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Active monitoring of long-eared owl (*Asio otus*) nestlings reveals widespread exposure to anticoagulant rodenticides across different agricultural landscapes

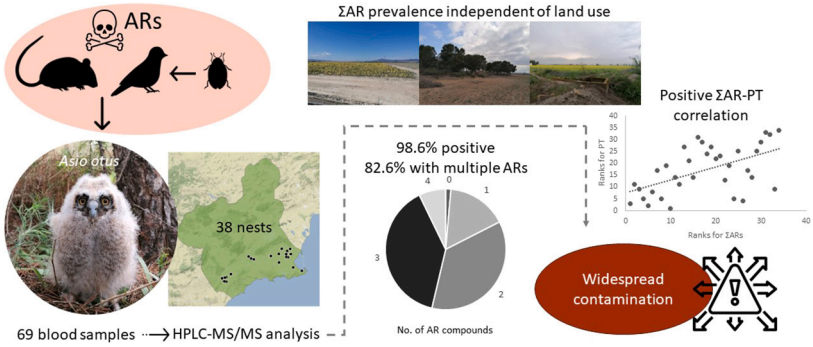
Livia Spadetto [a], Pilar Gómez-Ramírez [a,*], José Manuel Zamora-Marín [b,c,d], Mario León-Ortega [b], Sarah Díaz-García [b], Fernando Tecles [e], José Fenoll [f], Juana Cava [f], José Francisco Calvo [g], Antonio Juan García-Fernández [a]

[a] *Toxicology Research Group, 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 Applied Biology, Centro de Investigación e Innovación Agroalimentaria (CIAGRO-UMH), Miguel Hernández University of Elche, Elche, Spain*
[d] *Department of Zoology and Physical Anthropology, Faculty of Biology, University of Murcia, 30100 Murcia, 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*

HIGHLIGHTS

- Long-eared owl used as a model to assess AR exposure in agricultural landscapes
- ARs were detected in 98.6 % of the samples, with 82.6 % showing multiple compounds.
- Differences in diet composition suggest AR occurrence at various trophic levels.
- Direct effects on prothrombin time (PT) were linked to ΣAR levels in wild nestlings.
- Owlets are exposed from early life stages, which implies medium to long-term risks.

GRAPHICAL ABSTRACT



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ABSTRACT

The widespread use of anticoagulant rodenticides (ARs) poses a worldwide threat to farmland wildlife. These compounds accumulate in tissues of both target and non-target species, potentially endangering both direct consumers and their predators. However, investigations on ARs in blood of free-ranging predatory birds are rare. Here, the long-eared owl (*Asio otus*) has been used as a model predator to assess AR exposure in different agricultural landscapes from a Mediterranean semiarid region. A total of 69 owlets from 38 nests were blood-sampled over 2021 and 2022, aiming to detect AR residues and explore factors that determine their exposure, such as land uses. In addition, prothrombin time (PT) test was conducted to assess potential effects of AR

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

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Pesticides
Contamination

contamination. Overall, nearly all the samples (98.6 %) tested positive for at least one compound and multiple ARs were found in most of the individuals (82.6 %). Among the ARs detected, flocoumafen was the most common compound (88.4 % of the samples). AR total concentration (ΣARs) in blood ranged from 0.06 to 34.18 ng mL^{-1}, detecting the highest levels in the most intensively cultivated area. The analysis of owl pellets from 19 breeding territories showed relevant among-site differences in the contribution of rodents and birds into the diet of long-eared owls, supporting its high dietary plasticity and indicating AR presence at multiple trophic levels. Moreover, a positive and significant correlation was found between ΣARs and PT ($Rho = 0.547$, $p < 0.001$), which demonstrates the direct effect of ARs on free-living nestlings. Our results provide a preliminary overview of AR exposure in a little-studied owl species inhabiting agricultural and rural landscapes. Despite the low detected levels, these findings indicate widespread exposure -often to multiple compounds- from early life stages, which raises concern and draws attention to an ongoing and unresolved contamination issue.

1. Introduction

Anticoagulant rodenticides (ARs) are toxic compounds frequently used in agricultural lands to protect crops and also in urban areas to control rodent infestations (Jacob and Buckle, 2018). These substances act by interfering with blood clotting process as they disrupt the vitamin K cycle through the inhibition of the enzyme vitamin K epoxide reductase (VKOR). Reduced vitamin K (hydroquinone) activates clotting factors (II, VII, IX, and X), resulting in the conversion to its inactive oxidized form (epoxide) (Murphy, 2006). ARs hinder vitamin K reactivation via VKOR enzyme, causing depletion of activated clotting factors. Following the ingestion of a toxic dose of ARs, an individual can develop coagulopathy, thus even minor injuries can lead to uncontrollable bleeding and death (Feinstein et al., 2016; Horak et al., 2018).

First-generation ARs (FGARs, e.g., warfarin, coumatetralyl, coumafuryl, chlorophacinone, and diphacinone) were the first compounds developed and used for rodent control. They are characterized by moderate toxicity and require multiple feedings over several days to achieve lethal effects (King and Tran, 2015; Thijssen, 1995). However, target rodents became resistant to these compounds, resulting in a significant reduction in their effectiveness (Berny, 2011; Mcgee et al., 2020). Consequently, they were replaced by more potent second-generation ARs (SGARs, e.g., brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen), which exhibit higher toxicity and typically require only a single dose to cause lethal effects. Moreover, SGARs persist in rodent tissues for an extended period and can pose greater challenges for management due to their increased persistence in the environment (Erickson and Urban, 2004; Feinstein et al., 2016).

While ARs are effective in controlling rodent populations, they also pose a risk to non-target species, including pets, wildlife, and even humans (Lefebvre et al., 2017; Rattner et al., 2014b). In fact, accidental ingestion or consumption of poisoned rodents can lead to secondary poisoning (López-Perea and Mateo, 2018). Numerous studies assessed the exposure to both FGARs and SGARs in a wide range of non-target species, particularly mammal and avian predators and scavengers (Elmeros et al., 2018; Geduhn et al., 2015; Lohr, 2018; Murray, 2017; Rial-Berriel et al., 2021a; Thornton et al., 2022). Birds of prey appear to have the highest detection rates of AR residues in their livers (Nakayama et al., 2019) and are particularly susceptible to the toxic effects of these compounds (Nakayama et al., 2020; Rattner et al., 2012). Furthermore, while some avian predatory species primarily feed on target rodents, which is the more frequent exposure pathway to ARs (Geduhn et al., 2016), other taxa show generalist and varying diets, leading to a complex exposure scenario. In fact, some studies have shown that AR baits, intended for rodent control, are often accessible and consumed by non-target species, such as songbirds and invertebrates (Elliott et al., 2014; Walther et al., 2021). The consumption through predation or scavenging of these poisoned prey by higher-level organisms can lead to the distribution and accumulation of ARs throughout food webs. As a consequence, raptors that primarily rely on birds as their main food source are also vulnerable to AR exposure (Broughton et al., 2022; Hughes et al., 2013), which underscores the need to consider the broader ecological implications of AR use in rodent control programs.

Birds of prey have frequently been employed in biomonitoring studies of environmental contaminants (Badry et al., 2020; García-Fernández et al., 2023; Gómez-Ramírez et al., 2014). In fact, their position as apex predators, presence in various habitats, long lifespans for accumulation of contaminants and sensitivity to pollutants, collectively make them valuable indicators of environmental and ecosystem health (Movalli et al., 2017). ARs have predominantly been studied in liver samples from field-recovered carcasses (López-Perea and Mateo, 2018), while fewer studies have focused on active monitoring through the analysis of blood or serum/plasma samples in free-ranging birds of prey (Badry et al., 2022; Buechley et al., 2022; Martínez-Padilla et al., 2017; Murray, 2020; Oliva-Vidal et al., 2022). This type of sample, unlike those from opportunistically collected carcasses, allows documenting real-time exposure events, owing to the short half-life of ARs in plasma (Horak et al., 2018). Therefore, active monitoring programs offer the advantage of providing a detailed overview of contamination at the species and environmental level, enabling informed decision-making and the implementation of appropriate measures for wildlife conservation and management (Vyas, 2017).

In ecotoxicology, biomarkers are measurable, quantifiable, and observable molecular, cellular, histopathological and physiological indicators used to assess the exposure and/or effects of chemical agents on living organisms and ecosystems (De Coen et al., 2000; Eason and O'Halloran, 2002). Exposure biomarkers, such as the AR presence in animal tissues (e.g., liver, blood or plasma), can indicate the amount of chemical substance absorbed or accumulated over time in the organism and provide information about the type and intensity of exposure. On the other hand, biomarkers of effect allow to assess the toxic effects of ARs, such as haematological alterations on the coagulation process (Rached et al., 2020). Thus, the combined use of these two types of biomarkers can provide a more comprehensive overview of the impact of ARs on non-target species.

The long-eared owl (*Asio otus*) is a medium-sized bird of prey which shows markedly nocturnal habits, a generalist diet mostly based on small mammals and birds and a wide distribution range throughout the Holarctic region (both North America and Eurasia). In the Iberian Peninsula, this species usually breeds in forest edges or small woodland patches within agroforest environments, often near rural settlements (Martínez and Zuberogoitia, 2010). Therefore, the long-eared owl may act as a representative avian predator in such agricultural landscapes, due to its wide occurrence and moderate abundance (Emin et al., 2018). In such open agroecosystems, this species can easily capture rodents and birds from crops, making it likely to come into contact with ARs through secondary contamination. However, available information on AR exposure in the long-eared owl is extremely scarce and it is exclusively derived from field-recovered carcasses. Investigating ARs in blood of this species can thus provide valuable insights into the exposure and threats for the species itself, as well as for other farmland species, and reveal the extent of environmental contamination.

The aims of this study were (1) to provide data on AR exposure in the long-eared owl in four areas of the Region of Murcia (Spain) characterized by different land use compositions, (2) to assess the role of environmental variables, such as the study site and different land uses,

on the extent of exposure and (3) to evaluate specific toxic effects of these substances in their organism. These results may provide useful data for wildlife management and agri-environmental schemes (e.g., the EU Common Agricultural Policy) to account for the harmful effects of ARs on farmland biodiversity and contribute to regulate their widespread use.

2. Materials and methods

2.1. Study area

The study was conducted in the Region of Murcia (Spain), which is located in the southeast of the Iberian Peninsula (Fig. 1). This area is characterized by a Mediterranean semiarid climate, with mild winters and hot, dry summers, while rainfall is scarce throughout the year (about 300 mm per year, Spanish National Agency of Meteorology - AEMET, n.d.). Despite its semiarid climate, this region exhibits a pronounced environmental gradient that extends from the coastline to the interior. This gradient encompasses a range of settings marked by varying weather patterns, topography, and degree of human pressure.

Based on land use, we selected four different agricultural areas across the study region: "Campo de Cartagena", "Campo de Murcia", "Puerto Lumbreras" and "Saladares del Guadalentín" (Fig. 1). The "Campo de Cartagena" corresponds to a highly intensive agricultural area which has experienced a marked land use change over the last decades, whereby traditional rainfed crops (mostly olive, almond and carob groves as well as cereal crops) were replaced by conventional irrigated agriculture (mostly vegetable crops and citrus orchards), contributing to a rapid economic growth of the region (Rupérez-Moreno et al., 2017). The "Campo de Murcia" is represented by the northern part of the coastal plain of the "Campo de Cartagena" and is characterized by rainfed agriculture (mostly almond groves and cereal crops) with a low representation of intensive irrigated crops (though increasing in recent years), being this area crossed by numerous natural creeks and mostly dominated by monospecific pine woodlots (Esteve-Selma et al., 2015). The third study area is known as "Saladares del Guadalentín" and is mostly represented by a salt steppe associated to a highly torrential Mediterranean-type river (the Guadalentín River), which is interspersed with both rainfed and intensive farmland plots forming a mosaic landscape. This place is protected as a Site of Community Importance (SCI) and a Special Protection Area (SPA) and subject to specific management actions to preserve its ecological integrity and unique biodiversity. Lastly, an additional study site, "Puerto Lumbreras", was placed in the corresponding municipality situated eastward in the Region of Murcia. This site is characterized by extremely dry weather conditions, with average annual rainfall rarely exceeding 150 mm. Almost devoid of forest formations, the plain where the sampling was conducted is characterized by agricultural land and it features a mosaic of irrigated crops such as vegetables and orchards, often organized into small plots cultivated with various types of crops (Estadística Agraria Regional, n. d.).

2.2. Target species and sample collection

The long-eared owl is a nocturnal species, rather elusive and challenging to detect, thus its population trend at the European level remains unknown (Birdlife International, 2021). In Spain, an estimated population of 3300 breeding pairs was reported for the period 1998–2002 (Martí and Del Moral, 2003). However, more recent data indicate that this species has also occupied the southern plains of the Iberian

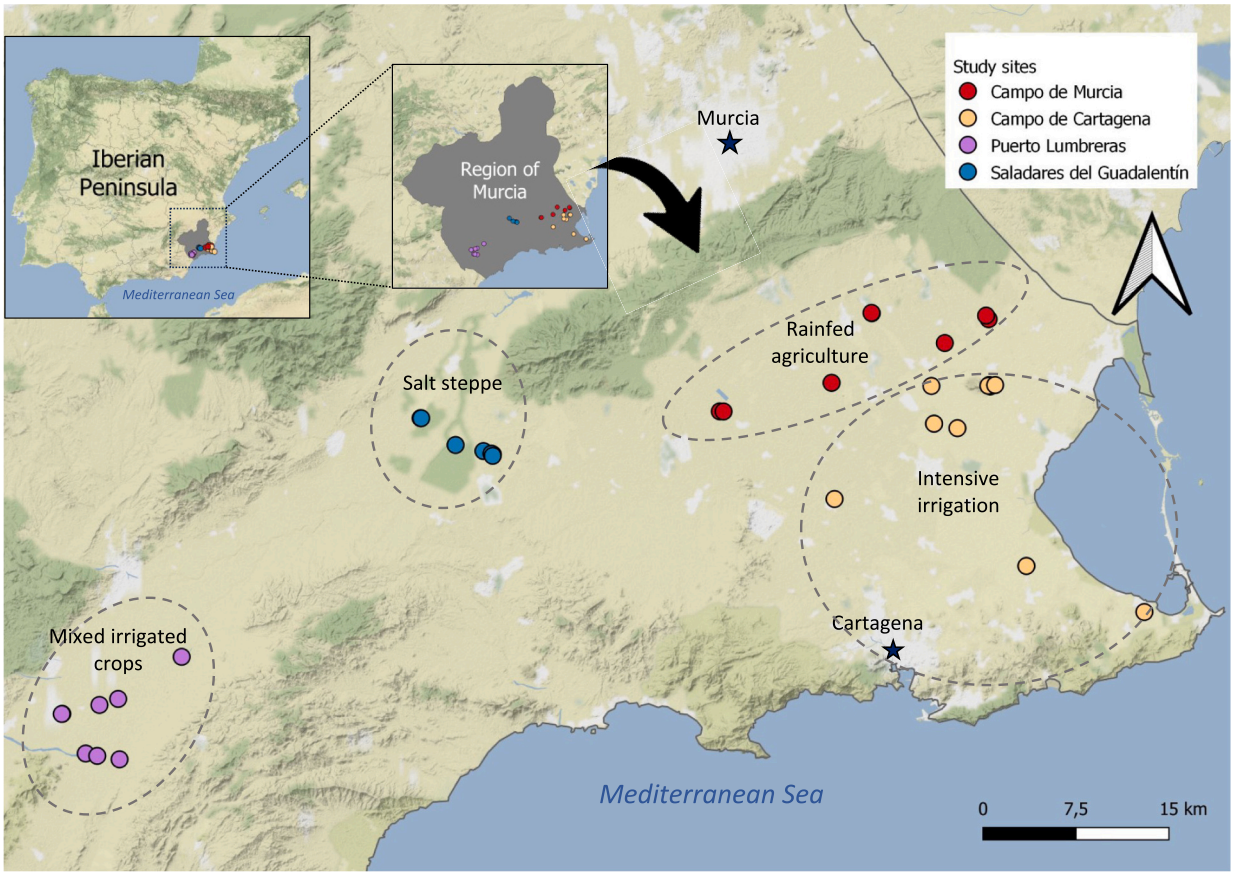

Fig. 1. Distribution of long-eared owl nests (dots) sampled in the Region of Murcia (southeastern Spain). Dot colour refers to a given agricultural area used for spatial comparisons in anticoagulant rodenticide exposure. Names of the two largest cities are provided for an easier geographic location of the target owl territories.

Peninsula in recent years (Salgado, 2022), although its current distribution remains uncertain. In our study area, the long-eared owl was listed as critically endangered due to the low number of breeding pairs (Robledano Aymerich et al., 2006), but the breeding population has notably increased during the last two decades (own data).

A total of 69 blood samples were obtained from free-living long-eared owl nestlings during the breeding season of 2021 ($n = 29$) and 2022 ($n = 40$). Sampled owlets corresponded to 38 nests visited during the study period, which belonged to 29 breeding territories. Nine nests were sampled in both years (2021 and 2022) whereas the remaining 20 territories were sampled only once. In those owl territories surveyed twice, the breeding pair usually selected the same nesting platform or a nearby alternative one (i.e., 20–50 m).

Long-eared owl territories were located based on a database containing nests found during the last two decades. Each owl territory was visited at least once during the pre-laying period (January–March) to record data on breeding site occupancy. When occupied, additional visits were conducted (March–June) to estimate the laying date, hatching date, and nestling age to safely access the nests and collect samples. Before owlets reached the fledging age (≈ 25–35 days old), owl nests were accessed to mark them with official metal rings, record biometric data (body weight, wing and third primary feather length) and take blood samples. A clinical examination of each owlet was performed by a veterinarian prior to blood sampling. These procedures were conducted as quickly as possible in order to minimize stress to the animals, following the protocol described by Espín et al. (2021) and the guidelines of EU Directive 2010/63/EU for animal experiments (approved by the Ethical Committee for Animal Experimentation at the University of Murcia; code 657/2020). About 1.5 mL of blood was drawn from the brachial vein using a sterile syringe with a 25G needle. A 450 μL aliquot of blood was preserved in a tube containing 50 μL of 0.109 M sodium citrate buffer, while the remaining volume (approximately 1 mL) was transferred to a heparinized tube. The blood samples were kept on ice during fieldwork and until arrival at the Toxicology Laboratory of the University of Murcia, which occurred within a few hours. The samples with sodium citrate buffer were centrifuged for 15 min at 2500*g* to obtain citrated plasma. Subsequently, all samples were stored at −80 °C until analysis.

Pellets were also collected between 2020 and 2021 in the vicinity of 19 surveyed breeding territories, and this material was analysed to characterize the diet of the long-eared owl based on reference literature (Moreno, 1986; Román, 2019).

2.3. Chemicals and standards

Acetonitrile and methanol (HPLC grade), sodium sulphate and sodium chloride (reagent grade) were obtained from Panreac Química S.L.U (Spain). Ethylenediamine-N-propyl phase that contains both primary and secondary amines (Supelclean PSA bonded silica), and C18 (Discovery DSC-18: octadecylsilane 18 % C) were purchased from Sigma-Aldrich (USA). Rodenticide reference standard difethialone was obtained from Dr. Ehrenstorfer GmbH (Germany), while bromadiolone, brodifacoum, chlorophacinone, coumatetralyl, coumachlor, difenacoum, diphacinone, flocoumafen, coumafuryl, and warfarin were purchased from Sigma-Aldrich (USA). The purity of the solvents and reagents used was >99 %.

To create stock solutions of the standard compounds, 10.0 mg of each compound was dissolved in 10 mL of methanol, yielding a concentration of 1.0 mg mL^{-1} for each individual compound. Subsequently, a standard mixture was prepared at a concentration of 1000 μg mL^{-1}, including all the ARs. To accomplish this, a portion of the stock solution for each compound was combined with a suitable volume of HPLC grade methanol. The resulting standard mix was then utilized to spike the chicken blood samples by adding a known amount of the standard mix to the samples.

2.4. Sample preparation and AR analysis

A modification of the method described by Taylor et al. (2019) was used for sample extraction. In the first step, 250 μL of blood (spiked chicken blood or owl blood) were transferred to a 5 mL plastic tube containing a ceramic homogenizer. Then, 1 mL of acetonitrile and 25 μL of coumachlor as an internal standard were added, and the tube was vortexed for 1 min. Next, extraction salts were added, namely 0.25 g NaCl and 1 g Na_2SO_4 for each sample. The tube was manually shaken for 1 min and centrifuged at 2500*g* for 5 min. The supernatant was collected and transferred to a 2 mL plastic tube containing purification products (12.5 mg PSA, 37.5 mg C18 and 225 mg Na_2SO_4). The tube was vortexed for 1 min and centrifuged at 2500*g* for 5 min. Finally, the supernatant was collected with a syringe and filtered through a 0.45 μm nylon syringe filter directly into a chromatography vial.

The vials containing the extract were transported under cold conditions to the laboratory of the Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental (IMIDA) of Murcia for analysis, following the method described by Rial-Berriel et al. (2020b). Ten ARs (coumafuryl, coumatetralyl, warfarin, diphacinone, chlorophacinone, brodifacoum, bromadiolone, difenacoum, flocoumafen, difethialone) were analysed using an HPLC 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 of 150 × 2.1 mm and 2.6-μm particle size (Phenomenex Kinetex R 2.6 μm EVO Polar C18 100 A) and an Ultivo 6465BA triple quadrupole mass spectrometer from Agilent, equipped with an electrospray ionization (ESI) interface operating in negative ion mode. The mobile phases were ammonium acetate 2 mM and 0.1 % formic acid in ultrapure water (A) and ammonium acetate 2 mM and 0.1 % formic acid in methanol (B). The gradient program for mobile phase A was: started with 95 % A, 70 % at min 1, 50 % at 2.5 min, 15 % at min 8, 0 % of mobile phase B from min 10–20. After this 20-min run time, 5 min of post-run time followed using initial 95 % of A. The flow rate was held constant (0.2 mL min^{-1}) during the whole process, and the injection volume was 10 μL in every case. Fragmentor voltages, collision energies applied to the compounds under study, along with their retention times and their optimized SRM transitions are described in Table SI-1. The calibration samples were prepared by spiking ARs (coumafuryl, coumatetralyl, warfarin, diphacinone, chlorophacinone, brodifacoum, bromadiolone, difenacoum, flocoumafen and difethialone) at 2.5–50 ng mL^{-1} levels into blank blood samples obtained from unexposed hens (*Gallus gallus domesticus*) from the Laboratory Animals section from University of Murcia, with authorization code CEEA 177/2015, in two replicates. The linearity (R) was >0.992; the limits of quantification (ng mL^{-1} wet weight) were 2.5 for coumafuryl, 0.01 for warfarin, 0.04 for coumatetralyl, 0.28 for diphacinone, 0.13 for chlorophacinone, 0.03 for bromadiolone, 0.05 for difenacoum, 0.02 for flocoumafen, 0.06 for brodifacoum, and 0.26 for difethialone and the recoveries and RSD ranged 80–109 % and 1.7–9.4 %, respectively.

2.5. Coagulation assays

Coagulation tests were performed at the Clinical Pathology Service of the Veterinary Clinical Hospital of the University of Murcia using a coagulometer (Clot 2B, RAL SA, Barcelona, Spain). The prothrombin time (PT) was chosen for analysis as this parameter is altered in birds of prey within 24–48 h following the ingestion of ARs, depending on the administered dose (Rattner et al., 2010a, 2010b, 2012, 2014a). Fibrinogen was measured to assess the quality of the blood sample, as a low fibrinogen level (<50 mg dL^{-1}) can influence blood coagulation function (Hindmarch et al., 2019; Rattner et al., 2010a, 2010b). In fact, fibrinogen is a crucial protein involved in clot formation and improper sample collection and handling can reduce its levels, affecting PT readings. Thus, measuring fibrinogen prior to PT allows detection of discrepancies, ensuring a more precise evaluation of coagulation

function and avoiding clinical interpretation errors. Tests were performed once due to the limited amount of available sample.

2.5.1. Fibrinogen assay

A commercial kit from Spinreact S.A.U (Spain), based on the Clauss method (Clauss, 1957), was employed for measuring the fibrinogen concentration in plasma. This technique involves the conversion of fibrinogen into fibrin by the enzymatic action of thrombin. Therefore, the time required for clot formation is inversely proportional to the amount of fibrinogen present in the sample. Citrated plasma samples were diluted 1:10 with Imidazole buffer. Then, 20 μL of kaolin were added to 200 μL of each dilution in a tube with a mixer. The tube was incubated for 3 min in the coagulometer at 37 °C. Afterwards, 100 μL of bovine thrombin were added, and the time required for clot formation was measured.

2.5.2. Prothrombin time (PT) assay

PT test is based on the addition of thromboplastin to the plasma sample, providing an indication of the blood coagulation factor function. Thromboplastin contains tissue factor, a protein released by damaged tissues following an injury, which activates the extrinsic coagulation pathway through a series of reactions leading to the activation of coagulation factors. The addition of calcium to the citrated plasma, along with thromboplastin, activates factor VII, which in turn converts factor X into activated factor Xa. Then, factor Xa converts prothrombin into thrombin, the enzyme responsible for converting fibrinogen present in the blood into fibrin, thereby leading to clot formation. The use of avian-derived thromboplastin is essential to obtain precise and relevant data in avian PT analysis. Chicken thromboplastin is usually employed for this purpose, as it has been observed that the use of mammalian thromboplastin significantly increases the estimated PT in birds (reviewed by Webster, 2009).

The thromboplastin reagent was prepared using the Quick method modified by Griminger et al. (1970), employing the brains of approximately two-week-old chickens (*Gallus gallus*) from the Laboratory Animals section from University of Murcia (authorization CEEA 547/2019). After sacrificing the chickens, their cerebral hemispheres were immediately excised and ground with pre-cooled acetone in a mortar for several minutes. The resulting mixture underwent suction filtration twice to separate the supernatant acetone and eliminate most of the lipids and water. The remaining brain tissue was then dried under reduced pressure overnight in a desiccator, followed by grinding in a mortar. The resultant thromboplastin extract was stored in cryotubes at −80 °C until further use.

To conduct the PT test, 50 mg of chicken thromboplastin were reconstituted in 2.5 mL of 25 mM $CaCl_2$ in a Falcon tube. The mixture was subjected to agitation for 15 min and centrifuged at 1800 rpm for 20 min. The supernatant was diluted 1:1 with 25 mM $CaCl_2$. For the PT analysis, 200 μL of the prepared reagent were transferred in a tube with a mixer and incubated for 3 min at 37 °C. Following this, 100 μL of citrated plasma sample, previously incubated at 37 °C for 3 min, were added. At that stage, the reaction started and the coagulometer recorded the time required for clot formation. Only samples obtained in 2022 breeding season were preserved adequately for this purpose (in citrate buffer). Hence, 40 blood samples were collected from 20 nests in that year, resulting in 35 citrated plasma samples. In five individuals, an aliquot for the coagulation assays was not separated due to insufficient sample volume.

2.6. Statistical and land use analysis

In order to investigate potential effects of land uses on AR exposure, we created 1-km and 1.5-km buffers using QGIS Geographic Information System open source (version 3.16.16). These buffer sizes were chosen according to prior research on the long-eared owl home range in agricultural, suburban, and mixed landscapes, which yielded similar results across different seasons of the year (Emin et al., 2018; Henrioux, 2000; Lövy and Riegert, 2013). Data on land uses were obtained from the CORINE Land Cover 2018 (European Environment Agency (EEA), 2018) and complemented with the SIOSE land use map (Instituto Geográfico Nacional (IGN), 2016) to acquire fine-scale information. Land uses were grouped into three main classes: natural vegetation, artificial areas and agricultural land. Within the agricultural land, two additional classes were defined: total non-irrigated agricultural land (including cereal crops, nut trees, vineyards, and olive groves) and total irrigated agricultural land (including irrigated arable land, citrus orchards and non-citrus orchards). For each land class and additional grouping, the percentage of surface area within each 1-km and 1.5-km buffer was calculated.

Median, mean ± SD, and range values were calculated for the ten ARs analysed in blood samples (see Table 2). To investigate the impact of environmental variables on the concentration of ARs, the detected compounds were summed for each individual (ΣARs). We used the information-theoretic methodology outlined by Burnham and Anderson (2002) to examine variations in blood ΣAR concentrations in relation to environmental factors such as monitoring year, study sites, and land uses (Table 1). Linear Mixed Models (LMM) were employed, using "lme" function from the "nlme" package (Pinheiro et al., 2023), with environmental variables considered as fixed effects and territory as a random factor. All models were compared against a null model, and these comparisons were conducted using the corrected Akaike information criterion (AICc). We calculated delta AICc to assess the strength of evidence and AICc weights to represent the relative likelihood of each model (Burnham and Anderson, 2002).

Descriptive statistics of diet composition were obtained by grouping the data according to the study site, in order to assess potential differences in the categories of prey consumed and discuss their relationship with AR exposure.

Spearman's correlation test was utilized to examine correlations between ΣARs and PT. Statistical significance was determined at $p < 0.05$. Statistical analyses were performed using R version 4.3.1 software.

3. Results

3.1. Concentrations and frequency of ARs in long-eared owls

A total of 69 blood samples from 38 nests were analysed, ranging from 1 to 3 samples per nest, with a mean ± SD of 1.82 ± 0.77 ng mL^{-1} (Table 2). Data resulting from the two monitoring years were grouped and analysed together, as no significant interannual differences were found in the total concentration (ΣARs), nor in the observed prevalence. Residues of ARs were detected in 98.6 % of long-eared owl nestlings (Table 2), with 68 out of 69 samples testing positive for at least one rodenticide. Six out of the 10 analysed ARs were detected in the sampled

Table 1
Models used to investigate the potential effects of selected environmental variables on total AR concentration (ΣARs). Land use variables were computed within 1 and 1.5 km radius buffers.

Model notation	Model description	Variable type	Assessment
m_study site	Corresponding to the study sites described above	Qualitative	Geographical differences
m_year	Year (2021−2022)	Qualitative	Inter-year differences
m_art_areas	Artificial areas	Quantitative	Land-use effect
m_nat_veg	Natural vegetation	Quantitative	Land-use effect
m_agr_land	Agricultural land	Quantitative	Land-use effect
m_non_irr_crops	Total non-irrigated crops	Quantitative	Land-use effect
m_irr_crops	Total irrigated crops	Quantitative	Land-use effect
m_null	Null model	–	Model comparison

Table 2
Detection frequency (%) and descriptive statistics of AR concentrations (ng mL^{-1}) detected in long-eared owl nestlings ($n = 69$) from the Region of Murcia.

	n+	%	Mean	SD	Median	Min.	Max.
FGARs							
Coumafuryl	0	0	–	–	–	–	–
Warfarin	0	0	–	–	–	–	–
Coumatetralyl	1	1.4	0.05	–	0.05	0.05	0.05
Diphacinone	0	0	–	–	–	–	–
Chlorophacinone	3	4.3	0.56	0.22	0.44	0.43	0.81
SGARs							
Bromadiolone	26	37.7	0.31	0.31	0.19	0.07	1.50
Difenacoum	27	39.1	0.38	0.42	0.18	0.13	1.71
Flocoumafen	61	88.4	1.30	4.62	0.25	0.03	33.57
Brodifacoum	44	63.8	1.05	1.29	0.44	0.10	5.85
Difethialone	0	0	–	–	–	–	–
ΣFGARs	4	5.8	0.43	0.31	0.43	0.05	0.81
ΣSGARs	68	98.6	2.11	4.72	0.72	0.06	34.18
ΣARs	68	98.6	2.13	4.72	0.77	0.06	34.18

owls. The prevalence of FGARs was rather low (5.8 %, $n = 4$) compared to that of SGARs, which accounted for 98.6 % ($n = 68$) and contributed to the high prevalence observed in the total AR concentration. Among the detected SGARs, flocoumafen showed the highest prevalence (88.4 %, $n = 61$), followed by brodifacoum (63.8 %, $n = 44$), difenacoum (39.1 %, $n = 27$), and bromadiolone (37.7 %, $n = 26$). As for the samples that tested positive, 1 to 4 compounds were identified in each of them. Indeed, 82.6 % (57/69) of the samples tested positive for multiple ARs. Specifically, within this group, 36.2 % displayed residues for two ARs, 39.1 % for three, and 7.2 % for four.

In owlets positive for at least one compound, the ΣARs ranged from 0.06 to 34.18 ng mL^{-1} (median = 0.77 ng mL^{-1}). When considering only SGARs, which exhibited the highest blood concentrations, the highest value corresponds to flocoumafen (33.57 ng mL^{-1}), followed by brodifacoum (5.85 ng mL^{-1}), difenacoum (1.71 ng mL^{-1}), and bromadiolone (1.50 ng mL^{-1}).

Taking the nest as a unit of analysis, the prevalence was 100 %, meaning that at least one compound was detected in all the sampled nests. Additionally, the highest ΣARs was again 34.18 ng mL^{-1}, which belonged to a nest where only one nestling was sampled. FGARs were detected in 5.23 % of nests (2/38), while SGARs were found in 100 % of nests.

3.2. Analysis of variables influencing AR levels

The mixed linear model used to analyse the different variables of study site, year, and land use classes showed that the "study site" was the highest ranked variable in relation to ΣAR results (Table 3). In fact, the highest AR levels (34.18 and 17.39 ng mL^{-1}) were recorded in two individuals from different nests present in the "Campo de Cartagena" study site (Fig. 2). In spite of the "study site" being the highest-ranking model, high unconditional standard errors suggest a significant level of uncertainty in the model.

3.3. Diet characterization

A total of 916 prey items have been identified, originating from the breeding territories of "Campo de Murcia" ($n = 86$), "Campo de Cartagena" ($n = 288$), "Saladares del Guadalentín" ($n = 432$), and "Puerto Lumbreras" ($n = 110$). Overall, we can confirm that the long-eared owl is a generalist species primarily feeding on rodents (51.4 % on average per study site, $n = 868$), followed by birds (44.2 %, $n = 432$) and to a lesser extent rabbits (3.3 %, $n = 21$) (see table SI-2). Prey remains of reptiles, invertebrates, and shrews were occasionally found. Most of the avian prey were granivorous and insectivorous species (21.5 % of the diet, $n = 218$), followed by exclusively granivorous (11.8 %, $n = 79$) and insectivorous birds (9.2 %, $n = 122$). Interestingly, considerable differences in the diet of this nocturnal raptor were observed depending on the study site, confirming that feeding habits can vary based on the prey availability in its hunting grounds. For instance, while in the "Campo de Cartagena" and "Campo de Murcia" the proportion of rodents and birds was quite similar (70.1 % vs 68.0 % rodents and 24.1 % vs 23.3 % birds), the proportion of avian prey increased in "Saladares del Guadalentín" (58.5 %) and reached 78.3 % in "Puerto Lumbreras".

3.4. Biomarker analysis

Regarding the analysis of blood coagulation parameters, the calculated fibrinogen concentration was above 70 mg dL^{-1} (range 77.9–186.2 mg dL^{-1}) in all samples, therefore sufficient to sustain blood clotting. This also suggests that the samples were properly collected and the studied individuals did not have any other clinical conditions that could negatively affect the coagulation process (such as liver or kidney failure).

The mean PT calculated for the long-eared owl nestlings was 11.6 ± 1.6 s, with a range of 6.4–15.3 s. The correlation between ΣAR values and PT was found to be positive and significant ($Rho = 0.547, p < 0.001$)

Table 3
Ranking of the models used to explain variations in blood AR levels (ΣARs) in long-eared owl nestlings, based on Akaike's information criterion (AIC).

	k	AICc	ΔAICc	*w*	cum. *w*	wt. PE	unc. SE
m_study_site	6	377.2686	0.0000	0.9249	0.9249	$\beta_{SG} = 0.96543$	2.435
						$\beta_{PL} = 4.4404$	2.276
						$\beta_{CC} = 1.7826$	2.307
						$\beta_{CM} = 1.0444$	2.486
m_null	3	384.3120	7.0434	0.0273	0.9523	$\beta = 0.13663$	0.746
m_year	4	385.3147	8.0461	0.0166	0.9688	$\beta_{2021} = 0.04195$	0.358
						$\beta_{2022} = 0.04292$	0.364
m_art_areas_1km	4	385.8406	8.5720	0.0127	0.9815	$\beta = 0.00370$	0.039
m_art_areas_1.5 km	4	387.5634	10.2948	0.0054	0.9869	$\beta = 0.00043$	0.018
m_nat_veg_1.5 km	4	389.0822	11.8136	0.0025	0.9894	$\beta = -0.00008$	0.006
m_non-irr_crops_1km	4	389.8253	12.5567	0.0017	0.9912	$\beta = -0.00003$	0.003
m_agr_land_1.5 km	4	389.8385	12.5699	0.0017	0.9929	$\beta = 0.00001$	0.003
m_non-irr_crops_1.5 km	4	389.8876	12.6190	0.0017	0.9946	$\beta = -0.00003$	0.003
m_nat_veg_1km	4	390.1314	12.8628	0.0015	0.9961	$\beta = -0.00002$	0.003
m_agr_land_1km	4	390.2082	12.9396	0.0014	0.9975	$\beta = -0.00002$	0.002
m_irr_crops_1.5 km	4	390.2812	13.0126	0.0014	0.9989	$\beta = 0.00005$	0.002
m_irr_crops_1km	4	390.6935	13.4249	0.0011	1.0000	$\beta = 0.02224$	0.002

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; cum. *w* = cumulative Akaike's weight; wt. PE = weighted parameter estimates; β = weighted parameter estimate; unc. SE = unconditional standard error; SG = Saladares del Guadalentín; PP = Puerto Lumbreras; CC = Campo de Cartagena; CM = Campo de Murcia.

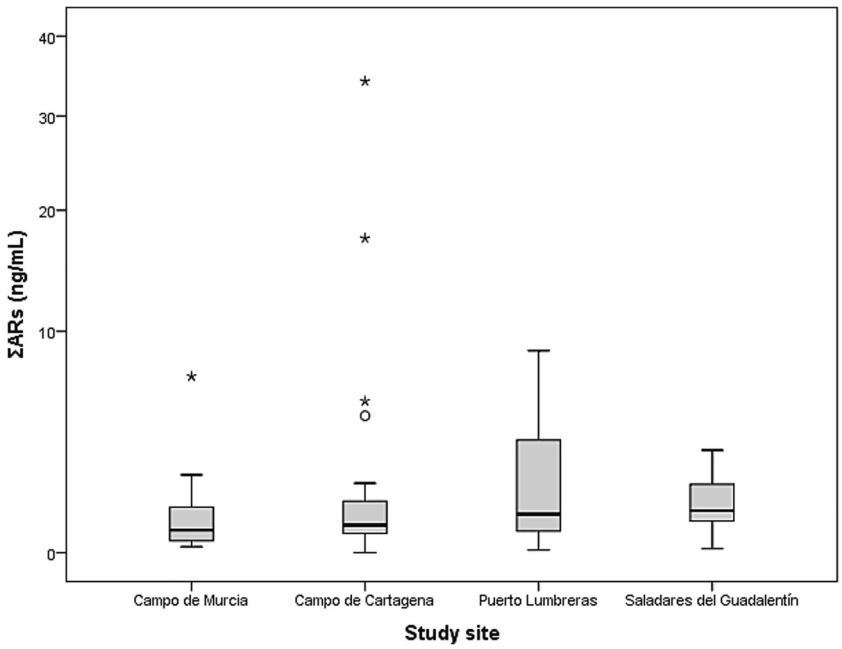

Fig. 2. Box plots showing AR total concentration (ΣARs) in the blood of long-eared owl nestlings across four different agricultural landscapes in the Region of Murcia (SE Spain). The y-axis is scaled using a square root transformation to improve visualization of the results.

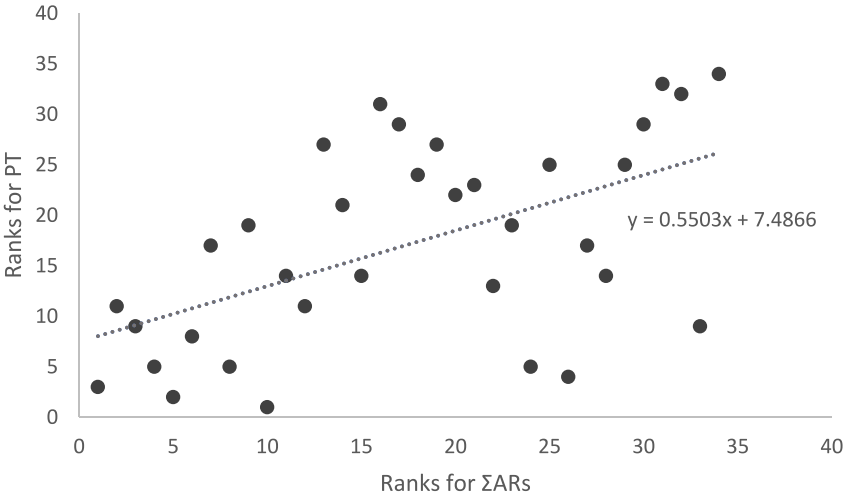

Fig. 3. Correlation between the ranks for prothrombin time (PT) (s) and detected ΣARs (ng mL^{-1}) in long-eared owl nestlings ($n = 34$) blood ($Rho = 0.547$, $p < 0.001$). The regression line represents the trend in the data, showing the general direction and strength of the relationship between ΣARs and PT.

(Fig. 3). A pair of values (ΣARs = 17.39 ng mL^{-1}; PT = 10 s) was excluded from the analysis as the concentration value was considered an outlier. By doing so, the positive and significant correlation was further enhanced.

4. Discussion

To our knowledge, this is the first study detecting ARs in blood samples from free-ranging long-eared owl nestlings through active biomonitoring. Overall, studies on AR exposure in the long-eared owl are scarce and rely on the detection of compounds in liver samples from individuals found dead in the field (López-Perea and Mateo, 2018; Nakayama et al., 2019). However, exposure rates in these investigations were consistently high, ranging from 60 to 100 %. In a study conducted in Denmark analysing only SGARs, the prevalence was 95 % ($n = 38$), and difenacoum (72.2 %) and brodifacoum (63.2 %) were the most detected compounds (Christensen et al., 2012). In the Canary Islands (Spain), residues of SGARs were detected in 100 % ($n = 34$) (Rial-Berriel et al., 2021b), 75 % ($n = 68$) (Rial-Berriel et al., 2021a) and 73.9 % ($n = 23$) (Ruiz-Suárez et al., 2014) of the analysed long-eared owl livers, where bromadiolone and brodifacoum were the most frequent compounds. Furthermore, in all cases of poisoning ($n = 8$) in *A. otus*, ARs were identified as the main cause of death and, overall, they were the most commonly detected compounds in the pesticide analysis across different species (Luzardo et al., 2014). Regarding mainland Spain, the AR prevalence in long-eared owls was lower, being 58.3 % ($n = 12$) in Catalonia (López-Perea et al., 2015) and 67 % ($n = 3$) in Aragon (López-Perea et al., 2019), although this could be due to the low number of samples analysed for this species.

Studies conducted in blood of predatory birds belonging to different age classes were almost exclusively carried out on diurnal and scavenger species (Abernathy et al., 2018; Badry et al., 2022; Herring et al., 2022;

Kwasnoski et al., 2019; Martínez-Padilla et al., 2017), with detection frequencies exceeding 50 % in the black kite (*Milvus migrans*), red kite (*Milvus milvus*), Egyptian vulture (*Neophron percnopterus*) (Oliva-Vidal et al., 2022), and American kestrel (*Falco sparverius*) (Buechley et al., 2022). As for owls, Gómez-Ramírez et al. (2012) did not find any AR residues in Eurasian eagle-owl (*Bubo bubo*) nestlings and adults from the same study area as ours, while Rial-Berriel et al. (2020a) detected the presence of ARs in 19.4 % of barn owls (*Tyto alba*) sampled in Castilla-León (Spain).

It is of significance to remember that, following repeated exposures, ARs progressively accumulate in the liver over time, making it the primary target organ for conducting toxicological analyses on these compounds (Valverde et al., 2021). After ingestion, ARs are absorbed by the gastrointestinal tract and enter the bloodstream, where they can persist for a few days (Watt et al., 2005). They bind to plasma proteins and are rapidly transported to tissues, mainly to the liver (Damin-Pernik et al., 2017). Moreover, the metabolism and toxicokinetic of each compound varies according to the species (Erickson and Urban, 2004; Watanabe et al., 2010). Therefore, it is presumable that the half-life (and consequently the probability of detection in case of exposure) of the ARs in the blood depends on species-specific physiological characteristics.

The low AR concentrations detected in our samples could be attributed to the short half-lives of these substances in the bloodstream (plasma half-lives of 27–34 h in chickens; Fisher, 2009; Watanabe et al., 2015) and may indicate recent exposure. Based on these considerations, it is therefore advisable to sample all chicks within each nest and to avoid the use of a blood pool, where possible, in order to increase chances of identifying cases of exposure. Nevertheless, our AR concentration ranges were comparable to those obtained in the blood of nestlings from other raptor species (see Table 4) (Badry et al., 2022; Martínez-Padilla et al., 2017; Oliva-Vidal et al., 2022). Interestingly, previous research found that non-nestling individuals exhibited higher concentrations and prevalence compared to chicks (Buechley et al., 2022; Oliva-Vidal et al., 2022), as also observed in studies based on AR liver analysis (Badry et al., 2021; Roos et al., 2021). The higher levels of ARs found in adult birds of prey compared to chicks may be attributed to prolonged exposure over time, leading to bioaccumulation in the liver. Furthermore, enterohepatic circulation may contribute to increasing blood levels in adult individuals, through the continuous reabsorption of compounds from the intestine into the bloodstream (Watt et al., 2005). These findings suggest that nestling exposure may sometimes be underestimated. It has also been hypothesized that this variation between adult and chick detection rates may be due to differences in the diet of nestlings compared to adults (Buechley et al., 2022). Therefore, the results obtained in our study appear noteworthy, even in the presence of low blood levels, as they indicate a high exposure probability in owl chicks throughout their developmental phases.

4.1. Analysis of variables influencing AR levels

Some studies showed that AR exposure was higher near urban areas (Nogeire et al., 2015) and at high human population density (Badry et al., 2021; López-Perea et al., 2015), while others found an association with the presence of intensive livestock farming in the area where the analysed carcasses were found (Geduhn et al., 2015; Ruiz-Suárez et al., 2016). The percentage of cultivated land was also identified as a risk factor for AR contamination (Sainsbury et al., 2018). In contrast, other studies highlighted that AR presence was not restricted to a specific land use, thus indicating their widespread occurrence across different landscapes (Cooke et al., 2022; Wiens et al., 2019).

In our study, the rate of AR occurrence in the assessed nests was absolute (100 %), which prevented the use of AR prevalence to study its potential associations with different sampling sites and land uses. It becomes evident that, despite geographical and land-use differences, ARs have been applied in various contexts and environments throughout the study area. Moreover, the long-eared owl commonly inhabits agricultural and rural landscapes in close proximity to human settlements in this region, which potentially represents a significant risk factor for AR contamination. We therefore opted to study the extent of AR contamination in relation to the aforementioned environmental variables. The only explanatory factor of AR levels seemed to be the "study site", indicating higher AR concentrations in the "Campo de Cartagena". Nevertheless, there was considerable uncertainty in the statistical model, indicating that the study site was not a suitable predictor of the extent of AR exposure. Furthermore, the results demonstrated that the extent of exposure was independent of all other factors examined, including those related to agricultural land classification. Consequently, we hypothesized that the higher concentrations observed in this area may be attributed to isolated instances of AR application near some of the sampled nests, rather than reflecting a widespread higher use across the entire study site. Hence, the concentration of ARs did not exhibit a discernible relationship with any of the environmental variables examined in this study. These results imply that blood ΣARs were probably influenced by additional factors, such as the time of exposure and the AR dose ingested. This is also consistent with the results reported by Badry et al. (2021) who analysed samples from four predatory bird species and proposed that AR levels are influenced by factors associated with the ecology and trophic traits of each species.

Due to growing concerns about human and environmental health, especially regarding the impact on wildlife, risk mitigation measures for ARs are in force in Europe and in other countries, such as the USA and Australia. These measures often include site-specific usage restrictions, concentration limits for authorized products, the obligation to use bait stations, and recommendations for a correct and safe use of products by the general public and professionals (e.g., removal of dead rodents and regular monitoring of bait stations). However, regulations are based on a cost-benefit estimate and do not completely prohibit the sale of AR products (Eisemann et al., 2018). Furthermore, their effect is not immediate, as demonstrated by wildlife biomonitoring studies, which show that AR contamination is still present with high prevalences, even years after the implementation of these strategies (Elmeros et al., 2018; Murray, 2020). Moreover, despite their hazardous nature, ARs are still permitted in Europe due to the lack of effective alternatives (*European Union*, 2012). The Regulation (EC) No. 1272/2008 (*European Union*, 2008) on the classification, labelling, and packaging of chemical substances and mixtures (CLP) was updated in 2018, reclassifying ARs with a concentration $\geq$ 30 ppm as reprotoxic products. As a result, new products with lower concentrations were introduced into the market, raising concerns about their unrestricted availability and the limited knowledge regarding their efficacy and potential development of rodent resistance. Studies have shown that ARs are extensively used in European farmlands (Geduhn et al., 2014; Hughes et al., 2013; Tosh et al., 2011). Although registered in Spain as biocides, AR compounds are not registered as plant protection products. Nevertheless, they can be used by professional staff (such as farmers) indoors and around buildings such as warehouses and other structures, which can explain their detection in agricultural environments. Additionally, formulations with low concentrations (<30 ppm) can be purchased by the general public and are not subject to any control over their use. This undoubtedly contributes to their widespread utilization and prevalence, closely related to human presence in various settings.

4.2. Implications of the diet

The results suggest that, even when the diet is predominantly composed of birds, exposure to ARs is possible, although they are non-target species. Similar results were obtained in studies conducted on specialized bird-eating predators, such as the Eurasian sparrowhawk (*Accipiter nisus*) (Broughton et al., 2022; Hughes et al., 2013; Ruiz-Suárez et al., 2014), supporting the existence of alternative exposure pathways involving avian prey. In fact, granivorous birds can directly come into contact with ARs used in agriculture that are presented as AR-

Table 4
Concentrations of ARs (ng mL^{-1}) in blood of raptor nestlings. The values shown for each AR compound refer to the detection rate and below are the concentrations in the form of median (or mean ± SD, when indicated with an asterisk) and range [min-max]. Concentrations are given for individuals with detected levels of ARs.

Species	Sampling year	Country	n	%	Warfarin	Coumatetralyl	Chlorophacinone	Bromadiolone	Difenacoum	Flocoumafen	Brodifacoum	Difethialone	Ref.[b]
Long-eared owl *Asio otus*	2021-2022	Spain	69	98.6	n.d.	1.4% (n+= 1) 0.05	4.3% (n+= 3) 0.44 [0.43-0.81]	37.7% (n+= 26) 0.19 [0.07-1.50]	39.1% (n+= 27) 0.18 [0.13-1.71]	88.4% (n+=61) 0.25 [0.03-33.57]	63.8% (n+= 44) 0.44 [0.10-5.85]	n.d.	1
Egyptian vulture *Neophron percnopterus*	2017-2021	Spain	33	45.5	-	-	-	ΣSGARs = 8.82* [0.12-28.02]					2
Bearded vulture *Gypaetus barbatus*	2017-2021	Spain	7	42.9	-	-	-	ΣSGARs = 2.53* [0.54-4.35]					2
Griffon vulture *Gyps fulvus*	2017-2021	Spain	7	0	-	-	-	-					2
Cinereous vulture *Aegypius monachus*	2017-2021	Spain	16	6.3	-	-	-	ΣSGARs = 0.17					2
Red kite *Milvus milvus*	2017-2021	Spain	20	55.0	-	-	-	ΣSGARs = 7.61* [0.49-18.44]					2
Common kestrel *Falco tinnunculus*	2014	Spain	112	16.9	-	-	-	16.9% (n+=19) 1.46* [0.01–6.55]	-	-	-	-	3
Eagle owl *Bubo bubo*	-	Spain	41	0	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	-	4
Common buzzard *Buteo buteo*	2019-2020	Germany	35	8.6	5.7% (n+=2) 1.0 [1.0-1.0]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.9% (n+=1) 27	5
Red kite *Milvus milvus*	2019-2020	Germany	53	22.6	1.9% (n+ =1) 1.0	5.7% (n+= 3) 1.0 [1.0-1.5]	n.d.	n.d.	7.6% (n+= 4) 6.5 [2.5-10.3]	n.d.	9.4% (n+= 5) 13.0 [8.0-13.0]	n.d.	5
Montagu's harrier *Circus pygargus*	2019-2020	Germany	29	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
White-tailed sea eagle *Haliaeetus albicilla*	2019-2020	Germany	64	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
Osprey *Pandion haliaetus*	2019-2020	Germany	23	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
Red kite *Milvus milvus*	2015	France	47	30	n.d.	n.d.	-	15% (n+=7) 16.9 [0.2–29.4]	9% (n+=4) 0.7 [0.5–2.5]	n.d.	9% (n+=4) 1.6 [0.6–3.0]	4% (n+=2) 6.9 [4.3–9.5]	6
American kestrel *Falco sparverius*	2019-2020	USA	59[a]	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7% (n+=1) 1.1 < QL	n.d.	7

"n" = number of sampled animals; "n.d." = not detected; "-"= no data available or not analysed. [a] the samples consisted of a pool of blood from different chicks of the same nest. In the present study, in Buechley et al. (2022), Gómez-Ramírez et al. (2012) and Powolny et al. (2020), diphacinone was also analysed but not detected, while in this study and in Buechley et al. (2022), coumafuryl was analysed but not detected. In Oliva-Vidal et al. (2022), all SGARs except difethialone were tested. [b] References: 1. This study; 2. Oliva-Vidal et al. (2022); 3. Martínez-Padilla et al. (2017); 4. Gómez-Ramírez et al. (2012); 5. Badry et al. (2022); 6. Powolny et al. (2020); 7. Buechley et al. (2022).

treated grains and seeds, thus facilitating their ingestion by small birds (Sánchez-Barbudo et al., 2012). Passerine birds can also enter bait boxes and consume the ARs present inside (Elliott et al., 2014; Walther et al., 2021). Additionally, it has been observed that invertebrates were able to access and feed on AR baits (Ogilvie et al., 1997; Spurr and Drew, 1999), involving a secondary exposure route for insectivorous mammals and birds (Dowding et al., 2010; Masuda et al., 2014), and a possible tertiary exposure route for the long-eared owl and other bird-eating raptors. In fact, as mentioned above, the prevalence of ARs was absolute (100 %) in the sampled nests, regardless of the study site and the main prey consumed. Exploring the accumulation and transfer of ARs along the food chain will be essential to corroborate these hypotheses and thoroughly evaluate the extent of AR spread and the potential risks these compounds pose to higher trophic levels.

4.3. Interpretation of prothrombin time findings

PT can be used to early detect AR intoxication in domestic mammals (Murphy, 2002) and in human medicine to assess blood coagulation function and monitor the effectiveness of anticoagulant medications (Levy et al., 2014). Establishing a blood threshold concentration for coagulopathy appears challenging because the baseline PT value is species-specific and unknown in most avian species. Controlled-feeding trials conducted on raptors indicate that this parameter is rapidly altered in a dose-dependent manner following exposure to ARs and returns to baseline values within a few days (Rattner et al., 2010a, 2012; Webster et al., 2015), thus serving as a valid biomarker of effect. Some experimental studies conducted under controlled conditions have found a relationship between the dose of ARs ingested or the hepatic AR concentration measured during experiments and the onset of coagulopathy. This allowed to establish a toxic dose for PT for 50 % of American kestrels, corresponding to 79.2 μg of chlorophacinone consumed/kg body weight/day (Rattner et al., 2015). Similarly, it has been possible to calculate a hepatic threshold concentration capable of causing pathological PT alteration in 90 % of Eastern screech owls (*Megascops asio*), corresponding to 0.638 μg/g wet weight (Rattner et al., 2014a). However, using PT as a biomarker of AR effect in free-ranging birds of prey is still challenging as there are no established physiological reference values for the long-eared owl or other nocturnal raptors. The only exception is represented by the Eastern-screech owl (mean ± SD equal to 9.3 ± 1.87 s, Rattner et al., 2014a), which was used by Hindmarch et al. (2019) as a reference species to evaluate coagulation in other nocturnal birds of prey. In the same study, it was observed that American barn owl (*Tyto furcata*) free-living nestlings generally did not show prolonged PT and therefore they appeared to have little to no exposure to ARs. It has also been proposed that a PT value that deviated from the mean by at least two standard deviations was indicative of coagulopathy (Rattner et al., 2012). Adopting this criterion and using the available baseline PT values for the Eastern screech owl, only three of our PT values (15.3, 13.7 and 13.1 s) would be indicative of coagulopathy in the long-eared owl nestlings. Probably, the owlets examined in the current study, although already exposed, had not yet accumulated toxic concentrations at the hepatic or blood level and were unlikely affected by haemostatic disorders. Similar results have been reported in other studies on nestling and adult free-ranging raptors, where prolonged PT was not detected (Herring et al., 2022; Webster et al., 2015). Regarding adult individuals, no association was identified between PT values and hepatic residues of ARs, possibly due to the small sample size (Hindmarch et al., 2019; Hopf-Dennis et al., 2022).

Nevertheless, in this study on free-living owlets, a significant and positive correlation between the detected blood ΣAR and PT was found, reflecting the direct effect of the presence of ARs in the nestlings' organism. This suggests that these individuals, if exposed to repeated doses of multiple compounds over time, may develop coagulopathy (Rattner and Harvey, 2021). Moreover, the inhibitory effect of ARs on the VKOR complex was much more prolonged (over 30 days in laboratory rats), and a subsequent dose of ARs caused a more severe coagulopathy, demonstrating the potential additive effect of repeated doses (Mosterd and Thijssen, 1991). Most adult raptors enter rehabilitation centres due to trauma (Cococcetta et al., 2022; Molina-López et al., 2011; Montesdeoca et al., 2016), and often AR effects are not recognized because they are not evident or the injuries can mask or confuse their symptoms (Murray, 2018). However, it should be noted that in the case of an increased coagulation time, even a minor trauma can be fatal. Nevertheless, coagulation tests are not routinely performed, not only due to the lack of reference intervals but also because the analytical methods are not standardized and specific reagents are required for birds (e.g., avian thromboplastin). In fact, commercial kits used in human medicine and for domestic mammals contain mammalian thromboplastin and are not suitable for avian sample analysis (Dickson et al., 2020; Tahira et al., 1977). Furthermore, it is not possible to ascertain when AR ingestion occurred or at what dose in wild animals, which adds complexity to the interpretation of the results. In conclusion, we recommend to standardize procedures for performing coagulation assays and investigate coagulation parameters in adult raptor individuals.

4.4. Risk assessment

A risk threshold for ARs has been proposed and widely used in the liver of predatory birds (20 ng g^{-1}, Thomas et al., 2011), although it has been more recently questioned (Rattner and Harvey, 2021). Regarding blood samples, there is no defined blood concentration threshold that can be used to diagnose poisoning. The toxicity of these compounds depends on various factors, such as the type of rodenticide, duration of exposure, individual susceptibility, and other variables (Rattner and Harvey, 2021). Therefore, simple exposure to ARs does not necessarily imply the occurrence of harmful effects or toxicosis. AR levels found in our study were very low, and it was unlikely that they could cause acute toxicity. However, these results show that the long-eared owl is exposed to ARs from early life stages, so continuous exposure is expected throughout the entire life history. It is also notable that multiple ARs were detected in most of the sampled owlets (82.6 %). Because most ARs are highly accumulative with long hepatic half-lives (Fisher et al., 2003; Watanabe et al., 2015), there could be a medium to long-term risk of exceeding the proposed hepatic threshold. Little is known about the sublethal effects that these toxics can cause in non-target wildlife that come into chronic contact with them. Some of the observed effects comprise an identified relationship between AR contamination and increased stress response (Fraser et al., 2018; Herring et al., 2023), impaired thermoregulatory function (Vyas et al., 2022), immune system disorders (Serieys et al., 2018), reduced body condition (Elmeros et al., 2011; Martínez-Padilla et al., 2017) and severeparasitic infestation (Serieys et al., 2015). By contrast, Kwasnoski et al. (2019) investigated the association between plasma occurrence of ARs and parasitaemia in juvenile free-living red-tailed hawks (*Buteo jamaicensis*) but found no significant relationship, which may suggest the need of further research in this field.

Regarding the identified ARs, it is not surprising that FGARs were present in only a small percentage of the samples, given the declining use of these compounds, which have been progressively replaced by more potent SGARs. Moreover, the only two compounds not detected (coumafuryl and diphacinone) are not registered in Europe (EC Regulation 528/2012, *European Union*, 2012). Conversely, we detected the presence of flocoumafen in most of the blood samples (88.4 %), contrasting with previous studies where this substance was either not detected or present at lower rates (Moriceau et al., 2022; Oliva-Vidal et al., 2022; Roos et al., 2021; Sánchez-Barbudo et al., 2012). Flocoumafen is a SGAR synthesized in the 1980s, with intermediate potency between bromadiolone and brodifacoum, and variable toxicity depending on the species. Initial toxicity assessments indicated that flocoumafen is more toxic to pigeons (*Columba livia*) than to chickens (Lund, 1988) and its hepatic half-life in the barn owl is >100 days

(Newton et al., 1994). A recent study demonstrated that this AR has the ability to bind to numerous target proteins and disrupt various physiological mechanisms, particularly interacting with endocrine disruption and carcinogenesis processes (Coronado-Posada et al., 2021).

It is evident, therefore, that ARs cause various harmful effects to non-target species, which involve the impairment of the coagulation process as well as systemic-level effects that are not immediately lethal but can damage the health and well-being of the animal in the long term. With early exposure from birth, there is a strong likelihood that the chicks will experience these toxic effects during adulthood. Further research is required to fully understand the long-term impacts of AR exposure on wildlife populations and to elucidate the combined effects of different ARs on the avian organism.

4.5. The long-eared owl as a biomonitoring species for AR exposure

The long-eared owl showed an exceptionally high prevalence of ARs in the blood of the sampled nestlings. This could indicate that this species is frequently exposed to environmental contamination in agricultural landscapes, particularly in relation to AR use. In support of this contention, the long-eared owl emerged as the bird species with the highest count of toxic substances detected in a recent study (41 out of 351 analysed compounds) (Rial-Berriel et al., 2021b). Moreover, as a medium-sized predator, its frequent contamination suggests that these toxic substances are accumulating in the food chain and clearly indicates potential risks not only to the long-eared owl but also to other wild species. For these reasons, this nocturnal predator can be considered an effective sentinel of the presence and persistence of pollutants in Mediterranean agroecosystems, which makes it a suitable candidate for biomonitoring studies. Its suitability as a biomonitoring species is further supported by its wide distribution range (throughout the Holarctic region), which could facilitate broad coordinated monitoring efforts (Badry et al., 2020). In fact, AR detection in owl populations could trigger further investigations into contamination sources and prompt timely measures to safeguard the health of non-target conservation-concern species.

5. Conclusions

This study demonstrated that long-eared owl nestlings were exposed to multiple ARs in the studied Mediterranean agroecosystems. Although the detected levels were unlikely to be associated to acute toxicity, repeated doses since early life stages could increase the risk of coagulopathies and other sublethal effects, potentially impacting the species' fitness and physiology. The risk of AR contamination and the extent of exposure did not appear to be dependent on any of the considered environmental variables (study site and land uses), thereby confirming the ubiquitous presence of these substances in the environment. Furthermore, through careful diet analysis, it was observed that the predominant diet (songbirds vs rodents) of this generalist predator depended on the area and available resources, but this did not affect the AR prevalence. As already observed in other bird-eating species (e.g., the sparrowhawk), it is likely that alternative exposure routes involving birds and/or invertebrates exist, leading to the propagation of ARs through the whole food chain. Lastly, the positive correlation between ARs and PT indicated that even nestling owls demonstrate a non-lethal but measurable effect of these substances on haemostasis.

Due to its biological characteristics and its key role in Mediterranean agroecosystems, the long-eared owl can provide valuable information about the ecosystem health and the presence of ARs. Our findings confirm that this nocturnal predator is an excellent sentinel species for environmental contamination from ARs and can be used in active surveillance studies to assess the risk of exposure for other threatened or declining species present in semi-arid agricultural landscapes.

CRediT authorship contribution statement

Livia Spadetto: Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pilar Gómez-Ramírez:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **José Manuel Zamora-Marín:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Mario León-Ortega:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Sarah Díaz-García:** Resources, Methodology, Investigation. **Fernando Tecles:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **José Fenoll:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Juana Cava:** Methodology, Investigation, Formal analysis, Data curation. **José Francisco Calvo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Antonio Juan 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.scitotenv.2024.170492.

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