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Exploring anticoagulant rodenticide exposure and effects in eagle owl (*Bubo bubo*) nestlings from a Mediterranean semiarid region

Livia Spadetto [a], Pilar Gómez-Ramírez [a,*], Mario León-Ortega [b], Antonio Zamora-López [c,b], Sarah Díaz-García [b], José Manuel Zamora-Marín [d,b,c], Fernando Tecles-Vicente [e], Luis Pardo-Marín [e], José Fenoll [f], José Francisco Calvo [g], Antonio Juan García-Fernández [a]

[a] Area of Toxicology, Faculty of Veterinary, Campus de Espinardo, University of Murcia, 30100, Murcia, Spain
[b] ULULA Association for Owl Study and Conservation, 30100, Murcia, Spain
[c] Department of Zoology and Physical Anthropology, Faculty of Biology, University of Murcia, 30100, Murcia, Spain
[d] Department of Applied Biology, Centro de Investigación e Innovación Agroalimentaria (CIAGRO-UMH), Miguel Hernández University of Elche, Elche, Spain
[e] Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Veterinary School, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, 30100, Murcia, Spain
[f] Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, IMIDA, 30150, Murcia, Spain
[g] Department of Ecology and Hydrology, Faculty of Biology, University of Murcia, 30100, Murcia, Spain

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ABSTRACT

Anticoagulant rodenticides (ARs) are widely used for pest control, resulting in their pervasive presence in the environment and posing significant toxicological risks to a range of predatory and scavenging species. Our study mainly aimed to evaluate AR exposure and effects in nestlings of eagle owl (*Bubo bubo*) from the Region of Murcia (southeastern Spain). We analysed ARs in blood samples ($n = 106$) using high-performance liquid chromatography-triple quadrupole (HPLC-TQ), assessed the influence of potential anthropogenic (presence of livestock farms, landfills and human population density) and environmental (land uses and proximity to watercourses) variables, and measured prothrombin time (PT) and plasma biochemical parameters as biomarkers of effects. Our results showed the presence of AR residues in 91.5% of the nestlings, with 70.8% exhibiting multiple ARs (up to six compounds in a single individual). Second-generation ARs (SGARs) were the most prevalent compounds. The analysis of biochemical parameters indicated that the sampled individuals were in good physiological condition. Although PT was positively correlated with total AR concentration (ΣARs), the relationship was not significant ($Rho = 0.04$; $p = 0.49$). Regarding environmental factors, higher ΣARs were associated with the most urbanised study site and the presence of landfills, likely due to the increased availability of rodent prey. The prevalence of two SGARs (brodifacoum and difenacoum) was linked to closer proximity to riverbeds, suggesting a contamination pathway associated with inland aquatic ecosystems, where these AR compounds may concentrate due to water scarcity. This study underscores the widespread exposure of eagle owls to ARs and highlights the importance of effective monitoring and management of these pollutants to protect conservation-concern wildlife in Mediterranean semiarid regions.

1. Introduction

The natural habitat of birds of prey is increasingly being taken over by human activities, leading to a variety of conservation issues. Urban expansion and agricultural intensification contribute to the degradation and fragmentation of their habitats and to the loss of nesting sites (Mainwaring, 2015; McClure et al., 2018). In addition, the widespread use of chemicals, particularly rodenticides and other pesticides, introduces toxic substances into the environment that can contaminate their food sources, leading to bioaccumulation and adverse health effects (Badry et al., 2021; González-Rubio et al., 2021; Shore and Taggart, 2019). Moreover, raptors often inhabit wildland-urban interface areas, where natural habitats intersect with human development (Radeloff et al., 2005), increasing their vulnerability to environmental hazards resulting from human activities.

A significant problem affecting raptors is their frequent and

* Corresponding author.
E-mail address: pilargomez@um.es (P. Gómez-Ramírez).

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widespread exposure to anticoagulant rodenticides (ARs) (García-Fernández et al., 2024; Gomez et al., 2022; Nakayama et al., 2019; Ruiz-Suárez et al., 2014). Birds of prey are particularly at risk because their feeding habits often include target rodents, and their biological characteristics make them more susceptible to the toxic effects of these compounds (Khidkhan et al., 2024; Nakayama et al., 2020; Rattner et al., 2012). It is therefore crucial to understand the extent of AR exposure, both in terms of spatial distribution and severity, in order to implement mitigation measures where necessary, particularly for species that are declining and for which AR contamination could be a significant risk factor (Spadetto et al., 2024b; Vicedo et al., 2024).

In an effort to mitigate the impact of these compounds on non-target wildlife, the European Union applied new regulations in 2018, classifying AR products exceeding concentrations of 30 ppm as reprotoxic (European Union, 2016). Consequently, in Spain and other countries of the European Union, the products available to the general public now contain lower concentrations, often involving second-generation ARs (SGARs), which are more potent and persistent in the environment than first-generation ARs (FGARs) (Erickson and Urban, 2004). Interestingly, it has recently been shown that AR baits with lower concentrations (25 ppm) were more palatable and therefore more consumed than those with higher concentrations (50 ppm). As a result, target rodents carried higher hepatic AR levels at the time of their death (Frankova et al., 2024). While the effects of these compounds at low doses on non-target fauna are still not fully understood, recent studies indicate that AR exposure and concentrations remain high in non-target predatory birds (Carrillo-Hidalgo et al., 2024; Moriceau et al., 2022; Spadetto et al., 2024b).

The Eurasian eagle owl (*Bubo bubo*, hereafter referred to as eagle owl) is a top predator with a diet primarily consisting of potentially pest species in the study area, such as European rabbits (*Oryctolagus cuniculus*) and rats. This large nocturnal raptor has a wide distribution across the Palearctic region, and it is monitored over the long term in numerous countries, which facilitates the replication of toxicological assessments on a large scale. Additionally, the eagle owl is considered a species of conservation interest, as it is included in the List of Species in Special Protection Regime (LESPRE) (Gobierno de España, 2011) and Annex I of the Birds Directive (European Union, 2009). With a high ecological plasticity in habitat selection, it breeds near highly anthropized areas such as intensive crops or landfills, potentially exposing it to ARs. Moreover, the ease of studying its diet and the abundant literature on its trophic ecology allow for a more thorough understanding of the spatiotemporal patterns in AR exposure.

The Region of Murcia, located in the southeast of the Iberian Peninsula, features a mix of agricultural lands, rural villages, and cities, creating a unique interface where wildlife habitats are increasingly intersected by human development. This region hosts high densities of eagle owls in areas where its primary prey, the European rabbit, is abundant. In areas with lower rabbit availability, the eagle owl diet diversifies, with species like hedgehogs, rats, and birds becoming more significant (León-Ortega et al., 2016; Pérez-García et al., 2012). Given its high territorial density in the study area, the eagle owl has already been used as a biomonitoring species for the presence of heavy metals and other environmental contaminants (Espín et al., 2014; Gómez-Ramírez et al., 2012a,b).

Previous studies have already highlighted the presence of ARs in various avian species across the southeastern Iberian Peninsula (Gómez-Ramírez et al., 2021; Spadetto et al., 2024b; Vicedo et al., 2024), particularly in populations breeding near human settlements, likely due to domestic use of these substances. The eagle owl occupies diverse environments, including territories located in natural areas as well as near potential exposure sources (e.g., livestock farms, landfills or human settlements). Additionally, this species often breeds and hunts in rocky gorges and along riverbeds, where its main prey is more abundant. However, the role of watercourses as pathways for AR exposure in top predators has been scarcely evaluated in semi-arid regions. Here, non-perennial watercourses could serve as collection points for run-off containing pesticides and toxic chemicals (Arenas-Sánchez et al., 2016), while also acting as vital water sources for wildlife during dry periods (Steward et al., 2012). As a consequence, the potential for biomagnification of ARs through the food chain increases. Additionally, recent studies suggest that raptors in semi-arid regions may depend on free water sources, particularly during droughts (Boal et al., 2023; O'Brien et al., 2006), which could further amplify their exposure to contaminants. Therefore, we set out to assess the influence of these riparian ecosystems on AR exposure, along with the presence of irrigation reservoirs near the breeding territories.

The aims of this study were 1) to evaluate AR exposure in eagle owl nestlings in a semi-arid Mediterranean landscape, 2) to determine if there was a relationship between potential risk factors (land uses, livestock farms, human population density, landfills and riverbed presence) with AR prevalence and concentration in blood, and 3) to assess the usefulness of a coagulation parameter as a biomarker of AR effect and study a plasma biochemical profile to evaluate the general health status of the chicks. Our hypotheses were that AR exposure could be widespread in this species, and that the rate of AR exposure is higher in eagle owls exposed to the considered risk variables, compared to those inhabiting more natural or protected areas. Additionally, we hypothesised that the coagulation capacity decreases proportionally to the levels of ARs detected in blood samples, as previously demonstrated in other bird of prey species (Spadetto et al., 2024a, 2024b). By providing critical insights into the extent of AR contamination and its impact on the eagle owl, this study seeks to provide information for the development of conservation strategies and regulatory measures to mitigate the risks posed by these chemicals.

2. Material and methods

2.1. Study area

The Region of Murcia is characterised by a typically warm and dry Mediterranean climate. Summer temperatures can usually reach 40 °C, while winters are mild with average temperatures around 10–15 °C. Precipitation is scarce, with an annual average of less than 300 mm (Spanish National Agency of Meteorology - AEMET, 2024). The region is known for its intensive cultivation of citrus fruits, vegetables, and orchards, highlighting its importance as an agricultural area. In fact, in 2022 agricultural land occupied 32% of the entire region, with 47% of irrigation agriculture (CARM Región de Murcia, 2022).

Within the region, eagle owl territories of our study were distributed across four geographic zones (Fig. 1), each with distinct geographic and environmental features.

1) Central-eastern badlands, referring to a badland area geologically characterized by extensive erosion and lack of vegetation, where non-perennial rivers supply water to the region. This study site surrounds the city of Murcia and it is characterized by a significant human presence, with a dense network of small settlements, rural houses, and agricultural structures scattered throughout the landscape. It hosts a high density of eagle owls, also due to the high availability of European rabbits.
2) Prelittoral mountain ranges, predominantly occupied by a regional park (Carrascoy y El Valle), along with a Site of Community Importance (SCI) and a Special Protection Area for Birds (SPA), which partially overlap. These areas are characterized by an arid mountainous landscape with a variety of rocky and hilly formations and are mainly covered by Mediterranean vegetation (Aleppo pine forests and scrubland). However, in some flat areas or wider valleys, limited agricultural activities such as fruit tree cultivation can be found. Human presence in these areas is limited, with few scattered settlements. The presence of eagle owls here is also considerable.

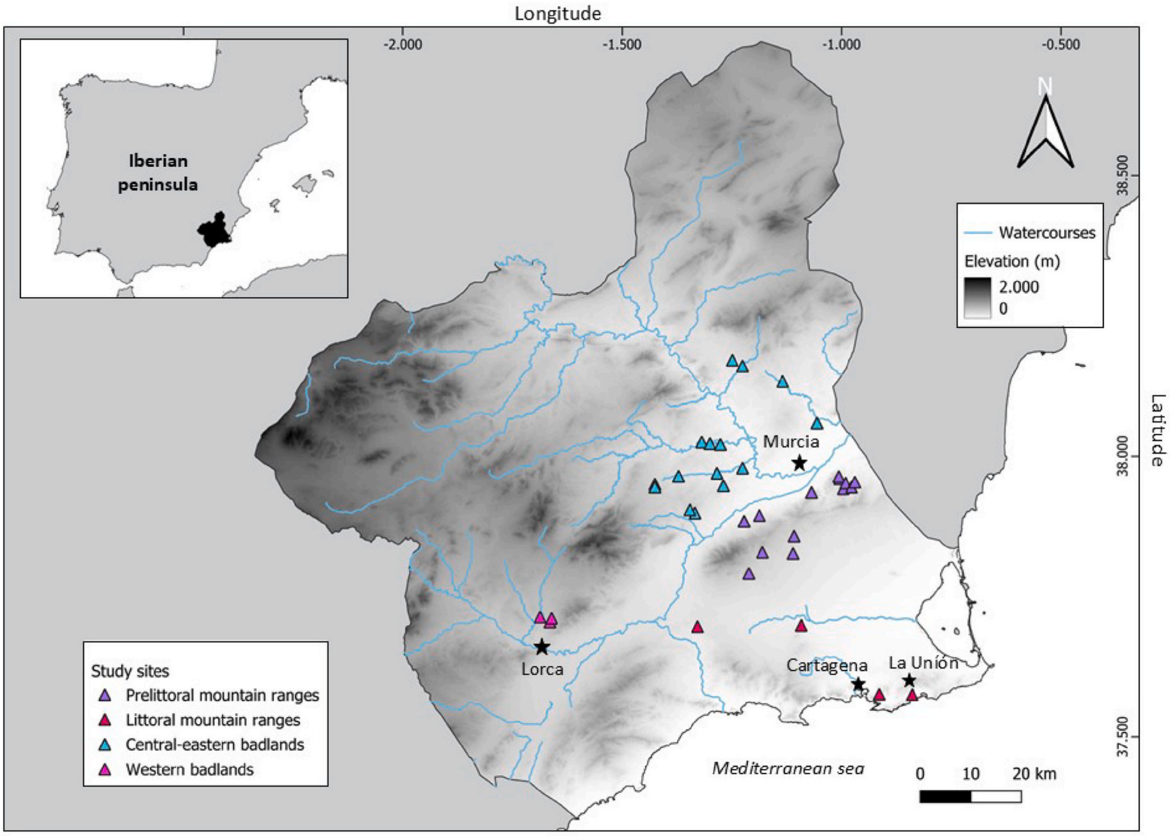

Fig. 1. Eagle owl territories (triangles) sampled in the Region of Murcia (southeastern Spain) in 2021–2022 for the purpose of assessing exposure to anticoagulant rodenticides (ARs). The main municipalities of the region are marked with a star.

3) Western badlands, within the municipality of Lorca. Agriculture in these areas may be limited due to soil conditions and water availability, but it is still present with rainfed crops including olive groves, almond trees, and cereal crops. The human population is generally sparse, with small and dispersed settlements throughout the territory. This area shows a high density of eagle owl breeding territories.
4) Littoral mountain ranges, an arid and mountainous terrain located south of an extensive coastal plain (Campo de Cartagena) and overlooking the coast of the Mediterranean Sea, with its climate and ecosystem influenced by the proximity to the sea. Human presence is mainly concentrated in urban centres such as Cartagena and La Unión, while the rest of the surrounding territory is characterized by small villages and scattered farms. The density of eagle owls here is much lower than in other areas, as is the presence of the European rabbit. Hence, they mainly feed on other prey items, predominantly rats (Sánchez-Virosta et al., 2020).

2.2. Sample collection

Sampling was conducted as part of a nocturnal raptor monitoring and marking program in the Region of Murcia. Territories were carefully monitored from December to June to estimate the egg laying, hatching, and fledging dates of the chicks and to choose the appropriate time to access the nests. Nests were accessed by specialized personnel once the chicks were approximately 30–45 days old, before reaching the fledging age. A total of 106 blood samples from eagle owl nestlings were collected during the breeding season of 2021 ($n = 39$) and 2022 ($n = 67$). Owlets belonged to a total of 34 territories, of which 10 were sampled in both study years, while the remaining 24 were sampled only once due to territorial occupation, either in 2021 or 2022, for a total of 44 sampling events.

The sample collection (approved by the Ethical Committee for Animal Experimentation of the University of Murcia; code 657/2020) was carried out following a protocol for monitoring contaminants in raptors (Espín et al., 2021). Blood samples were extracted from the brachial vein using a sterile syringe with a 23G needle and transferred into a heparinized tube (2 mL). In 2022, an aliquot of 450 μL was transferred to a tube with 50 μL of 0.109 M sodium citrate buffer. The samples were kept refrigerated until arrival at the laboratory, which occurred within a few hours. Then, an aliquot of 1 mL of heparinized blood ($n = 105$) was centrifuged at 2500 g for 10 min to obtain plasma, while the samples preserved in sodium citrate ($n = 62$) were centrifuged at the same speed for 15 min to obtain citrated plasma samples. All the samples were stored at −80 °C until the time of analysis.

2.3. Anticoagulant rodenticide analysis

Blood samples were analysed to detect the presence of 10 ARs (bromadiolone, brodifacoum, chlorophacinone, coumatetralyl, difenacoum, diphacinone, flocoumafen, coumafuryl, and warfarin), using the method outlined by Spadetto et al. (2024b). In summary, 1000 μL of acetonitrile and 25 μL of coumachlor (internal standard) were added to 250 μL of blood, in a tube with a ceramic homogeniser, followed by vortexing the tube for 1 min. Next, extraction salts, comprising 0.25 g of NaCl and 1 g of Na_2SO_4 per sample, were introduced. The tube was manually shaken for 1 min and then centrifuged at 2500 g for 5 min. The resulting supernatant was collected and transferred to a tube containing purification products (12.5 mg of PSA, 37.5 mg of C18, and 225 mg of Na_2SO_4). After vortexing for an additional minute, the tube was centrifuged at 2500 g for 5 min. The supernatant was then drawn using a syringe and transferred into a chromatography vial after filtering through a 0.45 μm nylon syringe filter. Detailed information on chemicals and standards can be found in Box 1 SI. The extracts underwent analysis for the aforementioned ARs using an HPLC-TQ system (consisting of vacuum degasser, autosampler and a binary pump; Agilent Series 1260, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C18 analytical column (150 × 2.1 mm and 2.6-μm particle size; Phenomenex Kinetex R 2.6 μm EVO Polar C18 100 A) and an Ultivo G6303 triple quadrupole mass spectrometer from Agilent, with an electrospray ionisation interface, following the procedure described by Spadetto et al. (2024b). The values of the SRM ratios from sample extracts for all of the two transitions selected were between 6% and 21% of average of calibration standards from same sequence. This is in accordance with the Analytical quality control and method validation procedures for pesticide residues analysis in food and feed (SANTE/11312/2021 v2; European Commission, 2021), based on ion-ratio statistics for the transitions monitored. Under the chromatographic conditions described above, the calibration graphs were constructed by plotting peak area vs. concentrations in the range 0.1–50 ng mL^{-1}. The limits of quantification (LOQ), calculated following the guidance in the document SANTE/11312/2021 v2 (European Commission, 2021), ranged between 0.01 and 2.5 ng mL^{-1}. The analytical technique exhibited recovery values between 76% and 105%, with a relative standard deviation < 14%. This complies with the recovery and precision accepted by the SANTE/11312/2021 v2 (European Commission, 2021).

2.4. Coagulation assays and biochemical analysis

The coagulation tests were conducted using a coagulometer, as detailed in Spadetto et al. (2024b). Fibrinogen was measured to assess the quality of the sample, as a decrease in its levels (less than 50–60 mg dL^{-1}; Rattner et al., 2010, 2020) may indicate improper sample collection or handling, as well as potential liver or kidney diseases or other pathologies. Briefly, a kit from Spinreact S.A.U (Spain) based on the Clauss method (Clauss, 1957) was utilized. Citrated plasma samples were diluted 1:10 with imidazole buffer. After that, 200 μL of the dilution were combined with 20 μL of kaolin in a tube containing a mixer. After incubating the tube for 3 min at 37 °C, 100 μL of bovine thrombin were added, and the clot formation time was measured.

Prothrombin time (PT) was chosen as an indicator of AR effect on blood coagulation, as it rapidly alters following the ingestion of ARs and has been previously used for this purpose (Hindmarch et al., 2019; Rattner et al., 2010, 2011, 2014a). To ensure reliable results in avian species, avian thromboplastin should be used as a reagent. In fact, commercially available kits typically contain mammalian thromboplastin, leading to inaccurate results (reviewed by Webster, 2009). The thromboplastin was obtained using the Quick method modified by Griminger et al. (1970), as described in Spadetto et al. (2024b). For the PT test, 50 mg of chicken thromboplastin were reconstituted in 2.5 mL of $CaCl_2$ in a Falcon tube. The mixture was agitated with a mixer for 15 min and centrifuged at 1800 rpm for 20 min. The supernatant was diluted 1:1 with $CaCl_2$ and 200 μL of the prepared reagent were added to a tube with a mixer and incubated for 3 min at 37 °C. Subsequently, 100 μL of citrated plasma were added to initiate the reaction, and the coagulometer measured the clotting time.

All analyses of biochemical parameters in plasma samples were performed using an Olympus A400 biochemical analyser with commercially available reagents (Beckman Co), with the exception of ovotransferrin, which was measured using an ELISA kit (Chicken Ovotransferrin ELISA Kit, ab157694). All assays had intra- and inter-assay imprecision <15% and were linear after serial dilutions.

2.5. Statistical analysis

Some environmental variables were selected and assessed as potential drivers of AR exposure. These factors were calculated within 1-km radius buffers constructed around each nest using QGIS Geographic Information System open source (version 3.32.3). The buffer radius was chosen as an approximation of the eagle owl's home range in the study area, as reported in the literature (León-Ortega, 2016; Pérez-García et al., 2012). Land use data were extracted from the CORINE Land Cover 2018; EEA, 2018), supplemented with information from the SIOSE Land Use Map (IGN, 2016) to provide a more detailed land use categorization. To allow for a more reliable analyses, land uses were classified into the five main classes: artificial areas, agricultural land, natural vegetation, wetlands, and water bodies. Additional subclasses were calculated within the "agricultural land" category: total irrigated and non-irrigated crops and the percentage of land occupied by irrigated orchards. In order to evaluate the role of the hydrological network in AR contamination, we calculated the minimum distance of each nest to the nearest watercourse (using a hydrological network layer; Gobierno de España, 2024), and the number of irrigation reservoirs within the buffer. Moreover, the human population density of the census section where the nest is located was used, extracting data from the latest population census conducted in 2021 (National Institute of Statistics, 2021). Finally, livestock farm data for 2021 in the Region of Murcia were provided by the Spanish Ministry of Agriculture, Fisheries, and Food, allowing for the calculation of animal density (i.e., of pigs, sheep and goats, poultry, equines, and total livestock) as well as the total number of farms within the buffer. Poultry and equines were excluded from the analysis, as they were present in very low numbers near the sampled territories. The variables used are described in detail in Table 1 SI.

Descriptive statistics were computed for each compound, as well as for the sum of SGARs, FGARs, the 10 analysed ARs (ΣARs), PT and biochemical parameters. To examine the association between the selected environmental factors (refer to Table 2 SI) with the concentration and prevalence of ARs, we utilized the information-theoretic method pioneered by Burnham and Anderson (2002). Linear Mixed Models (LMM) were constructed using the "lme" function from the "nlme" package (Pinheiro et al., 2023), treating environmental variables as fixed effects and territory as a random factor. Model comparisons were based on the bias-corrected version of Akaike's information criterion (AICc). Models were ranked using AICc differences (ΔAICc) and Akaike weights (*w*). ΔAICc was calculated as the difference between the AICc of each model and the AICc of the best model. Models with ΔAICc <2 can be alternative models to the selected model. Akaike weights may be interpreted as the probability that a given model is the actual best model of the set (Burnham and Anderson, 2002). To compare the prevalence and concentration of ΣARs across years, we employed the "glmer" function from the "lme4" package (Bates et al., 2015), with a logit link and binomial error distribution, regarding the breeding territory as a random factor.

Spearman's correlation test was employed to evaluate the relationships between ΣARs and PT and between ΣARs and the plasma biochemical parameters. All statistical analyses were conducted using R software version 4.3.1, and significance levels were set at $p < 0.05$.

3. Results

3.1. AR levels and prevalence

The data resulting from the two years of study were grouped and analysed together since no significant interannual differences were found in either AR prevalence or ΣARs. At least one AR was detected in 91.5% of the samples ($n = 106$). The prevalence of FGARs was markedly lower (11.3%) compared to that of SGARs (91.5%). All the analysed compounds were detected, except coumafuryl. The compound with the highest prevalence was flocoumafen (79.2%), followed by difenacoum (49.1%), brodifacoum (41.5%), and bromadiolone (28.3%) (Table 1). Considering the breeding territory as a unit of analysis, compounds were detected in 90.9% of the sampling events (40/44).

Multiple ARs were detected in 70.8% of the nestlings. Indeed, in individuals testing positive, one to six compounds were detected (Fig. 2). The total AR concentration (ΣARs) in positive individuals ranged from 0.03 to 57.81 ng mL^{-1} (with a median of 0.77 ng mL^{-1}).

Table 1
Prevalence (%) and levels (ng mL^{-1}) of anticoagulant rodenticides (ARs) detected in eagle owl nestlings (*n* = 106) sampled in the Region of Murcia (southeastern Spain). Descriptive statistics are provided for individuals with detected levels of ARs (expressed as *n*+ in the table).

	n+	%	Mean	Median	SD	Min.	Max.
FGARs							
Chlorophacinone	2	1.9	0.24	0.24	0.15	0.13	0.34
Coumafuryl	0	0	–	–	–	–	–
Coumatetralyl	4	3.8	0.14	0.08	0.14	0.04	0.34
Diphacinone	2	1.9	0.60	0.60	0.45	0.28	0.92
Warfarin	5	4.7	0.02	0.02	0.01	0.01	0.04
SGARs							
Brodifacoum	44	41.5	0.72	0.27	1.63	0.06	9.87
Bromadiolone	30	28.3	0.39	0.24	0.44	0.03	2.09
Difenacoum	52	49.1	1.34	0.22	7.02	0.05	50.83
Difethialone	4	3.8	0.74	0.65	0.49	0.26	1.40
Flocoumafen	84	79.2	1.26	0.16	6.32	0.02	57.43
ΣFGARs	12	11.3	0.19	0.08	0.26	0.01	0.92
ΣSGARs	97	91.5	2.34	0.74	7.81	0.03	57.81
ΣARs	97	91.5	2.37	0.77	7.81	0.03	57.81

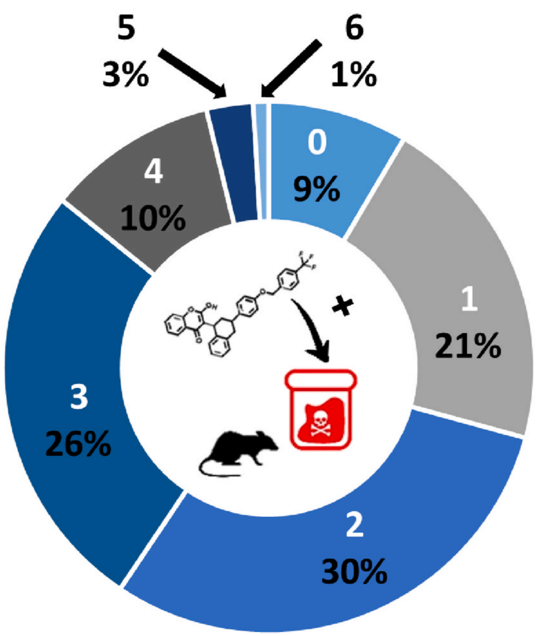

Fig. 2. Ring plot showing the percentage of eagle owl nestlings in the Region of Murcia (southeastern Spain) for which varying numbers of anticoagulant rodenticide (AR) compounds were detected. The number at the top of each segment indicates the specific number of AR compounds found, including cases where no compounds (0) were detected.

Coinciding with the prevalence, the compounds yielding the highest values were flocoumafen (57.43 ng mL^{-1}), difenacoum (50.83 ng mL^{-1}), and brodifacoum (9.87 ng mL^{-1}).

3.2. Analysis of factors that may influence AR exposure

Based on statistical analyses, it appears that none of the selected variables were related to AR prevalence (understood as the overall prevalence of all ARs analysed collectively) (Table 3 SI). Applying the same model to the prevalence of SGARs with the highest detection rates (flocoumafen, bromadiolone, difenacoum, and brodifacoum), we found that brodifacoum and difenacoum were associated with the distance from the nearest river (Table 4 SI and 5 SI; Fig. 4). Sheep-goat and swine density were slightly higher than the null model in the case of flocoumafen and bromadiolone, respectively (Table 6 SI and 7 SI). However, these models differ only slightly from the null model. Similarly, for brodifacoum, two variables (livestock density and study site) were above the null model (Table 4 SI), but the difference was minimal and thus not truly explanatory of the detected brodifacoum prevalence.

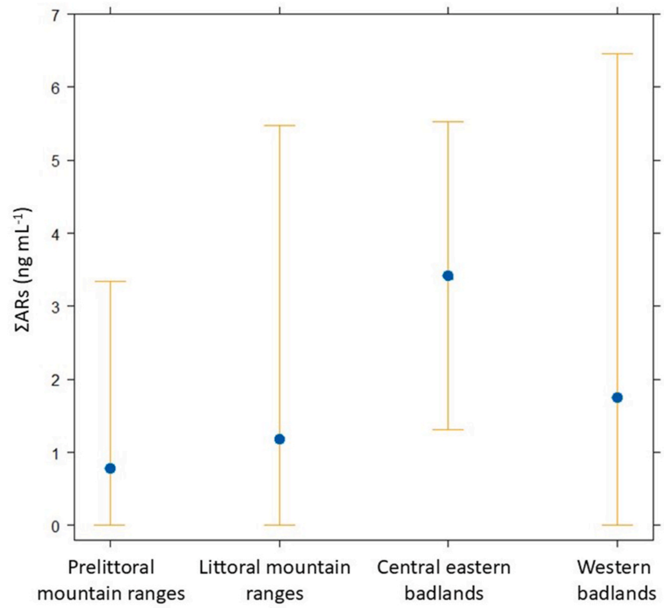

Fig. 3. Effect of different study sites on total concentration of anticoagulant rodenticides (ΣARs) in eagle owls (*Bubo bubo*) nestlings from the Region of Murcia (southeastern Spain). The plot shows concentration estimates (ng mL^{-1}) and 95% confidence intervals.

Regarding AR levels, these were related to the study site (the highest ranked variable) and the proximity to a landfill (Table 2). Indeed, the highest concentrations of ARs were detected in the Central-eastern badlands study site (Fig. 3). The remaining variables considered did not prove to be explanatory of the observed ΣAR concentrations.

3.3. Coagulation tests and biochemical analysis

The fibrinogen level was found to be above 100 mg dL^{-1} in all analysed samples (range 108.9–589.2 mg dL^{-1}), indicating that the samples were collected and handled correctly and that the individuals examined had sufficient fibrinogen levels to ensure normal blood coagulation. PT in eagle owl nestlings averaged 13.03 ± 1.83 s (range 8.8–17.5 s). A pair of values was considered an outlier and excluded from the analysis (57.81 ng mL^{-1}; 12.5 s). Although positive, no significant relationship was detected between ΣARs and PT ($Rho = 0.04$; $p = 0.49$), as shown in Fig. 1 SI.

Regarding the analysis of biochemical parameters in plasma samples, descriptive statistics were calculated, excluding a single ALT value, considered an outlier due to its significantly higher level (373 UI L^{-1}). Results are presented in Table 3. ΣARs was not found to be correlated with any of the plasma biochemical parameters analysed, except for glucose ($Rho = 0.225$; $p = 0.029$).

4. Discussion

As mentioned above, the eagle owl is considered an ideal biomonitoring species in the study area (Gómez-Ramírez et al., 2021). However, as far as we know, this is only the second biomonitoring study of AR exposure using blood samples from eagle owl nestlings. In the first study, Gómez-Ramírez et al. (2012b) analysed 50 blood samples from free-living eagle owls (both adults and nestlings) from southeastern Spain, but no individuals tested positive. However, it is possible that the technique used was not sufficiently sensitive to detect low levels of ARs.

The presence of ARs in the eagle owl has been predominantly studied

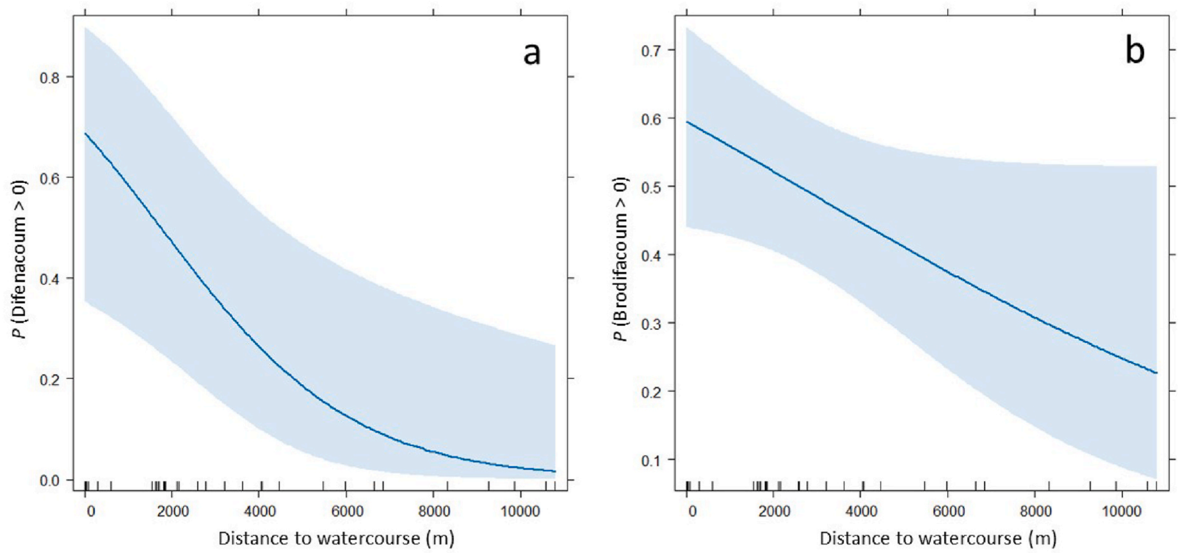

Fig. 4. The plots show the effect of the distance to the nearest watercourse on difenacoum (a) and brodifacoum (b) prevalence in eagle owl (*Bubo bubo*) nestlings in the Region of Murcia (southeastern Spain). Shadow areas represent 95% confidence intervals.

Table 2
Ranking of the factors used to explain the total concentration of ARs (ΣARs) detected in nestling eagle owls (*Bubo bubo*) in the Region of Murcia (southeastern Spain).

	k	AICc	ΔAICc	*w*
Study site	6	725.745	0.000	0.796
Landfill	4	729.201	3.456	0.141
Null model	3	731.968	6.223	0.035
Farm number	4	734.245	8.500	0.011
Irrigation reservoirs	4	735.647	9.902	0.006
Artificial areas	4	736.646	10.901	0.003
Orchards	4	738.466	12.721	0.001
Non irrigated crops	4	738.516	12.771	0.001
Irrigated crops	4	738.676	12.931	0.001
Natural vegetation	4	739.029	13.284	0.001
Agricultural land	4	739.157	13.412	0.001
Human density	4	740.947	15.202	0.000
Swine density	4	744.633	18.888	0.000
Sheep-goat density	4	745.658	19.913	0.000
Watercourse distance	4	748.845	23.100	0.000
Livestock density	4	749.750	24.005	0.000

k = number of parameters estimated; AICc = corrected Akaike's Information Criterion; ΔAICc = difference between AICc of each model and the minimum AICc; w = Akaike's weight.

using liver samples from carcasses found in various European countries, although the number of samples is generally low (i.e. < 20 individuals). Nonetheless, the detection rate is notably high, ranging from 50 to 100% (Christensen et al., 2012; Fourel et al., 2024; Langford et al., 2013; López-Perea et al., 2015, 2019; Moriceau et al., 2022; Sánchez-Barbudo et al., 2012). In fact, AR residues were detected in 83% of liver samples from adult individuals (n = 18) found in an area adjacent to ours (Gómez-Ramírez et al., 2021). Additionally, Fourel et al. (2024) recently found that the median sum of SGARs in eagle owls (180 ng g^{-1} ww, n = 14) was the highest among the species from France and Réunion Island examined in the study. Similarly, Christensen et al. (2012) found a substantial median concentration (241 ng g^{-1} ww, n = 10) in eagle owl liver samples from Denmark, a value closely approaching the highest concentration recorded in the red kite (*Milvus milvus*; 260 ng g-^{1}, n = 3). In López-Perea et al. (2015), 64.3% of the individuals (n = 14) from Catalonia (Spain) had hepatic concentrations of SGARs higher than 200 ng g^{-1} ww. Such concentrations exceed the assumed toxicity level for ARs (100–200 ng g^{-1}, Newton et al., 1999; Thomas et al., 2011) and are therefore considered potentially lethal. These data are significant because they do not only highlight that the eagle owl is a species easily exposed to ARs, but also may show higher capacity than other species to accumulate these compounds, which increases the risk of adverse effects.

Overall, AR concentrations detected here were low and fell within the range found in other studies conducted on blood samples for nestlings of other birds of prey (Badry et al., 2022; Martínez-Padilla et al., 2017; Oliva-Vidal et al., 2022; Powolny et al., 2020; Spadetto et al., 2024b). However, two of our owlets presented unusually elevated ΣAR concentrations, exceeding 50 ng mL^{-1} (50.9 and 57.8 ng mL^{-1}). These AR levels have been the highest detected so far in blood samples from free-living raptor nestlings, and presumably indicate the recent consumption of a prey with high concentrations of SGARs (flocoumafen and difenacoum respectively). These data are quite alarming, as they suggest that episodes of exposure to high AR doses might be occurring more frequently than expected, with possible acute or subacute effects in the short term.

The prevalent compounds were SGARs, as expected, due to their greater potency and effectiveness compared to FGARs. They are also preferred for their ability to overcome the widespread resistance that rodent populations have developed against FGARs. Flocoumafen was also detected at very high prevalence in other nocturnal and diurnal raptors in the Region of Murcia sampled during the same study years (Spadetto et al., 2024a, 2024b). These results are remarkable given that, in Spain, only one product containing flocoumafen is registered for non-professional use, and two are intended for use by specialized personnel (Carrera et al., 2024). Difenacoum and brodifacoum were also detected with very high prevalences, which likely indicates their widespread use in the area, possibly due to the large number of registered products for both professional and non-professional use (69 for difenacoum and 129 for brodifacoum) (Carrera et al., 2024). The low prevalence of difethialone (3.8%) suggests that it may be the only second-generation compound scarcely used in the study area. FGAR diphacinone is not authorized for use in Europe (EC Regulation 528/2012) (European Union, 2012), yet it was detected in two eagle owl nestlings. Similar findings were reported for other raptor (Vicedo et al., 2024) and mammal (Carrera et al., 2024) species sampled in the same area (SE Spain), suggesting illegal use of this compound.

Table 3
Descriptive statistics (median, mean, SD, minimum and maximum) of biochemical parameters in the plasma of eagle owl nestlings ($n = 105$) from the Region of Murcia (southeastern Spain).

	Median	Mean	SD	Minimum	Maximum
Creatinine (mg dL^{-1})	0.36	0.39	0.19	0.05	1.83
Total proteins (g dL^{-1})	3.14	2.90	0.64	1.05	4.30
Albumin (g dL^{-1})	1.21	1.22	0.10	0.95	1.52
Calcium (mg dL^{-1})	9.77	10.04	2.46	8.35	34.27
Amylase (UI L^{-1})	529.40	516.54	121.93	175.00	810.70
Creatine kinase (UI L^{-1})	1516.10	1616.39	772.50	268.40	3817.80
Cholesterol (mg dL^{-1})	199.11	202.57	34.93	127.16	291.47
Alkaline phosphatase (UI L^{-1})	600.20	623.53	140.00	330.40	1250.60
Phosphorus (mg dL^{-1})	7.14	7.12	0.79	5.24	9.03
γ-glutamyltransferase (UI L^{-1})	4.40	5.19	4.85	0.70	43.70
Glucose (mg dL^{-1})	409.90	413.26	27.62	357.20	523.60
Aspartate aminotransferase (UI L^{-1})	225.60	244.04	92.42	113.30	748.70
Alanine aminotransferase (UI L^{-1})	37.60	43.94	36.31	11.00	373.00
Urea (mg dL^{-1})	13.70	14.33	6.27	4.00	45.20
Total bilirubin (mg dL^{-1})	0.10	0.11	0.04	0.03	0.39
Uric acid (mg dL^{-1})	12.99	13.88	4.57	5.47	25.42
Magnesium (mg dL^{-1})	2.34	2.42	0.45	1.75	4.96
Lactate dehydrogenase (UI L^{-1})	882.60	969.17	453.90	292.00	2304.50
Ovotransferrin (μg mL^{-1})	12137.00	12607.46	6058.91	1155.00	34400.00
Butyrylcholinesterase (mol mL^{-1} min^{-1})	1.10	1.17	0.33	0.10	2.60
Acetylcholinesterase (mol mL^{-1} min^{-1})	1.76	1.81	0.38	0.30	3.14

4.1. Variables affecting AR exposure

Significantly higher ΣAR concentrations have been detected in the study site of the Central-eastern badlands, where the level of urbanisation near eagle owl territories is the highest among the study sites in the region. Eagle owls in our study area often reside in the wildland-urban interface (zones where urban or residential development is directly adjacent to or mixed with natural vegetation), where it has been recently shown that the risk of AR exposure can be significant (Hofstadter et al., 2021; Silveira et al., 2024). In another research conducted on common kestrels in the same study area, higher ΣSGAR levels have been observed in the more urbanised study site, surrounding the city of Murcia (Spadetto et al., 2024a), which suggests that in heavily anthropized areas, the residential use of ARs is widespread among the local population. These findings are also consistent with other studies in which the degree of urbanisation has been found to be a strong predictor of AR exposure (Geduhn et al., 2015; Lohr, 2018; López-Perea et al., 2019; Musto et al., 2024). It is worth noting that in the other study sites, particularly in the western badlands and littoral mountain ranges, the number of nests is notably lower, which could be a limitation of our study.

Furthermore, we identified a correlation between ΣAR levels and the proximity of landfills and waste treatment facilities to eagle owl territories. Waste dumps serve as environments with an abundant food supply, attracting numerous wildlife species, particularly rodents. The consequent proliferation of these animals often necessitates the increased use of ARs for population control (Berny et al., 2014; Coeurdassier et al., 2018). As a result, eagle owls that consume these AR contaminated rodents are likely exposed to elevated doses of these compounds. Avian scavengers, such as kites and vultures, often exploit these facilities and were also reported to bioaccumulate ARs in their bodies (Badry et al., 2022; López-Perea et al., 2019; Oliva-Vidal et al., 2022). These findings underscore the critical need for rigorous monitoring of AR application near landfills and the consideration of alternative management strategies to mitigate the impact on non-target raptor and scavenger species.

On the other hand, AR detection in blood essentially indicates recent exposure, as the compounds have a short-lived presence in the bloodstream (e.g., 16.5 ± 10.0 h for warfarin in Eastern barn owl *Tyto javanica*, Khidkhan et al., 2024). We do not know the exact timing of the exposure nor the initial dose. Although it is unclear whether some degree of bioaccumulation in the blood might occur, higher concentrations in a particular study site could indicate repeated exposures or at least massive application of these substances in the considered area. It is also likely that these products are not correctly applied (e.g., due to lack of bait boxes, incorrect amounts, use in inappropriate areas, and lack of post-application monitoring) and that dead rodents are not promptly removed. Additionally, the prevalence of ARs was high (≥75%) in all four study sites and could not be related to any of the environmental variables considered, indicating that, regardless of any factor taken into account, and even in nests located in natural and protected areas, the risk for the eagle owl of being exposed to ARs remains considerable.

Interestingly, the higher likelihood of finding certain SGAR compounds (brodifacoum and difenacoum) in territories near watercourses can be attributed to a combination of ecological, behavioural, and anthropogenic factors. ARs can be transported from application areas to watercourses through surface runoff, especially during rains or irrigations (Regnery et al., 2018). Once in watercourses, they can be further transported downstream and accumulate in nearby areas. Zones adjacent to water bodies, particularly around ponds or riverbeds in semiarid environments, tend to harbour greater biodiversity and rodent density (Shenbrot et al., 2010; Williams, 2005; Zamora-Marín et al., 2021), exposing them to AR contamination. Furthermore, these areas may experience intensive use of ARs to control rodent populations, especially in agricultural zones or near human settlements. Predatory birds such as the eagle owl, in arid conditions, are attracted by the abundance of prey and the presence of water (Boal et al., 2023), which may facilitate secondary AR exposure and would make these areas an indicator of the presence and distribution of ARs in the environment. It is also worth noting that, in Mediterranean semi-arid regions, watercourses are often represented by riverbeds and ravines where the presence of water is often limited or even absent. Therefore, ARs may easily concentrate in these areas due to the reduced flow and limited dispersion. In general, SGARs have lower water solubility than FGARs and degrade within a few hours when exposed to light (reviewed by Regnery et al., 2018). However, they can persist in organic matter and sediment and accumulate in the tissues of aquatic organisms (Regnery et al., 2018, 2020). Indeed, the same SGARs that were found associated with watercourses in our study were detected in faeces of Eurasian otter (*Lutra lutra*) and other mammals linked to riparian ecosystems from the Region of Murcia between 2020 and 2021 (Andrés-Esteso et al., 2023), confirming the relationship of these compounds with the aquatic environment. Residues of ARs in predators linked to aquatic environments have also been detected in other countries such as Germany (Regnery et al., 2024) and France

(Fournier-Chambrillon et al., 2004), suggesting that watercourses represent an important pathway for AR exposure and dissemination.

The eagle owl primarily feeds on rabbits in the study area. Therefore, given the high AR prevalence found, it is likely that this mammal frequently comes into contact with these compounds. In the Region of Murcia, as well as in other areas of Spain and the world, the rabbit is considered a pest by farmers, mainly because it directly damages various types of crops (Delibes-Mateos et al., 2018, 2020). For this reason, the rabbit is often a target species of ARs (Berny et al., 2010; Lohr and Davis, 2018), which in Spain are likely used for this purpose illegally (Colomina et al., 2024). In fact, ARs are not authorised as plant protection products, so they should not be used in agriculture and open spaces by untrained personnel. Further studies are recommended to confirm this hypothesis. Additionally, since the presence of rabbits is a key factor for the sustenance of eagle owl populations, it is critically necessary to know if the widespread presence of ARs in the environment is altering the presence of this species in the study area. It should also be noted that, in the absence of this prey species, the eagle owl can hunt other prey, including rats, which are target species for ARs and may also have contributed to the high AR prevalence found, especially in the littoral mountain ranges study site, where rabbits are scarcer.

4.2. *Biomarker of effect and general health status of eagle owl nestlings*

PT is considered a valid biomarker of the toxic effect that ARs cause by altering the blood's coagulative capacity (Rached et al., 2020), as this parameter changes rapidly following AR exposure in relation to the dose, and returns to baseline values after few days post-exposure (Rattner et al., 2010, 2014a). Unfortunately, PT is a species-specific parameter and reference values are not available for the eagle owl and most birds of prey (Hindmarch et al., 2019). Moreover, there is no standardisation of analysis procedures among different laboratories, partly because a specific reagent, avian thromboplastin, is required for PT analysis (Webster, 2009). This reagent is not commercially available and must be produced in the lab. All these factors make it difficult to compare results across different studies, and to perform interspecific comparisons. In our case, PT was evaluated using the same method as in samples from other nocturnal raptors in the Region of Murcia, namely the long-eared owl (*Asio otus*) and the barn owl (*Tyto alba*). In these two species, it was possible to establish a positive and significant correlation between the total concentration of ARs detected and the PT, indicating a non-acute but measurable adverse effect produced by ARs (Spadetto et al., 2024a, 2024b). In the case of eagle owl chicks, a positive but not significant relationship between the two variables was observed. This data may indicate that in this species, despite repeated exposure, nestlings had not accumulated sufficiently high hepatic AR concentrations to impair blood coagulation. Further investigations are needed to establish reference PT values in raptor species. Moreover, the standardization of laboratory protocols for analysing coagulation parameters would be critically important for assessing the immediate effects of ARs on the health of non-target wildlife.

Similarly to PT, interpreting the results obtained from the analysis of plasma biochemical parameters requires species-specific reference values. Additionally, it should be noted that these values in avian species can vary depending on the age and sex of the individuals (Agusti Montolio et al., 2018; Casado et al., 2002; Scholtz et al., 2009). The analysed biochemical parameters fall within the normal range when compared to those previously obtained in free-ranging chicks of the same species (Gómez-Ramírez et al., 2016). Some of the evaluated parameters have never been studied in the eagle owl. Where such information was unavailable, we compared the results with those obtained in nestlings or adults of other owl species (Agusti Montolio et al., 2018; Ammersbach et al., 2015; Jones and Chitty, 2020; Szabo et al., 2014).

Interestingly, the only parameters that appear higher compared to those reported for other owls are total bilirubin and magnesium (Jones and Chitty, 2020). However, bilirubin is not considered a useful marker of liver damage in birds, due to the almost complete absence of biliverdin reductase in the liver (Jones and Chitty, 2020; Raidal, 2020), so these higher levels could be due to species and/or age differences of the subjects. Nonetheless, they are not accompanied by an alteration of other parameters indicative of liver damage. Similarly, no alterations were found in other electrolyte levels related to magnesium, indicating that the detected differences are likely interspecific. Furthermore, age is an important factor to consider since several parameters have been found to be higher in nestlings compared to juveniles/adults of the same species (Agusti Montolio et al., 2018; Gómez-Ramírez et al., 2016). Ovotransferrin was evaluated as an acute-phase protein biomarker of the activation of the immune system, as its levels increase significantly in response to infections, inflammation or trauma (Giansanti et al., 2012). It should be noted that baseline levels of ovotransferrin vary significantly depending on the species and are slightly higher in nestlings compared to adults (Horrocks et al., 2011). In the case of the eagle owl, the average found is higher than that observed in nestlings of various wild bird (non-raptor) species (Horrocks et al., 2011). However, since the rest of parameters fall within normality, we cannot state with certainty whether this is an alteration. We can therefore conclude that the eagle owl nestlings in our study were in good physiological condition. It is known that ARs can damage the liver over time and alter liver parameters (reviewed by Popov Aleksandrov et al., 2024). However, hepatic parameters were not altered, and no correlation was found between them and the total concentration of ARs detected in blood samples. Moreover, the correlation between ΣARs and glucose is weak and unlikely to be meaningful. Overall, although these individuals are exposed to ARs, the concentrations or duration of exposure were probably not sufficient to cause a detectable toxic effect.

These biochemical analysis results confirm the values reported by Gómez-Ramírez et al. (2016) and complement the panel with additional parameters that are not routinely evaluated but can be useful in research contexts or for assessing the health status of individuals admitted in wildlife rescue and rehabilitation centres.

5. Conclusions

Apex predators like the eagle owl are essential to ecosystems as they regulate prey populations, preventing resource overexploitation and maintaining balance among various species within their environment (Wallach et al., 2015). However, these species are often exposed to environmental contaminants through bioaccumulation and biomagnification along the food chain. This is particularly critical for predatory birds living in human-impacted environments, where exposure to harmful substances becomes inevitable. In our study, we found widespread AR contamination in the eagle owl, raising significant concerns for the conservation, not only of this species, but also of other predators and scavengers sharing the same habitat. Our results also suggest the potential presence and accumulation of certain ARs in the hydrological network, representing an exposure pathway that should be carefully considered, especially in semi-arid regions where water scarcity can result in reduced dilution and increased concentration of these compounds.

Although there are no clear signs of acute intoxication, chronic exposure to these compounds over time can compromise individuals' health, affecting their coagulative capacity and causing other sublethal effects. This could lead to a decrease in their biological fitness, making them more susceptible to diseases and other environmental threats (Rattner et al., 2014b). Due to the high prevalence found, further studies are needed to monitor these effects in the long term. Ultimately, this study on the eagle owl, along with others (Spadetto et al., 2024b), serves as an important starting point for further research into other top predator species such as the golden eagle (*Aquila chrysaetos*) or Bonelli's eagle (*Aquila fasciata*), which share habitat and prey and have higher protection status. In fact, to effectively conserve raptor species, it is essential to implement concrete actions aimed at mitigating the impact of ARs and protecting the biodiversity of their ecosystems.

CRediT authorship contribution statement

Livia Spadetto: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Pilar Gómez-Ramírez:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mario León-Ortega:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Antonio Zamora-López:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Sarah Díaz-García:** Writing – review & editing, Methodology, Investigation, Conceptualization. **José Manuel Zamora-Marín:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Fernando Tecles-Vicente:** Writing – review & editing, Methodology, Formal analysis. **Luis Pardo-Marín:** Writing – review & editing, Methodology, Formal analysis. **José Fenoll:** Writing – review & editing, Methodology, Formal analysis, Data curation. **José Francisco Calvo:** Methodology, Formal analysis, Data curation. **Antonio Juan García-Fernández:** Writing – review & editing, Visualization, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.120382.

Data availability

Data will be made available on request.

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