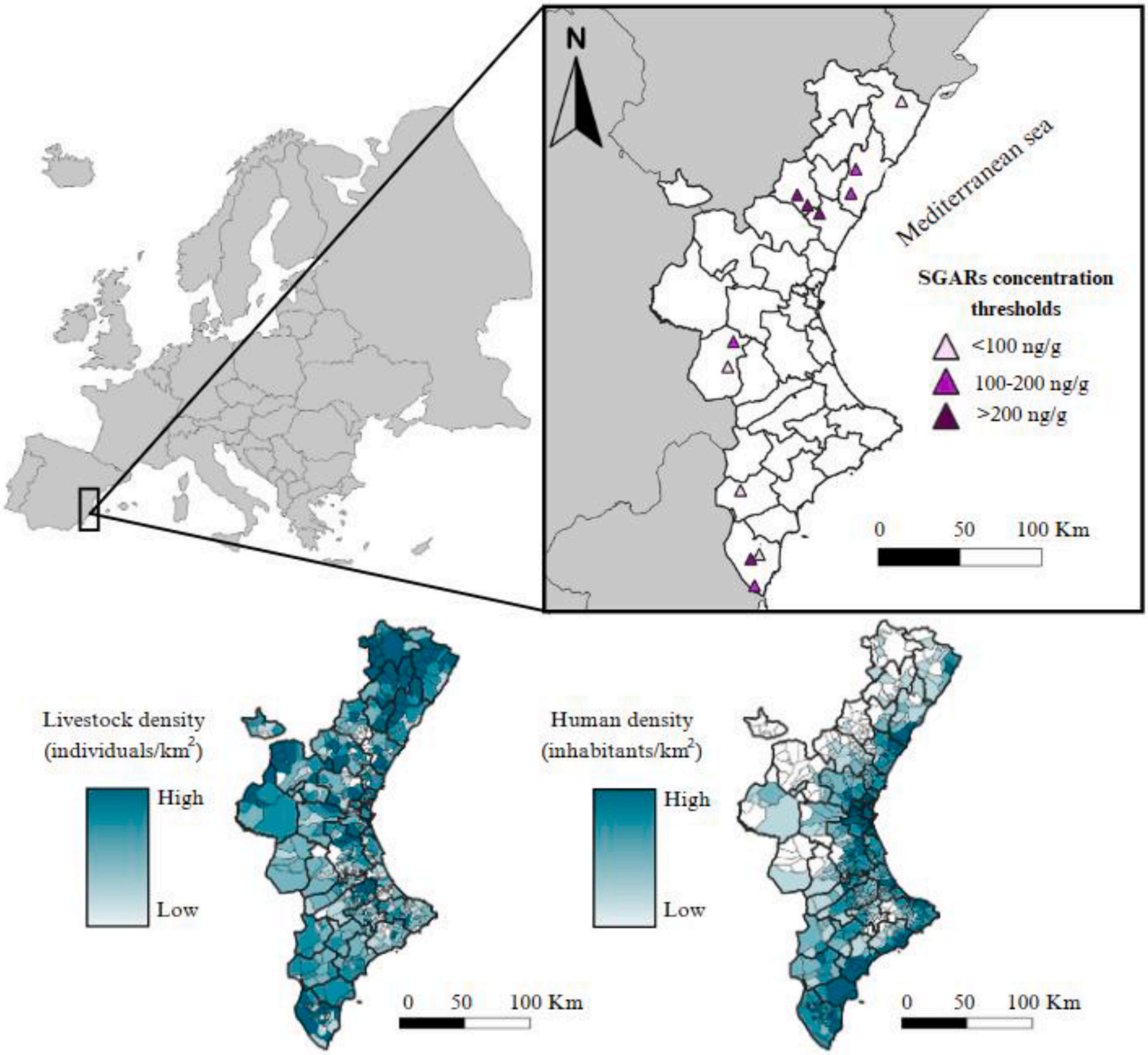

**Fig. 1.** Map of the Valencian Community showing the locations and sum of SGARs concentrations in each Bonelli's eagle by district (the second administrative division). Livestock and population densities are plotted according to municipal gradient. The triangles represent the Bonelli's eagle locations.

red-legged partridges which are frequent in the study area, with rabbits being so abundant that in several municipalities near the study area they have been considered a pest due to the economic damage caused to crops. In 2020, the population of this raptor in the study area was estimated to be 55 reproductive breeding pairs (Generalitat Valenciana, 2020).

### 2.3. Sample collection

All the Bonelli's eagle carcasses came from three wildlife rehabilitation centers in the Valencian Community (Valencia, Castellón de la Plana and Alicante), which either collected the carcasses from the field a few days after their death or took in ill birds, which died within a few days. None of them were treated with vitamin K because no clinical signs of AR poisoning were observed. Between 2015 and 2022, 17 carcasses of Bonelli's eagle were frozen until necropsy in the Toxicology and Forensic Veterinary Service at the University of Murcia. The eagles were necropsied to establish the cause of death and to collect samples for toxicological and monitoring analysis. For each specimen, the history of the carcass was provided (place of collection, date of discovery, description of the area) and information was collected on the species and sex, including descriptions of the external and internal examination of the carcasses during the necropsy. Samples of liver, kidney, muscle, brain, feathers, and bone were taken for toxicological and biomonitoring studies of environmental contaminants. Accurate information about the location of the carcasses was only available for 13 of the 17 eagles; only liver samples were used to develop this study.

### 2.4. Reagents, solvents, and standards

The HPLC-quality acetonitrile and methanol used were from Lab-Scan®, while the purifying agents PSA and C18 were from Supelco® (Sigma-Aldrich, Merck, USA). The analytical-grade salts, sodium sulphate and sodium chloride, were from Panreac®.

Standards for difenacoum, bromadiolone, brodifacoum, flocoumafen, warfarin, coumatetralyl, coumafuryl and coumachlor were purchased from Sigma-Aldrich (Merck, USA); and for difethialone, chlorophacinone and diphacinone were purchased from Dr. Ehrenstorfer GmbH (Germany).

### 2.5. Extraction procedure

Whole livers, the primary organ for rodenticide accumulation (Dowding et al., 2010), were removed and stored at −20 °C until sample preparation. To extract the anticoagulant rodenticides present in the livers of the Bonelli's eagles in this study, a modified QUECHERS technique (Anastassiades et al., 2003), adapted by Taylor et al. (2019) was used.

One g of liver sample was extracted and placed in a 50 mL Falcon tube along with a ceramic homogenizer (Agilent). Next, 20 μL of a 1000 ppb coumachlor solution was added as an internal standard (IS) for anticoagulant rodenticides. Next, 4.98 mL of ACN was added to make a final volume of 5 mL. To this mixture, 4 g $NaSO_4$ and 1 g NaCl were added to act as extraction aids. The prepared Falcon tube was then shaken vigorously using a vortex at high speed for 1 min. It was Subsequently centrifuged for 5 min at 3000 rpm and the tube was kept frozen at −20 °C for 15–20 min.

After 20 min of freezing, if the supernatant was not transparent, a new preparation was centrifuged under the same conditions as described above. At this point, 3 mL of the supernatant was transferred to a tube containing the purification agents (125 mg of PSA, 375 mg of C18 and 2.25 g of $NaSO_4$). The tube was vigorously vortexed for 1 min and then centrifuged for 5 min at 3000 rpm. Finally, 1 mL of the purified supernatant was filtered through a 0.45 μm pore nylon membrane filter and transferred to a chromatography vial. The chromatography vial was kept refrigerated until further analysis using HPLC-MS-TOF.

Calibration curves were made analyzing samples of chicken livers from healthy hens (*Gallus gallus domesticus*) free of residues obtained from the Educational Farm of the Faculty of Veterinary of the University of Murcia. These hens were only fed with non-medicated feed, and they were never treated with pharmaceuticals. Control liver samples were fortified with increasing concentrations of each rodenticide between 0 and 100 ng/mL and were processed in the same way as the problem samples. From the curves and the 5 replicates of the 10 and 50 ng/mL concentrations, the quality assurance and control parameters of the technique (recovery percentages, coefficients of variation and linearity) were calculated (Carrera et al., 2024, Table S1, Supporting Information (SI)).

### 2.6. Analyses of anticoagulant rodenticides by HPLC-MS-TOF

All anticoagulant rodenticides were detected and quantified using an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated Multisampler module and a high-speed binary pump and connected to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual System (AJS-Dual ESI) interface (Agilent Technologies, Santa Clara, CA, USA). The experimental parameters for HPLC and Q-TOF were set using MassHunter Workstation data acquisition software (Agilent Technologies, Rev. B.08.00).

The standards and samples (20 μL injection volume) were injected into a Zorbax Eclipse XDB C8, 5 μm, 150 × 4.6 mm, at a flow rate of 0.7 mL/min. The column was equalized at a temperature of 25 °C. Solvents A (MilliQ water with 20 mM ammonium acetate) and B (methanol with 20 mM ammonium acetate) were used as a mobile phase to separate the compounds through the column. The initial conditions were 50% solvent A and 50% solvent B. After injection, the compounds were eluted using a 50–95% B linear gradient for 22 min. This was followed by a 95-50% B linear gradient over 3 min and finally the system was equilibrated at initial conditions (50% B) for 10 min before further injection.

### 2.7. Statistical analysis

Firstly, descriptive statistics were conducted for each compound analyzed (mean, median, standard deviation, minimum and maximum) and Shapiro-Wilk normality tests revealed that the distributions were not normal ($p < 0.05$). Due to the small sample size, we decided not to conduct an analysis with demographic factors such as age and sex, which are commonly analyzed in other studies.

Additionally, the individuals were classified according their cause of death and we applied a Mann-Whitney test for the two most common, electrocution and collision. The results of these analyses were considered significant if $p < 0.05$ and for non-detected values we used the half of the limit of detection. We did one test for each AR (anticoagulant rodenticide) individually and collectively, grouping them into first-generation anticoagulants (FGARs), second-generation anticoagulants (SGARs). Lastly, the Spearman correlation test was performed to confirm or refute possible correlations between different rodenticide concentrations. All analyses were performed with R.4.3 software and Excel.

### 2.8. Spatial analysis

Due to low sample sizes available for this study and the massive home ranges of this species in the study area (Bosch et al., 2010; Morollón et al., 2022b), their large daily movements (Cadahía et al., 2007), and linked to the toxicodynamic of SGARs, which kill 4–5 days after ingestion of a lethal dose (Brakes and Smith, 2005; Eason et al., 2002; Hadler and Buckle, 1992; Mason and Littin, 2003), we conducted the analysis at the district level. We employed a generalized linear model (GLM) in the form of a gamma logistic regression to predict which environmental variables could determine the total concentration of all ARs in individuals at district level.

We selected variables related to urbanization and agriculture as descriptors of SGARs concentration. These variables included human population density, distance from major cities (defined as cities with over 50,000 inhabitants), the percentage of urban habitat coverage (including the total and the subdivisions, urban and industrial), and agricultural surface percentage (comprising rainfed, and irrigated land). Additionally, we considered farm and livestock density, differentiating between intensive and extensive livestock farming. We also included specific types of livestock, in both forms, farm and animal density (such as cattle, sheep, goats, horses, pigs, and poultry) in our analysis. To standardize the livestock units, we utilized a weight-based system, where each unit represented 100 kg of body weight. This approach, commonly used in comparative livestock studies (Geduhn et al., 2015), allowed for a more accurate comparison across different livestock types.

The same process was repeated for FGARs (first-generation anticoagulant rodenticides) as a response variable, using a GLM with Gaussian logistic regression due to the distribution of the response variable, employing identical predictor variables as in the previous step.

Specimens with unknown or imprecise locations were excluded from the analysis. The studied models were constructed following a forward-backward stepwise procedure according to the corrected Akaike's Information criteria (AICc). Those models with significant descriptor variables and those showing a $\Delta AICc < 2$ and descriptors with slopes different from zero (i.e., $|\beta \pm error| > 0$) were selected to explain the presence of ARs in animals. The variance inflation factor (VIF) and Spearman's correlations were performed to determine the collinearity among independent conditioning factors. If $VIF \geq 5$ and the correlation $\geq 0.7$, then that variable was ignored and not entered into the final GLM analysis.

The analyses were performed with RStudio version 4.3.1. We used freely available land cover maps for 2018 from CORINE (Coordination of Information on the Environment) land cover data from Copernicus land cover monitoring services (European Environment Agency, 2018).

## 3. Results

### 3.1. AR detection frequency

The presence of FGARs in the analyzed samples was considerably lower than that of SGARs. From this second group, difenacoum, bromadiolone, flocoumafen and brodifacoum were detected in all eagle samples, followed by difethialone, detected in 88.2% of the samples. Chlorophacinone was the most prevalent and the only FGAR found in more than 50% of the samples (Fig. 2).

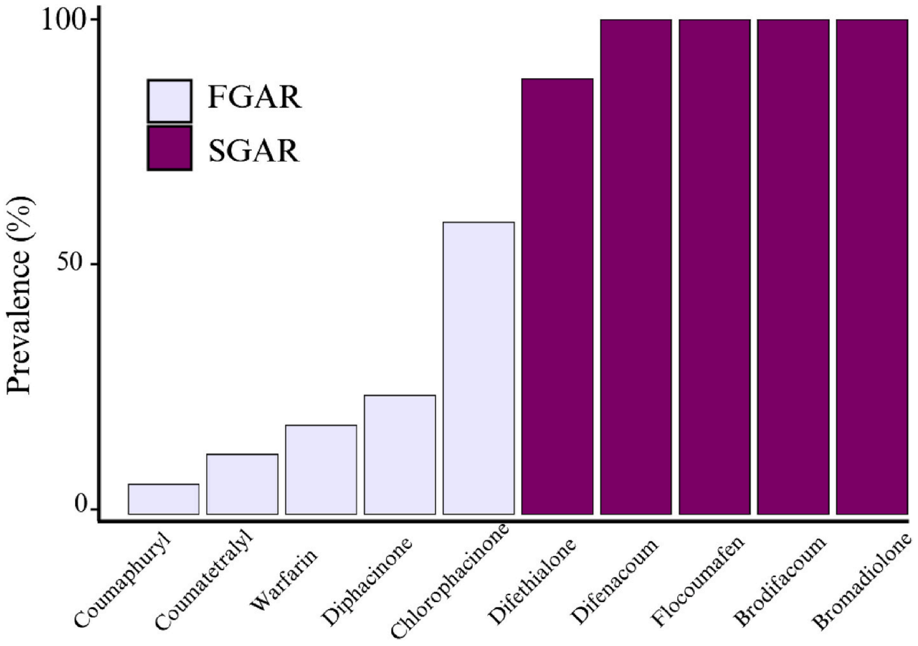

**Fig. 2.** Prevalence of anticoagulant rodenticides in Bonelli's eagle from the Valencian Community (eastern Spain) found dead between 2015 and 2022. FGAR: first generation anticoagulant rodenticides; SGAR: second generation anticoagulant rodenticides.

### 3.2. *AR concentrations in Bonelli's eagle livers*

All the individuals analyzed in this study were positive for at least 5 different compounds. SGARs were present in all the samples whilst FGARs were not detected in 17% of the eagles. A maximum of two FGAR compounds per sample was detected in the Bonelli's eagles; despite this, all of the analyzed compounds were detected in different individuals (Table S2).

In terms of mean AR concentrations, SGARs were found in higher concentrations than FGARs. Specifically, the rodenticide bromadiolone had the highest mean concentrations (118.55 ± 101.72 ng/g), followed by brodifacoum (90.93 ± 94.46 ng/g). The highest concentration detected in any of the eagles was for bromadiolone (307.50 ng/g). Moreover, brodifacoum and bromadiolone were the most prevalent ARs, being found in 94% of the samples ($n = 16$), although flocoumafen did disrupt this pattern (Table S2).

### 3.3. *Significance of AR concentrations*

More than a third (35%) of the eagles ($n = 6$) had AR levels of over 200 ng/g in their livers (Table S2), a threshold value reported to trigger adverse effects (Geduhn et al., 2015; López-Perea et al., 2019). Moreover, 70% of the samples exceeded the 100 ng/g threshold value for AR concentrations, a figure that has been linked to the appearance of symptoms compatible with toxicosis (Berny et al., 1997; Murray, 2011; Rattner et al., 2014a). According to the hepatic values mentioned, the population studied could be divided into three degrees of exposure: low (hepatic AR concentration below 100 ng/g); medium (hepatic AR concentration between 100 and 200 ng/g); and high (hepatic AR concentration higher than 200 ng/g) (Table 1).

### 3.4. *Statistical analysis*

We found positive correlations between the different concentrations of the following AR (Difenacoum, brodifacoum, bromadiolone and difethialone). Brodifacoum and bromadiolone were the compounds that exhibited the highest correlation ($\rho = 0.931$; p=<0.001) (Table 2).

Regarding the two main causes of death, no significant differences were detected for the individual compounds or for the sum of FGARs and SGARs. However, slight differentiation between the cause of death and the rodenticide generation was observed; their p values are close to 0.05 although they are not below (Table 3).

**Table 1**
Distribution of the Bonelli's eagles according to cause of death and threshold concentrations (low, medium and high).

| Cause of death[1] | Low <100 ng/g | Medium 100–200 ng/g | High >200 ng/g |
|---|---|---|---|
| Collision (6) | 3 | 2 | 1 |
| Electrocution (7) | - | 3 | 4 |
| Shooting (1) | - | 1 | - |
| Predation (1) | - | - | 1 |
| Indeterminate (2) | 2 | - | - |
| Total (17) | 5 | 6 | 6 |
| | 29% | 35% | 35% |

[1] Cause of death was established according to clinical signs founded during the necropsy carried out by the vets from the wildlife rehabilitation centers.

**Table 2**
Correlations between hepatic concentrations of anticoagulant rodenticides in the Bonelli's eagles found dead in the Valencian Community.

| Anticoagulant rodenticides | | |
|---|---|---|
| Difenacoum | Brodifacoum ($n = 17$) | $\rho = 0.818^{c}$ |
| | Bromadiolone ($n = 17$) | $\rho = 0.799^{c}$ |
| | Difethialone ($n = 15$) | $\rho = 0.788^{c}$ |
| Brodifacoum | Difethialone ($n = 15$) | $\rho = 0.584^{b}$ |
| | Bromadiolone ($n = 17$) | $\rho = 0.931^{c}$ |
| Bromadiolone | Difethialone ($n = 15$) | $\rho = 0.540^{a}$ |

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

#### 3.4.1. *Spatial analysis*

No significant differences were detected between samples found in districts affected by rabbits as pests and those that were not (Table S3).

The best models based on Akaike's Information Criteria indicates that percentage of rainfed, and intensive farm density respectively, were

**Table 3**
Descriptive statistics (mean ± standard deviation; median) related to concentrations of SGARs (ng/g) in the individuals associated with cause of death (Electrocution and Collision).

| | Cause of death | | |
|---|---|---|---|
| | Electrocution | Collision | p-value |
| Coumatetralyl | 0.006 ± 0.2; 0.00 | 0.01 ± 0.02; 0.00 | 0.91 |
| Coumafuryl | 0.02 ± 0.04; 0.00 | NA | 0.44 |
| Warfarin | 0.03 ± 0.06; 0.00 | NA | 0.10 |
| Diphacinone | NA | 0.15 ± 0.24; 0.00 | 0.14 |
| Chlorophacinone | 0.12 ± 0.2; 0.00 | 0.32 ± 0.27; 0.39 | 0.14 |
| Difenacoum | 11.42 ± 18.24; 2.74 | 5.20 ± 6.11; 3.44 | 0.84 |
| Brodifacoum | 130.45 ± 104.89; 142.28 | 50.42 ± 45.10; 35.36 | 0.18 |
| Bromadiolone | 144.92 ± 106.76; 149.53 | 100.68 ± 103.17; 68.56 | 0.53 |
| Difethialone | 0.93 ± 0.96; 1.50 | 1.50 ± 1.38; 1.23 | 0.35 |
| Flocoumafen | 19.03 ± 44.68; 1.49 | 3.67 ± 6.05; 1.37 | 0.73 |
| Total FGARs | 0.17 ± 0.18; 0.15 | 0.489 ± 0.28; 0.50 | 0.07 |
| Total SGARs | 306.74 ± 195.42; 306.47 | 161.49 ± 153.86; 105.10 | 0.14 |

the variables that best determined the SGARs in the animals analyzed (Table S4).

Considering the statistical principle of parsimony (Burnham and Anderson, 2004) and the <2 units of AIC differences, the model that best explained the total SGAR concentrations was Model 3, which incorporated only one variable, the percentage of rainfed surface, despite having higher AIC values, see Table S4 for more details.

On the other hand, the models that best explained the sum of FGARs included percentage of industrial surface and pig density as significant predictors, respectively. Table S5 shows how whereas porcine densities influenced positively FGARs, sheep densities had a negative influence in FGARs concentration.

## 4. Discussion

Our results revealed that all the Bonelli's eagles in the study were exposed to anticoagulant rodenticides, principally SGARs. At least 5 out of the 10 different compounds analyzed were detected in the livers of these eagles, and 7 compounds (the 5 SGARs plus chlorophacinone and diphacinone) were detected in three of the eagles. Therefore, second generation anticoagulant rodenticides (SGARs) were the most frequently detected in Bonelli's eagle and at concentrations higher than those of first-generation anticoagulant rodenticides (FGARs) suggesting that they are the most widely used today in the study area and it is related to the quantity of products that are registered for pest control in Spain. In spite of this, the detection of some FGARs, like warfarin, diphacinone, and coumafuryl, indicates that baits containing unauthorized compounds have also been used. The concentrations of anticoagulant rodenticides in the liver of Bonelli's eagles suggest that many of them could have suffered sublethal effects prior to death, which could partly explain their high mortality in collisions with power lines; it could even be considered another cause of mortality, since approximately a third of the eagles analyzed in this study presented concentrations of higher than 200 ng/g.

### *4.1. AR prevalence*

SGARs were not only the most frequently detected compounds (in 100% of the individuals, except for difethialone in 88.2%, see Fig. 2), they were found in the highest concentrations. This contrasts with FGARs, which were very infrequent (in less than 25% of individuals, except for chlorophacinone in 59%) and at very low concentrations. Similar patterns were described in other studies of different species in Spain (López-Perea et al., 2015; Carrera et al., 2024), as well as in other parts of the world (Cypher et al., 2014; Lohr, 2018; Murray, 2020, 2017). Along the same lines, brodifacoum and bromadiolone were the most widely detected and abundant compounds in this study and their concentrations in liver were also highly correlated (ρ = 0.931) (Table 2), findings that agree perfectly with those described in a recent study of red foxes (*Vulpes vulpes*) from the same area (Carrera et al., 2024). This combination of brodifacoum and bromadiolone seems to be the most frequent pair observed in studies with raptors, some of which have a similar size and ethology to the Bonelli's eagle (Stone et al., 2003; Albert et al., 2010; Ruiz-Suárez et al., 2014; Slankard et al., 2019; Niedringhaus et al., 2021; Elliott et al., 2022), although it is also seen in other animal groups (Gabriel et al., 2012; Sánchez-Barbudo et al., 2012; Alomar et al., 2018; Seljetun et al., 2020; Cerri et al., 2023; Carrera et al., 2024). In this study, this finding could be explained by the huge number of legal commercial baits approved for use in Spain that contain brodifacoum or bromadiolone in comparison with the other ARs. According to the Spanish Official Register of Biocides (Ministerio de Sanidad, n.d.) only 7 of the 10 anticoagulant rodenticides analyzed in this study are included in legally registered commercial products (all 5 SGARs plus 2 FGARs, coumatetralyl and chlorophacinone), with SGARs representing 98% of the registered products, with 75.6% of them containing brodifacoum or bromadiolone as the active ingredient (Fig. 3).

Despite the apparent lack of symptoms directly related to SGARs in this study, the exposure is comparable to similar species in other studies. Niedringhaus et al. (2021) found SGARs in 83% of bald eagles (*Haliaetus albicilla*) n = 96, and 77% of golden eagles (*Aquila chrysaetos*) n = 13. In Australia, AR compounds were detected in 74% of Tasmanian wedge-tailed eagles (*Aquila audax fleayi*) n = 50 (Pay et al., 2021). In the US, Viner et al. (2022) detected ARs in the livers of 38.7% of golden eagles (n = 62) found dead under power lines or wind turbines. In a similar study in the UK, 100% of Eurasian kestrels (*Falco tinnunculus*) n = 20 had detectable SGARs in their livers (Walker et al., 2013). While exposure to anticoagulant rodenticides seems widespread globally, instances of 100% exposure are rare and often occur in studies with small sample sizes. In France, 75% of Eurasian kestrels (*Falco tinnunculus*) n = 4, 70% of barn owls (*Tyto alba*) n = 10, 20% of tawny owls (*Strix aluco*) n = 5, and 91% of common buzzards (*Buteo buteo*) n = 11 had liver SGAR residues (Lambert et al., 2007) and, recently, SGARs were detected in the livers of 100% of golden eagle (*Aquila chrysaetos*) carcasses, n = 7, and 80% of Bonelli's eagles n = 7 in an area that is relatively close to our study area (southeastern France) (Moriceau et al., 2022). This fact could indicate the massive use of these compounds in the Mediterranean area, a trend probably associated with higher densities of human populations along the Mediterranean coast (López-Perea et al., 2015). This would also be consistent with high exposures in coastal areas and lower exposures in inland areas, and it could be that the same pattern is repeated worldwide (Koivisto et al., 2018; Lohr, 2018; Serieys et al., 2019).

### *4.2. AR concentrations in liver related to potential adverse effects*

Although toxicosis was not considered as the presumptive diagnosis, previous studies proposed 200 ng/g as the threshold of SGARs in liver beyond which poisoning would begin to be observed (Berny et al., 1997; Walker et al., 2008). In line with the results of the study by Murray (2011), who observed that red-tailed hawks (*Buteo jamaicensis)* with brodifacoum concentrations between 12 and 269 ng/g in their livers showed clinical signs of depression, apathy, and non-responsiveness to stimuli (Murray, 2011), and considering that Stone et al. (2003) described a case of acute poisoning in a golden eagle with a liver concentration of above 30 ng/g, the results obtained in our study indicate that approximately 65% of our specimens may have been suffering acute AR intoxication and that 95% may have had some symptoms related to the presence of ARs that could have influenced their movement coordination or reaction to stimuli. Five Bonelli's eagles had concentrations above 200 ng/g, which indicates that their death is likely to be linked to these compounds. It is important to note that compounds with the same

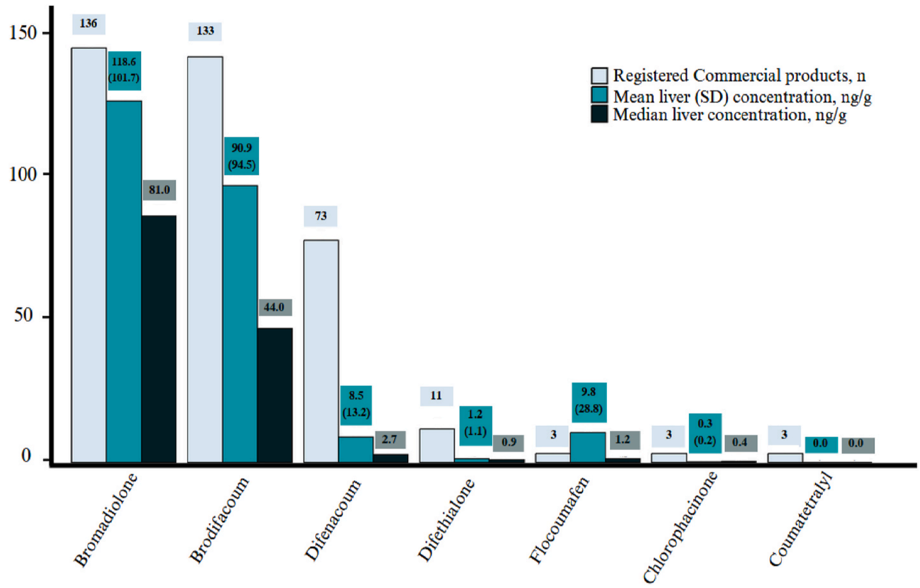

**Fig. 3.** Number of commercial AR products registered in the Spanish Official Registry of Biocidal Products, January 3rd, 2024 (Ministerio de Sanidad, n.d.) versus mean and median liver concentrations (ng/g) of anticoagulant rodenticides in Bonelli's eagles from eastern Spain.

mechanism of toxic action may have an additive effect (Aleksunes and Eaton, 2021), meaning that the mix of SGARs could have had synergistic effects with one another, thereby enhancing their effects. It must be considered that the more days that have passed after the death of the animal, the more the tissue concentration of the ARs may suffer variations, particularly in the liver. A study carried out on common kestrels (*Falco tinnunculus)* demonstrated that under summer weather conditions, the rapid dehydration of the liver led to a threefold increase in bromadiolone detection in the liver compared to the initial concentration (Valverde et al., 2020).

Coagulopathy is probably more dangerous for wild birds, due to their high mobility and greater possibility of injury during prey capture. This may have synergistic interactions with environmental stressors thus increasing the likelihood of mortality. Furthermore, hepatic concentrations of rodenticides may affect the coordination of movements when flying, hunting or perching on structures (Rattner et al., 2014a). For this reason, the way in which Bonelli's eagles hunt their prey (often by impact hunting) could result in traumas and coagulopathies that make it difficult for them to coordinate their movements (Rattner et al., 2014a); this could partially explain the high mortality of the species associated with power lines, due to both collisions and electrocution (Mañosa and Real, 2001; Real et al., 2001).

This observation might suggest the widespread use of these compounds in the Mediterranean region, particularly in highly populated areas, as reported in the same region by López-Perea et al. (2015) and observed worldwide in several studies (Koivisto et al., 2018; Lohr, 2018; Serieys et al., 2019). The densely populated nature of the Mediterranean coast could further accentuate the distinctions between the coastal and inland areas.

### *4.3. AR generation and cause of death*

Despite finding no significant differences between electrocution and collision (Table 3), it does seem that there is a different pattern according which generation of AR is involved, with FGARs tending to be more related to collision and SGARs to electrocution. These differences could be explained by the toxicodynamics of each group —FGARs are less toxic and require multiple feeds over several days to result in death (Rattner et al., 2014b)— however, some of these compounds, such as chlorophacinone and diphacinone are more potent in a single feed (Clark, 1978), possibly causing rapid movement coordination alterations that would explain the tendency towards collision. In addition, there have been some secondary exposure incidents in which the death of raptors was attributed to chlorophacinone (Berny et al., 1997), and as this is used principally in agricultural and urban settings and is highly toxic for birds, it would not be unreasonable to think that it has a strong effect on this species of eagle. It is known that diphacinone, disappear rapidly from the liver and, at the same time, it is linked to secondary poisoning (Stone et al., 2003, 1999); some studies of raptors have indicated that they are more sensitive to diphacinone than other bird species (Rattner et al., 2011). These facts could permit us to suppose that both compounds could be determinant in the collision and death of the Bonelli's eagles, even if their concentrations in liver were lower than other compounds, such as SGARs.

With regard to SGARs and their effects over several days, their toxicity and the high concentration of these found in the liver could have been key factors in causing the eagles to spend more time perched on electric towers or even to simply perch more often, (considering that the symptoms include apathy and fatigue), which increases their chances of being electrocuted. The continued sampling of this species will help to understanding the possible relation between AR generation exposure and their consequences on eagle death.

### *4.4. Land-use and agriculture*

In the spatial analysis, a positive correlation between total SGAR concentration and human density was expected due to the widespread use of rodenticides in commercial and residential settings. This exposure pattern was suggested following the detection of elevated exposure rates in densely populated areas. It is well known that high population and farm densities correlate positively with increased rodenticide use (López-Perea et al., 2019; Rial-Berriel et al., 2021), especially pig farms. Rodent pest control is mandatory in pig farms in European countries as a measure against potential transmission vectors able to spread epizootic diseases via rodents. Additionally, Geduhn et al. (2015) detected a positive relationship between pig farms and AR exposure in red foxes, for which reason we expected to see a positive correlation with intensive farm density, specifically for pig farms.

In our results, the significance of rainfed crops in the results of this study is noteworthy, indicating widespread rodenticide use across all agricultural environments. With respect to total FGAR concentration, the results are along the same lines as those of SGARs, with a few differences. Sheep densities were negatively related to FGARs while pig densities were positively related, indicating the importance for which type of farming is more frequent the use of these compounds (the majority of pig farming is intensive whereas sheep farming tend to be extensive). Moreover, industrial surface area was significantly more important than urban surface area, likely indicating the frequent use of FGARs in those environments, where food is abundant and rodenticide treatments are common (Hindmarch and Elliott, 2018).

Finally, as we noted previously, human density has been related to exposure to anticoagulant rodenticides. Some studies point to high AR exposure levels in urban and densely populated areas (Riley et al., 2007; Koivisto et al., 2018; Lohr, 2018; Serieys et al., 2019). This could imply hotspots of rodenticide presence in areas near large population centers and agricultural activities, both intensive and rainfed, aligning perfectly with this Mediterranean region.

### 4.5. *Bonelli's eagle as a sentinel species*

AR exposure pathways in Bonelli's eagle seem to be clear and warn of a possible hazard for human health. According to their predatory behavior (principally feeding on live prey), it seems that AR exposure in Bonelli's eagle is secondary through their ingestion of rabbits, red-legged partridges and doves that would have been subject to non-target primary AR poisoning via ingestion of commercial baits. This hypothesis was corroborated by a simultaneous study conducted on raptors exhibiting diverse dietary compositions and diminished interaction with the primary prey species of Bonelli's eagle, specifically an osprey (*Pandion haliaetus*) and a short-toed eagle (*Circaetus gallicus*) from the same study area (unpublished data), where it was observed that the total rodenticide liver concentrations were much lower than those in the Bonelli's eagles (16.33 ng/g and 29.12 ng/g, respectively). Small game hunting in the study area and the consumption of wild rabbits, hares, red partridges, and wood pigeons (including their livers) is a deeply rooted custom among hunters and their families. In this sense, the risk suggested in this study for the Bonelli's eagle should be considered a warning for consumers of these foods. This is not the first time that ARs present in wildlife species have been linked to a risk for human health. A study in wild boars sampled near large urban centers in Spain found ARs in their livers, which the authors considered a potential risk to public health (Alabau et al., 2020).

## 5. Conclusions

The widespread use of anticoagulant rodenticides in eastern Spain poses a significant threat to the viability of wildlife species in this region. The current presence of ARs in the environment is affecting the health and survival of populations of critically important predators, such as the Bonelli's eagle (*Aquila fasciata*), and could be considered another principal cause of mortality for this species.

Our results suggest that anticoagulant rodenticides are ingested by non-target species, which are prey for Bonelli's eagle (rabbits, partridges, turtledoves, etc.), leading to a greater risk of secondary poisoning in this species. At the same time, this fact may reveal the unintentional poisoning or persecution of this bird of prey.

Moreover, the consumption of the small game cited above, which is prey for both Bonelli's eagle and humans, may pose a health risk to consumers of this type of meat. This study allows us to suggest the usefulness of the Bonelli's eagle as a sentinel species of this risk for humans.

For these reasons we claim the importance of monitoring and managing the way ARs are used in order to prevent further declines of endangered predators.

## 6. Caveats

It is important to acknowledge that the number of specimens analyzed in this study is rather limited, n = 17; however, collecting a higher number of carcasses of a critically endangered species is challenging. In 2022 in the Autonomous Community of Valencia, our study area, the Bonelli's eagle was reclassified as critically endangered. The estimated population is 50 breeding pairs, and this number is rapidly declining due to anthropogenic factors, including electrocution and collision with power lines, making it virtually impossible to obtain a greater number of specimens.

Predictions regarding the factors that may determine and explain the concentrations of rodenticide residues in the liver are suggested as part of a statistical process. While these predictions can offer a general idea of where the problem lies, caution must be exercised when utilizing them for conservation measures, given the small sample size which might not be adequate for making significant or drastic decisions.

Simultaneously, it is crucial to note that 100% of the Bonelli's eagles analyzed presented AR residues in their livers. This data must be taken into consideration as exposure to these compounds poses a real threat to the conservation of this species and wildlife in general.

## CRediT authorship contribution statement

**T. Vicedo:** Writing – original draft, Visualization, Investigation, Formal analysis. **I. Navas:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **P. María-Mojica:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Conceptualization. **A.J. García-Fernández:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

## 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.2024.124530.

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