UNIVERSIDAD DE MURCIA

Escuela de Doctorado

TESIS DOCTORAL

Nuevos avances en la evaluación del riesgo de los rodenticidas anticoagulantes en aves rapaces del sureste Ibérico

New insights into anticoagulant rodenticide risk assessment in raptors from the southeastern Iberian Peninsula

AUTOR/A Livia Spadetto

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In loving memory of my grandmothers, Lina and Silvia, whose strength and values inspired and guided me throughout this journey.

Thesis structure

This thesis addresses the exposure and effects of anticoagulant rodenticides (ARs) on certain birds of prey species inhabiting a semi-arid region located in southeastern Iberia (Region of Murcia, Spain). Raptors serve as excellent sentinels of environmental contamination. They provide crucial information to guide targeted measures aimed at mitigating the impacts of these toxic substances on both the studied raptors and other non-target species sharing the same habitats.

The thesis is structured as follows:

- The General Introduction provides a comprehensive review of the literature on ARs, framing the research problem and justifying the methodological choices made in this study.
- Aims and objectives. The general aim of the thesis and the specific objectives addressed in each chapter are outlined in this section.
- Chapters I-IV. Each chapter corresponds to an original, standalone research study. Chapters I, II, and III have already been published in peer-reviewed journals in the field of *Environmental Sciences*, while Chapter IV is currently being prepared for submission. The cover page of each chapter specifies its publication status.
- A General discussion integrates and synthesizes the main findings from all chapters, offering a comprehensive perspective on the research outcomes and suggesting directions for future research and mitigation measures to reduce the impact of ARs on non-target species.
- The thesis concludes with General conclusions, summarizing the overall results.
- Additionally, this thesis comprises an Extended abstract along with its Spanish translation (Resumen).

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Abbreviations

AEMET: Agencia Estatal de Meteorología (Spanish State Meteorological Agency)

AICc: Corrected Akaike Information Criterion

AR: Anticoagulant Rodenticide

BCI: Body Condition Index

BGP: Bone Gla Protein

BLAST: Basic Local Alignment Search Tool

BPR: Biocidal Products Regulation

C18: Octadecylsilane (18% Carbon Content)

CARM: Comunidad Autónoma de la Región de Murcia (Regional Government of the Autonomous Community of Murcia)

CCorA: Canonical Correlation Analysis

CE: Collision Energy

CI: Confidence Interval

CLP: Classification, Labelling, and Packaging of substances and mixtures

Ca^{2+}: Calcium ions

EC: European Commission

ECHA: European Chemicals Agency

EDTA: Ethylenediaminetetraacetic Acid

EEA: European Environment Agency

EPA: Environmental Protection Agency

ESI: Electrospray Ionization Source

EU: European Union

FGAR: First-Generation Anticoagulant Rodenticide

FV: Fragmentation Voltages

GGCX: Gamma-Glutamyl Carboxylase

Gla: Gamma-carboxyglutamic acid

HPLC: High-Performance Liquid Chromatography

INE: Instituto Nacional de Estadística (Spanish National Statistics Institute)

IPM: Integrated Pest Management

LMM: Linear Mixed Model

LOD: Limit of Detection

LOQ: Limit of Quantification

N: Total Number of Samples/Individuals

NA: Not Available

ND: Not Detected

NaCl: Sodium Chloride

Na_2SO_4: Anhydrous Sodium Sulphate

nt: Nucleotide

p: Probability value (in statistical context)

PPP: Plant Protection Product

PSA: Primary Secondary Amine

PT: Prothrombin Time

PTB: Persistent, Toxic, and Bioaccumulative

ppm: Parts Per Million

qPCR: Quantitative Polymerase Chain Reaction

RDA: Redundancy Analysis

RT: Retention Time (in HPLC context) / Retro Transcription (in molecular biology context, clarified where needed)

RVVT: Russel's Viper Venom Time

s: Seconds

SANTE: Directorate-General for Health and Food Safety (EU)

SCI: Site of Community Importance

SD: Standard Deviation

SEO: Sociedad Española de Ornitología (Spanish Ornithological Society)

SGAR: Second-Generation Anticoagulant Rodenticide

SPA: Special Protection Area

SRM: Selected Reaction Monitoring

TF: Tissue Factor

TQ: Triple Quadrupole

UI: International Units

UK: United Kingdom

USA: United States of America

v: Volts

VKDP: Vitamin K Dependent Protein

VKOR: Vitamin K 2,3-Epoxide Reductase

VKORC1: Vitamin K 2,3-Epoxide Reductase Complex Subunit 1

VKORC1L1: Vitamin K 2,3-Epoxide Reductase Complex Subunit 1-Like 1

VKR: Vitamin K Quinone Reductase

w: Weight

ΔAIC: Difference in Akaike Information Criterion

Σ: Summed Concentrations

General introduction

1. Anticoagulant rodenticides: role, effectiveness, and challenges in rodent control

Anticoagulant rodenticides (ARs) are chemical compounds used to manage pest rodent populations, primarily house mice (*Mus musculus*) and rats (*Rattus rattus* and *R. norvegicus*), which pose significant threats to agriculture, public health, ecosystems, and forests (Capizzi et al., 2014). Rodents constitute the most diverse order of mammals globally, encompassing over 2,000 species (Huchon et al., 2002) and accounting for more than 40% of extant mammal species. Although only a small fraction of these species (approximately 7%) is classified as pests (Capizzi et al., 2023), their impact is substantial, causing extensive damage annually.

In agricultural settings, rodents destroy crops and contaminate food reserves, while in urban environment, they compromise building integrity and pose public health risks (Jacob and Buckle, 2018). Economically, the losses caused by rodents in agriculture can be devastating. For instance, it has been estimated that pre-harvest losses of rice in Asia due to rodent damage amount to 5%, a quantity sufficient to feed 180 million people for an entire year (Singleton, 2003). Moreover, rodents are vectors of numerous bacterial and viral diseases that can be transmitted directly or indirectly to livestock and humans, such as leptospirosis, salmonellosis, and hantavirus (Kazemi-Moghaddam et al., 2019; Meerburg et al., 2009; Morand et al., 2019). Rodent-induced damage to forests has also been reported in several countries (Imholt et al., 2017; Wood and Singleton, 2015), particularly by invasive rodent species that harm native vegetation and alter ecosystem structures (Walker et al., 2019). Furthermore, rodent introduction poses a severe risk to the survival of already vulnerable species through predation and competition for food resources. In some cases, invasive rodents have driven endemic species to extinction (Doherty et al., 2016), reducing biodiversity, especially in fragile island ecosystems (Harper and Bunbury, 2015).

For these reasons, the rodent control business is vast, relying primarily on chemical methods, traps, and habitat management (reviewed by Capizzi et al., 2023). Rodents' responses to control measures can vary significantly. On one hand, genetic responses,

such as resistance to ARs, occur when genetic mutations lead to the selection of resistant rodent populations, complicating management through traditional chemical methods (Mcgee et al., 2020). On the other hand, behavioural adaptations, such as neophobia and bait shyness, further hinder effective rodent management. Neophobia refers to rodents' innate fear or reluctance to approach new objects or environmental changes. This behaviour has been observed in various rodent species, especially in scenarios where new traps or bait stations are introduced. As a result, rodents may initially avoid traps or poisoned bait, delaying their exposure to control measures (Raj, 2018). Bait shyness occurs when rodents associate a specific bait with a negative experience, such as illness caused by sublethal exposure to a toxic substance. Once this association is formed, rodents actively avoid consuming the bait, even when faced with food scarcity. Unlike neophobia, which is a transient response, bait shyness can persist over time, significantly reducing the effectiveness of bait-based control methods (Berny et al., 2018; Raj, 2018). These genetic and behavioural adaptations make it challenging to develop control methods that are effective, safe, and species-specific targeting only pest species without affecting non-target wildlife or posing risks to humans.

ARs are currently the most widely used method for rodent control worldwide, despite the associated risks. This widespread use is primarily due to their effectiveness, practicality, and relatively low cost compared to alternative solutions (see Table 1). In fact, ARs are highly effective against a wide range of rodent species. Their mode of action causes a gradual death in affected rodents, typically within a few days (Rattner and Mastrota, 2018). This delay in symptom onset allows more individuals to consume the bait before developing any immediate suspicion, thereby reducing the risk of behavioural resistance, such as bait shyness (Berny et al., 2018).

ARs are also easily accessible and can be applied with relative simplicity and safety by professional operators, as well as by farmers and non-specialists. Furthermore, in cases of accidental poisoning, an effective antidote is available: vitamin K. AR baits are often pre-packaged and ready to use, making their application less complex than other techniques, such as mechanical traps or biological control systems. Additionally, ARs are widely available and marketed in many countries, facilitating their large-scale use. Their relatively low cost compared to other rodent control strategies makes them an attractive choice for

managing infestations over large areas and often provides temporary resolution. Lastly, their long environmental persistence enables sustained effectiveness.

Table 1. Comparison of three main rodent control methods: ARs, mechanical traps, and biological methods (e.g. the introduction of natural predators). The green background represents an advantage, the red background a drawback, and the yellow background an intermediate condition.

Characteristic	ARs	Mechanical traps	Biological methods
Effectiveness	High against a wide range of target species; delayed action avoids bait shyness	Moderate, ineffective on a large scale	Variable
Accessibility	Widely available and easy to use by non-professionals	Widely available	Limited, requires advanced knowledge
Cost	Relatively low compared to other solutions	Low per unit but expensive on a large scale	Moderate/high (habitat construction or predator introduction)
Environmental impact	Potential contamination and risks to non-target species	No contamination	Careful evaluation is needed to preserve ecological balance
Maintenance requirements	Baits and dead rodents must be removed after use to prevent non-target exposure	Requires supervision and removal of captured rodents	Requires continuous monitoring
Duration of effect	Prolonged due to long environmental persistence	Limited to the period traps are active	Potentially long, but not always predictable
Operator safety	Risk of chemical exposure if handled improperly	Safe	Safe
Behavioral resistance	Reduced due to delayed symptom onset (e.g., counters bait shyness)	High: rodents can learn to avoid traps	Not applicable

Despite the growing awareness of the risks associated with ARs, such as toxicity to non-target species (including natural predators and domestic animals), no equally efficient and widely applicable alternatives currently exist (Rattner et al., 2014b). In many regions of the world, the use of ARs has been entrenched in pest control practices for decades, meaning there is an established infrastructure for their distribution and use (Jacob and Buckle,

2018). Changing this infrastructure and training operators in new control techniques may require significant time and resources, which is why ARs remain the preferred choice in many situations. Finally, in some contexts, the risks associated with the use of ARs are often perceived as lower than the potential damage caused by an uncontrolled rodent population, leading to an underestimation of their ecological consequences (Quinn, 2019).

2. Types of anticoagulant rodenticides

In the early 1930s, scientists discovered and isolated dicoumarol, a natural anticoagulant found in poorly preserved clover hay (*Melilotus officinalis* and *M. alba*). This substance caused "sweet clover disease" in cattle, leading to severe haemorrhages and death within 30–50 days (Link, 1959). By the mid-20th century, the first synthetic anticoagulant, warfarin, was developed (Schein, 1950). Warfarin quickly gained widespread use both as a rodenticide and as an oral anticoagulant drug in human medicine. Later, second-generation ARs (SGARs), such as brodifacoum and difenacoum, were developed. These compounds are more potent and have a longer duration of action, making them effective against rodent populations that had developed resistance to warfarin and other first-generation ARs (FGARs) (Murphy, 2006). ARs are classified based on their chemical structure and potency (Murphy, 2006). This classification helps to understand the specific features, mode of action, and practical applications of each type.

ARs are divided into two main chemical categories (Figure 1):

4-Hydroxycoumarins: These compounds are derived from coumarin, a natural substance found in various plants and a precursor of dicoumarol. Chemically, they are synthesized by adding a hydroxy group at the 4-position and a large aromatic substituent at the 3-position of the coumarin molecule, which gives them anticoagulant properties. Warfarin is the most well-known compound in this group. Other examples of ARs in this category include bromadiolone, brodifacoum, coumafuryl, coumatetralyl, difenacoum, and flocoumafen.

Indandiones: These compounds share a similar mechanism of action with 4-hydroxycoumarins but have a different chemical structure. They are based on a 1,3-indandione (or indanedione) core with various side-chain substituents at the 2-position.

Indandiones are less common than hydroxycoumarins but are still used, particularly in first-generation formulations. Examples include pindone, chlorophacinone, and diphacinone.

Benzothiopyranone: Difethialone, a distinct anticoagulant, does not fall strictly into the hydroxycoumarin or indandione categories (Mcgee et al., 2020). In fact, it is derived from benzothiopyranone, a structural analogue of coumarin with the addition of a sulfur ring. While it shares the same mechanism of action, disrupting the vitamin K cycle and impairing blood clotting, its benzothiopyranone-based structure enhances its potency and prolongs its duration of action (Lechevin and Poché, 1988).

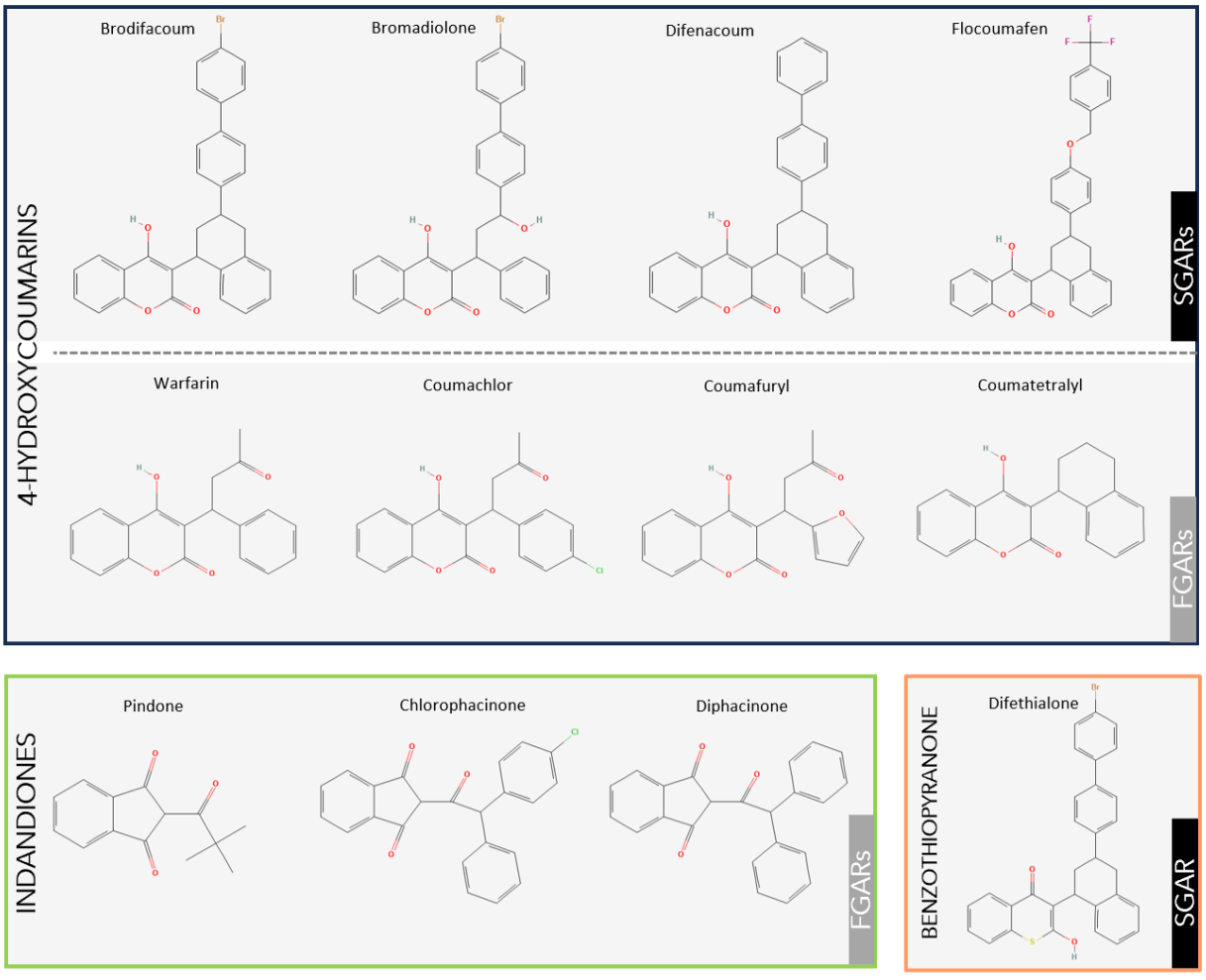

Figure 1. Chemical structure of first- and second-generation anticoagulant rodenticides (FGARs and SGARs). (Source: chemicals structures from PubChem, 2024).

Although both indandione- and coumarin-based ARs act by interfering with the vitamin K cycle and impairing blood clotting, indandiones have a simpler chemical structure. This

difference affects their duration of action and metabolism in organisms, with indandiones generally having a shorter hepatic half-life compared to second-generation hydroxycoumarins (Horak et al., 2018).

In addition to their chemical classification, ARs are distinguished by their potency and efficacy, dividing them into first-generation and second-generation products.

First-Generation ARs: These compounds are generally less potent than second-generation products and often require repeated doses to be effective, meaning that target rodents must consume the poison multiple times to accumulate a lethal dose (Jacob and Buckle, 2018). FGARs include warfarin, chlorophacinone, diphacinone, coumachlor, dicumarol, coumafuryl, and coumatetralyl. These products were the first to be developed and introduced to the market, beginning in the 1940s and 1950s. However, rodents quickly developed resistance, reducing their effectiveness in areas where they were widely used (Buckle et al., 1994; Jacob and Buckle, 2018).

Second-Generation ARs: These compounds were designed to be more potent and effective against resistant rodents, generally requiring only a single dose to be lethal. They have a longer duration of action within the rodent's body, primarily due to their higher lipophilicity and persistence in the tissues (Mcgee et al., 2020; Murphy, 2006). SGARs include brodifacoum, bromadiolone, difenacoum, flocoumafen, and difethialone. They became more common in the early 1970s (Jacob and Buckle, 2018). Although more effective, these ARs are also more persistent, posing a greater risk to non-target wildlife and requiring careful management and usage. Moreover, cases of resistance to SGARs are now widely documented in several countries (Mcgee et al., 2020).

3. Regulatory framework for anticoagulant rodenticides

The use of ARs is strictly regulated worldwide due to the potential risks these compounds pose to public health, non-target wildlife, and the environment. Regulations vary by country but are typically based on guidelines established by internationally recognized health and environmental authorities, such as the European Chemicals Agency (ECHA) or the United States Environmental Protection Agency (EPA).

The regulatory framework for ARs is continuously evolving as new evidence emerges regarding their ecological impacts and the resistance developed by rodent populations. Regulatory bodies have been working to strike a balance between the need to control rodent infestations and the protection of the environment (van den Brink et al., 2018). In the future, further restrictions on the use of these compounds and increased adoption of safer and more sustainable alternatives are expected.

3.1. Global regulatory framework

Globally, the regulation of ARs varies significantly. In the United States, for example, the EPA oversees the regulation of these products under the Federal Insecticide, Fungicide, and Rodenticide Act. The registration and approval of rodenticides require evidence that the product is effective and that its use does not pose unacceptable risks to human health or the environment. Additionally, restrictions on the use of SGARs to reduce exposure risks for wildlife and other non-target animals have been recently implemented (EPA, 2024).

In other countries, such as Canada, Australia, and Japan, similar regulations require risk assessments for ARs before their approval for use. These regulations aim to balance the efficacy of ARs in controlling rodent populations with the need to protect public health and the environment (Eisemann et al., 2018).

3.2. Regulatory framework in Europe

In Europe, the use of ARs is primarily regulated under Regulation (EU) No. 528/2012 (European Union, 2012), known as the Biocidal Products Regulation (BPR). Currently, eight ARs are approved for use as biocides in the EU: three FGARs (chlorophacinone, coumatetralyl, and warfarin) and five SGARs (brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen), all included in the Union list of approved active substances. After approval at the European level, products containing these active substances are assessed at the national level for authorization.

The BPR also governs the placing on the market and use of biocidal products, including ARs, aiming to ensure a high level of protection for human health and the environment.

According to the BPR, all biocidal products, including ARs, must be approved before being marketed and used. Companies intending to commercialize ARs must submit a detailed dossier demonstrating the safety and efficacy of their products. This dossier is evaluated by the competent authority of the relevant EU Member State, which provides a scientific opinion. If approved, the product may be sold and used with specific restrictions, labelling, and packaging requirements.

Under the BPR, ARs are considered candidates for substitution due to their high toxicity, persistence, bioaccumulation, and associated risks to human health and the environment. While these products do not fully meet the approval conditions outlined in Article 4 of the BPR, they have nevertheless been approved because effective rodent control is deemed essential, particularly to prevent rodent-borne diseases. At present, no equally effective and less harmful alternatives are available. As such, the environmental risks have been weighed against public health and hygiene benefits.

Additionally, new regulations came into effect in 2018 (Regulation (EU) No. 2016/1179; European Union, 2016) following updates to Regulation (EC) No. 1272/2008 (European Union, 2008) on the classification, labelling, and packaging of substances and mixtures (CLP). These regulations mandate that products containing active substance concentrations above 0.003% must be labelled as reprotoxic (Categories 1A and 1B), due to evidence from animal studies suggesting a risk of adverse reproductive health effects. This classification is based on evidence indicating that warfarin is teratogenic in mammals (Chetot et al., 2020). Consequently, since 2018, new products for non-professional use have been marketed with active substance concentrations below 0.003%.

The EU has also introduced the concept of mitigated use of ARs, promoting Integrated Pest Management (IPM) measures to reduce the indiscriminate use of these products and minimize associated risks (as required by Article 19 of the BPR). Furthermore, the European regulation is reviewed every five years to assess whether these products can be withdrawn and replaced with eco-friendly or less risky alternatives or whether their approval should be renewed. The most recent evaluation was initially scheduled for 2024. However, since the approvals were likely to expire before a decision could be reached, the EU extended the authorization deadlines to December 31, 2026 (European Union, 2024).

Some ARs (chlorophacinone, bromadiolone, and difenacoum) are also used as plant protection products (PPPs) to prevent crop damage, under the Plant Protection Products Regulation (EC) No 1107/2009 (PPPR; European Union, 2009). Their use targets specific scenarios where rodents pose a direct threat to crops or food reserves and is subject to country-specific regulations, similar to the framework under the BPR.

3.3. Regulatory framework in Spain

In Spain, the regulatory framework governing the use of ARs aligns with European Union directives, particularly BPR (European Union, 2012), while also incorporating specific national regulations overseeing the authorization, distribution, and application of these products. Under the BPR, ARs used in Spain must be registered and authorized before they can be marketed or applied. The Spanish Agency for Medicines and Medical Devices (*Agencia Española de Medicamentos y Productos Sanitarios* - AEMPS) serves as the competent authority for the approval and oversight of biocidal products in Spain, including ARs. These products are subject to strict regulations concerning active ingredient concentrations, labelling, and usage. Products must comply with the CLP Regulation (European Union, 2008), which mandates clear warnings about health and environmental risks.

In Spain, the use of ARs is tightly controlled, with a clear distinction between professional use (specialized personnel) and general public use. Products with higher concentrations of active ingredients (>0.003%), deemed more hazardous, are restricted to qualified pest control professionals. These specialized professionals must have undergone specific training on the safe use of such products. Additionally, Spanish regulations require distributors and professional users to maintain detailed records on AR sales and use, including application sites, quantities used, and measures taken to prevent unintended exposure of non-target species and humans. Another important aspect of Spain's regulatory framework is the protection of wildlife and domestic animals. To minimize risks, regulations mandate the use of protected bait stations and tamper-proof containers, along with specific guidelines for outdoor applications (e.g., labelled bait boxes, prohibition of permanent baiting, prompt removal of carcasses, and follow-up measures).

As of now, Spain has registered 209 products for trained professional use and 146 for professional (e.g., farmers, livestock breeders, sewage workers without specialized rodent control training) and general public use (Carrera et al., 2024). Most commercial products contain SGARs brodifacoum (*n*=129) and bromadiolone (*n*=132), as active ingredients. Unlike some other countries, ARs in Spain are not approved as PPPs but only as biocides.

4. Toxicokinetics and toxicodynamics of ARs

4.1. AR toxicokinetics

Toxicokinetic data are abundant for target rodent species but scarce for non-target wildlife species (Horak et al., 2018). After ingestion, ARs are rapidly absorbed in the gastrointestinal tract within a few hours. Once absorbed, ARs effectively bind to plasma proteins, particularly albumin (Watt et al., 2005), although the protein binding may differ between species. For instance, studies suggest that in chickens (*Gallus gallus*), warfarin's longer half-life compared to most mammals may be due to a stronger binding affinity to albumin, despite birds having lower albumin levels overall (Watanabe et al., 2015). This binding could reduce toxicity while extending the compound's half-life.

Afterwards, AR compounds reach the liver via the hepatic circulation, where they are mainly metabolized through cytochrome P450. Ring hydroxylation is another critical biotransformation step for coumarin-based anticoagulants, producing hydroxylated metabolites that may undergo glucuronic acid conjugation before entering systemic circulation. Metabolism is generally biphasic, with a rapid initial phase during the first days following exposure and a slower clearance phase thereafter. In birds, part of the blood from the intestine is transported directly to the kidneys via the coccygeomesenteric vein, which may contribute reducing AR plasma half-life (Horak et al., 2018). Variations in warfarin metabolism have been observed across bird species, with higher activity in chicken and crow liver microsomes compared to those of mallard or ostrich, indicating that the pharmacokinetics of ARs may differ significantly among avian species (Watanabe et al., 2015). The metabolic rate varies between first- and second-generation compounds. SGARs tend to be metabolized more slowly, contributing to their higher potency and prolonged duration of action (Vandenbroucke et al., 2008). For SGARs, some compounds are primarily excreted as parent compounds in the faeces without metabolite formation,

whereas FGARs are largely eliminated as metabolites in the urine (Horak et al., 2018). Furthermore, ARs may undergo enterohepatic recirculation, especially SGARs, potentially contributing to their increased persistence in the body.

In general, the elimination of ARs occurs mainly via faeces and, to a lesser extent, via urine. In birds, excretion into eggs has also been documented (Fisher, 2009; Na'Im et al., 2012; Salim et al., 2015). Some studies have demonstrated that these compounds can also be eliminated through hair. This has been reported in humans (Carelli et al., 2020; Leporati et al., 2016) and recently confirmed in wild species such as foxes (*Vulpes vulpes*) (Picone et al., 2025). Moreover, it is expected that elimination might also occur in avian species through feathers, although further research is needed to confirm this hypothesis. Due to their long half-life, particularly for SGARs, these compounds can persist in the organism for weeks or even months (Horak et al., 2018), which increases the risk of accumulation and toxicity in non-target animals.

4.2. Blood coagulation in mammal and avian species

The blood coagulation mechanism, both in birds and mammals, is a complex process involving the sequential activation of proteins called coagulation factors, to facilitate clot formation and prevent further blood loss. This process is divided into two main pathways: the intrinsic pathway and the extrinsic pathway, which ultimately converge into the common pathway. The common pathway is responsible for the conversion of prothrombin into thrombin, an enzyme that transforms fibrinogen into fibrin, forming the essential meshwork for clot stabilization. While these pathways achieve the same goal, they do so in different ways and are triggered by different stimuli.

- Intrinsic pathway: In mammals, this pathway is activated when blood comes into contact with negatively charged surfaces, such as exposed collagen following vascular injury. This pathway involves factors XII, XI, IX, and the sequential activation of these factors leads to the activation of factor X, initiating the common phase of the cascade. Unlike the extrinsic pathway, the intrinsic pathway is slower and requires more steps. In mammals, it involves factors XI and XII, which are absent in birds (Edwards et al., 2002; Nevill, 2009). Despite this absence, birds can still coagulate because their coagulation mainly depends on the extrinsic pathway. The

intrinsic pathway in birds is still a topic of discussion (Buzala et al., 2017), but it seems to involve a residual intrinsic pathway activated by factor IX via the activated tissue factor (TF)-factor VII complex (capable of activating both factors IX and X). Factor IXa, along with factor VIIIa, Ca^{2+}, and phospholipids, form the tenase complex (Childers et al., 2022), which can activate factor X. This tenase complex is also formed as a result of feedback from thrombin, which activates factor VIII (Edwards et al., 2002; Gentry, 2004; Ponczek et al., 2008).

- Extrinsic pathway: This pathway is activated by external tissue damage, such as an injury or trauma, which exposes TF (factor III or thromboplastin) on the cell membrane of monocytes, blood vessel cells, and other cells (Buzala et al., 2017). TF then activates factor VII present in plasma, followed by other factors in the coagulation cascade. Once activated, the TF-factor VII complex activates factor X, initiating the common phase of the coagulation cascade and leading to fibrin production. The extrinsic pathway is rapid, being the first response to external damage. This pathway is essential in both birds and mammals and is the primary pathway for coagulation in birds.
- Common phase: Both pathways converge on the activation of factor X, which leads to the conversion of prothrombin into thrombin. Thrombin subsequently transforms fibrinogen into fibrin, forming a stable clot (Yeh, 2008). Fibrin is a protein that forms a network of filaments trapping blood cells, such as platelets and red blood cells, creating a stable clot that seals the site of injury, halting blood loss until tissue regeneration occurs.

One of the main differences between birds and mammals involves avian thrombocytes (nucleated cells, 5–8 μm in diameter), which play a role analogous to mammalian platelets (non-nucleated, 2–3 μm in diameter) in the formation of the platelet plug but show significantly lower reactivity (Gallo et al., 2015). Despite differences, the coagulation process remains functional in both mammal and avian species, adapted to the specific needs of each class of animals.

4.3. AR toxicodynamics

ARs act by inhibiting vitamin K epoxide reductase (VKOR), an enzyme that plays a key role in the vitamin K cycle. This cycle is essential for regenerating active vitamin K, a critical cofactor required for the activation of blood clotting factors, specifically factors II (prothrombin), VII, IX, and X. Under normal conditions, when blood coagulation is triggered, vitamin K is oxidized during the activation of clotting factors, converting into its inactive form (vitamin K epoxide). The function of VKOR is to regenerate vitamin K in its reduced form (hydroquinone, the active form), thus enabling the continuous synthesis of clotting factors (Garcia and Reitsma, 2008).

In the coagulation process, vitamin K serves as a cofactor for the enzyme gamma-glutamyl carboxylase (GGCX), which plays a critical role in the post-translational modification of clotting factors II, VII, IX, and X (Rattner and Mastrota, 2018). These factors contain glutamic acid (Glu) residues, which must be converted into gamma-carboxyglutamic acid (Gla) to become functional. This modification is known as gamma-carboxylation and is essential because Gla residues enable clotting factors to bind to calcium ions (Ca^{2+}), a key step in the blood coagulation process. Calcium ions stabilize interactions between clotting factors and cell membranes during clot formation (Furie and Furie, 1990).

When ARs inhibit the VKOR enzyme, they prevent the regeneration of vitamin K in its active form (hydroquinone) (Figure 2). Without active vitamin K, GGCX cannot add carboxyl groups to the glutamate residues on clotting factors. Consequently, these factors cannot bind to Ca^{2+} and become inactive, disrupting the entire coagulation process. This leads to the inability of the blood to clot, causing fatal haemorrhages in poisoned animals. Since ARs have a delayed action, their lethal effects are not immediate. Rodents develop internal bleeding that may manifest several days after ingesting the poisoned bait. This delay often prevents rodents from associating the bait with the symptoms, allowing multiple individuals to consume it (reviewed by Murphy, 2006).

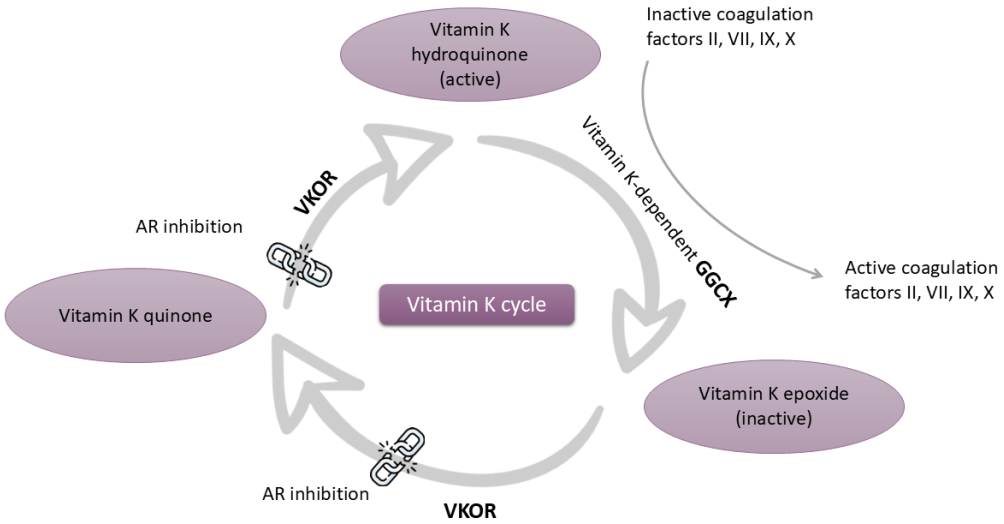

Figure 2. Schematic representation of the vitamin K cycle and the mechanism of action of anticoagulant rodenticides (ARs). Vitamin K undergoes cyclical oxidation and reduction to regenerate its active form (vitamin K hydroquinone), which serves as a cofactor for gamma-glutamyl carboxylase (GGCX). This enzyme carboxylates clotting factors II, VII, IX, and X, converting them from their inactive to active forms. ARs inhibit vitamin K epoxide reductase (VKOR), blocking the regeneration of active vitamin K and disrupting the coagulation process.

5. Health effects and ecological impacts

5.1. Coagulation disorders and internal haemorrhages.

As previously mentioned, VKOR inhibition causes a depletion of vitamin K-dependent coagulation factors (such as factors II, VII, IX, and X), leading to reduced haemostatic capacity, commonly referred to as coagulopathy. Animals exposed to ARs develop spontaneous internal haemorrhages, which may affect various organs and tissues, causing anaemia, debilitation, and, in the absence of treatment, death (Rattner and Mastrota, 2018). Even minor traumas that would not normally be fatal can become lethal due to the impaired blood coagulation capacity. For instance, raptors admitted to wildlife rescue centres are frequently injured (Montesdeoca et al., 2016; Panter et al., 2022; Silva et al., 2023). However, it is often challenging to determine whether the trauma itself or AR poisoning contributed to the lethality of the event, partly due to the significant interspecific differences in susceptibility to these compounds (Erickson and Urban, 2004; Nakayama et al., 2020; Rattner et al., 2010a). Moreover, contamination by ARs is rarely, if ever, assessed in wildlife rehabilitation centres through specific tests (e.g., coagulation

assays) or post-mortem analyses for residue detection in biological samples. Observed symptoms include bleeding, haematomas, pallor, anorexia, lethargy, and ataxia (Murray, 2018). AR-induced coagulopathy also manifests as alterations in coagulation parameters, such as prolonged prothrombin time (PT) and Russell's Viper Venom Time (RVVT) in birds (Hindmarch et al., 2019; Rattner et al., 2010a, 2014a; Webster et al., 2015). These parameters provide critical insights into the physiological impact of AR exposure, the extent of coagulopathy, and the potential risk of haemorrhagic events in affected individuals.

5.2. Reproductive effects.

Exposure to ARs can significantly impact the reproduction of exposed animals. The teratogenic effects of warfarin are well documented in humans and include congenital malformations, haemorrhages, and cognitive and respiratory impairments (collectively known as "Foetal Warfarin Syndrome"; Abadie et al., 2023; Chetot et al., 2020; Ginsberg and Hirsh, 1989). In fact, this compound readily crosses the placenta, leading to these adverse effects. Chronic exposure-induced coagulopathy has also been associated with spontaneous abortions and foetal mortality (Abadie et al., 2023). In birds, reproductive impairments have been documented in barn owls (Salim et al., 2014), although these findings might also be influenced by reduced prey availability following AR applications. While ARs are transferred to eggs (Na'Im et al., 2012), there is no documented reduction in eggshell thickness, shape, or mass (Salim et al., 2015). These combined effects, along with reduced fitness in adult individuals, can possibly compromise the reproductive capacity of exposed populations, negatively affecting population dynamics and long-term sustainability.

5.3. Sublethal toxicological effects

Beyond their lethal effects, sublethal exposure to ARs can cause a range of adverse physiological and behavioural effects. These include:

Immune system alterations. Exposure to ARs can suppress the immune response in animals, making them more susceptible to infections and parasitic diseases (Fraser et al., 2018; Serieys et al., 2018). Immunosuppression may result from both the direct effects of

rodenticides on lymphatic tissues and from malnutrition or anaemia caused by chronic haemorrhaging. Immunocompromised individuals exhibit a higher incidence of parasitic infestations, which have been associated with AR exposure (Lemus et al., 2011; Riley et al., 2007; Serieys et al., 2015). Increased parasitic loads can lead to debilitating conditions, further reducing the survival potential of affected animals.

Thermoregulatory dysfunctions. Chronic anaemia and haemorrhaging can impair the ability of animals to maintain a stable body temperature, an effect documented in free-ranging individuals exposed to sublethal AR concentrations (Vyas et al., 2022). Anaemia and blood loss reduce blood volume, limiting the body's capacity to maintain physiological body temperature. Additionally, oxygen deprivation in tissues disrupts metabolic activity, impairing heat production and compromising thermal homeostasis.

Reduced Body Condition Index (BCI): Chronic blood loss and impaired nutrient absorption can result in weight loss and a lower BCI (Elmeros et al., 2011; Herring et al., 2023; Martínez-Padilla et al., 2017; Smallwood et al., 2024). Low BCI in wildlife species has been linked to decreased physical endurance, reduced hunting efficiency, increased vulnerability to predators, impaired reproductive performance, and weakened immune function (Brodie et al., 1995; Genovart et al., 2010).

Other vitamin K-dependent proteins (VKDPs) impairment: ARs can also inhibit VKDPs other than coagulation factors. Among these, the inhibition of matrix Gla-protein can contribute to vascular calcification, a condition that leads to the hardening of arterial walls and impairs normal blood flow (Andrews et al., 2018; Krüger et al., 2013; Schurgers et al., 2004). Additionally, the suppression of bone Gla-protein (BGP), also known as osteocalcin, may result in bone abnormalities, including reduced bone density, osteoporosis, and increased susceptibility to fractures (Popov Aleksandrov et al., 2024; Stock and Schett, 2021). However, a study investigating bone fragility in common kestrels (*Falco tinnunculus*) and barn owls (*Tyto alba*) exposed to ARs did not detect significant alterations in bone density or strength, indicating that the impact of ARs on bone integrity might require prolonged exposure for observable effects or may vary across species (Knopper et al., 2007). It is important to note that most available information on the effects of ARs in non-target wildlife focuses on the inhibition of coagulation factors, while the potential impacts on other VKDPs remain less well documented.

Behavioural alterations in target rodents and invertebrates: ARs not only cause physical harm but also induce behavioural changes. In target rodents, impaired neurological function and general debilitation make them less active and more susceptible to predation (Cox and Smith, 1992; Littin et al., 2000). These behavioural changes can have dual ecological impacts: on one hand, they reduce rodent populations, but on the other, they increase predator exposure to ARs, exacerbating secondary and tertiary poisoning effects. Invertebrates are also affected by AR exposure through the ingestion of baits. Behavioural changes in invertebrates include reduced mobility, faster emergence, and increased vulnerability to predation (Parli et al., 2020). Such alterations can trigger cascading effects throughout ecosystems, affecting predator-prey interactions and reshaping food web structures (Elliott et al., 2014; Masuda et al., 2014).

5.4. Impact of ARs on population dynamics

Research linking AR exposure to alterations in population dynamics remains limited, highlighting the need for further investigation. Recently, the annual abundance of common kestrels in the United Kingdom (UK) was negatively correlated with SGAR levels detected during the same year (Roos et al., 2021). Coeurdassier et al. (2014) hypothesized that bromadiolone, which had been widely used in agriculture for vole control, negatively impacted the reproduction of red kites (*Milvus milvus*). Similar findings have been reported in carnivorous mammals, such as foxes and badgers, whose abundance was higher in areas with less intensive rodent control (Jacquot et al., 2013; Proulx and Mackenzie, 2012). A more recent study showed that the trapping effort required to capture fishers (*Pekania pennanti*) in the USA increased as the proportion of the population exposed to at least one AR grew, suggesting a potential impact of ARs on the species' population dynamics (Silveira et al., 2025). Although the effects of ARs on predator populations have not been thoroughly investigated, their widespread use and high toxicity still represent a significant threat, particularly for species with precarious conservation status or for long-lived predators with low reproductive rates (Gomez et al., 2022).

6. Exposure of non-target wildlife to ARs

ARs are primarily employed for rodent control, yet their pervasive presence in diverse environments– ranging from urban and rural to natural settings –poses substantial risks to wildlife. These compounds can seep into the environment and create a range of challenges, from direct toxic effects on individual animals to cascading effects throughout ecosystems. Exposure to these compounds in non-target wildlife can be classified as primary, secondary, and tertiary (see Figure 3), leading to bioaccumulation and biomagnification effects.

Primary exposure occurs when an individual comes into direct contact with the rodenticide. This happens when an animal ingests directly the AR, mainly contained in a poisoned bait or occasionally present in contaminated water (Regnery et al., 2018), which, depending on the amount consumed, can result in direct toxic effects such as internal bleeding, coagulopathy, and death in severe cases (Shore and Coeurdassier, 2018). Invertebrates can also easily access the bait and ingest the rodenticide (Elliott et al., 2014; Spurr and Drew, 1999). It is important to note that primary exposure not only affects wildlife but also poses risks to domestic animals and humans.

Secondary exposure occurs when animals consume poisoned rodents or other contaminated prey. Consequently, predators and scavengers that feed on contaminated rodents would accumulate ARs in their bodies. This transfer of toxicity through the food chain poses significant risks to predator species, which may show toxic symptoms similar to those of the poisoned rodents, such as coagulopathy and mortality (Shore and Coeurdassier, 2018).

Tertiary exposure is less studied but equally significant. In this scenario, a predator that feeds on other poisoned predators accumulates the rodenticide through multiple trophic levels (Hindmarch and Elliott, 2018). Another example of tertiary exposure occurs when a predator consumes insectivorous species. This type of exposure can severely impact high trophic level species, intensifying the toxic effects already observed at lower levels of the food chain.

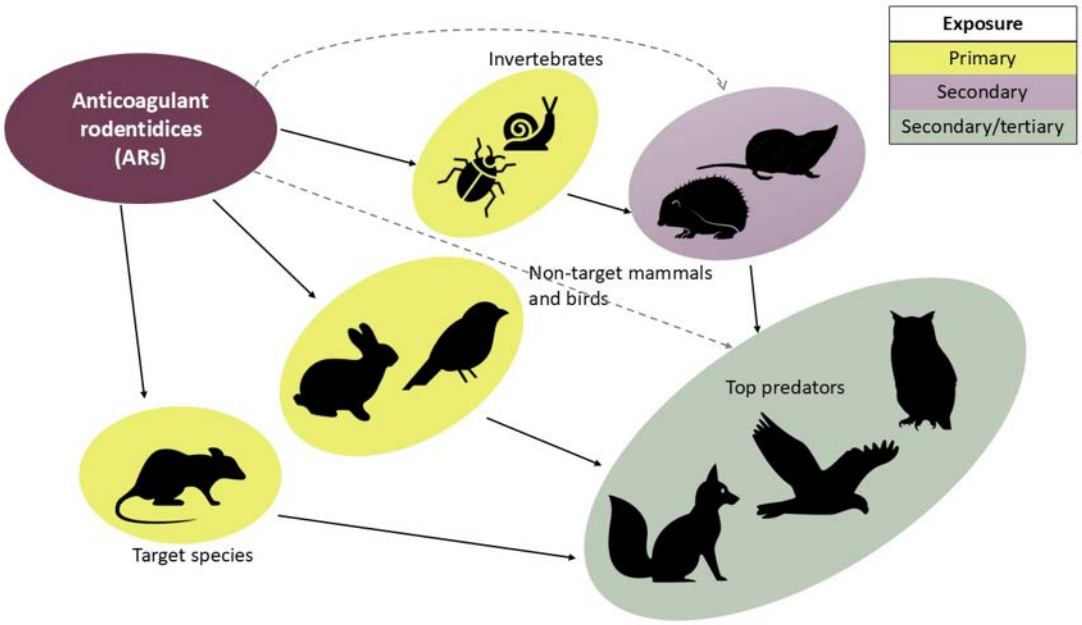

Figure 3. Examples of exposure pathways to anticoagulant rodenticides (ARs) in terrestrial ecosystems. The color-coded ovals represent the different exposure levels: yellow for primary, purple for secondary, and light green for secondary/tertiary. The dashed grey arrows represent other possible exposure pathways.

Massive and repeated exposure to ARs can result in bioaccumulation and biomagnification phenomena:

Bioaccumulation refers to the gradual accumulation of toxic substances in an organism's tissues over time, due to repeated ingestion and the difficulty in metabolizing and excreting them. This typically occurs when the uptake of a substance exceeds its elimination (Chormare and Kumar, 2022). ARs (especially SGARs), being lipophilic compounds, accumulate in the liver and adipose tissues of organisms, leading to toxic concentrations that can cause adverse health effects.

Biomagnification, on the other hand, describes the increase in concentration of a toxic substance as it moves up through successive levels of the food chain, indicating the trophic transfer of the toxic compound. Each higher trophic level consumes a greater number of organisms from lower levels, thereby accumulating higher amounts of the toxic substance (Miller et al., 2020). An example of biomagnification occurs when invertebrates, after ingesting AR baits, are consumed by insectivorous mammals or birds. These, in turn, are preyed upon by higher trophic-level predators, such as foxes or raptors, which accumulate increasing concentrations of ARs as they consume multiple contaminated

prey from lower trophic levels. This process can lead to severe toxicity in apex predators, negatively impacting their health and survival.

7. Ecotoxicological studies on non-target species exposed to ARs

The study of non-target species' exposure to ARs began since the late 1970s, following the first reports of secondary poisoning in apex predators and other wildlife (Colvin et al., 1988; Harradine, 1976; Mendenhall and Pank, 1980). These early studies documented exposure to ARs in predatory birds and mammals, highlighting the risks existing for these species. For instance, in the UK, extensive research on AR exposure in barn owls has been conducted since the 1980s using liver samples, as part of the "Predatory Bird Monitoring Scheme," motivated by the species' population decline. Newton and Wyllie (2002) reported the results of a 15-year study, showing an increase in positive cases over time, from 5% in the 1980s to 40% by the late 1990s, reflecting the growing use of these compounds. The authors hypothesized that this trend might be linked to shifts in barn owl diet and the emergence of AR resistance, which could lead to an increased presence of live, contaminated rodents and heightened risk for their predators. By 2006, the prevalence of SGARs in barn owls had risen to 62.9%, a trend accompanied by advancements in the sensitivity of analytical techniques (Walker et al., 2010).

Recent reviews have pointed out significant findings on AR exposure. For example, López-Perea and Mateo (2018) examined data on predator species (both mammals and birds) from various geographic regions, spanning from 1984 to 2015, revealing a generally high prevalence across these species (58%, n = 4,187). Moreover, predator species that are specialists and generalists showed similar AR prevalence (58%), although generalist species often exhibited higher sum AR (ΣAR) concentrations. Nakayama et al. (2019) reviewed 30 global studies conducted between 1998 and 2015, reporting an average prevalence of 55% across nearly 5,000 liver tissue samples analysed (n = 4,891). Raptors were among the most frequently exposed taxa, with 17 species showing over 60% prevalence of ARs. These included nocturnal raptors, such as *Asio otus* (75%) and *Bubo bubo* (85%), as well as diurnal raptors like the common kestrel (75%), golden eagle (*Aquila chrysaetos*, 64%), and sparrowhawk (*Accipiter nisus*, 85%). Keating et al. (2024) focused on AR exposure in non-target mammals worldwide, reporting an average

prevalence rate of 32.8% across 78 studies reviewed spanning from the 1990s to 2024. Brodifacoum and bromadiolone, both SGARs, were the most frequently detected compounds, identified in over 66% of the studies. Their work highlighted that, while sublethal concentrations are common, 33.9% of the studied species included at least one individual with hepatic levels exceeding the threshold considered lethal for some species.

Over the last five years, numerous studies evidenced alarming rates of AR exposure in raptors worldwide, based primarily on liver samples collected opportunistically from deceased individuals. High prevalence rates have been documented across different regions, including the Americas, Europe, Asia, and Australia. In the Americas, studies have revealed extensive AR contamination. For example, 100% of red-tailed hawks (*Buteo jamaicensis*) sampled in the USA showed SGAR residues in their livers (Murray, 2020), while 93% of turkey vultures (*Cathartes aura*) tested positive (Herring et al., 2022). Similarly, in Canada, 62% of raptors representing 12 species were found exposed to ARs (Thornton et al., 2022). Studies in Asia and Australia also reported concerning results, with 84% prevalence of black-shouldered kites (*Elanus caeruleus*) in Asia (Lin et al., 2022) and 74% of wedge-tailed eagles (*Aquila audax*) in Australia found contaminated (Pay et al., 2021). Moreover, ARs may pose a significant threat to endangered raptor species, such as the *Ninox novaeseelandiae undulata* in New Zealand (Sperring et al., 2024).

In Europe, high prevalence rates have been reported across several species. In Spain, 83% of Eurasian eagle-owls showed AR residues in liver samples (Gómez-Ramírez et al., 2021), and 100% of Bonelli's eagles (*Aquila fasciata*) were found AR positive (Vicedo et al., 2024). In addition, recent studies in Spain and France documented SGAR prevalence rates above 50% in some raptor species, raising significant conservation and ecotoxicological concerns (Fourel et al., 2024; Moriceau et al., 2022; Rial-Berriel et al., 2021). Comparable trends were observed in other countries, such as the UK, where 81% of sparrowhawks (Broughton et al., 2022) and 79% of barn owls (Ozaki et al., 2022) were found contaminated; in Germany, over 80% of Northern goshawks (*Accipiter gentilis*) and red kites tested positive for ARs (Badry et al., 2021).

Studies conducted using blood, plasma, or serum samples are fewer in number but often yield equally alarming results. Nestlings are more frequently sampled, as accessing and handling them is simpler than capturing and managing adult birds. Typically, AR levels in

these matrices are lower than those detected in liver tissues, and residues are often challenging to identify. Interestingly, three studies have assessed AR presence in migrating red-tailed hawks, sampling non-nestling individuals (juveniles and adults) with detection rates ranging from 8% to 32% (Abernathy et al., 2018; Kwasnoski et al., 2019; Murray, 2020). Murray (2020) conducted a comparative analysis of serum and liver samples and found that birds that died from AR toxicosis also had AR residues in serum, whereas sublethally exposed individuals tested negative in serum samples. Moreover, in American kestrels (*Falco sparverius*), adult individuals showed a prevalence of 58%, which was significantly higher than AR prevalence detected in nestlings (1.7%) from the same study (Buechley et al., 2022). A recent study conducted on non-nestling griffon vultures (*Gyps fulvus*) in the southeast Iberian Peninsula found the presence of ARs (mainly SGARs, with a prevalence of 93%) in 95% of the blood samples analysed (García-Fernández et al., 2024). A summary of studies on raptor nestlings conducted before this thesis is presented in Table 2.

Table 2. 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].

Species	Sampling year	Country	n	%	Warfarin	Coumatetralyl	Chlorophacinone	Bromadiolone	Difenacoum	Flocoumafen	Brodifacoum	Difethialone	Ref.[b]
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 are available or not analysed. [a] samples consist of a pool of blood from different chicks of the same nest. [b] References: 1. Oliva-Vidal et al. (2022); 2. Martínez-Padilla et al. (2017); 3. Gómez-Ramírez et al. (2012); 4. Badry et al. (2022); 5. Powolny et al. (2020); 6. Buechley et al. (2022). In 3, 5 and 6, diphacinone was analysed but not detected, while in 6, coumafuryl was analysed but not detected. In 1, all SGARs except difethialone were tested.

8. Biomonitoring and role of birds of prey in environmental surveillance

Biomonitoring is the practice of using living organisms to evaluate environmental health by detecting and assessing the presence and effects of chemical contaminants in ecosystems. It involves analysing tissues, fluids, or other biological components to measure chemical exposure and, often, to identify biological changes that indicate environmental stress (Karr, 1987; Maroni et al., 2000). Biomonitoring is crucial for understanding the impacts of human activities on ecosystems and for informing environmental management policies and public health protection.

In active biomonitoring, researchers collect samples from living organisms selected directly from nature. This approach allows the specific selection of the timing and location of sampling, ensuring that samples are representative of the population under study. Passive biomonitoring relies on the collection of samples from animals found dead in the field (often road-killed or electrocuted) or deceased shortly after being brought to recovery centres. This method is often used to study cases of mortality caused by poisoning or infectious diseases (Guberti et al., 2014; Descalzo et al., 2021). Below are the main pros and cons of both approaches (Table 3).

Table 3. Pros and cons of active and passive biomonitoring approaches in environmental health assessment. Active biomonitoring involves sampling living organisms, allowing for controlled sampling and detailed data collection, while passive biomonitoring uses deceased animals to study mortality caused by contaminants, with lower costs but potential sampling biases.

Active biomonitoring	Passive biomonitoring
Pros	
- Decision-based sampling: time and location are planned to obtain representative samples, reducing the risk of bias.	- Cost-effectiveness: Generally, less expensive, as it does not require the capture of live animals.
- Sample quality: Samples are more stable, of higher quality, and easier to preserve under controlled conditions.	- Non-invasive sample collection: Provides biological samples without the need for handling live animals.
- Detailed information: Complete data on live animals, including parameters such as weight, age, general health can be obtained. Possibility to study sublethal and chronic effects.	- Sample accessibility: Provides access to elusive or rare species, especially if found dead. No need of certain ethical authorizations.
- Versatility: Allows sampling of different species and monitoring populations over time for longitudinal studies.	- Study of severe cases: Allows identification of acute poisoning incidents and analysis of lethal contaminants in the environment.
Cons	
- Costs and logistics: Requires resources for animal capture and sampling.	- Sampling bias: Only severely intoxicated or debilitated animals are found, introducing bias and potentially overstating lethal effects.
- Interference: Handling animals may cause stress or alter their natural behaviour.	- Limited information: Often lacks the animal's history and environmental conditions complicate data interpretation.
- Ethical concerns: The capture and handling of live animals can raise ethical issues and require specific permits.	- Uncertain origin: Dead animals may come from different areas, making it difficult to link contaminant exposure to a specific geographic region.

Birds of prey are considered excellent biomonitoring species due to a range of characteristics (Badry et al., 2020; García-Fernández et al., 2023; Gómez-Ramírez et al., 2014; Movalli et al., 2018). In fact, these birds are often utilized in coordinated biomonitoring programs across different countries, such as the COST Action program ERBFacility (Dulsat-Masvidal et al., 2021). Raptors occupy the top of the food chain, and as apex predators, they tend to bioaccumulate and biomagnify toxic substances present in their prey, making the impact of pollutants at higher trophic levels more apparent (Ratajc

et al., 2022). Moreover, many raptors have relatively long lifespans, making them valuable indicators for monitoring the accumulation of contaminants over time and studying chronic exposure. Raptors are found across a wide range of habitats, from urban to wilderness areas, on almost every continent, allowing for environmental pollution monitoring in various ecosystems (Gómez-Ramírez et al., 2014). Furthermore, they are particularly sensitive to different classes of pollutants, such as insecticides, rodenticides, and heavy metals, making them early indicators of environmental problems (García-Fernández et al., 2023; Ortiz-Santaliestra et al., 2015; Rattner et al., 2012; Sánchez-Virosta et al., 2020). Raptors are often charismatic and protected species (Donázar et al., 2016), so their biomonitoring is not only important for assessing ecosystem health but also for supporting conservation efforts. In addition, their diverse diet, hunting a wide variety of prey from small mammals to birds and insects, enables the evaluation of contaminant accumulation across different components of the food chain (Badry et al., 2020; Geduhn et al., 2016). Finally, collecting samples from wild raptors, such as blood, feathers, or tissues, can be done with minimal harm, making biomonitoring less invasive.

9. Biomarkers as tools for the assessment of AR exposure and effects

Biomarkers are measurable biological indicators that reflect the interaction between an organism and a chemical substance. They are used in ecotoxicology to monitor exposure, evaluate biological effects, and understand the mechanisms of action of toxicants (De Coen et al., 2000; López-Barea, 1995). These tools are essential for the early detection of the impact of ARs, even in the absence of evident clinical symptoms (Rached et al., 2020; Silva et al., 2022). Biomarkers can be classified into three main categories: exposure biomarkers, effect biomarkers, and susceptibility biomarkers.

Exposure biomarkers reveal the presence of ARs in the organism, providing quantitative and qualitative information on the exposure (Rached et al., 2020). Among the most commonly used exposure biomarkers, is the direct measurement of AR concentration in the blood, liver, or other biological matrices, using highly sensitive analytical techniques such as high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The blood concentration of ARs reflects recent exposure (due to their short plasma half-life; Horak et al., 2018), whereas tissue levels, such as in the

liver, can indicate chronic or cumulative exposures. In addition to AR levels, the analysis of specific metabolites in plasma serves as a biomarker of exposure to ARs as some of them are active and toxic to the organism (Fourel et al., 2020; López-García et al., 2017).

Effect biomarkers assess the biological consequences of AR exposure and can be used to infer the risk of impaired haemostatic function (López-Barea, 1995). The PT test is one of the most established methods, as it is the first coagulation parameter to be altered in case of AR exposure (Rattner et al., 2014a, 2010b; Webster, 2009). Prolonged PT indicates a deficit in vitamin K-dependent coagulation factors, suggesting impaired haemostatic capacity. Another relevant effect biomarker is the RVVT (Rattner et al., 2015, 2014a, 2011), which allows the detection of abnormalities in the common coagulation pathway. Additionally, measuring the activity of the VKOR enzyme, the primary target of ARs, provides direct insights into enzymatic inhibition and the associated risk of coagulopathy (Nakayama et al., 2020; Rached et al., 2020; Watanabe et al., 2010). Recent studies are investigating new effect biomarkers, such as genomic and metabolomic profiles (Deguchi et al., 2014; Fraser et al., 2018; Yan et al., 2016), which can offer a more detailed view of the physiological and molecular responses to exposure.

Susceptibility biomarkers provide useful information on individual or population-level variability in AR toxicity (Schlenk, 1999). For example, mutations in the gene encoding the enzyme VKOR, which is the molecular target of ARs, can significantly influence the level of resistance to ARs. Genetic analysis of these polymorphisms is crucial for understanding the dynamics of AR resistance in rodents (Bermejo-Nogales et al., 2022; Ruiz-López et al., 2022; Yiğit et al., 2023) and the associated risks for apex predators.

Research is now exploring new monitoring strategies, including omics approaches (genomics, transcriptomics, proteomics, and metabolomics), to enhance the precision and effectiveness of ecotoxicological monitoring (Rached et al., 2020). Furthermore, the study of cytochrome P450 enzymes as potential biomarkers focuses on their role in the metabolism of ARs and other xenobiotics. Changes in the expression or activity of specific P450 isoforms could provide insights into individual or species-specific susceptibility to ARs and help to assess exposure and detoxification capacity (Watanabe et al., 2015, 2010). These advanced tools promise to identify more specific and sensitive biomarkers, improving our ability to detect sublethal effects of ARs in ecosystems and apex predators.

10. Key environmental variables influencing AR exposure risk

Exposure to environmental contaminants, such as ARs, can vary significantly depending on several environmental factors. These factors determine the likelihood of organisms coming into contact with these substances and the severity of the effects they may experience. Recent studies conducted in different parts of the world have identified key environmental variables that influence exposure risk, including:

Human population density can influence the risk of exposure to ARs (Carrillo-Hidalgo et al., 2024; López-Perea et al., 2015; Pay et al., 2021; Szapu et al., 2024). In areas with high population density, the use of ARs is often more widespread to control rodent infestations, thereby increasing the likelihood of wildlife exposure (Silveira et al., 2024). Animals living in densely populated human areas, such as predatory birds or carnivores, are particularly vulnerable to secondary contamination through the food chain. ARs are often applied without adhering to regulations that mandate the use of bait boxes, the removal of dead rodents, and post-application monitoring, which exposes wildlife to higher risks of contamination and poisoning.

Presence of urban or residential areas. Urban and peri-urban areas are critical factors influencing AR exposure risk (Alabau et al., 2020; Badry et al., 2021; Geduhn et al., 2015; Lohr, 2018; López-Perea et al., 2019; Riley et al., 2007; Serieys et al., 2015; Silveira et al., 2024). These areas are often associated with higher AR use, both for rodent population control and the management of public green spaces. Additionally, ARs are frequently applied in sewer systems in cities to prevent rat infestations. Wild animals venturing into these areas or approaching them to hunt, such as foxes, raptors, and small mammals, can easily come into contact with poisoned baits or contaminated prey, thereby increasing their risk of poisoning.

Intensive livestock farming can significantly contribute to the risk of exposure to ARs (Badry et al., 2021; Geduhn et al., 2015; López-Perea et al., 2019; Rial-Berriel et al., 2021). In such facilities, rodenticides are commonly used by professional personnel to prevent feed contamination and reduce the transmission of diseases. However, this poses a high risk to wildlife, especially predators that may feed on poisoned rodents near livestock farms.

Intensive agriculture often relies on ARs to protect crops from rodent attacks (Fritsch et al., 2024). This type of agriculture, characterized by large-scale monocultures and heavy use of chemicals, can serve as a significant source of wildlife exposure (Hughes et al., 2013; Martínez-Padilla et al., 2017). In Spain, the use of ARs is not permitted as PPP, meaning they are classified as biocides and are not officially approved for agricultural purposes. Nevertheless, illegal applications of these products in open fields cannot be ruled out. Furthermore, agricultural workers may apply baits around or inside storage facilities for agricultural products. Wildlife in cultivated fields or near agricultural infrastructure can hunt prey that have consumed poisoned baits, leading to high risks of poisoning (Serieys et al., 2015).

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Aims and objectives

General aim

The general aim of this doctoral thesis is to increase the knowledge about the impact of anticoagulant rodenticides (ARs) on non-target species through the study of AR exposure and effects in four birds of prey species inhabiting a semi-arid region of the Iberian Peninsula. Particularly, the study was conducted on three nocturnal (long-eared owl *Asio otus*, Eurasian eagle owl *Bubo bubo*, and barn owl *Tyto alba*) and one diurnal (common kestrel *Falco tinnunculus*) raptor species from the Region of Murcia (southeastern Spain). This study encompasses the analysis of AR residues in blood samples, the evaluation of effect biomarkers, the assessment of the study species' diet, and the identification of environmental factors (e.g., agricultural intensification, urbanization, and the presence of livestock farms) influencing exposure risk. Ultimately, this thesis aims to contribute to the conservation of these and other non-target fauna, using predatory birds as sentinels of environmental contamination.

Specific Objectives

Obective 1. (Chapter I) To evaluate AR exposure in long-eared owl nestlings sampled across study sites in the Region of Murcia with varying levels of agricultural intensification; to investigate key environmental factors influencing AR exposure and validate prothrombin time (PT) as a biomarker of AR effect in this species.

Objective 2. (Chapter II) To assess AR exposure in common kestrels (nestlings) and barn owls (nestlings and adults) from areas with varying levels of agricultural intensification and urbanization; to compare exposure levels between these species, examining how environmental factors and ecological traits influence AR exposure risk; to validate PT as an effect biomarker in barn owls.

Objective 3. (Chapter III) To study AR exposure in eagle owl nestlings sampled in the Region of Murcia, assessing biomarkers such as PT and a plasma biochemical panel to

evaluate their general health; to identify environmental drivers influencing AR exposure risk while accounting for the species' ecological traits.

Objective 4. (Chapter IV) To analyse mRNA expression of *vkorc1* and *vkorc1l1* genes in blood samples from eagle owl nestlings as biomarkers of AR exposure and effect; to investigate molecular mechanisms underlying AR toxicity and validate previously identified environmental risk factors.

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

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Photo by Livia Spadetto

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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 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. The findings 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.

Chapter II. Comparing anticoagulant rodenticide exposure in barn owl (*Tyto alba*) and common kestrel (*Falco tinnunculus*): a biomonitoring study in an agricultural region of Southeastern Spain

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Abstract

Second-generation anticoagulant rodenticides (SGARs) are commonly used for rodent control, affecting various non-target wildlife species. Here, blood samples from common kestrels (*Falco tinnunculus*, *n* = 70 chicks) and barn owls (*Tyto alba, n* = 54 chicks and 12 adults) from Southeastern Spain were analysed using HPLC-TQ. SGAR prevalence was 68.6% in kestrel chicks, 50% in barn owl chicks and 100% in adult barn owls, with multiple SGARs in both species. Prothrombin time analysis in barn owls revealed a positive correlation with blood ΣSGARs, suggesting a potential adverse effect on coagulation. Analysis of variables potentially influencing SGAR prevalence indicated that, for kestrels, it was only related to the extent of artificial surface, showing no differences across study sites. In owlets, the highest prevalence occurred in the most urbanized study site, with human population density being a key factor. This study highlights species-specific differences in SGAR exposure, likely influenced by ecological traits. Barn owls probably encounter contaminated prey near anthropized areas, with widespread SGAR use and higher presence of target rodents. Conversely, kestrels, hunting a variety of prey often near human settlements, face consistently elevated exposure from multiple sources. Understanding these variations is crucial for effective conservation and minimizing SGAR impact on non-target wildlife.

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

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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. This study mainly aimed to evaluate AR exposure and effects in nestlings of eagle owl (*Bubo bubo*) from the Region of Murcia (southeastern Spain). ARs in blood samples ($n = 106$) were analysed using high-performance liquid chromatography-triple quadrupole (HPLC-TQ), the influence of potential anthropogenic (presence of livestock farms, landfills and human population density) and environmental (land uses and proximity to watercourses) variables on AR exposure was assessed, and prothrombin time (PT) and plasma biochemical parameters were measured as biomarkers of effects. The 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 urbanized 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.

Chapter IV. Molecular biomarkers in wildlife ecotoxicology: *vkorc1* and *vkorc1l1* expression changes in response to anticoagulant rodenticides

Photo by Kiko Abad

This chapter is based on original research, currently in preparation for submission.

Abstract

Anticoagulant rodenticides (ARs), globally used for rodent control, pose significant risks to non-target predators and scavengers through secondary poisoning. While chemical analyses have extensively documented AR exposure in wildlife, sublethal effects remain understudied, particularly through the use of minimally invasive biomarkers. The *vkorc1* and *vkorc1l1* genes encode components of the vitamin K epoxide reductase complex (VKOR), recognized as the molecular target of ARs. This study investigates the expression of these genes in a population of free-ranging eagle owls (*Bubo bubo*). Blood samples (0.3–0.5 mL) were collected from 26 breeding territories (n= 72 nestlings) in 2022 to analyse mRNA levels of the paralogous genes *vkorc1* and *vkorc1l1* using RT-qPCR. Additionally, Canonical Correlation Analysis was performed to explore relationships between environmental variables and VKOR gene expression. Results revealed that *vkorc1* is the predominantly expressed gene in eagle owl blood (median = 5.16 RU), while *vkorc1l1* exhibits lower expression levels (median = 2.49 RU). Nevertheless, in one-third of the samples *vkorc1l1* was the predominant gene. *Vkorc1l1* expression likely acts as a compensatory mechanism, increasing in response to recent AR exposure. This pattern was particularly evident in anthropogenic environments, such as urban areas and landfills. Conversely, *vkorc1* appears to respond adaptively over time, with higher expression associated with natural watercourses and livestock farms. This is the first study to report *vkorc1* and *vkorc1l1* gene expression in a raptor species, demonstrating their suitability as biomarkers of AR exposure and effect. The integration of molecular and environmental data provides valuable insights into AR exposure dynamics in apex predators, informing management strategies to reduce risks to wildlife and ecosystem functioning.

1. Introduction

Vertebrate genomes contain two paralogous enzymes with VKOR activity, vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) and VKORC1-like 1 (VKORC1L1), which are thought to have arisen from a gene duplication event of an ancestral *vkor* gene (Rost et al., 2004). Therefore, the VKOR enzyme complex consists of two isoenzymes that have the same enzymatic activity but with structural differences and variations in tissue distribution (Caspers et al., 2015; Lacombe et al., 2017). VKORC1 is the main enzyme responsible for the reduction of vitamin K epoxide in the liver of mammals, which is essential for the activation of clotting factors and -consequently- for haemostasis. It is also the primary molecular target of ARs and anticoagulant drugs like warfarin in humans and other mammals (Oldenburg et al., 2007). In fact, genetic variations in *vkorc1* can be associated to alterations in the sensitivity of individuals and populations to these anticoagulants and have often been studied in AR target species, such as rats (*Rattus* spp.) and mice (*Mus musculus*), to identify mutations that confer resistance to ARs (Bermejo-Nogales et al., 2022; Goulois et al., 2017; Huang et al., 2022; Krijger et al., 2022). VKORC1L1 is an alternative form of the VKOR enzyme, encoded by a paralogous gene, and shares significant homology with VKORC1 (Oldenburg et al., 2015). It is thought to have a similar function to VKORC1 in the reduction of vitamin K epoxide, although it is less extensively studied and its role in coagulation is not fully understood (Oldenburg 2015). Furthermore, it may play a more significant role in extrahepatic tissues, where its gene expression level is generally similar to that of *vkorc1* (Caspers et al., 2015; Lacombe and Ferron, 2018). In fact, Caspers et al. (2015) highlighted a differential expression of both genes in 29 distinct mouse tissues, concluding that *vkorc1* is primarily expressed in exocrine tissues, especially the liver. In contrast, *vkorc1l1* showed high expression levels in the brain and uniform basal levels across all other tissues analysed (Caspers et al., 2015). A study conducted on rats *in vivo* reveals that VKORC1L1 is less sensitive to warfarin than VKORC1 and it is capable of at least temporarily compensating for VKORC1 functions when the latter is inhibited (Lacombe and Ferron, 2018). Interestingly, in rats exposed to bromadiolone, baseline levels of *vkorc1* were found to be lower in resistant rats, while *vkorc1* expression remained practically unchanged following AR exposure in both susceptible and resistant individuals (Markussen et al., 2007). Despite gene expression

studies conducted on avian species are still limited, available information supports that the distribution and expression of both genes vary significantly depending on the species considered. For example, *vkorc1*:*vkorc1l1* ratio is much lower in chickens (*Gallus gallus*; 2:3) compared to turkeys (*Meleagris gallopavo*; 8:1) (Nakayama et al., 2020). These interspecific differences in the expression of both genes and in VKOR activity is thought to explain the greater sensitivity to ARs observed in birds of prey compared to other avian species (Nakayama et al., 2020; Rattner et al., 2010; Watanabe et al., 2010).

In this study, the expression of *vkorc1* and *vkorc1l1* genes were tested as biomarkers of exposure and effect in a free-living population of Eurasian eagle owls (*Bubo bubo*, hereafter referred as to eagle owl) from southeastern Spain. More specifically, the following aims were established: 1) to analyse the expression of *vkorc1* and *vkorc1l1* in blood of eagle owl nestlings from breeding territories located in potentially contaminated and non-contaminated areas, and 2) to assess the role of environmental and anthropogenic factors (e.g., land use, livestock farms, landfills and riverbed proximity) as factors explaining potential differences observed in gene expression. The hypothesis was that after recent exposure to ARs, *vkor* gene expression would increase as an adaptive-compensatory mechanism. Compensatory and adaptive responses are essential in maintaining homeostasis when an organism is exposed to stressors. Compensatory responses are immediate and reversible mechanisms aimed at counteracting functional impairments by upregulating alternative genes or pathways (Gioeli et al., 2011; Lopez et al., 1998; Lu et al., 2015). In contrast, adaptive responses involve long-term changes that result from selective pressures, allowing the organism to better cope with chronic stressors through permanent adjustments in gene expression or function (Hoffmann and Hercus, 2000; Koch and Guillaume, 2020; Swindell et al., 2007; Van Der Meer et al., 2005). Both responses are critical for understanding how organisms respond to environmental challenges, including exposure to toxic substances or pharmacological agents. On the other hand, certain environmental variables, such as the presence of urbanized areas and landfills, may influence the genetic expression of both markers. This expectation is based on previous studies identifying these land-use types as key determinants of AR exposure (e.g., Spadetto et al., 2024a, 2025). Understanding how these factors impact *vkor* expression could provide valuable insights into the mechanisms underlying wildlife responses to AR exposure.

2. Materials and methods

2.1. Study area

This study was conducted in the Region of Murcia (southeastern Spain), a province characterized by a broad environmental gradient and mixed land use. The climate in the study area is mostly semi-arid, with dry summers and mild winters (AEMET, 2024), which significantly influences its ecological dynamics. The environmental gradient spans from the arid steppe lands of littoral areas to the continental Mediterranean forests in the inner mountainous regions, encompassing low mountain ranges, river valleys, and extensive plains across the landscape. Badlands are one of the most representative habitats in semi-arid zones, such as the study area. They are characterized by steep slopes, sparse vegetation, and originate from natural processes such as water runoff and wind erosion. (Alonso-Sarria et al., 2011; Moreno-de Las Heras and Gallart, 2018). This varied topography creates a unique environmental mosaic that influences both human activities and wildlife distribution.

Agriculture is one of the major activities supporting the regional economy (ca. 32% of the territory is occupied by agricultural land; CARM Región de Murcia, 2022), with the fertile lands along the Segura River being extensively utilized as crop fields, mostly stone fruit trees and vegetables. This intense agricultural activity is a major factor in shaping the local habitats, with important consequences on species presence and abundance. Despite the ongoing growth of urban areas (Pérez Morales et al., 2016), large extensions of natural and semi-natural areas still remain, providing important habitats for wildlife. One of the most representative landscape features of the study area is the presence of "ramblas" (e.g., ephemeral streams or creeks), which are intermittent watercourses that only flow during or after events of heavy rainfall. These natural formations are essential in shaping natural and semi-natural landscapes, and provide key resources for wildlife (such as surface water), thus supporting biodiversity in an otherwise dry environment (Gómez et al., 2005). Overall, this variety within the region allows for a comprehensive analysis of how different environments and human activities can influence the presence and impact of environmental contaminants such as ARs, on local wildlife.

2.2. Study species and sampling

The eagle owl is a large nocturnal raptor distributed throughout the Palearctic region, showing a widespread presence and high population density in southeastern Spain, particularly in the Region of Murcia (León-Ortega, 2016; Pérez-García et al., 2012). In this semi-arid Mediterranean region, the eagle owl displays a remarkable ecological plasticity and it is able to occupy a variety of habitats, from heavily human-modified areas such as urban centres to farmlands and predominantly natural environments. Despite its diet in the study area mostly consists of rabbits (*Oryctolagus cuniculus*) (León-Ortega et al., 2017), it can alternatively feed on a wide array of medium-sized species -e.g., small mammals and birds- depending on prey availability in the environment (Lourenço et al., 2015). The eagle owl is considered a conservation-concern species at regional (Robledano Aymerich et al. 2006), national (Gobierno de España, 2011) and international (European Union, 2009) scales, mostly because its top predatory habits make it particularly sensitive to fluctuations in prey abundance, as those caused by the rabbit haemorrhagic disease (Wissenschaftsverlag et al., 2001). Additionally, its position in the food chain and its feeding habits make it particularly susceptible to the bioaccumulation of toxic substances like ARs (Spadetto et al., 2025), making it an optimal biomonitoring species for environmental contaminants (Gómez-Ramírez et al., 2021).

Blood sampling was carried out in the framework of a regular monitoring scheme targeting the breeding owl population in the Region of Murcia. Blood samples used in this study were collected during the fieldwork described in Spadetto et al. (2025), where various types of samples were collected from the same individuals for different research purposes. From December 2021 to June 2022, breeding territories were monitored to estimate egg-laying, hatching, and fledging dates, and to determine the optimal period to access the nests. Trained technicians accessed the nests when owlets were approximately 30-45 days old, just before reaching the fledging stage (ca. 50-65 days old, Penteriani et al., 2005). A total of 72 blood samples were collected from eagle owl nestlings during the 2022 breeding season, across 26 breeding territories. Sample collection was performed according to a protocol for monitoring contaminants in raptors (Espín et al., 2021), with approval from the Ethical Committee for Animal Experimentation

at the University of Murcia (code 657/2020). Blood samples were drawn from the brachial vein using a sterile syringe fitted with a 23G needle. A 0.3-0.5 ml aliquot of blood was immediately transferred into a 0.5 ml EDTA tube. The tube was gently inverted 3-5 times to ensure the anticoagulant thoroughly mixed with the sample. After that, the blood was transferred into a sterile 2 ml tube containing 1300 µL of RNA*later*® solution (Sigma-Aldrich, USA). The samples were kept refrigerated until arrival at the laboratory, which occurred within a few hours after sample collection, and then stored at -80°C until further analysis.

2.3. Sequence analysis and primer design

The sequences of *vkorc1* and *vkorc1l1* genes into the eagle owl whole genome shotgun sequence bioproject (PRJNA431699) were searched. A partial sequence of *vkorc1* (accession number ML985836.1:6890:7004) and two partial sequences of *vkorc1l1* (accession number ML982884.1:1639227-1639345 and 1638296-1638523) were found. The obtained sequences were aligned, curated, and compared with the reference sequences of *Gallus gallus vkorc1* (Accession No. NM_001395922) and *vkorc1l1* (Accession No. NM_001001328.3). Additionally, they were compared with the *vkorc1* (Accession No. ENSACCT00020025251.1) and *vkorc1l1* (Accession No. ENSACCT00020007277) sequences of the golden eagle (*Aquila chrysaetos chrysaetos*). Partial sequences of *vkorc1* (113 nucleotides) and *vkorc1l1* (342 nucleotides) from the eagle owl were successfully obtained. To confirm the gene identity and to assess the homology with known sequences in related species, the retrieved sequences using BLAST (Basic Local Alignment Search Tool) (Table 1) were aligned. Additionally, to further infer the identity of both gene sequences, a phylogenetic analysis using the Neighbor-Joining method with 26 nucleotide sequences. was performed. There was a total of 542 positions in the final dataset. These analyses were conducted with MEGA7 software (Kumar et al., 2016). Primers for real-time PCR were designed using Primer3plus web application (https://www.primer3plus.com/index.html) (Table 2).

Table 1. Characteristics of *vkorc1* and *vkorc1l1* sequences of eagle owl matched with those of other avian species through BLAST search.

Species (Family)	Accession No[a]	Size (nt)	Query cover (%)	E[b]	Identity (%)
vkorc1					
(Gruidae)					
Grus americana	XM_054808744.1	606	50	2e-60	87.85
vkorc1l1					
(Galliformes)					
Gallus gallus	NM_001001328.3	2491	98	2e-163	97.94
(Accipitrinae)					
Haliaeetus leucocephalus	XM_010573578.1	3622	99	3e-172	99.41
Aquila chrysaetos chrysaetos	XM_030028180.2	4441	99	3e-172	99.41
(Gruidae)					
Grus americana	XM_054847279.1	2531	99	3e-172	99.41

[a]GenBank accession number.
[b]Expectation value.
Nt = nucleotides

Table 2. Forward and reverse primers for real-time PCR of *vkorc1* and *vkor1l1* genes.

Gene name	Symbol		Primer sequence
Vitamin K 2,3-epoxide reductase complex subunit 1	*vkorc1*	F	CTG GTC TTC GGC CTG CAG
		R	CAG TTG AGG GCA AGG AGG AC
Vitamin K 2,3-epoxide reductase complex subunit 1-like1	*vkorc1l1*	F	AGG ATT CGG TCT GTT GGG TTC
		R	CTG CAC TTG CTG TCA TAC CAA G

2.4. RNA extraction and gene expression analysis

Total RNA was extracted from whole blood using the DNA/RNA extraction kit (Biotools) with a DNase step, following the manufacturer's instructions. First, 50 µL of each blood sample in RNA*later*® were centrifuged for 30 seconds at 11,000 *g* to remove the RNA*later* solution. To allow cell lysis, a lysis mix was prepared (600 µL of lysis buffer and 6 µL of 20mM DDT for each sample) and added to 2 mL Eppendorf safe-lock tubes containing one steel ball and the blood sample. Samples were then homogenized for 10 seconds at

a frequency of 30 Hz using a Tissue Lyser II (QUIAGEN, Hilden, Germany). Briefly, the lysate was filtered through a column by centrifugation at 11,000 *g*. Binding conditions were established by adding 350 µL of 70% molecular-grade ethanol to the filtrate, vortexing, and loading the mixture onto a column, followed by centrifugation for 30 seconds at 11,000 *g*. DNA was washed twice with 500 µL of DNA Wash Buffer and centrifuged for 1 minute each time. After drying the membrane, DNA was eluted with 50 µL of DNA Elute Buffer, incubated for 15 minutes, and centrifuged for 1 minute. Residual DNA was digested with 10 µL DNase and 90 µL DNase Buffer, followed by incubation for 15 minutes. RNA purification included three washes with two different buffers and three centrifugation steps (two for 30 seconds and one for 2 minutes). Finally, RNA was eluted with 40 µL of RNase-free water by centrifugation at 11,000 *g* for 1 minute. The RNA yield ranged from 20 to 230 ng/µL, with absorbance ratios (A260/280) between 1.9 and 2.1.

Reverse transcription (RT) to obtain cDNA was performed using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-rad, USA) with random decamers. For RT reaction, 1000 ng of total RNA was used in a final volume of 250 µL. Negative control reactions were conducted without the reverse transcriptase. Additionally, 1 µL of luciferase RNA (0.5 ng μL^{-1}) was added to the reaction as an external control to monitor the efficiency of each step of the process (Jiwaji et al., 2010; Johnston et al., 2012).

Real-time quantitative PCR (RT-qPCR) was performed on RT reactions (200 ng of cDNA) in a final volume of 25 µL, using SYBR Green Master Mix (Biotools, Spain) and specific primers for *vkorc1* and *vkorc1l1* at a final concentration of 0.9 µM (Table 2). The reactions were run on a 7500 Real-Time PCR System (Applied Biosystems, USA). The program used for PCR amplification included an initial denaturation step at 95 °C for 3 minutes, followed by 40 cycles of denaturation for 15 seconds (s) at 95 °C and annealing/extension for 60 s at 60 °C. The specificity of reactions was verified by melting curves analysis (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C) and the linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta-delta Ct method (Livak and Schmittgen, 2001) using luciferase as a housekeeping gene. Gene expression results are presented in Relative Units (RU), which represent normalized values obtained from qPCR data.

2.5. Analysis of environmental variables

ArcGis 10 has been used to characterize some environmental variables within the area of influence of 26 owl nests. A 2-km buffer area was generated around each nest to calculate the surface (m^2) of each land use type, classified as forest (mainly pine trees), watercourses, scrubland, non-irrigated crops, irrigated crops and urban areas (Figure 1). This buffer was selected as an approximation of the home range of the eagle owl in the study area, based on available information (León-Ortega, 2016; Martínez et al., 2003; Pérez-García et al., 2012). Cartographic resources were obtained from the official information system of the Region of Murcia (SITMurcia, 2024). Buffers were also used to identify landfill sites and quantify the number of livestock farms within the home range of each owl territory (Figure 2), distinguishing between equine, cattle, swine, sheep, goat and poultry farms. Data on livestock farms for 2021 in the Region of Murcia were supplied by the Spanish Ministry of Agriculture, Fisheries, and Food.

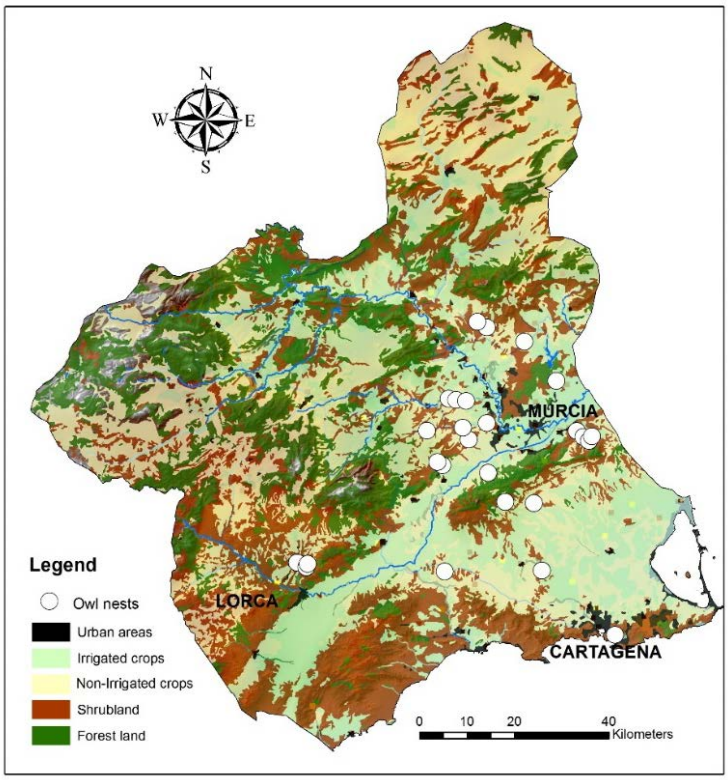

Figure 1. Map of the Region of Murcia (southeastern Spain) depicting the distribution of the different land uses established in this study, as well as the location of the 26 breeding territories (white dots) of the Eurasian eagle owl sampled in 2022.

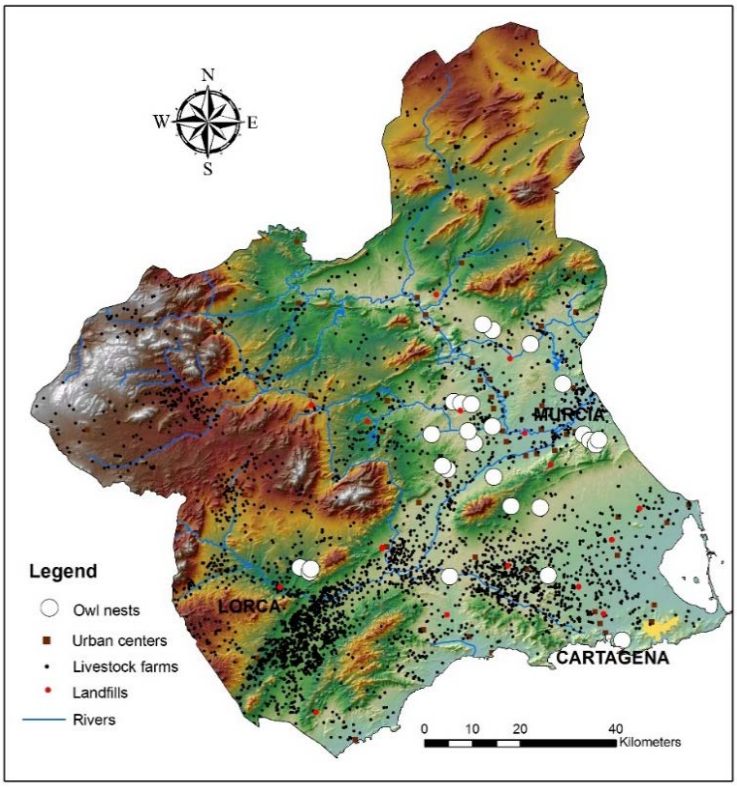

Figure 2. Map of the Region of Murcia (southeastern Spain), showing the locations of livestock farms (black dots), landfills (yellow squares), and the 26 breeding territories (white dots) of the Eurasian eagle owl sampled in 2022.

2.6. Statistical analysis

Basic descriptive statistics, including mean, median, and standard deviation (SD), were calculated for the mRNA expression levels of the *vkorc1*, *vkorc1l1* genes, and their total expression. For comparative analysis, the nest was considered the statistical unit, with nestlings as replicates. The number of replicates (nestlings) per nest varied depending on the number of individuals sampled in each case (range 1-4 chicks per nest, mean ± SD = 2.77 ± 0.71).

Canonical Correlation Analysis (CCorA) was used to explore the relationships between environmental variables (land uses, number and types of farms around owl nests, proximity to a landfill site) and genes implicated in resistance to ARs (mRNA expression levels of *vkorc1* and *vkorc1l1* genes). This analytical approach is widely used in ecological studies and, unlike the redundancy analysis (RDA), it is symmetrical. Let Y1 and Y2 represent two datasets: Y1 contains the levels of *vkorc1* and *vkorc1l1* gene expression and total *vkor* expression, while Y2 includes response variables such as land use types, landfill sites, and livestock farms, with Y1 and Y2 comprising p and q variables, respectively.

Canonical Correlation Analysis aims at obtaining two vectors $a_{(i)}$ and $b_{(i)}$ as follows:

$$\rho(i) = cor(Y1a(i), Y2b(i)) = \frac{\text{cov}(Y1a(i), Y2b(i))}{\text{var}(Y1a(i)).\text{var}(Y2b(i))}$$

The CCorA provides two vectors, *a(i)* and *b(i)*, which are maximised. Constraints must be introduced so that the solution for *a(i)* and *b(i)* is unique because the ultimate intention is to maximise the covariance between *Y1a(i)* and *Y2b(i)* and to minimise their respective variance (Jobson, 1992; Takoutsing et al., 2018). The number of vectors that can be extracted is to the maximum equal to min (p, q). The CCorA results are presented as graphical bi-plot scaling to evaluate the relationship between expression levels of *vkorc1* and *vkorc1l1* and environmental variables. Canonical correlation coefficients range from -1 to 1; values close to ±1 indicate a strong relationship, while mid-range values (e.g., 0.3–0.5) suggest moderate relationships. Wilks' Lambda was used to evaluate statistical significance of canonical correlation. All the statistical analyses were carried out using the XLSTAT (Addinsoft version 2012.2.02)

3. Results and discussion

3.1. *Vkorc1* and *vkorc1l1* expression in eagle owl blood: results and existing research.

Vkor gene expression has been extensively studied in various mammal tissues, such as liver, brain, and lungs. However, avian whole blood represents a valuable sample matrix for gene expression studies due to the presence of nucleated erythrocytes. Unlike mammals, birds, as well as fish and reptiles, have nucleated erythrocytes, which makes whole blood an important tissue for gene expression analysis in environmental and toxicological studies (Farag and Alagawany, 2018; Martos-Sitcha et al., 2017).

To the best of current knowledge, this is the first work to analyse the gene expression of the *vkor* complex in whole blood samples from eagle owls. The results revealed that, in this biological matrix, *vkorc1* is the predominantly expressed gene (median= 5.16 RU), while its paralog *vkorc1l1* shows markedly lower expression levels in blood (median = 2.49 RU), with a median in the ratio *vkorc1*:*vkorc1l1* of 1.79 (Table 3). Consequently, the

total expression of the VKOR complex in whole blood of eagle owls is primarily driven by *vkorc1*, with *vkorc1l1* playing a secondary role (Figure 3). Nevertheless, it is worth to note that the ratio was lower than 1 in 31.9% of the assessed blood samples, thus indicating that *vkorc1l1* predominates in blood expression in approximately one-third of the owl nestlings.

Table 3. Basic statistics of mRNA expression levels of *vkorc1*, *vkorc1l1*, total expression (sum of both genes' expression) and the *vkorc1*:*vkorc1l1* ratio for eagle owl nestlings (*n*=72) sampled in the Region of Murcia (southeastern Spain) in 2022.

	Mean	SD	Median	Min.	Max.
Vkorc1	5.16	3.88	4.22	0.55	21.54
Vkorc1l1	2.49	1.38	2.25	0.63	9.15
Total expression	7.65	4.15	6.28	1.88	22.50
Ratio *vkorc1*:*vkorc1l1*	2.71	3.14	1.79	0.23	22.49

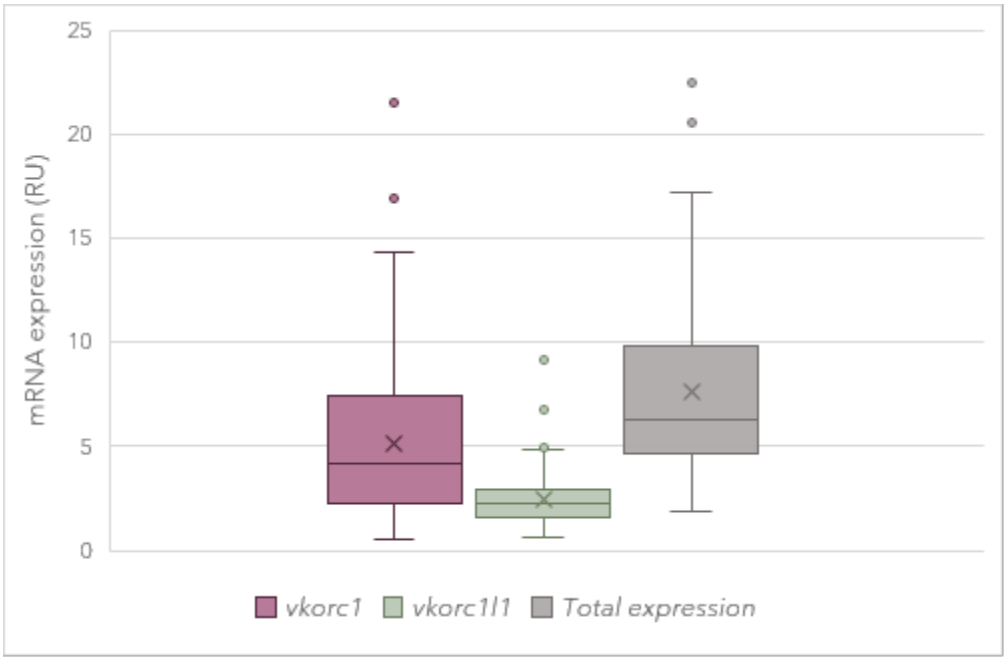

Figure 3. Expression of the *vkorc1* and *vkorc1l1* genes (median= 4.22 and 2.25 RU, respectively), as well as the total expression (sum of the expression of both genes; median= 6.28 RU) in blood of eagle owl nestlings (*n* = 72) from the Region of Murcia (southeastern Spain). In each box, the horizontal line represents the median, while the "X" indicates the mean. Whiskers show the range of data excluding outliers, which are displayed as individual points.

The VKOR complex has been studied since the 1970s (Bell and Matschiner, 1970; Oldenburg et al., 2007) and, up to the late 2000s, VKORC1 was considered the main, and likely unique, component with VKOR activity (Oldenburg et al., 2007). Spohn et al. (2009) generated *vkorc1* knockout mice to investigate the primary *in vivo* function of this enzyme. These mice died between 2 and 20 days after birth due to severe internal haemorrhages caused by the depletion of activated coagulation factors. This study ruled out the presence of other enzymes with VKOR activity capable of compensating for VKORC1 suppression or inhibition and highlighted the essential role of this enzyme in the vitamin K cycle. However, it is noteworthy that the mice studied by Spohn et al. (2009) did not exhibit significant embryonic mortality following *vkorc1* silencing, unlike findings from other studies involving mice with silenced genes for prothrombin and Factor X (prothrombin-/- and FX-/-) (Dewerchin et al., 2000; Sun et al., 1998). These authors observed that *vkorc1* knockout reduced γ-carboxylation efficiency but did not inhibit alternative pathways preventing embryonic death.

Subsequent research conducted on human embryonic cells confirmed that VKORC1L1 has VKOR and vitamin K quinone reductase (VKR) enzymatic activities, functions that were previously attributed only to VKORC1 (Westhofen et al., 2011). However, it was observed that VKORC1L1 exhibits 2.2- and 7.3-fold lower affinity for the two oxidized vitamin K substrates compared to VKORC1. Additionally, warfarin inhibition of VKORC1L1 was measured and found to be 1.8 times lower than that of VKORC1 in the same cell line (HEK 293T; Westhofen et al., 2011). It has been also observed that mice deficient in both *vkorc1* and *vkorc1l1* (*vkorc1*–/–; *vkorc1l1*–/–) died shortly after birth due to severe haemorrhaging (Lacombe et al., 2017), demonstrating that VKORC1L1 could support the carboxylation process during pre- and perinatal stages. In addition, the overexpression of the *vkorc1l1* gene supports coagulation in adult mice lacking *vkorc1* and VKORC1L1 was confirmed to be less sensitive to warfarin inhibition *in vivo*. In conclusion, the available data suggest that although *vkorc1l1* is expressed at lower levels than *vkorc1* in the adult liver, it can contribute to some extent to the production of functional coagulation factors when VKORC1 is completely inhibited (Lacombe et al., 2017; Lacombe and Ferron, 2018).

Hammed et al. (2013) observed that vitamin K antagonists also inhibit VKORC1L1, but this enzyme was found to be approximately 30–50 times more resistant compared to

VKORC1, which is consistent with subsequent studies on the sensitivity of both enzymes to ARs (Czogalla et al., 2018). From the studies of Hammed et al. (2013) and Caspers et al. (2015), it also emerged that VKOR activity in extrahepatic tissues likely supported by both isoenzymes in a complementary manner. *Vkorc1* transcription was notably higher in mouse liver compared to other tissues, with glandular tissues such as the salivary and mammary glands also showing relatively elevated levels. In contrast, *vkorc1l1* showed consistent expression across all mouse tissues analysed, with the highest levels observed in the brain and the lowest in the liver (Caspers et al., 2015). Another study on mice and rats demonstrated that *vkorc1* had predominant expression in the liver, with significantly lower levels observed in other tissues such as the kidney, lung, testis, and brain. In contrast, *vkorc1l1* was expressed more uniformly across all tissues analysed. In the brain and testis, *vkorc1* expression was slightly lower than *vkorc1l1* (Hammed et al., 2013). The discovery of high extrahepatic mRNA expression of VKORC1L1 (Hammed et al., 2013), together with a high inhibition constant (Ki) of VKOR for warfarin (indicating lower warfarin affinity), suggests that higher expression of this gene could lead to warfarin resistance, at least in mammals. Consequently, susceptibility to warfarin and ARs in general may also depend on the expression ratio of *vkorc1* and *vkorc1l1* mRNA.

As highlighted by Oldenburg et al. (2007), studies conducted on mice, rats, humans, and zebrafish revealed that the expression ratio of the two genes can vary significantly depending on the species, although high hepatic expression of *vkorc1* is generally confirmed. However, it is worth to note that in some species *vkorc1l1* is expressed in significant amounts, including at the hepatic level. For example, *vkorc1*:*vkorc1l1* ratio in the liver is approximately 2:3 in chickens (Nakayama et al., 2020). Unfortunately, studies on VKOR genes and activity in avian species remain limited. The nucleotide sequence of *vkorc1* in GeneBank was only available for nine avian species (including turkey, brown roatelo, chicken, ostrich, canary, hummingbird, sandgrouse, crested ibis, and emperor penguin) and no *vkorc1* sequence from raptor species had been reported before the study by Nakayama et al. (2020). In contrast, the *vkorc1l1* nucleotide sequence was available for over 100 bird species. The authors identified key amino acid variations in both *vkorc1* and *vkorc1l1*, including mutations associated with warfarin resistance in mammals, suggesting potential functional differences in these genes and a role in varying sensitivity to ARs across avian species. In fact, the high hepatic expression of *vkorc1l1*

observed in chickens may indicate that this enzyme supports VKOR activity and contributes to their greater resistance to anticoagulants compared to other avian species and mammals (Nakayama et al., 2020).

Based on this previous research, it is reasonable to hypothesize that increased *vkorc1l1* gene expression acts as a compensatory mechanism in the event of VKORC1 inhibition and may play a significant role in birds, depending on the species. Increased expression of this gene must not necessarily improve long-term fitness but could at least be associated with short-term protection to the organism. This might explain why, in a number of samples analysed in this study, *vkorc1l1* expression predominates in the blood of eagle owls, suggesting that the organism is attempting to compensate for recent AR exposure. Conversely, variations in *vkorc1* expression, the primary target of ARs, could reflect an adaptive response, indicating a functional adaptation developed over time to cope with higher levels of AR exposure.

Furthermore, it is important to point out that the avian species for which *vkorc1* and *vkorc1l1* sequences have been defined, belong to distant phylogenetic groups. For instance, chickens and turkeys belong to the Galliformes order, that diverged from the Neoaves clade (including diurnal Falconiformes, Accipitriformes and nocturnal Strigiformes raptors) approximately 90 million years ago (Jarvis et al., 2014). This phylogenetic distance is relevant because genetic differences in the *vkorc1* and *vkorc1l1* genes could reflect adaptive variations that evolved in response to distinct ecological niches and dietary habits. Nakayama et al. (2020) provided gene expression data for both *vkorc1* and *vkorc1l1* only for chickens and turkeys, highlighting the need for further research on raptors to better understand the role of these genes in AR sensitivity across different avian groups.

3.2. Analysis of environmental variables in relation to *vkor* gene expression

Considering previous research on *vkorc1* and *vkorc1l1* gene expression, the aim of this research was to evaluate the suitability of these parameters as biomarkers of exposure and effect to ARs. Specifically, potential correlations between gene expression and various environmental variables selected for their potential role as risk factors associated to the presence of ARs in wildlife were investigated. CCorAs were used to study the

relationship between the expression levels of *vkor1* and *vkorc1l1* genes and the selected environmental variables. In particular, ARs have often been associated with agricultural land use (Elliott et al., 2022; Fernandez-de-Simon et al., 2022; Hughes et al., 2013; Pay et al., 2021), the presence of intensive livestock farming (Geduhn et al., 2015; López-Perea et al., 2019; Rial-Berriel et al., 2021), and urbanization or human population density (Carrillo-Hidalgo et al., 2024; Elmeros et al., 2011; Musto et al., 2024; Serieys et al., 2015; Szapu et al., 2024).

The land-use analysis revealed that *vkorc1l1* expression in owl nestlings was significantly higher in areas with a greater presence of urban zones (Figure 4a). In fact, anthropization is considered one of the major risk factors for AR exposure (Spadetto et al., 2025, 2024). In residential areas, AR application is often massive and recurrent, so the increase in mRNA expression of this specific gene observed in this study suggests that owlets had recently been exposed to ARs, showing a short-term response to the exposure stimulus. Furthermore, higher AR concentrations were detected in eagle owl chicks from the most urbanized area of the Region of Murcia (Spadetto et al., 2025), while the extent of artificial surface (e.g., urban areas and industrial facilities) and human population density were identified as the main risk factors for AR exposure in kestrel and barn owl chicks from the same study area (Spadetto et al., 2024).

Agricultural land did not appear to be related to *vkorc1* and *vkorc1l1* gene expression, with similar results for natural land uses (i.e., forest and scrubland). In related studies conducted in the study area and other regions of Spain (López-Perea et al., 2019; Spadetto et al., 2025, 2024), no relationship was detected between agricultural land use and AR contamination, which is consistent with the fact that ARs in Spain are classified as biocides and should not be directly applied in open spaces by untrained personnel (Ministerio de Sanidad, 2024). Furthermore, when land use was grouped into three broad categories (=natural, agricultural, and urban areas; see Figure 4b) a negative relationship emerged between *vkorc1* expression and the proportion of natural land uses in the owl's home range, likely reflecting lower anthropogenic impact and reduced AR use in these areas. Once again, *vkorc1l1* gene expression was associated with urban areas, while a certain link between *vkorc1* and agricultural land in general seemed to exist, perhaps suggesting that these compounds had been used over time on agricultural fields. This

result is also evident in the analysis of different land uses (Figure 4a) and specifically concerns irrigated lands (intensive agriculture), although it appears to have less significance compared to other variables.

River margins are not often considered a relevant land use type affecting AR exposure in wildlife. However, a recent study targeting the same eagle owl population found that the prevalence of two second-generation ARs (difenacoum and brodifacoum) was linked to the proximity to the nearest ephemeral stream (Spadetto et al., 2025). In this context, other authors have already hypothesized that aquatic environments may serve as a pathway of AR exposure in wildlife, particularly in semiarid regions where surface water availability is scarce and these watercourses, often intermittent, turn into pools of high ecological value (Steward et al., 2012). The scarcity of water resources may increase the concentration of AR compounds and other toxins contained in it (Arenas-Sánchez et al., 2016), derived from the runoff of nearby urban, residential or industrial areas or livestock facilities. This scenario increases the contamination risk for wildlife that depends on these ecosystems for a wide variety of functions (Sánchez-Montoya et al., 2022; Zamora-Marín et al., 2024), creating a contamination source that propagates through the food chain via the biomagnification of these toxic compounds. In the present study, this variable is also associated with increased *vkorc1* expression (Figure 4a), which could indicate repeated exposure events over time. The eagle owl may have responded to this continuous AR exposure through an adaptive regulatory mechanism, increasing *vkorc1* transcription.

Regarding the assessment of the presence of intensive livestock farming on AR exposure (Figure 4c), it can be observed that the types of farms with the greatest influence on *vkorc1* expression were sheep and goat farms, followed by cattle farms, with swine farms likely having a lesser impact on AR exposure. Poultry and equine farms were less represented in the Region of Murcia and therefore did not appear to be related to VKOR related genes expression. In this case, *vkorc1l1* did not seem to be associated with the presence of farms in the proximity of the nests, suggesting that these products were unlikely misused in the context of livestock farming, at least during the sampling period, as no compensatory response of this gene was detected in relation to these facilities. Moreover, in other studies conducted on AR residues in blood samples of eagle owls and other raptors (Spadetto et al., 2025, 2024), livestock farms have not been identified as a

significant risk factor, and only a weak relationship was detected between the number of livestock farms and AR prevalence in barn owl chicks (Spadetto et al., 2024). Indeed, using only the number of farms and the total number of livestock animals as variables within the 2-km buffer, a relationship between *vkorc1* expression and the total number of farms in the buffer emerged once again (Figure 4d). These findings could indicate an adaptive *vkorc1* response linked to these environments where rodent control is often necessary but may not have been adequately managed in the past. Conversely, the total number of animals did not appear to be a variable correlated with *vkor* gene expression. Interestingly, increased *vkorc1* expression was observed in relation to specific environmental variables associated with a higher risk of AR exposure. These results are likely associated to an adaptive and inherited response of this gene, possibly resulting from prolonged selective pressure on a population exposed to an environmental stressor such as ARs. Therefore, *vkorc1* response could reflect a genetic background adapted to the presence of ARs in the environment.

Regarding landfills, it is well known that they are inhabited or extensively used by rodents (e.g., rats), which are often controlled through the application of ARs. The higher availability of these frequently poisoned prey items can result in increased consumption by predators such as the eagle owl. In a previous study on the same target species (Spadetto et al., 2025), higher blood AR levels were found to be associated with the presence of a landfill near the nest. It is therefore unsurprising to observe a relationship between *vkorc1l1* expression and this variable (Figure 4d), suggesting recent and repeated exposure to ARs. In Spain, there was no uniform regulation governing the use of ARs in landfills until 2024. In fact, rodent control practices in landfills depended on local policies and the specific needs of each facility, where the use of ARs was regulated by general biocide regulations (Ministerio de Sanidad, 2024). With the introduction of Commission Implementing Decision (EU) 2024/816 (European Commission, 2024), new guidelines for the use of ARs have been established, emphasizing the use of ready-to-use baits in tamper-resistant bait stations. These devices aim to ensure a safer and more controlled use of ARs, reducing risks to human and non-target animal health. However, the implementation and enforcement of these regulations can vary, and the presence of ARs in the vicinity of landfills remains a concern. Because these regulations were

established after the study period of the present research, future research in the study area would be useful to assess the effectiveness of the recently implemented measures.

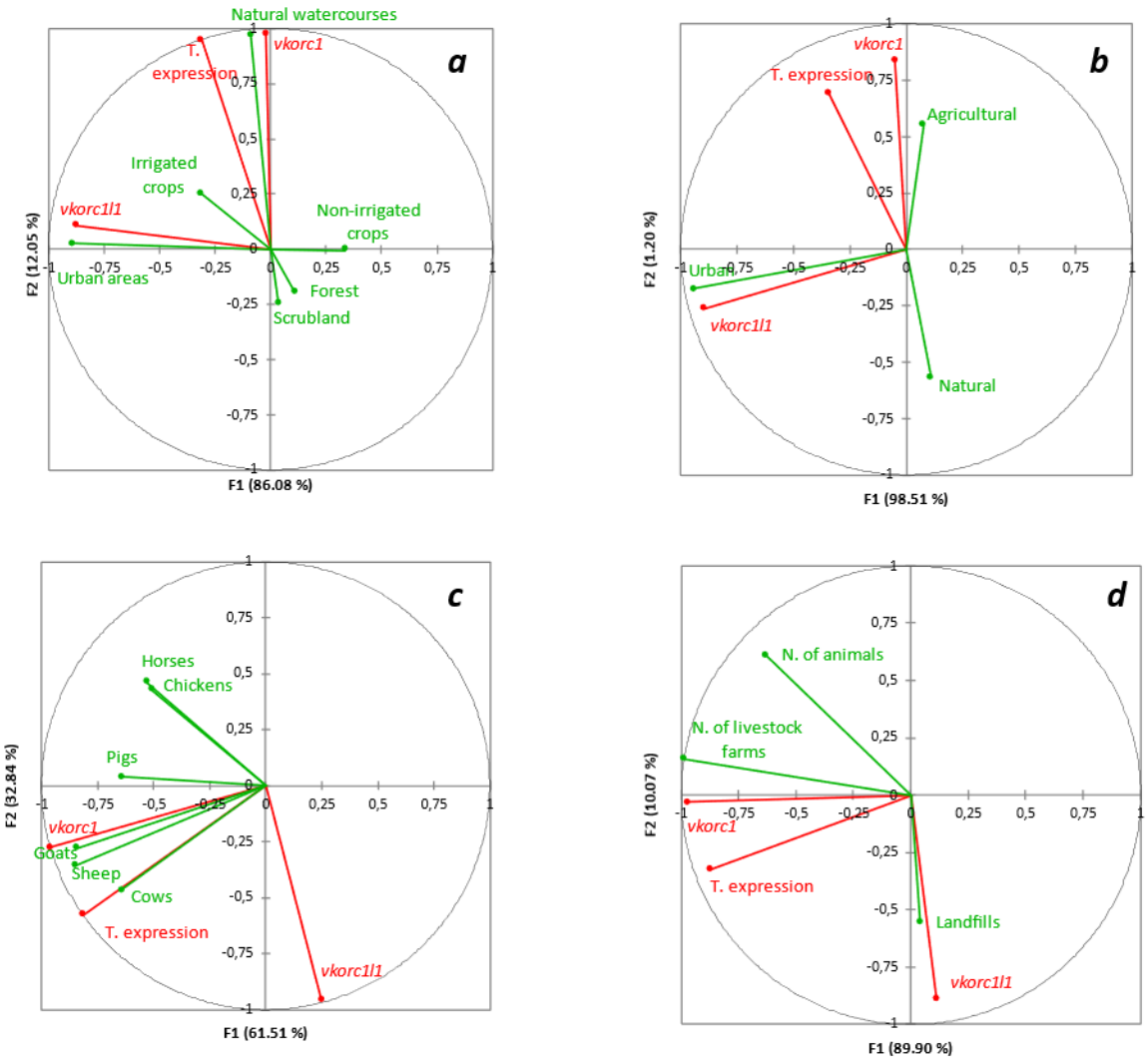

Figure 4. Ordination diagrams based on the Canonical Correspondence Analyses of *vkorc1* and *vkorc1l1* and total gene expression (T. expression; i.e., sum of mRNA expression of both genes) in eagle owl nestlings versus main land uses, landfills and livestock farms in the home range (2-km buffer) of each owl territory. Land use cover was quantified as the surface area (m^2) occupied by each land use type. Fig. 4a considered different land uses; In Fig. 4b land uses were grouped into three main categories; Fig. 4c refers to the total number of animals from different livestock species present within the buffer; In Fig. 4d the total number of livestock farms, animals and landfills within the buffer was considered.

4. Conclusions

This study provides novel information on the gene expression of *vkorc1* and *vkorc1l1* in the blood of a raptor species. Moreover, it highlights the significant role of *vkorc1* and *vkorc1l1* genes in the biochemical response to AR exposure, suggesting potential compensatory and adaptive mechanisms in non-target species such as the Eurasian eagle

owl. The results also suggest that the gene expression of *vkorc1* and *vkorc1l1* in eagle owl nestlings is significantly influenced by various environmental variables associated with anthropogenic activities and land use, where the recurrent and intensive use of ARs poses a tangible risk to wildlife. The increased expression of *vkorc1l1* near urban areas and landfills underscore the importance of these facilities as potential sources of AR exposure. Conversely, the weak relationship observed with agricultural areas and natural vegetation suggests a less significant impact of these land uses within the studied context. Natural watercourses and livestock farms emerged as likely long-term sources of exposure, possibly leading to the upregulation of *vkorc1* as an adaptive response. This approach, combining environmental and molecular data, provides insights into AR exposure dynamics in apex predators, and supports the hypothesis that VKOR related genes may serve as biomarkers for assessing the impact of ARs on wildlife. Further research should explore the genetic adaptations to AR exposure across different species and environmental conditions. By understanding these mechanisms, more effective management conservation strategies can be developed to protect sensitive species and mitigate the adverse effects of these toxic compounds in natural ecosystems.

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General discussion

Based on the analysis of anticoagulant rodenticide (AR) residues in the blood of four raptor species (long-eared owl, barn owl, eagle owl and common kestrel), together with the exploration of species-specific ecological traits and the study of potential biomarkers such as prothrombin time (PT) and *vitamin K 2,3-epoxide reductase* (*vkor*) gene expression, this research aims to contribute to improving knowledge about the risk of exposure and the effects of ARs in non-target species, especially in birds of prey inhabiting the semi-arid Mediterranean landscapes of the Region of Murcia (Southeastern Spain). The study area, characterized by a mosaic of anthropized and natural habitats, provides a unique opportunity to investigate how landscape features influence AR contamination. The results highlighted the widespread presence of ARs in the studied species within the study area and revealed potential ecological and environmental factors that influence exposure. This discussion will synthesize the key findings, starting with an overview of AR exposure across species, before exploring specific ecological drivers and testing possible biomarkers of effect.

1. AR exposure in raptors of the Region of Murcia

The first step in assessing AR contamination within the selected study area was to evaluate the overall exposure levels and prevalence across four raptor species by analysing ARs in whole blood of nestlings from all the studied species. Notably, second-generation ARs (SGARs) showed higher concentrations compared to first-generation ARs (FGARs) (median 0.43 ng mL^{-1} and 0, respectively) (Figure 1). This pattern aligns with the known persistence and bioaccumulative properties of SGARs and with their prevalent use in current rodent control practices, which pose a greater risk to non-target wildlife. As expected, the prevalence of SGARs was significantly higher than that of FGARs (80.3% vs. 7.7%), clearly indicating that the overall prevalence of ARs (81.3%) is primarily driven by the widespread use of SGARs (Figure 2). This highlights the critical role of SGARs in the contamination profile and their dominant contribution to the exposure risks for birds of prey.

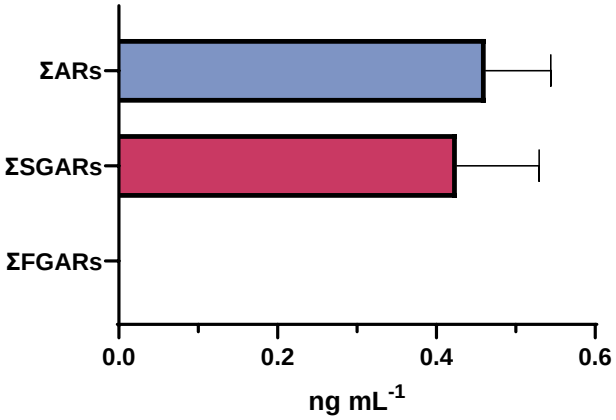

Figure 1. Median concentrations (ng mL^{-1}) and 95% confidence intervals (CI) of total anticoagulant rodenticides (ΣARs), summed second-generation anticoagulant rodenticides (ΣSGARs), and summed first-generation anticoagulant rodenticides (ΣFGARs) detected in the blood of nestling raptors sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

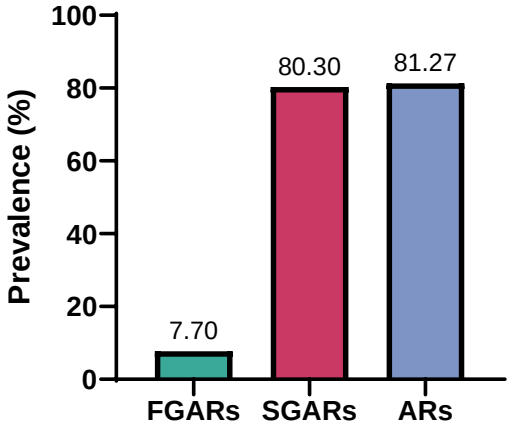

Figure 2. Prevalence (%) of FGARs, SGARs and ARs in general in the blood of nestlings belonging to four raptor species sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

Further insight is provided in Figure 3 by comparing the prevalence of SGARs and FGARs across species. Among the studied raptors, the long-eared owl and the eagle owl showed the highest prevalence of SGAR contamination (98.6% and 91.5%, respectively), likely reflecting their significant exposure through dietary habits and suggesting they are particularly vulnerable to SGAR contamination in their habitats. However, SGAR prevalence was consistently high across all four species, suggesting the possibility of widespread environmental contamination in the study area. These findings underscore the potential risks posed by SGARs to non-target wildlife, particularly to top predators such as raptors, which accumulate these compounds through trophic transfer

(biomagnification) and repeated exposure events (bioaccumulation) (Elliott et al., 2014; López-Perea et al., 2019; López-Perea and Mateo, 2018). Conversely, FGARs were generally not detected or detected at low levels in all species, likely due to their reduced use in rodent control programs following the emergence of resistance in rodent populations (Berny et al., 2018). Despite this, the presence of FGARs, even at low levels, indicates that these older rodenticides are still in use and may sometimes contribute to combined exposure risks for wildlife.

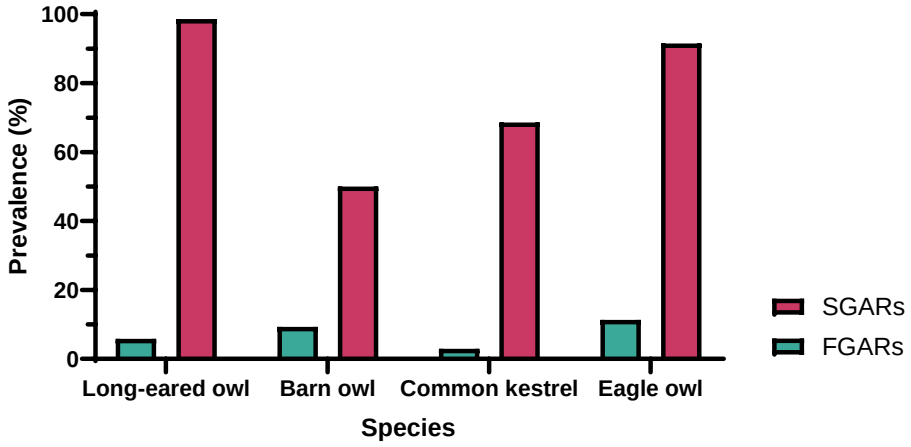

Figure 3. Prevalence (%) of SGARs and FGARs in the blood of nestlings belonging to four raptor species sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

In fact, focusing on second-generation compounds (Figure 4), it can be observed that the prevalent compound was flocoumafen in all species except the barn owl, for which bromadiolone prevails. Brodifacoum and flocoumafen exhibited the highest prevalence, particularly in long-eared owls (63.8% and 88.4%, respectively) and eagle owls (79.2% for flocoumafen), indicating significant exposure for these species. Bromadiolone showed a more uniform distribution across all species, while difenacoum prevalence varied, being notably higher in eagle owls (49.1%). All SGARs were therefore present and used in the study area, except for difethialone, which was scarcely detected in any species, suggesting limited environmental presence.

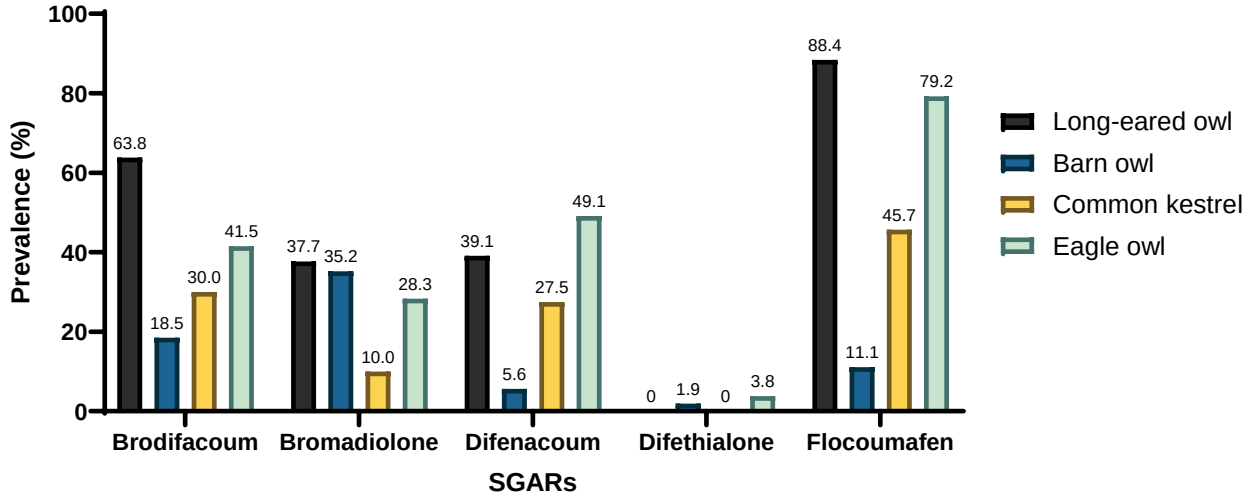

Figure 4. Prevalence (%) of five SGARs studied in the blood of nestlings of the four raptor species sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

The total concentration of SGARs in the blood, as shown in Figure 5, was remarkably low across all bird of prey species, with minimal levels detected in barn owls and common kestrels (median 0.18 and 0.49 ng mL^{-1} respectively). This finding aligns with the pharmacokinetics of SGARs, which are characterized by a short half-life in blood (Horak et al., 2018; Khidkhan et al., 2024). After ingestion, ARs are rapidly distributed to tissues, particularly the liver, where they tend to accumulate, rather than persisting in the bloodstream (Horak et al., 2018). One key factor likely influencing this dynamic is the enterohepatic recirculation of ARs, a process that may temporarily prolong their presence in the body (Watt et al., 2005). However, even with this mechanism, the elimination of these compounds from the liver appears to follow a biphasic pattern (Horak et al., 2018). In the initial phase, blood concentrations decline rapidly as the compounds are redistributed to tissues, while the second phase reflects slower metabolism and excretion. The low blood concentrations observed in this study are more likely to represent a "snapshot" of exposure at the time of sampling rather than a cumulative measure of contamination. This underscores the importance of interpreting blood levels in conjunction with other indicators, such as prevalence rates, which provide a more comprehensive picture of AR exposure in the population. The high prevalence of SGAR detection across the sampled individuals, despite the low blood concentrations, reinforces the idea that SGAR contamination is widespread and persistent in the

environment. It also highlights the necessity of monitoring SGAR residues in both blood and other tissues to fully understand the exposure dynamics and risks to raptor species.

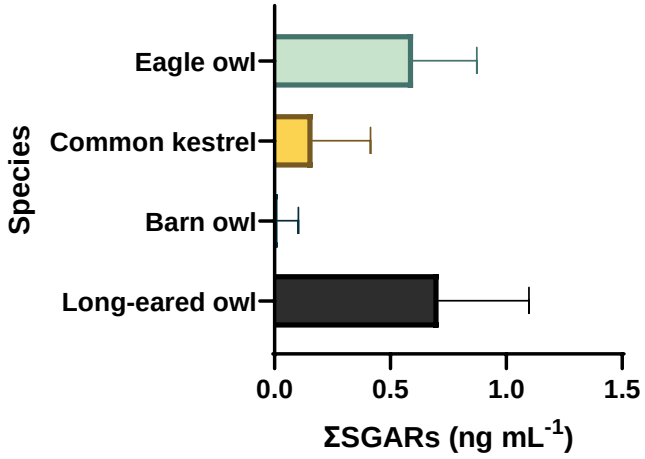

Figure 5. Median concentrations (ng mL^{-1}) and 95% confidence intervals (CI) of summed second-generation ARs (ΣSGARs) detected in the blood of nestling raptors sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

Across all species, a high percentage of individuals exposed to multiple SGARs was observed (Figure 6), with the long-eared owl and eagle owl exhibiting particularly striking rates of 70.8% and 82.6%, respectively. These results are consistent with numerous studies conducted on liver samples from various raptor species (Christensen et al., 2012; George et al., 2024; Moriceau et al., 2022; Murray, 2020). Given that most commercial AR products contain a single active ingredient, these results strongly suggest that multiple products are being used, leading to repeated exposure of prey to various compounds over time. Interestingly, even among nestlings of the common kestrel and barn owl, the presence of multiple SGARs was detected in 32.9% and 16% of cases, respectively. These findings highlight that, despite their relatively short lifespans and limited mobility, nestlings are already subjected to repeated exposure via parental provisioning. This underscores the extent of contamination within the prey base, as adults are likely capturing prey items from various locations, each potentially contaminated with different SGARs. This repeated AR exposure may be attributed to the persistence of SGARs in the environment and their bioaccumulation in different prey species. Additionally, the high rates of multiple compound exposure in top predators reflect their position at the apex of

the food chain, where bioaccumulation and biomagnification of contaminants are more pronounced.

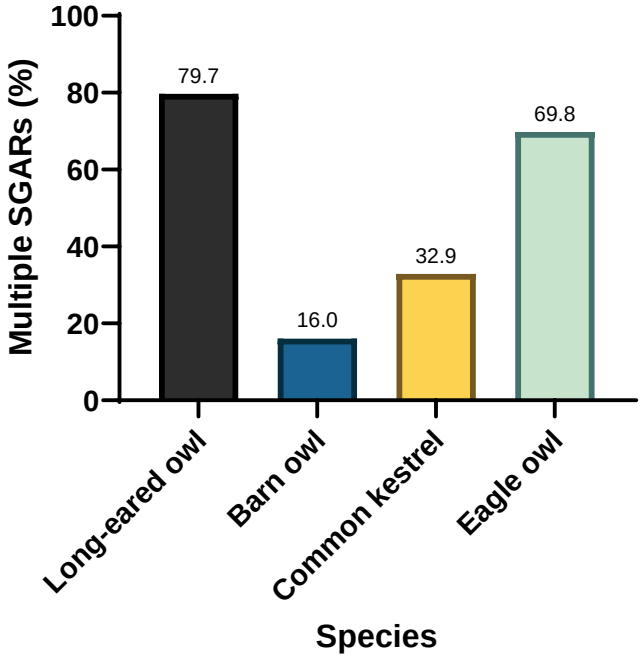

Figure 6. Prevalence (%) of multiple SGARs in the blood of nestlings belonging to four raptor species sampled in the Region of Murcia (southeastern Spain) in 2021-2022.

The effect of age on the prevalence and concentration of ARs could only be partially studied in the barn owl, as it was not possible to collect samples from adult individuals of other species. Even for the barn owl, only 12 samples from non-juvenile individuals were collected and analysed. Nonetheless, the finding of higher concentrations (median = 1.21 ng mL^{-1}) and absolute prevalence (100%) of SGARs in adult individuals of this species is significant. This suggests that adults may have been exposed to these compounds over time, aligning with evidence from previous studies that analysed blood and liver samples (Badry et al., 2021; Buechley et al., 2022; Oliva-Vidal et al., 2022; Roos et al., 2021). These results underscore the importance of considering age as a factor in biomonitoring studies, particularly given the potential for bioaccumulation of persistent compounds like SGARs. Further research involving larger sample sizes and multiple species is essential to better understand the dynamics of age-related exposure and its implications for the health and conservation of raptor populations.

The levels and prevalence of AR exposure found in this study raise concerns about the ecological consequences for these top predators, as well as the potential to cause

sublethal effects that may compromise their health and fitness. Furthermore, the exposure of nestlings to multiple compounds emphasizes the persistent contamination of their prey base and the potential for early-life exposure to accumulate over time. These findings call for enhanced monitoring efforts and mitigation strategies to reduce SGAR exposure in non-target wildlife.

2. Environmental variables influencing AR exposure in nestling raptors

The summarized table (Table 1) highlights the complex interplay between environmental variables and the prevalence and concentration of ARs in nestling raptors in the Region of Murcia. It is evident that exposure pathways to ARs differ among species due to their behaviour, dietary habits, and preferred habitats. In fact, certain factors influence the risk of exposure in some species but not in others. The current results clearly show that the use of ARs, and consequently the risk of contamination for non-target species, is primarily associated with human activities across various environments.

One of the most significant factors of exposure for both the barn owl and the common kestrel was the percentage of artificial surfaces within the buffers considered in the analysis. This suggests that increased urbanization and land-use changes may heighten the risk of AR exposure for these raptors. These two species were sampled within the same study sites but exhibit distinct dietary habits and behaviours. The common kestrel, as previously noted, is a generalist species with a varied diet (Montoya et al., 2021; Riegert and Fuchs, 2011) and tends to breed and hunt near buildings and urban areas. As a result, its exposure to SGARs was relatively uniform across the study sites, with SGAR prevalence primarily influenced by the higher presence of artificial surfaces as a land-use type. In contrast, the barn owl in the Region of Murcia is a rodent specialist that prefers open and dark spaces for hunting, avoiding urban areas whenever possible (Séchaud et al., 2021). In this case, individuals from territories located in areas with small, cultivated plots and gardens (referred to as mixed crops) interspersed with residential zones appear to be more exposed due to the widespread use of ARs in these contexts. Additionally, urbanized zones tend to experience an increase in rat populations, which are primary targets of ARs and are frequently contaminated. The greater availability of these prey items was reflected in the increased presence of rats in the diet of barn owls, as observed

by Spadetto et al. (2024a). For these reasons, another key factor associated with the prevalence of ARs in barn owls was human population density, which was significantly higher in the urban-agricultural area surrounding the city of Murcia, compared to other study sites. This finding aligns with various studies conducted in Spain and other countries, which also link human presence directly to higher risks of contamination by ARs (Badry et al., 2021; Broughton et al., 2022; López-Perea et al., 2019; Musto et al., 2024). The area surrounding Murcia city proved to be more contaminated than other parts of the region, resulting in a higher SGAR prevalence in barn owls and elevated levels of SGARs in kestrels and ARs in eagle owls (Spadetto et al., 2024a, 2025).

Environmental factor analysis was more challenging for the long-eared owl (Spadetto et al., 2024b), a species with a highly varied diet that often breeds near inhabited or rural areas (Birrer, 2009). AR prevalence was almost absolute, and the highest ΣAR levels were recorded in a study site characterized by intensive agriculture. However, no clear pattern of increased exposure or risk in this area could be detected. What did emerge from the analysis of individual SGAR levels was a correlation with the presence of intensive livestock farming near nesting sites (own data). Unlike other species, long-eared owl breeding territories are often located in areas with high densities of livestock farms, including cattle, pigs, sheep, goats, and horses, particularly in the municipality of Puerto Lumbreras, located in the southwestern part of the region. It is likely that ARs were used in these facilities for rodent control, which may contribute to the exposure risk for non-target species, such as the long-eared owls living in the proximity of these farms. Studies conducted in Spain and other regions have also identified this relationship between the presence of intensive farming and AR contamination in non-target species (Geduhn et al., 2015; López-Perea et al., 2019; Rial-Berriel et al., 2021). Rodents attracted by livestock feed could provide a consistent prey base for raptors, thereby increasing their risk of exposure. It is possible that this relationship was not clearly observed for other bird of prey species due to the lower density of livestock farms near their breeding territories.

The last two variables found to significantly influence AR exposure risk were evaluated exclusively for the eagle owl, a species with a high density of breeding territories in the region (León-Ortega, 2016; Pérez-García et al., 2012). Some of these territories were located near landfills or waste treatment plants, where rodents are abundant due to the

presence of waste, and chemical control methods are essential. This factor was correlated with the total concentration of ARs (ΣARs) in eagle owl chicks. Similarly, the idea of assessing the influence of inland riparian network on AR exposure arose from the observation that eagle owls often breed and hunt in *ramblas* or ephemeral riverbeds, which are abundant in the semi-arid landscape of the region. These ecosystems, both vital for biodiversity and sensitive to contamination, can concentrate toxic substances like ARs, which spread and biomagnify through the food web. The findings of this study suggest that two SGAR compounds (difenacoum and brodifacoum) may have reached the eagle owl chicks through this exposure pathway.

Table 1. Summary table showing the environmental factors found to influence the prevalence and levels of ARs in the blood of nestling raptors from the Region of Murcia (southeastern Spain).

		Artificial surface	Human population density	Study site	Livestock farms	Landfills	Watercourse proximity
AR prevalence	Long-eared owl				SGARs*		
	Barn owl	SGARs	SGARs	SGARs[1]	SGARs		
	Common kestrel	SGARs					
	Eagle owl						Brodifacoum and difenacoum
AR levels	Long-eared owl			ΣARs[2]			
	Barn owl						
	Common kestrel			ΣSGARs[1]			
	Eagle owl			ΣARs[1]		ΣARs	

The green background indicates no correlation, red indicates a positive correlation, yellow indicates a weak/uncertain correlation, and gray indicates not applicable. Within the red or yellow cells, individual compounds or compound groups for which a correlation was found are specified.
[1]Higher prevalence or concentration values in the most urbanized study site.
[2]Higher AR levels in an area dedicated to intensive agriculture.
* Flocoumafen, bromadiolone and difenacoum prevalence was found to be related to livestock density (own data).

Together, these results provide valuable data for evaluating AR exposure risk to both wildlife and broader ecosystem health and emphasize the need for a species-specific approach to understanding and mitigating the impacts of ARs on non-target wildlife in anthropogenically influenced environments.

3. Biomarkers of AR effect

Regarding biomarkers of effect to ARs, PT was used as it is considered a sensitive indicator of AR exposure and its effects on blood coagulation (Hindmarch et al., 2019; Rattner et al., 2011; Webster et al., 2015). It was observed that PT values varied depending on the species, with median values of 11.9, 15.3, and 12.7 seconds (s) for the long-eared owl, barn owl, and eagle owl, respectively (Spadetto et al., 2025, 2024a, 2024b) (see Fig. 7). Furthermore, the relationship between blood AR concentrations and PT was positive in all three species and statistically significant in the long-eared owl and barn owl. This finding is crucial, as it demonstrates that PT is an effective biomarker of AR effects, and that even at low blood AR concentrations, adverse effects can be detected in raptor chicks.

As previously highlighted, it remains essential to study baseline PT levels in both juvenile and adult individuals of various raptor species. Additionally, standardizing procedures for PT measurement in birds is critical to obtain comparable results and establish physiological PT ranges. These ranges would enable early detection of AR exposure in intoxicated animals admitted to wildlife rehabilitation centres, where currently PT measurement is not a routine test. However, it could be a valuable tool, as most birds of prey are admitted due to trauma (Cococcetta et al., 2022; Montesdeoca et al., 2016), and concurrent AR intoxication leading to impaired coagulation represents a risk factor that reduces their chances of survival and recovery.

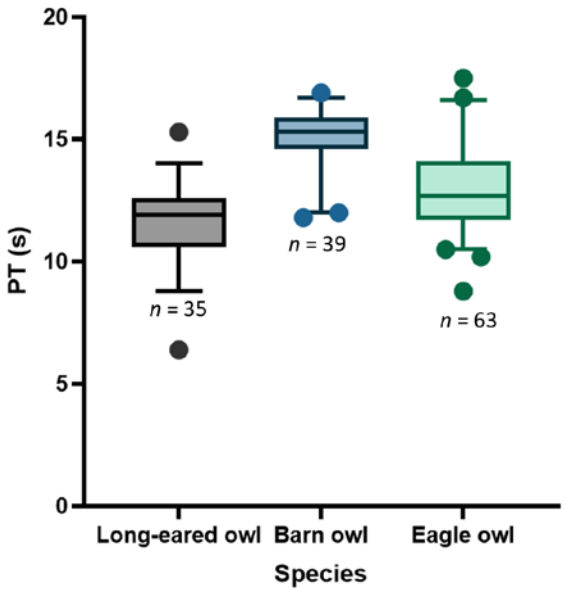

Figure 7. Box plot of PT in seconds for three raptor species sampled in the Region of Murcia (Southeastern Spain). The boxes represent the interquartile range, with whiskers extending to the 5th and 95th percentiles. The line inside each box indicates the median and individual points represent outliers.

As part of this thesis, a novel biomarker of AR effect was also tested: the mRNA expression of *vitamin K 2,3-epoxide reductase complex subunit 1* (*vkorc1*) and *vkorc1-like 1* (*vkorc1l1*) genes in

blood samples from nestling eagle owls. This biomarker allows valuable insights into the short- and long-term responses of raptors to ARs using a small amount of blood (50 µL). Through this study, the first-ever data on *vkor* gene expression in the blood of a raptor species were obtained. These results revealed that *vkorc1* is generally more highly expressed in this tissue. However, in about one-third of the cases, *vkorc1l1* was the predominant gene expressed. It was hypothesized that VKORC1L1, which is less sensitive to anticoagulants, may act as a compensatory mechanism when its paralog enzyme is inhibited by ARs. Therefore, the increased mRNA expression of *vkorc1l1* could indicate a transient response to recent AR exposure. In contrast, VKORC1 is the primary target of ARs, due to their strong affinity for this enzyme. Changes in *vkorc1* gene expression may indicate resistance or susceptibility to ARs (Markussen et al., 2007), and mutations in *vkorc1* sequence have been shown to underlie AR resistance in rodents (Krijger et al., 2022; Maltsev et al., 2021; Yao et al., 2023).

Moreover, the gene expression levels were correlated with environmental variables thought to influence the risk of exposure to ARs, yielding revealing and consistent results. These findings aligned with previous results obtained analysing AR residues in blood samples (Spadetto et al., 2025, 2024a) and indicated recent and significant exposure near urban areas and landfills (increased *vkorc1l1* expression), where AR use is widespread and contaminated prey is more readily available. Interestingly, *vkorc1* mRNA expression increased in association with intensive livestock farms, riparian margins, and, to a lesser extent, agricultural lands. This increased *vkorc1* expression suggests possible historical misuse of ARs in these contexts and an adaptive response shaped by prolonged selective pressure. Notably, the association with proximity to non-perennial riverbeds in semi-arid regions was confirmed by this study, highlighting the need to further investigate contamination pathways linked to riparian ecosystems in Mediterranean areas such as the study site of this research.

Further research into the VKOR complex in birds of prey is essential, as it has been suggested as a key factor underlying the heightened sensitivity to ARs showed by these species (Khidkhan et al., 2024; Nakayama et al., 2020; Watanabe et al., 2010). Obtaining the full sequence of these genes will be decisive for studying mutations that explain inter- and intraspecific differences in sensitivity and resistance to ARs.

4. Role of raptors as biomonitoring species for AR contamination

Birds of prey species, such as the barn owl, common kestrel, long-eared owl and eagle owl, are widely recognized as important biomonitoring species for assessing environmental contamination (Badry et al., 2020; García-Fernández et al., 2023; Gómez-Ramírez et al., 2014). Their widespread distribution, varied diets, and ecological sensitivity make these raptors invaluable allies in monitoring environmental health, underlining the need for continued research and protection of their populations (Kovács et al., 2008). Their role is particularly significant due to their position at the top of the food chain, which makes them highly susceptible to bioaccumulation and biomagnification of pollutants such as ARs. By preying on rodents, other small mammals, birds and invertebrates, these raptors integrate contamination from lower trophic levels, providing valuable insights into the extent and distribution of toxicants within ecosystems (Movalli et al., 2017).

The findings of this thesis further reinforce their value as biomonitors, given the strong correlations observed between AR exposure and specific environmental variables, such as urbanization and proximity to livestock farms or landfill sites (Spadetto et al., 2025, 2024a, 2024b). Nestlings, in particular, serve as sensitive indicators of local contamination levels because their diets and habitats are restricted to their parents' breeding territories, specifically the immediate surroundings of their nests during development. This localized exposure makes them ideal for detecting spatial patterns of pollutant distribution.

In addition to their ecological importance, the use of these raptors as biomonitoring species is instrumental for wildlife conservation and environmental risk assessment (Ratajc et al., 2022). Monitoring AR prevalence and concentrations in these species provides early warnings about the widespread use and potential misuse of ARs, which could otherwise remain undetected. These data can inform management strategies to mitigate risks to both wildlife and human health, ensuring a more sustainable coexistence between pest control measures and biodiversity conservation (Movalli et al., 2018).

5. AR mitigation strategies and implications for conservation

Reducing AR exposure through sustainable pest management. The widespread detection of ARs in nestlings of different species of raptors underscores the urgent need to

implement more sustainable pest management strategies to mitigate secondary poisoning. Integrated Pest Management approaches that prioritize non-chemical methods, such as habitat management (cultural control), trapping (physical control), and the use of rodent predators or anti-fertility products (biological control) (Stuart et al., 2024), can reduce reliance on ARs. Where no alternatives to chemical control are available, rodenticides should be used responsibly, ensuring proper application to minimize risks, and priority should be given to eco-friendly options (Burke et al., 2021; Damin-Pernik et al., 2016; Jokić and Blažić, 2021). Moreover, the application of ARs should be strictly regulated, particularly in areas of high biodiversity or where sensitive species, such as raptors, are present. This includes spatial restrictions near breeding sites and controlled distribution to licensed operators.

Targeted measures for high-risk areas. The results of this study indicate that AR exposure in nestling raptors is strongly influenced by environmental variables such as urbanization and proximity to landfill sites or livestock farms. Targeted mitigation strategies should focus on high-risk areas where AR prevalence and concentrations are particularly elevated. For example, in urban and peri-urban zones, public awareness campaigns about the environmental impact of ARs could promote responsible use among residents. In agricultural landscapes, collaboration with farmers to reduce AR use through improved grain storage, mechanical traps, or natural predator conservation can lower rodent populations without extensive chemical input.

Wildlife-friendly policies and regulations. To reduce AR-related risks, stronger policies and enforcement mechanisms are essential at local, national, and international levels. Wildlife-focused regulations should ensure that AR products are marketed and applied responsibly, with consideration of their environmental impact. This could include restrictions on the use of SGARs to limit their availability and substitution with less hazardous rodent control methods. Periodic review of AR formulations, labels, and application guidelines should be required to align pest control practices with biodiversity conservation goals.

Implications for future research. This thesis highlights critical knowledge gaps that should be addressed in future research. First, additional work is needed to investigate the long-term sublethal effects of AR exposure on raptor health, reproduction, and survival. While

this study focused on blood concentrations, monitoring AR residues in other tissues (e.g., liver) and their impact on fitness metrics would provide a more comprehensive understanding of the risks. Second, exploring the potential for population-level impacts, particularly in heavily exposed species like eagle owls and long-eared owls, will help quantify the broader conservation implications.

A key component of future research should also include the continuation of long-term monitoring programs. These programs are essential to track trends in AR contamination, population dynamics, and reproductive parameters over time. Regular biomonitoring can provide critical insights into the effectiveness of mitigation measures and help identify emerging risks or hotspots of contamination, enabling timely interventions.

Another important approach is the development of biomarkers for AR exposure and toxicity. Advanced molecular techniques, such as transcriptomic or proteomic profiling, could aid in identifying early warning signals of AR effects (Rached et al., 2020), allowing for more proactive conservation measures. Furthermore, studies investigating how AR residues interact with other environmental stressors (e.g., habitat loss, climate change, or other pollutants) could provide insight into cumulative impacts on raptor populations.

Education, training, and cross-sector collaboration. Public education and training programs are indispensable in raising awareness about the risks of ARs and promoting alternative pest management practices. Outreach efforts targeted at key stakeholders, such as farmers, pest control operators, and urban communities, can encourage practices that benefit both wildlife and human health. Training programs for professionals can improve the implementation of mitigation strategies and ensure their alignment with conservation objectives.

Cross-sector collaboration is equally crucial. Effective mitigation requires the involvement of diverse stakeholders, including government agencies, conservation organizations, agricultural and livestock sectors, and local communities. Building partnerships between these groups can enhance the design and implementation of AR management plans while fostering the exchange of knowledge and resources. International cooperation is also vital, as AR contamination is a global issue that transcends political boundaries.

Promoting ecosystem-based conservation approaches. Finally, conserving raptors as apex predators involves not only reducing AR exposure but also protecting the ecosystems on which they depend. Restoring natural prey populations, ensuring the availability of suitable nesting sites, and reducing habitat fragmentation are integral components of a holistic conservation strategy. Promoting the role of raptors as natural pest controllers could shift societal attitudes towards these species, leading to coexistence and reducing the demand for chemical rodenticides.

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General conclusions

This thesis provides comprehensive insights into the widespread contamination by anticoagulant rodenticides (ARs) in a Mediterranean semiarid environment and highlights their impact on non-target raptors species. Through an integrative approach combining ecological, biochemical, and genetic analyses, key risk factors, biomarkers of effect, and pathways for future monitoring and mitigation were identified. The following conclusions summarize the main findings and their implications.

1. The results of this thesis reveal significant and widespread AR contamination in the target area (Region of Murcia, southeastern Spain). Both terrestrial and aquatic ecosystems were affected, underscoring the pervasive nature of AR residues in Mediterranean semiarid landscapes. The persistence and bioaccumulation of ARs, especially second-generation compounds, in the environment pose a serious threat to local biodiversity, particularly to species at the top of the food chain, such as birds of prey.

2. Data demonstrate that exposure to ARs begins at early life stages for raptors. Nestlings exhibited detectable levels of AR residues, often involving multiple AR compounds. This repeated and cumulative exposure throughout life can lead to chronic health effects, such as impaired reproduction, weakened immunity, and increased mortality, compromising population stability in the long term.

3. Human activities such as increasing urbanization, livestock farming and waste management play a pivotal role in shaping AR exposure risk. In fact, raptors breeding or foraging near urbanized areas, intensive livestock farms and landfill sites face increased AR exposure due to the massive use of ARs to control rodent populations. Additionally, this study highlights the role of aquatic ecosystems, particularly non-perennial riverbeds, which are characteristic of Mediterranean semiarid landscapes. These ecosystems may act as pathways for AR contamination, allowing toxic substances to accumulate and biomagnify through the trophic chain.

4. Prothrombin time (PT) was confirmed as an effective and reliable biomarker for detecting sublethal effects of AR exposure. This biochemical marker reflects

disturbances in blood coagulation pathways caused by ARs, providing valuable information on the adverse impact of these compounds on exposed wildlife. Monitoring PT in non-target species can therefore serve as an important tool for assessing AR exposure and associated health risks.

5. Analysis of *vkorc1* and *vkorc1l1* gene expression has provided further insights into response to AR exposure in the eagle owl (*Bubo bubo*). These genes, which are involved in vitamin K cycle and AR sensitivity, could offer a deeper understanding of individual and species-specific responses to ARs. By integrating genetic data with contaminant levels and ecological information, previously identified environmental risk factors could be confirmed to better assess the risks posed by ARs.

6. Raptors have proven to be highly effective biomonitoring species for AR contamination in Mediterranean semiarid regions. Their role as apex predators, combined with their reliance on prey species that may carry AR residues, makes them sensitive indicators of environmental contamination. This study demonstrates how raptors can serve as sentinels for identifying areas of concern, monitoring contaminant trends, and assessing the broader ecological impact of ARs in semi-arid ecosystems.

7. Long-term biomonitoring programs are essential to fully understand the temporal dynamics of AR contamination and its impact on non-target wildlife. By continuing to monitor contaminant levels in biological samples, biomarkers of AR contamination, and population trends over time, changes in contamination patterns can be identified, the effectiveness of mitigation strategies can be evaluated, and evidence-based conservation measures can be developed. Such efforts will be critical to safeguarding vulnerable species and preserving ecosystem health in Mediterranean regions.

8. Immediate and targeted mitigation measures are needed to reduce the exposure of non-target species to ARs. This includes implementing alternative rodent control methods, such as integrated pest management and non-chemical approaches, which minimize the environmental impact of AR use. Additionally, stricter

regulations on AR application near sensitive wildlife habitats and information campaigns on the correct use of ARs could help mitigate exposure risks. Collaborative efforts among stakeholders, including professionals in the agricultural and livestock sector, policymakers and local communities, will be essential to protect biodiversity and ensure the sustainable coexistence of human activities and wildlife.

Extended abstract

General introduction

Anticoagulant rodenticides (ARs) are widely used for the control of rodent populations, primarily in agricultural, urban, and industrial settings (Capizzi et al., 2014). In fact, rodents pose a major concern due to the extensive damage they cause to crops, forests and infrastructure, as well as their role as vectors of diseases transmissible to humans and livestock (Imholt et al., 2017; Jacob and Buckle, 2018; Morand et al., 2019). The effectiveness of ARs in reducing rodent populations has made them one of the most common tools in pest control strategies (Capizzi et al., 2023). However, despite their efficacy, the use of ARs presents significant challenges, particularly regarding their environmental impact and the risks they pose to non-target wildlife (López-Perea and Mateo, 2018).

ARs work by reducing the ability of blood to clot, causing internal bleeding and eventually death in exposed rodents. Their delayed-action mechanism increases the likelihood that a rodent will return to the bait multiple times, enhancing the effectiveness of the poison (Berny et al., 2018). However, the same mechanism of action also poses a substantial risk to non-target species, including predators and scavengers that feed on poisoned rodents. Non-target wildlife exposure to ARs has been documented worldwide, raising concerns about the broader ecological consequences of their use (Nakayama et al., 2019).

ARs are divided into two main categories: first-generation ARs (FGARs) and second-generation ARs (SGARs). FGARs, such as warfarin, chlorophacinone or coumatetralyl, were developed in the mid-20th century and require multiple feedings to be lethal. Their effectiveness in controlling rodents soon diminished due to the emergence of resistance, which prompted the development of more potent SGARs (Jacob and Buckle, 2018). SGARs, including brodifacoum, difenacoum, flocoumafen, bromadiolone, and difethialone, are highly toxic and can be lethal after a single ingestion (Mcgee et al., 2020; Murphy, 2006). Their prolonged persistence in animal tissues increases the risk of secondary poisoning in predators and scavengers. SGARs are currently the most commonly used ARs due to their effectiveness in managing rodents, despite the fact that

resistance has been widely documented for these compounds as well (Mcgee et al., 2020).

The primary mode of action of ARs involves the inhibition of the enzyme vitamin K 2, 3-epoxide reductase (VKOR), which is critical for the recycling of vitamin K. Vitamin K is an essential cofactor in the synthesis of clotting factors II, VII, IX, and X, which are necessary for the blood coagulation cascade. By blocking the VKOR enzyme, ARs prevent the activation of these clotting factors, leading to prolonged bleeding and eventually death from haemorrhage (Rattner and Mastrota, 2018). The pharmacokinetics and pharmacodynamics of ARs vary across species, with notable differences between mammals and birds. Birds, unlike mammals, lack certain clotting factors, such as factors XI and XII, and rely primarily on the extrinsic pathway to initiate coagulation (Buzala et al., 2017). Moreover, birds possess thrombocytes which are nucleated cells that play a role in blood clotting, being less reactive compared to mammalian platelets (Gallo et al., 2015). Significant interspecific differences have been observed in both the metabolism of ARs and VKOR activity (Nakayama et al., 2020; Watanabe et al., 2015). These variations are essential to consider when evaluating the risks and impacts of AR exposure in avian species.

The use of ARs is regulated at both national and international levels to balance pest control efficacy with environmental protection. In Europe, the regulatory framework for ARs is governed by the European Union's Biocidal Products Regulation (EU BPR No. 528/2012; European Union, 2012), which requires the authorization of biocidal products based on their efficacy, safety, and environmental impact. This regulation aims to minimize risks to human health, animals, and the environment by enforcing strict guidelines on the use, labelling, and packaging of AR products. In Spain, the use of ARs is further regulated by national laws that implement the EU directives. Despite these regulations, biomonitoring studies indicate that AR residues are still frequently detected in wildlife, suggesting that current measures may not be sufficient to prevent environmental contamination (Carrillo-Hidalgo et al., 2024).

Non-target wildlife is exposed to ARs primarily through two pathways: primary exposure, which occurs when animals directly consume AR bait, and secondary exposure, which occurs when predators and scavengers feed on poisoned prey (Shore and Coeurdassier,

2018). Tertiary exposure is also possible, for example, through the ingestion of insectivorous species, as invertebrate can also access AR baits (Elliott et al., 2014). Birds of prey are particularly vulnerable to secondary poisoning due to their reliance on rodents (AR target species) as a primary food source. Studies have shown that AR residues are commonly detected in the liver of raptors, indicating widespread exposure in various ecosystems (López-Perea and Mateo, 2018; Nakayama et al., 2019). Other non-target species, including mammals (Keating et al., 2024), as well as reptiles (Martín-Cruz et al., 2024) and amphibians (Rowley et al., 2024), have also been found to carry AR residues. The widespread presence of ARs in non-target wildlife raises concerns about the potential for population-level effects, particularly in species that are already threatened or endangered (Cooke et al., 2022; Roos et al., 2021).

The health effects of AR exposure in non-target wildlife vary depending on the level and duration of exposure. Acute exposure can cause severe haemorrhaging and death (Rattner and Mastrota, 2018), while sub-lethal exposure may result in impaired reproduction, reduced fitness, and increased susceptibility to diseases (Herring et al., 2023; Salim et al., 2014; Serieys et al., 2018). Chronic exposure to low doses of ARs can have cumulative effects, leading to long-term health effects that may not be immediately apparent. On an ecological level, the impacts of ARs may extend beyond individual animals to entire populations. The decline of predator populations due to AR poisoning can potentially have cascading effects on ecosystem stability and biodiversity (Gomez et al., 2022), leading to increased rodent populations and further use of ARs.

Birds of prey are considered suitable sentinel species for monitoring environmental contamination due to their position at the top of the food chain, long lifespan, wide distribution and sensitivity to pollutants (García-Fernández et al., 2024; Gómez-Ramírez et al., 2014). Biomonitoring studies involving raptors can provide valuable insights into the prevalence and distribution of ARs in the environment (Badry et al., 2022). By analysing AR residues in liver, blood or plasma samples of birds of prey, researchers can assess the effectiveness of regulatory measures and identify high-risk areas for AR exposure. Furthermore, biomonitoring data can assess the usefulness of conservation strategies and guide policy decisions to reduce the environmental impact of ARs (Movalli et al., 2017). Active biomonitoring involves the proactive surveillance of environmental contaminants

by regularly collecting and analysing biological samples from selected species (Guberti et al., 2014). This approach ensures sample quality, allows for obtaining representative samples, gathering detailed information, and monitoring populations over time.

In the context of AR exposure, biomarkers can be used to monitor both exposure and effects in non-target species. In fact, biomarkers are defined as measurable biological indicators that reflect the interaction between an organism and a chemical substance such as ARs (De Coen et al., 2000). Common biomarkers of exposure include the detection of AR residues in blood, liver or other tissues, which can indicate recent or chronic exposure (Rached et al., 2020). Prolonged prothrombin time (PT) or the Russell viper venom time (RVVT) are effect biomarkers that measure the impact of ARs on the coagulation process (Rattner et al., 2010). These tests can reveal sub-lethal effects that may compromise the animal's ability to survive and reproduce. The use of biomarkers is essential for understanding the extent of AR exposure and its potential impacts on non-target wildlife (Rached et al., 2020).

In the study of exposure routes, it is important to consider human-related activities as key drivers of AR exposure in wildlife. In fact, areas with high human density often show widespread AR use to control rodent infestations (Badry et al., 2021). The lack of compliance with safety regulations, such as using bait boxes or removing poisoned rodents, further raises the risk of secondary exposure. Cities and surrounding urban areas are hotspots for AR application as they are frequently used in sewer systems, parks, and residential areas. In addition, livestock farms commonly use ARs to prevent rodent infestations and reduce disease transmission (López-Perea et al., 2015). Finally, large-scale agricultural practices often use ARs to protect crops from rodents (Fritsch et al., 2024). While ARs are not legally approved as plant protection products (PPP) in some countries like Spain, AR applications near storage facilities remain a concern. Wildlife in these areas may consume poisoned prey, leading to high risks of secondary poisoning.

Aim and objectives

General aim

The general aim of this doctoral thesis is to increase the knowledge about the impact of ARs on non-target species through the study of the exposure and effects in four species of birds of prey inhabiting a semi-arid region of the Iberian Peninsula. Particularly, the study was conducted on three nocturnal (long-eared owl *Asio otus*, Eurasian eagle owl *Bubo bubo*, and barn owl *Tyto alba*) and one diurnal (common kestrel *Falco tinnunculus*) raptor species from the Region of Murcia (southeastern Spain). Ultimately, this thesis aims to contribute to the conservation of these and other non-target fauna, using predatory birds as sentinels of environmental contamination.

Specific objectives

Objective 1. (Chapter I) To evaluate AR exposure in long-eared owl nestlings sampled across study sites in the Region of Murcia with varying levels of agricultural intensification, to investigate key environmental factors influencing AR exposure, and to validate PT as a biomarker of AR effect in this species.

Objective 2. (Chapter II) To assess AR exposure in common kestrels (nestlings) and barn owls (nestlings and adults) from areas with varying levels of agricultural intensification and urbanization, to compare exposure levels between these species, examining how environmental factors and ecological traits influence AR exposure risk and to validate PT as an effect biomarker in barn owls.

Objective 3. (Chapter III) To study AR exposure in eagle owl nestlings sampled in the Region of Murcia, assessing biomarkers such as PT and a plasma biochemical panel to evaluate their general health and to identify environmental drivers influencing AR exposure risk while accounting for the species' ecological traits.

Objective 4. (Chapter IV) To analyse mRNA expression of *vkorc1* and *vkorc1l1* genes in blood samples from eagle owl nestlings as biomarkers of AR exposure and effect, to investigate molecular mechanisms underlying AR toxicity and to validate previously identified environmental risk factors.

Materials and methods

Study area

The study area is represented by the Region of Murcia in southeastern Spain, characterized by a semi-arid climate, diverse topography, and mixed land use. Its landscape includes low mountain ranges, river valleys, plains, and badlands formed by erosion, creating a unique environmental mosaic that hosts both human activities and wildlife habitats. Agriculture is a key economic activity, occupying 32% of the land (CARM, 2022), with particular concentration in the fertile plains surrounding the Segura River. The region's hydrology features non-perennial watercourses, which are crucial for biodiversity in the dry environment (Gómez et al., 2005). This diversity makes the Region of Murcia an ideal location to study the influence of human activities and different landscapes on AR exposure in wildlife. Within the study area, some study sites were selected for each study species, characterized by different land uses, topography, and degrees of urbanization, in order to investigate potential variations in AR use and exposure in the four raptor species studied.

Sampling procedures

Sampling was conducted as part of a nocturnal raptor monitoring and marking program in the Region of Murcia during the 2021 and 2022 breeding seasons. Breeding territories were closely monitored to estimate egg laying, hatching, and fledging dates, enabling the selection of optimal moments for nest access. Specialized personnel accessed the nests before the nestlings reached fledging age. Additionally, some adult barn owls were captured inside the nests alongside the owlets.

Blood sampling procedures, approved by the Ethical Committee for Animal Experimentation of the University of Murcia (code 657/2020), followed established protocols for contaminant monitoring in raptors (Espín et al., 2021). Blood was extracted from the brachial vein using a sterile syringe and transferred into heparinized tubes. For citrated plasma samples, 450 µL of blood was mixed with 50 µL of 0.109 M sodium citrate buffer and then centrifuged at 2,500 *g* for 15 minutes to obtain citrated plasma. To obtain plasma from eagle owl blood, a 1 mL aliquot of heparinized blood was centrifuged at 2,500 *g* for 10 minutes. Additionally, a 0.3-0.5 mL aliquot of eagle owl blood was placed into a 0.5 mL EDTA tube before being transferred into a sterile 2 mL tube containing

1,300 µL of RNA*later*® solution. All samples were stored at −80 °C until analysis. The number of blood and plasma samples collected during the study is summarized in Table 1.

To characterize the diet, pellets were collected from 19 long-eared owl and 16 barn owl breeding territories and analysed according to reference literature (Moreno, 1986; Román, 2019).

Table 1. Number of blood samples collected from each raptor species during the 2021-2022 breeding season, including heparinized whole blood, sodium citrate plasma, plasma from heparinized blood, and EDTA-preserved whole blood stored in RNA*later*.

	Sample			
Species	Whole blood	Citrated plasma	Plasma	RNA*later*
Long-eared owl	69	35	-	-
Barn owl	62	39	-	-
Common kestrel	70	-	-	-
Eagle owl	106	62	105	72

AR analyses in blood samples

A modified version of the method described by Taylor et al. (2019) was used for sample extraction, as detailed in Spadetto et al (2024b). The extracts in the vials were analysed following the protocol by Rial-Berriel et al. (2020). Ten ARs (coumafuryl, coumatetralyl, warfarin, diphacinone, chlorophacinone, brodifacoum, bromadiolone, difenacoum, flocoumafen, difethialone) were quantified using a high-performance liquid chromatography coupled with triple quadrupole (HPLC-TQ) system equipped with a reversed phase C18 analytical column of 150 × 2.1 mm and 2.6-µm particle size. Calibration samples were prepared by spiking ARs at concentrations ranging from 0.1 to 50 ng mL^{-1} into blank blood obtained from unexposed hens (*Gallus gallus*). The limits of quantification (LOQ) ranged from 0.01 to 2.5 ng mL^{-1}. The analytical technique exhibited recovery values between 76% and 105%, with a relative standard deviation below 14%.

Coagulation assays and biochemical analysis

Coagulation tests (PT and fibrinogen) were performed using a coagulometer. PT was chosen for analysis as this parameter is altered in birds of prey within 24-48 hours

following the ingestion of ARs, depending on the administered dose (Rattner et al., 2010). 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). A commercial kit, based on the Clauss method (Clauss, 1957), was employed for measuring the fibrinogen concentration in citrated plasma. PT test is based on the addition of thromboplastin to the plasma sample. The use of avian-derived thromboplastin is essential to obtain precise and relevant data in avian PT analysis (Webster, 2009). The thromboplastin reagent was prepared using the Quick method modified by Griminger et al. (1970). All analyses of biochemical parameters in eagle owl plasma samples were performed using a biochemical analyser with commercially available reagents, with the exception of ovotransferrin, which was measured using an ELISA kit. All assays had intra- and inter-assay imprecision <15% and were linear after serial dilutions.

Gene expression analysis of *vkor* in the eagle owl

Partial sequences of the *vkorc1* (113 nucleotides) and *vkorc1l1* (342 nucleotides) genes from the eagle owl genome were identified by aligning them with reference sequences from chicken (*Gallus gallus*) and golden eagle (*Aquila chrysaetos*). To confirm their identity, a BLAST search was performed and conducted a phylogenetic analysis using the Neighbor-Joining method with 26 sequences. A final dataset with 542 positions was obtained using MEGA7 software.

Total RNA was extracted from whole blood using a DNA/RNA extraction kit with a DNase step. Reverse transcription (RT) to obtain cDNA was performed with a RT-qPCR mix (Bio-Rad, USA) using random decamers. To monitor process efficiency, 1 µL of luciferase RNA (0.5 ng μL^{-1}) was added as an external control (Jiwaji et al., 2010; Johnston et al., 2012). RT-qPCR was performed using specific primers for *vkorc1* and *vkorc1l1*. The qPCR results were normalized using the delta–delta Ct method (Livak and Schmittgen, 2001), with luciferase as a housekeeping gene.

Land use analysis

To evaluate the land use configuration around each sampled nest, buffers were generated using Quantum GIS software version 3.16.16 (QGIS Development Team, 2022). The buffer size was determined based on previous studies on the home ranges of

the target raptor species (Arlettaz et al., 2010; Boileau et al., 2006; Taylor, 1994; Village, 1982). Data on land use cover were extracted and categorized into four main classes: natural vegetation, artificial areas, agricultural land, and water bodies. Within the agricultural land category, two additional distinctions were made: non-irrigated agricultural land and irrigated agricultural land. Furthermore, a 'mixed crops' category was established, referring to areas characterized by small cultivated plots and gardens, an agricultural practice widespread in specific areas of the Region of Murcia. The percentage of land use area within each buffer was calculated for all classes and subgroups. Data on human population density (based on the 2021 census) were retrieved for each census tract from the National Institute of Statistics (INE, 2021). Additionally, data on livestock load in 2021 for the Region of Murcia (Ministerio de Agricultura, Pesca y Alimentación) were used to calculate animal density and the total number of farms within each buffer. The role of the hydrological network in AR contamination was also assessed for the eagle owl, a species often nesting in gorges and ephemeral riverbeds. To do this, the minimum distance of each nest to the nearest watercourse was calculated. The proximity of some eagle owl territories to landfills and waste treatment plants, where rodent control is a priority, was also considered.

ArcGIS 10 was used to analyse environmental variables within the influence area of the 26 eagle owl breeding territories considered in chapter IV. A 2 km buffer zone was created around each nest, and land use surfaces (m^2) were calculated, including forest, riparian margins, scrubland, non-irrigated crops, irrigated crops, and urban areas. This buffer was chosen to approximate the eagle owl's home range in the study area (León-Ortega, 2016). The same buffer was used to assess the presence of landfill sites and calculate the number of livestock farms.

Statistical analysis

Basic statistics were computed for AR concentrations in blood samples and eagle owl biochemical parameters. For barn owls and common kestrels, the analysis focused on SGARs due to the low prevalence of FGARs. To investigate the relationship between AR prevalence and blood ΣAR concentrations with environmental factors, the information-theoretic approach described by Burnham and Anderson (2002) was employed. Linear Mixed Models (LMMs) were applied (Pinheiro et al., 2023), with environmental variables as

fixed effects and territory as a random factor. All models were compared to a null model using the corrected Akaike information criterion (AICc).

ΣAR concentration and prevalence were compared across sampling years. ΣSGARs and SGAR prevalence were compared between common kestrels and barn owls. Similar analyses were conducted to compare ΣSGARs and prevalence across age classes (chicks and adults) exclusively for barn owls. In all cases, breeding territory was treated as a random factor.

Diet composition data for long-eared owls were analysed by study site to explore differences in prey categories and their association with AR exposure. For barn owls, regression analyses were conducted to examine the relationship between ΣSGAR concentrations and the proportion of *Rattus spp.* in their diet, considering both prey number and biomass.

The Spearman's correlation test was applied to evaluate the relationships between ΣARs and PT, as well as ΣARs and the plasma biochemical parameters of eagle owls. All statistical analyses were performed using R software version 4.3.1, with significance levels set at $p < 0.05$.

Descriptive statistics were calculated for mRNA expression levels of the *vkorc1* and *vkorc1l1* genes, as well as their total expression. For comparative analyses, nests were considered the statistical units, with nestlings as replicates. Canonical Correlation Analysis (CCorA) was used to explore the relationships between environmental variables and mRNA expression levels of *vkorc1* and *vkorc1l1*.

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

Introduction

Birds of prey, including owls, are highly susceptible to ARs due to their position in food webs and their reliance on prey species that may have been exposed to these chemicals. While several studies have assessed AR contamination in avian predators, most have focused on liver samples from field-recovered carcasses (López-Perea and Mateo, 2018), with fewer studies examining real-time exposure through blood samples (Badry et al.,

2022; Martínez-Padilla et al., 2017; Oliva-Vidal et al., 2022). Blood analysis offers the advantage of documenting recent contamination events due to the short half-life of ARs in plasma (Khidkhan et al., 2024).

The long-eared owl is a nocturnal, generalist predator that preys on small mammals and birds in agroforestry environments. Its feeding habits increase the likelihood of secondary AR contamination, making it an ideal species for assessing AR exposure in agricultural landscapes. Despite being listed as critically endangered in the region, the long-eared owl population has increased in recent years. However, data on AR exposure in this species are limited. The aim of this study was to assess AR exposure in long-eared owl nestlings by analysing blood samples collected in four different areas of the Region of Murcia. The role of environmental factors, such as land uses, in AR contamination levels was also explored. Additionally, PT was evaluated as a specific toxic response to ARs. This study provides valuable information on the threats ARs pose to farmland biodiversity and aims to support wildlife management strategies and agri-environmental policies.

Results and discussion

A total of 69 blood samples were collected from long-eared owl nestlings during the 2021 and 2022 breeding seasons, spanning 29 nesting territories in four areas with different land-use characteristics. AR residues were detected in 98.6% of the samples, with absolute prevalence in nests (100%). SGARs were dominant, with flocoumafen being the most frequently detected compound (88.4%), followed by brodifacoum, difenacoum, and bromadiolone. Multiple AR residues were found in 82.6% of the samples, highlighting widespread contamination. Total plasma AR levels (ΣARs) ranged from 0.06 to 34.18 ng mL^{-1}.

Diet analysis confirmed the long-eared owl as a generalist predator, feeding predominantly on rodents (51.4%) and birds (44.2%), with diet profiles varying among study sites. Despite the predominance of avian prey in some study sites, AR exposure was ubiquitous, suggesting both secondary and tertiary exposure pathways, including the consumption of granivorous-insectivorous birds, which may also be exposed primarily or through contaminated invertebrates. A significant correlation between ΣARs and PT indicated a direct impact of ARs on the haemostatic system of nestlings. Detected AR

levels were generally low and not associated with acute toxicity, although they likely pose long-term risks.

Main conclusions

1. The elevated prevalence of ARs in nestlings, even at sublethal levels, is of concern due to the cumulative and sublethal effects of these compounds, such as coagulopathies and other alterations that could impair long-term fitness.
2. The predominant diet (songbirds vs. rodents) of this generalist predator varied by area and available resources, but this did not affect the prevalence of ARs. As seen in other bird-eating species (e.g., sparrowhawks), alternative exposure routes likely involve the consumption of birds and/or invertebrates.
3. The long-eared owl emerges as an ideal sentinel species for biomonitoring ARs in Mediterranean agroforestry systems due to its broad distribution and the feasibility of data collection.
4. These findings emphasize the urgent need to regulate the widespread use of ARs and implement management measures to safeguard biodiversity in agricultural environments.

Chapter II. Comparing anticoagulant rodenticide exposure in barn owl (*Tyto alba*) and common kestrel (*Falco tinnunculus*): a biomonitoring study in an agricultural region of Southeastern Spain

Introduction

The common kestrel and the barn owl are two key predatory species in Eurasian agroecosystems, playing an important role in controlling rodent populations. However, agricultural intensification, habitat loss, and pesticide use, including ARs, have led to population declines in both species (Buck et al., 2020; Grande et al., 2018). The common kestrel is a generalist predator hunting in open habitats, while the barn owl is more specialized, primarily preying on rodents in farmlands. Both species are currently experiencing population declines, with the kestrel classified as endangered in Spain and the barn owl as nearly threatened (Martínez-Padilla et al., 2021; SEO/BirdLife, 2021).

In this study, the aim was to assess environmental exposure to ARs in both diurnal (*F. tinnunculus*) and nocturnal (*T. alba*) raptors from a Mediterranean agricultural region. Specifically, interspecific, interannual, and age-related differences in AR prevalence and concentrations were investigated. The potential associations between ARs and environmental variables such as land use, human population density, and livestock farming were explored and the relationship between PT and blood AR concentrations as a biomarker of effect was examined.

Results and discussion

A total of 136 blood samples were collected from adult barn owls (*n*= 12), barn owl chicks (*n*= 54), and kestrel chicks (*n*= 70) across 42 breeding territories in 2021–2022. While FGARs were detected infrequently and at low concentrations, SGARs were prevalent. In barn owls, 50% of nestlings and 100% of adults tested positive for SGARs, with higher concentrations observed in adults (median = 1.21 vs. 0.18 ng mL^{-1}). Among kestrel nestlings, 68.6% were positive for SGARs, with a median concentration of 0.25 ng mL^{-1} and a peak value of 11.26 ng mL^{-1} for flocoumafen. Brodifacoum and flocoumafen were the most frequently detected SGARs in both species. Sixteen percent barn owl and 33% kestrel nestlings exhibited multiple SGARs, indicating a cumulative exposure risk.

Environmental variables influenced AR exposure differently across species. In kestrels, SGAR prevalence correlated with the percentage of artificial surfaces within breeding territories, while no significant differences were observed between study sites. Conversely, barn owl exposure was primarily influenced by human density, the percentage of artificial areas, and mixed crops, which were particularly present in urban-agricultural landscapes. Notably, the highest prevalence (78.6%) occurred in the area surrounding the city of Murcia, where mixed crops and human settlements likely increase AR use.

Dietary habits and ecological traits also influenced exposure probability. Kestrels' diversified diets and proximity to human activity may heighten exposure, while barn owls' rodent-specialist diet varied by site, with higher dietary presence of *Rattus spp.* prey in the urban-agricultural study site. SGAR prevalence in barn owls correlated with the presence of *Rattus* spp. in their diet, with these target rodents being highly susceptible to AR contamination and linked to urban areas.

PT was significantly prolonged in individuals with higher SGAR concentrations, suggesting ongoing sublethal adverse effects.

Main conclusions

1. Both barn owls and common kestrels inhabiting Mediterranean agricultural regions were extensively exposed to SGARs. Although concentrations were generally low, high SGAR prevalence and frequent detection of multiple compounds suggested repeated exposure over time.
2. Exposure risk varied between species based on their ecological characteristics. Common kestrels, as generalist raptors that forage in urban areas, showed a slightly higher likelihood of exposure, potentially linked to contamination pathways involving non-target species. Barn owls, which primarily hunt in open and natural spaces, were at greater risk near urban centres with high rodent populations, where the presence of target prey may amplify exposure.
3. Measuring coagulation function offers a promising method to evaluate the adverse effects of SGARs in wild birds of prey.
4. The study highlights the need to integrate ecological factors, such as breeding biology and foraging behaviour, with exposure assessments to develop targeted conservation and management strategies for mitigating the impact of environmental contaminants on raptor populations.

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

Introduction

The Eurasian eagle owl, a top predator with ecological plasticity, serves as a valuable biomonitoring species due to its dietary diversity and established use in environmental toxicology studies (Espín et al., 2014; Gómez-Ramírez et al., 2021). In the semi-arid Region of Murcia, eagle owls inhabit a variety of landscapes, where prey availability and human activities overlap. Moreover, riparian ecosystems and irrigation reservoirs near eagle owl territories may serve as alternative pathways for AR contamination. In fact, in semi-arid

regions, urban and agricultural runoff carrying pesticides and ARs often accumulates in riverbeds, creating concentrated points of exposure (Arenas-Sánchez et al., 2016). These areas, which retain water and vegetation even during dry periods, can act as hotspots for interactions between contaminants and wildlife (Steward et al., 2012). Eagle owls frequently breed in rocky gorges and along riverbeds, where their prey is abundant. This habitat selection, combined with the reliance of wildlife on these water sources, amplifies the potential for AR biomagnification.

This study focuses on evaluating AR exposure in eagle owl nestlings and its relationship with ecological and anthropogenic factors, including land use, proximity to riverbeds, livestock farms, and landfills. Additionally, coagulation function and biochemical parameters were assessed to explore potential sublethal effects of ARs. These findings aim to provide critical insights into AR contamination pathways and contribute to the development of conservation strategies aimed at reducing risks to avian predators in Mediterranean semi-arid landscapes.

Results and discussion

A total of 106 blood samples from eagle owl nestlings were collected across 34 breeding territories during 2021 and 2022. ARs were detected in 91.5% of samples, with SGARs being significantly more prevalent (91.5%) than FGARs (11.3%). Flocoumafen, difenacoum, and brodifacoum were the most frequently detected compounds, with flocoumafen also reaching the highest concentration (57.43 ng mL^{-1}). Multiple ARs were present in 70.8% of nestlings and ΣAR concentrations ranged 0.03-57.81 ng mL^{-1}.

No significant relationship was found between AR prevalence and the selected environmental variables, except for brodifacoum and difenacoum, which were associated with proximity to watercourses. ARs can reach non-perennial riverbeds through surface runoff, particularly following rainfall or irrigation events. Predators like the eagle owl are drawn to these areas for prey availability and water access, increasing their risk of secondary AR exposure. Furthermore, SGARs can persist in organic matter and sediment, accumulating in aquatic organisms (Regnery et al., 2018). Studies have confirmed the presence of SGARs in mammals linked to riparian ecosystems in Murcia (Andrés-Esteso et al., 2023), as well as in aquatic predators across Europe (Fournier-Chambrillon et al.,

2004; Regnery et al., 2024), indicating that watercourses play an important role in AR exposure.

The highest ΣAR concentrations were detected the Central-eastern badlands study site, where urbanisation and improper AR use probably contribute to exposure. Proximity to landfills also correlated with elevated ΣARs, likely due to increased rodent populations and AR application. These patterns align with findings from other studies linking anthropized environments to higher AR exposure risks in non-target predators (Geduhn et al., 2015; López-Perea et al., 2019).

Although AR presence in blood indicates recent exposure, the study did not reveal a significant relationship between ΣARs and PT, with no evidence of coagulopathy. Biochemical parameters fell within expected ranges for healthy eagle owl nestlings, coinciding with those reported by Gómez-Ramírez et al. (2016) and expand the panel with additional parameters valuable for research and assessing the health of wildlife in rescue and rehabilitation centres.

Main conclusions

1. The study revealed a widespread and significant exposure to ARs in eagle owls, with many individuals showing contamination by multiple compounds. These results underscore the vulnerability of this top predator to ARs and the potential implications for their population dynamics and conservation status in Mediterranean landscapes.
2. Evidence points to the accumulation of ARs in hydrological networks, particularly in semi-arid regions where the scarcity of water can lead to the concentration of these compounds. These findings suggest that watercourses and surrounding areas act as hotspots for AR contamination, emphasizing the need for targeted mitigation strategies in these environments.
3. Although no acute intoxication signs were observed, the high AR prevalence found in their blood raises concerns about chronic exposure. Such exposure could compromise their health, inducing sublethal effects like impaired coagulation and reduced biological fitness, which may negatively impact their survival and reproduction in the long term.

4. Additional long-term studies are necessary to monitor AR impacts on eagle owls and other raptor species of conservation concern sharing habitats and prey.

Chapter IV. Molecular biomarkers in wildlife ecotoxicology: *vkorc1* and *vkorc1l1* gene expression changes in response to anticoagulant rodenticides.

Introduction

The mechanisms underlying raptor sensitivity to ARs remain poorly understood. Studies suggest that birds of prey exhibit reduced hepatic VKOR activity and lower cytochrome P450-mediated metabolism of ARs compared to domestic birds and mammals (Khidkhan et al., 2024; Nakayama et al., 2020; Watanabe et al., 2010). The VKOR enzyme complex, critical for blood coagulation, consists of two paralogous enzymes: VKORC1 and VKORC1-like 1 (VKORC1L1), which differ in tissue distribution and function (Caspers et al., 2015; Lacombe et al., 2017). VKORC1 is the primary target of ARs, and genetic variations in *vkorc1* are associated with resistance in AR-target species (Bermejo-Nogales et al., 2022; Goulois et al., 2017). On the other hand, VKORC1L1 appears to play a compensatory role in extrahepatic tissues (Lacombe and Ferron, 2018). Differences in *vkorc1* and *vkorc1l1* expression among avian species may explain the heightened AR sensitivity observed in raptors (Nakayama et al., 2020).

In this study, the expression of *vkorc1* and *vkorc1l1* was evaluated as biomarkers of AR exposure and effect in a free-living population of Eurasian eagle owls in southeastern Spain. Specific objectives were: (1) to assess gene expression in blood samples of owl nestlings from 26 breeding territories and (2) to determine whether environmental and anthropogenic factors (e.g., land use, livestock farms, landfills, riverbeds) influence gene expression. An increase of *vkor* gene expression was expected following AR exposure as a compensatory mechanism or an adaptive response (Gioeli et al., 2011; Lopez et al., 1998; Lu et al., 2015). Additionally, it was hypothesized that some anthropogenic factors, such as urban areas and landfills, would significantly influence gene expression, given their association with AR exposure in previous studies (Spadetto et al., 2024a, 2025). Understanding these dynamics could provide valuable insights into how raptors respond to AR exposure and the environmental drivers shaping these responses.

Results and discussion

Previous research has extensively investigated *vkor* gene expression in mammalian tissues such as the liver, brain, and lungs (Oldenburg et al., 2015). However, avian whole blood is a valuable matrix for gene expression studies due to the presence of nucleated erythrocytes, a characteristic shared by birds, fish, and reptiles, making whole blood an important tissue for environmental and toxicological studies (Farag and Alagawany, 2018; Martos-Sitcha et al., 2017). The results indicate that *vkorc1* is the predominantly expressed gene in eagle owl blood (median = 5.16 RU), while its paralog *vkorc1l1* shows lower expression levels (median = 2.49 RU), with a median *vkorc1:vkorc1l1* ratio of 1.79. Consequently, the total *vkor* complex expression in whole blood is mainly driven by *vkorc1*, with *vkorc1l1* playing a secondary role. However, in 31.9% of the analysed samples, a ratio below 1 was observed, suggesting that *vkorc1l1* predominates in a subset of nestlings.

Previous research on mammals and birds has highlighted interspecific differences in the *vkorc1:vkorc1l1* expression ratios. In chickens, for instance, hepatic *vkorc1:vkorc1l1* expression ratios are approximately 2:3, indicating a significant role of *vkorc1l1* in vitamin K metabolism (Nakayama et al., 2020). The limited availability of avian *vkor* gene sequences, especially in raptors, further underscores the need for additional research on the evolutionary and functional differences in these genes. However, given previous findings (Hammed et al., 2013; Lacombe and Ferron, 2018), *vkorc1l1* upregulation was hypothesized as a compensatory response to VKORC1 inhibition, offering short-term protection to AR exposure without necessarily improving long-term fitness.

Statistical analysis revealed that *vkorc1l1* expression was significantly higher in urbanized areas, supporting previous findings reporting that urban environments pose a major risk for AR exposure due to frequent applications (López-Perea et al., 2019; Spadetto et al., 2024a). In contrast, no association was observed between agricultural land use and *vkor* gene expression, which is consistent with the regulatory framework in Spain that restricts AR application in open fields (Ministerio de Sanidad, 2023). When land-use categories were grouped, a negative relationship was found between *vkorc1* expression and the proportion of natural habitats, suggesting a reduced AR exposure in these areas. The presence of natural watercourses also emerged as a factor associated with *vkorc1*

upregulation, potentially indicating repeated AR exposure over time. Ephemeral streams may serve as AR exposure pathways for wildlife, as previously suggested (Spadetto et al., 2025). Similarly, the presence of livestock farms was linked to *vkorc1* expression, while no association with *vkorc1l1* was detected, indicating no immediate compensatory response. The increased expression of *vkorc1* in the presence of factors associated with AR-exposure risk could be the consequence of an inherited adaptive response developed over time. Finally, landfills were again identified as a potential source of AR exposure, with *vkorc1l1* expression showing a significant association. The lack of standardized regulations on AR use in landfills prior to 2024 may have exacerbated this issue.

Main conclusions

1. This study provides novel insights into the gene expression of *vkorc1* and *vkorc1l1* in the blood of a raptor species.
2. The *vkorc1* and *vkorc1l1* genes play a key role in the biochemical response to AR exposure, suggesting compensatory and adaptive mechanisms in non-target species such as the eagle owl.
3. The expression of *vkorc1* and *vkorc1l1* in eagle owl nestlings is significantly influenced by environmental variables related to anthropogenic activities and land use, where the recurrent and intensive use of ARs poses a tangible risk to wildlife.
4. This integrated approach, combining environmental and molecular data, provides insights into AR exposure dynamics in apex predators and supports the hypothesis that VKOR-related genes may serve as biomarkers for assessing the impact of ARs on wildlife.

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Resumen (Spanish extended abstract)

Introducción general

Los rodenticidas anticoagulantes (RAs) se utilizan ampliamente para el control de poblaciones de roedores, principalmente en entornos agrícolas, urbanos e industriales (Capizzi et al., 2014). Los roedores representan una preocupación significativa debido a los daños extensos que causan a cultivos, bosques e infraestructuras, así como a su papel como vectores de enfermedades transmisibles a humanos y ganado (Imholt et al., 2017; Jacob y Buckle, 2018; Morand et al., 2019). La efectividad de los RAs para reducir las poblaciones de roedores los ha convertido en una de las herramientas más comunes en las estrategias de control de plagas (Capizzi et al., 2023). Sin embargo, a pesar de su eficacia, el uso de ARs plantea importantes desafíos, particularmente en relación con su impacto ambiental y los riesgos que representan para la fauna no diana (López-Perea y Mateo, 2018).

Los RAs actúan reduciendo la capacidad de la sangre para coagular, lo que causa hemorragias internas y eventualmente la muerte en los roedores expuestos. Su mecanismo de acción retardado aumenta la probabilidad de que un roedor consuma varias veces el cebo, mejorando la efectividad del veneno (Berny et al., 2018). Sin embargo, el mismo mecanismo de acción también representa un riesgo sustancial para las especies no objetivo, incluidos depredadores y carroñeros que se alimentan de roedores envenenados. La exposición de la fauna silvestre a los RAs se ha documentado en todo el mundo, generando preocupaciones sobre las consecuencias ecológicas más amplias de su uso (Nakayama et al., 2019).

Los RAs se dividen en dos categorías principales: RAs de primera generación (FGARs) y RAs de segunda generación (SGARs). Los FGARs, como la warfarina, la clorofacinona o el coumatetralilo, se desarrollaron a mediados del siglo XX y requieren múltiples ingestas para ser letales. Su efectividad disminuyó rápidamente debido a la aparición de resistencia, lo que llevó al desarrollo de los más potentes SGARs (Jacob y Buckle, 2018). Los SGARs, como el brodifacum, el difenacum, el flocumafen, la bromadiolona y la difetialona, son altamente tóxicos y pueden ser letales con una sola ingesta (Mcgee et al.,

2020; Murphy, 2006). Su persistencia prolongada en los tejidos animales incrementa el riesgo de intoxicación secundaria en depredadores y carroñeros.

El principal modo de acción de los RAs implica la inhibición de la enzima vitamina K 2,3-epóxido reductasa (VKOR), que es crítica para el reciclaje de la vitamina K. La vitamina K es un cofactor esencial en la síntesis de los factores de coagulación II, VII, IX y X, necesarios para la cascada de coagulación sanguínea. Al bloquear la enzima VKOR, los RAs evitan la activación de estos factores, causando hemorragias prolongadas y eventualmente la muerte (Rattner y Mastrota, 2018). Las diferencias en la farmacocinética y farmacodinámica entre mamíferos y aves son notables. Las aves, a diferencia de los mamíferos, carecen de ciertos factores de coagulación, como los factores XI y XII, y dependen principalmente de la vía extrínseca para iniciar la coagulación (Buzala et al., 2017). Además, las aves poseen trombocitos, células nucleadas que desempeñan un papel en la coagulación sanguínea, siendo menos reactivas en comparación con las plaquetas de los mamíferos (Gallo et al., 2015). Se han observado diferencias interespecíficas significativas tanto en el metabolismo de los RAs como en la actividad de VKOR (Nakayama et al., 2020; Watanabe et al., 2015). Por tanto, es esencial tener en cuenta estas variaciones al evaluar los riesgos e impactos de la exposición a los RAs en las especies aviares.

El uso de los RAs está regulado a nivel nacional e internacional para equilibrar la eficacia del control de plagas con la protección ambiental. En Europa, el marco regulador está gobernado por el Reglamento de Productos Biocidas de la Unión Europea (EU BPR No. 528/2012; European Union, 2012), que exige la autorización de productos biocidas basada en su eficacia, seguridad e impacto ambiental. En España, el uso de RAs está regulado adicionalmente por leyes nacionales que implementan las directivas de la UE. Sin embargo, estudios de monitorización indican que los residuos de RAs aún se detectan con frecuencia en la fauna silvestre, lo que sugiere que las medidas regulatorias actuales podrían no ser suficientes para prevenir la contaminación ambiental (Carrillo-Hidalgo et al., 2024).

La fauna no diana está expuesta a los RAs principalmente a través de dos vías: la exposición primaria, que ocurre cuando los animales consumen directamente los cebos envenenados, y la exposición secundaria, que sucede cuando los depredadores y

carroñeros se alimentan de presas intoxicadas (Shore y Coeurdassier, 2018). También es posible la exposición terciaria, por ejemplo, mediante la ingestión de especies insectívoras, ya que los invertebrados también pueden acceder a los cebos (Elliott et al., 2014). Las aves de presa son particularmente vulnerables al envenenamiento secundario debido a su dependencia de los roedores (especies diana de los RAs) como principal fuente de alimento. Estudios han demostrado que los residuos de RAs se detectan comúnmente en el tejido hepático de las rapaces, lo que indica una exposición generalizada en diversos ecosistemas (López-Perea y Mateo, 2018; Nakayama et al., 2019). Otras especies no objetivo, incluyendo mamíferos (Keating et al., 2024), así como reptiles (Martín-Cruz et al., 2024) y anfibios (Rowley et al., 2024), también han mostrado presencia de residuos de RAs. La amplia detección de RAs en la fauna no objetivo genera preocupación por los posibles efectos a nivel poblacional, especialmente en especies que ya están amenazadas o en peligro de extinción (Cooke et al., 2022; Roos et al., 2021).

Los efectos sobre la salud derivados de la exposición a RAs en la fauna no objetivo varían dependiendo del nivel y la duración de la exposición. La exposición aguda puede causar hemorragias severas y muerte (Rattner y Mastrota, 2018), mientras que la exposición subletal puede resultar en alteraciones en la reproducción, disminución de la aptitud física y mayor susceptibilidad a enfermedades (Herring et al., 2023; Salim et al., 2014; Serieys et al., 2018). La exposición crónica a bajas dosis de RAs puede tener efectos acumulativos, dando lugar a problemas de salud a largo plazo que pueden no ser evidentes de inmediato. A nivel ecológico, los impactos de los RAs pueden extenderse más allá de los individuos, afectando a poblaciones enteras. La disminución de poblaciones de depredadores debido al envenenamiento por RAs puede tener efectos en cascada sobre la estabilidad del ecosistema y la biodiversidad (Gómez et al., 2022), lo que llevaría al aumento de las poblaciones de roedores y al uso continuo de RAs.

Las aves de presa son consideradas buenas especies centinela para la monitorización de la contaminación ambiental debido a su posición en lo alto de la cadena trófica, su larga vida, amplia distribución y sensibilidad a los contaminantes (García-Fernández et al., 2024; Gómez-Ramírez et al., 2014). Los estudios de biomonitorización que involucran a las rapaces pueden proporcionar información valiosa sobre la prevalencia y distribución de los RAs en el medio ambiente (Badry et al., 2022). Analizando los residuos de RAs en

muestras de hígado, sangre o plasma de aves de presa, los investigadores pueden evaluar la eficacia de las medidas regulatorias e identificar áreas de alto riesgo de exposición a los RAs. Además, los datos de biomonitorización pueden aportar información para diseñar estrategias de conservación y guiar decisiones políticas para reducir el impacto ambiental de estos contaminantes (Movalli et al., 2017). La biomonitorización activa implica la vigilancia proactiva de contaminantes ambientales mediante la recolección y análisis regular de muestras biológicas de especies seleccionadas (Guberti et al., 2014). Este enfoque garantiza la calidad de las muestras, permite obtener muestras representativas, recopilar información detallada y monitorear poblaciones a lo largo del tiempo.

En el contexto de la exposición a los RAs, los biomarcadores pueden ser utilizados para monitorizar tanto la exposición como los efectos en especies no diana. De hecho, los biomarcadores se definen como indicadores biológicos medibles que reflejan la interacción entre un organismo y una sustancia química como los RAs (De Coen et al., 2000). Los biomarcadores comunes de exposición incluyen la detección de residuos de RAs en sangre, hígado u otros tejidos, lo que puede indicar una exposición reciente o crónica (Rached et al., 2020). El tiempo de protrombina (PT) o el tiempo de veneno de víbora de Russell son biomarcadores de efecto que miden el impacto de los RAs en el proceso de coagulación (Rattner et al., 2010). Estas pruebas pueden revelar efectos subletales que comprometan la capacidad del animal para sobrevivir y reproducirse. El uso de biomarcadores es esencial para comprender el alcance de la exposición a los RAs y sus posibles impactos en la fauna no diana (Rached et al., 2020).

En el estudio de las vías de exposición, es importante considerar las actividades humanas como factores clave que impulsan la exposición a los RAs en la fauna. De hecho, las áreas con alta densidad humana a menudo muestran un uso generalizado de rodenticidas para controlar infestaciones de roedores (Badry et al., 2021). La falta de cumplimiento con las normativas de seguridad, como el uso de cajas cebo o la eliminación de roedores envenenados, aumenta aún más el riesgo de exposición secundaria. Las ciudades y áreas urbanas circundantes son puntos críticos de aplicación de RAs, ya que frecuentemente se utilizan en sistemas de alcantarillado, parques y áreas residenciales. Además, las granjas ganaderas suelen utilizar RAs para prevenir infestaciones de roedores y reducir la

transmisión de enfermedades (López-Perea et al., 2015). Por último, las prácticas agrícolas a gran escala utilizan a menudo RAs para proteger los cultivos de los roedores (Fritsch et al., 2024). Aunque los RAs no están aprobados legalmente como productos fitosanitarios en algunos países como España, las aplicaciones de RAs cerca de instalaciones de almacenamiento siguen siendo motivo de preocupación. La fauna en estas áreas puede consumir presas envenenadas, lo que conlleva altos riesgos de envenenamiento.

Objetivos

Objetivo general

El objetivo general de esta tesis doctoral es ampliar el conocimiento sobre el impacto de los RAs en especies no diana a través del estudio de la exposición y los efectos en cuatro especies de aves de presa que habitan una región semiárida de la Península Ibérica. En concreto, el estudio se realizó en tres especies nocturnas (el búho chico *Asio otus*, el búho real *Bubo bubo* y la lechuza común *Tyto alba*) y una diurna (el cernícalo vulgar *Falco tinnunculus*) de la Región de Murcia (sureste de España). En última instancia, esta tesis tiene como propósito contribuir a la conservación de estas y otras especies no diana, utilizando a las aves depredadoras como centinelas de la contaminación ambiental.

Objetivos específicos

Objetivo 1. (Capítulo I) Evaluar la exposición a RAs en pollos de búho chico muestreados en diferentes áreas de estudio en la Región de Murcia con niveles variables de intensificación agrícola, investigar los factores ambientales clave que influyen en la exposición a RAs y validar el PT como biomarcador de efecto de los RAs en esta especie.

Objetivo 2. (Capítulo II) Evaluar la exposición a RAs en cernícalos vulgares (pollos) y lechuzas comunes (pollos y adultos) de áreas con distintos niveles de intensificación agrícola y urbanización, comparar los niveles de exposición entre estas especies, examinando cómo los factores ambientales y las características ecológicas influyen en el riesgo de exposición y validar el PT como biomarcador de efecto en la lechuza común.

Objetivo 3. (Capítulo III) Estudiar la exposición a RAs en pollos de búho real muestreados en la Región de Murcia, evaluando biomarcadores como el PT y un panel bioquímico plasmático para valorar su estado general de salud e identificar los factores ambientales

que influyen en el riesgo de exposición a RAs, teniendo en cuenta las características ecológicas de la especie.

Objetivo 4. (Capítulo IV) Analizar la expresión de ARNm de los genes *vkorc1* y *vkorc1l1* en muestras de sangre de pollos de búho real como biomarcadores de exposición y efecto de RAs, investigar los mecanismos moleculares subyacentes a la toxicidad de los RAs y validar los factores de riesgo ambiental previamente identificados.

Materiales y métodos

Área de estudio

El área de estudio corresponde a la Región de Murcia, en el sureste de España, caracterizada por un clima semiárido, topografía diversa y un uso mixto del suelo. Su paisaje incluye sierras bajas, valles fluviales, llanuras y badlands formados por erosión, creando un mosaico ambiental único que alberga tanto actividades humanas como hábitats para la fauna silvestre. La agricultura es una actividad económica clave, ocupando el 32% del territorio (CARM, 2022), con especial concentración en las fértiles llanuras que rodean el río Segura. La hidrología de la región presenta cursos de agua no permanentes, cruciales para la biodiversidad en este entorno seco (Gómez et al., 2005). Esta diversidad convierte a la Región de Murcia en un lugar idóneo para estudiar la influencia de las actividades humanas y los diferentes paisajes en la exposición a RAs en la fauna. Dentro del área de estudio, se seleccionaron sitios específicos para cada especie de estudio, caracterizados por distintos usos del suelo, topografía y grados de urbanización, con el fin de investigar posibles variaciones en el uso y la exposición a RAs en las cuatro especies de rapaces estudiadas.

Procedimientos de muestreo

El muestreo se llevó a cabo como parte de un programa de monitoreo y marcaje de rapaces nocturnas en la Región de Murcia durante las temporadas reproductivas de 2021 y 2022. Los territorios reproductivos se monitorizaron para estimar las fechas de puesta, eclosión y salida del nido, lo que permitió seleccionar los momentos óptimos para acceder a los nidos. Personal especializado accedió a los nidos antes de que los pollos

alcanzaran la edad de vuelo. Además, se capturaron algunas lechuzas adultas dentro de los nidos junto con sus crías.

Los procedimientos de toma de muestras de sangre, aprobados por el Comité Ético de Experimentación Animal de la Universidad de Murcia (código 657/2020), siguieron protocolos establecidos para la monitorización de contaminantes en rapaces (Espín et al., 2021). La sangre se extrajo de la vena braquial utilizando una jeringa estéril y se transfirió a tubos con heparina. Para las muestras de plasma citratado, se mezclaron 450 µL de sangre con 50 µL de tampón de citrato de sodio 0.109 M y luego se centrifugaron a 2500 *g* durante 15 minutos para obtener el plasma. Para conseguir el plasma de búhos reales, se centrifugó una alícuota de 1 mL de sangre heparinizada a 2500 *g* durante 10 minutos. Adicionalmente, una alícuota de 0.3-0.5 mL de sangre de búho real se colocó en un tubo con EDTA de 0.5 mL antes de transferirse a un tubo estéril de 2 mL que contenía 1300 µL de solución RNA*later*®. Todas las muestras se almacenaron a −80 °C hasta su análisis. El número de muestras de sangre y plasma recolectadas durante el estudio se resume en la Tabla 1.

Tabla 1. Número de muestras de sangre recolectadas de cada especie durante la temporada reproductiva 2021-2022, incluyendo sangre entera heparinizada, plasma citratado, plasma de sangre heparinizada y sangre total preservada en EDTA y almacenada en RNA*later*®.

	Muestra			
Especie	Sangre entera	Plasma citrato	Plasma	RNA*later*
Búho chico	69	35	-	-
Lechuza común	62	39	-	-
Cernícalo vulgar	70	-	-	-
Búho real	106	62	105	72

Para caracterizar la dieta, se recolectaron egagrópilas de 19 territorios reproductivos de búho chico y 16 de lechuza común, y se analizaron según la literatura de referencia (Moreno, 1986; Román, 2019).

Análisis de RAs en muestras de sangre

Para la extracción de muestras, se utilizó una versión modificada del método descrito por Taylor et al. (2019), detallada en Spadetto et al. (2024b). Los extractos en los viales se analizaron siguiendo el protocolo de Rial-Berriel et al. (2020). Se cuantificaron diez RAs

(coumafuril, coumatetralil, warfarina, difacinona, clorofacinona, brodifacum, bromadiolona, difenacum, flocumafen, difetialona) mediante un sistema de cromatografía líquida de alta resolución acoplada con triple cuadrupolo (HPLC-TQ) equipado con una columna analítica de fase reversa C18 de 150 × 2.1 mm y un tamaño de partícula de 2.6 µm. Las curvas de calibración se prepararon fortificando sangre en blanco obtenida de gallinas (*Gallus gallus*) no expuestas a RAs en concentraciones que iban de 0.1 a 50 ng mL^{-1}. Los límites de cuantificación oscilaron entre 0.01 y 2.5 ng mL^{-1}. La técnica analítica mostró valores de recuperación entre el 76% y el 105%, con una desviación estándar relativa inferior al 14%.

Pruebas de coagulación y análisis bioquímico

Las pruebas de coagulación (PT y fibrinógeno) se realizaron utilizando un coagulómetro. Se seleccionó el PT para el análisis, ya que este parámetro se altera en aves rapaces dentro de las 24-48 horas posteriores a la ingestión de RAs, dependiendo de la dosis administrada (Rattner et al., 2010). El fibrinógeno se midió para evaluar la calidad de la muestra de sangre, dado que un nivel bajo (<50 mg dL^{-1}) puede alterar la coagulación sanguínea (Hindmarch et al., 2019). Para la determinación de la concentración de fibrinógeno en plasma citratado, se utilizó un kit comercial basado en el método de Clauss (Clauss, 1957). El ensayo de PT se basa en la adición de tromboplastina a la muestra de plasma. El uso de tromboplastina derivada de aves es fundamental para obtener datos precisos y relevantes en el análisis de PT en aves (Webster, 2009). El reactivo de tromboplastina se preparó utilizando el método de Quick modificado por Griminger et al. (1970). Todos los análisis de parámetros bioquímicos en muestras de plasma de búho real se realizaron con un analizador bioquímico y reactivos comerciales, con la excepción de la ovotransferrina, que se midió mediante un kit ELISA. Todos los ensayos presentaron una imprecisión intra- e interensayo <15% y fueron lineales tras diluciones seriadas.

Análisis de expresión génica de *vkor* en el búho real

Se identificaron secuencias parciales de los genes *vkorc1* (113 nucleótidos) y *vkorc1l1* (342 nucleótidos) del genoma del búho real alineándolas con secuencias de referencia de gallina (*Gallus gallus*) y águila real (*Aquila chrysaetos*). Para confirmar su identidad, se realizó una búsqueda BLAST y un análisis filogenético utilizando el método Neighbor-

Joining con 26 secuencias. Se obtuvo un conjunto final de datos con 542 posiciones, utilizando el software MEGA7.

El ARN total se extrajo de la sangre entera utilizando un kit de extracción de ADN/ARN con un paso de DNasa. La transcripción inversa (RT) para obtener cDNA se realizó con un mix de RT-qPCR utilizando decámeros aleatorios. Para monitorizar la eficiencia del proceso, se añadió 1 µL de ARN de luciferasa (0.5 ng μL^{-1}) como control externo (Jiwaji et al., 2010; Johnston et al., 2012). Se llevó a cabo RT-qPCR utilizando cebadores específicos para *vkorc1* y *vkorc1l1*. Los resultados de qPCR se normalizaron utilizando el método delta–delta Ct (Livak y Schmittgen, 2001), con la luciferasa como gen de referencia.

Análisis del uso del suelo

Para evaluar la configuración del uso del suelo alrededor de cada nido muestreado, se generaron buffers utilizando el software Quantum GIS versión 3.16.16 (QGIS Development Team, 2022). El tamaño de los buffers se determinó según estudios previos sobre las áreas de campeo de las especies de rapaces objetivo (Arlettaz et al., 2010; Boileau et al., 2006; Taylor, 1994; Village, 1982). Los datos de cobertura de uso del suelo se extrajeron y categorizaron en cuatro clases principales: vegetación natural, áreas artificiales, terrenos agrícolas y cuerpos de agua. Dentro de la categoría de terrenos agrícolas, se realizaron dos distinciones adicionales: terrenos agrícolas de secano y de regadío. Además, se estableció una categoría de "cultivos mixtos", que se refiere a áreas caracterizadas por pequeñas parcelas cultivadas y huertos, una práctica particularmente extendida en áreas específicas de la Región de Murcia. Se calculó el porcentaje de área de uso del suelo dentro de cada buffer para todas las clases y subgrupos.

Se recuperaron datos de densidad de población humana (según el censo de 2021) para cada sección censal del Instituto Nacional de Estadística (INE, 2021). Asimismo, se utilizaron datos sobre las granjas ganaderas en 2021 de la Región de Murcia (Ministerio de Agricultura, Pesca y Alimentación) para calcular la densidad de animales y el número total de granjas dentro de cada buffer. También se evaluó el papel de la red hidrológica en la contaminación por RAs en el búho real, una especie que a menudo cría en barrancos y cauces fluviales efímeros. Para ello, se calculó la distancia mínima de cada nido al curso de agua más cercano. Además, se consideró la proximidad de algunos

territorios de búho real a vertederos y plantas de tratamiento de residuos, donde el control de roedores es prioritario.

Se utilizó ArcGIS 10 para analizar las variables ambientales dentro del área de influencia de 26 territorios reproductivos de búho real considerados en el capítulo IV. Se creó una zona buffer de 2 km alrededor de cada nido y se calcularon las superficies de uso del suelo (m^2), incluyendo bosque, márgenes riparios, matorrales, cultivos de secano, cultivos de regadío y áreas urbanas. Este buffer se eligió para aproximar el área de campeo del búho real en el área de estudio (León-Ortega, 2016). El mismo buffer se utilizó para determinar la presencia de vertederos y calcular el número granjas ganaderas alrededor de los nidos.

Análisis estadístico

Se calcularon estadísticas básicas para las concentraciones de RAs en muestras de sangre y parámetros bioquímicos del plasma de los búhos reales. Para las lechuzas comunes y los cernícalos, el análisis se centró en los SGARs debido a la baja prevalencia de FGARs. Para investigar la relación entre la prevalencia de RAs y las concentraciones sanguíneas de ΣAR con factores ambientales, se empleó el enfoque descrito por Burnham y Anderson (2002). Se aplicaron Modelos Lineales Mixtos (LMMs) (Pinheiro et al., 2023), con variables ambientales como efectos fijos y el territorio como un factor aleatorio. Todos los modelos se compararon con un modelo nulo utilizando el criterio de información de Akaike corregido (AICc).

Las concentraciones de ΣAR y su prevalencia se compararon entre años de muestreo. La suma de concentraciones de SGARs (ΣSGARs) y la prevalencia de SGARs se compararon entre cernícalos y lechuzas comunes. Se realizaron análisis similares para comparar ΣSGARs y prevalencia entre clases de edad (pollos y adultos) exclusivamente para lechuzas comunes. En todos los casos, el territorio reproductivo se trató como un factor aleatorio.

Los datos de composición de dieta de los búhos chicos se analizaron por sitio de estudio para explorar diferencias en las categorías de presas y su asociación con la exposición a RAs. Para las lechuzas comunes, se realizaron análisis de regresión para examinar la

relación entre las concentraciones de ΣSGAR y la proporción de *Rattus* sp. en su dieta, considerando tanto el número como la biomasa de las presas.

Se aplicó la prueba de correlación de Spearman para evaluar las relaciones entre ΣARs y PT, así como ΣARs y los parámetros bioquímicos plasmáticos de los búhos reales. Todos los análisis estadísticos se realizaron utilizando el software R versión 4.3.1, con niveles de significancia establecidos en $p < 0.05$.

Se calcularon estadísticas descriptivas para los niveles de expresión de ARNm de los genes *vkorc1* y *vkorc1l1*, así como su expresión total. Para los análisis comparativos, los nidos se consideraron las unidades estadísticas, con los pollos como réplicas. Se utilizó el Análisis de Correlación Canónica (CCorA) para explorar las relaciones entre las variables ambientales y los niveles de expresión de ARNm de *vkorc1* y *vkorc1l1*.

Capítulo I. El monitoreo activo de los pollos de búho chico (*Asio otus*) revela una exposición generalizada a rodenticidas anticoagulantes en distintos paisajes agrícolas

Introducción

Las aves rapaces, incluidas las lechuzas y los búhos, son altamente susceptibles a los RAs debido a su posición en las redes tróficas y a su dependencia de presas que pueden haber estado expuestas a estos químicos. Aunque varios estudios han evaluado la contaminación por RAs en aves de presa, la mayoría se ha centrado en muestras de hígado de cadáveres recuperados en campo (López-Perea y Mateo, 2018), mientras que menos estudios han examinado la exposición en tiempo real mediante muestras de sangre (Badry et al., 2022; Martínez-Padilla et al., 2017; Oliva-Vidal et al., 2022). El análisis de sangre ofrece la ventaja de documentar eventos recientes de contaminación debido a la corta vida media de los RAs en el plasma (Khidkhan et al., 2024).

El búho chico, un depredador nocturno y generalista, es una especie ideal para evaluar la exposición a RAs en paisajes agrícolas. Esta rapaz nocturna caza pequeños mamíferos y aves en entornos agroforestales, lo que incrementa la probabilidad de contaminación secundaria. Aunque está catalogado como críticamente amenazado en la región, la población de búho chico ha aumentado en los últimos años. Sin embargo, los datos sobre la exposición a AR en esta especie son limitados. En este estudio, se evalúa la exposición a AR en pollos de búho chico analizando muestras de sangre recolectadas en

cuatro áreas diferentes de la Región de Murcia. También se explora el papel de factores ambientales, como los usos del suelo, en los niveles de contaminación por RAs. Además, se evalúa el PT como un efecto adverso específico de los RAs. Este estudio proporciona información valiosa sobre las amenazas que representan los RAs para la biodiversidad en tierras agrícolas y busca apoyar estrategias de gestión de vida silvestre y políticas agroambientales.

Resultados y discusión

Se recolectaron un total de 69 muestras de sangre de pollos de búho chico durante las temporadas de cría de 2021 y 2022, abarcando 29 territorios reproductivos en cuatro áreas con características de uso del suelo diferentes. Se detectaron residuos de RAs en el 98.6% de las muestras, con una prevalencia absoluta en los nidos (100%). Los SGARs dominaron, siendo el flocumafén el compuesto más frecuentemente detectado (88.4%), seguido por el brodifacum, el difenacum y la bromadiolona. Se encontraron residuos de múltiples RAs en el 82.6% de las muestras, lo que resalta una exposición repetida. Los niveles totales de RAs (ΣARs) en sangre oscilaron entre 0.06 y 34.18 ng mL^{-1}.

El análisis de la dieta confirmó que el búho chico es un depredador generalista, alimentándose principalmente de roedores (51.4%) y aves (44.2%), con hábitos alimentarios que variaron entre los sitios de estudio. A pesar del predominio de las aves en la dieta en algunos sitios, la exposición a RAs fue ubicua, lo que sugiere vías de exposición secundarias y terciarias, incluida la ingestión de aves granívoras e insectívoras, que también pueden estar expuestas de forma primaria o a través de invertebrados contaminados. Una correlación significativa entre ΣRAs y el PT indica un impacto directo de los RAs en el sistema hemostático de los pollos. Los niveles detectados de RAs fueron generalmente bajos y no se asociaron con toxicidad aguda, aunque probablemente representen riesgos a largo plazo.

Conclusiones principales

1. La elevada prevalencia de RAs en pollos, incluso en niveles subletales, es preocupante debido a los efectos acumulativos y subletales de estos compuestos, como coagulopatías y otras alteraciones que podrían afectar la aptitud a largo plazo.

2. La dieta predominante (aves paseriformes frente a roedores) de este depredador generalista varió según el sitio de estudio y los recursos disponibles, pero esto no afectó la prevalencia de los RAs. Como se ha observado en otras especies de rapaces (e.g., gavilanes), las rutas de exposición alternativas probablemente implican el consumo de aves y/o invertebrados.
3. El búho chico surge como una especie centinela ideal para el biomonitorización en sistemas agroforestales mediterráneos debido a su amplia distribución y la viabilidad de la recolección de datos.
4. Estos hallazgos subrayan la necesidad urgente de regular el uso generalizado de RAs e implementar medidas de gestión para salvaguardar la biodiversidad en entornos agrícolas.

Capítulo II. Comparación de la exposición a rodenticidas anticoagulantes en la lechuza común (*Tyto alba*) y el cernícalo vulgar (*Falco tinnunculus*): un estudio de biomonitorización en una región agrícola del sureste de España

Introducción

El cernícalo vulgar (*Falco tinnunculus*) y la lechuza común (*Tyto alba*) son dos especies depredadoras clave en los agroecosistemas eurasiáticos, desempeñando un papel importante en el control de las poblaciones de roedores. Sin embargo, la intensificación agrícola, la pérdida de hábitat y el uso de pesticidas, incluidos los RAs, han provocado el declive poblacional en ambas especies (Buck et al., 2020; Grande et al., 2018). El cernícalo vulgar es un depredador generalista que caza en hábitats abiertos, mientras que la lechuza común es más especializada, alimentándose principalmente de roedores en terrenos agrícolas. Ambas especies están experimentando disminuciones poblacionales, con el cernícalo clasificado como en peligro de extinción en España y la lechuza como casi amenazada (Martínez-Padilla et al., 2021; SEO/BirdLife, 2021).

En este estudio, se evalúa la exposición ambiental a RAs en rapaces diurnas (*F. tinnunculus*) y nocturnas (*T. alba*) en una región agrícola mediterránea. Específicamente, se investiga diferencias interespecíficas, interanuales y relacionadas con la edad en la prevalencia y concentraciones de RAs, se explora posibles asociaciones entre los RAs y

variables ambientales, como los usos del suelo, la densidad de población humana y la ganadería y se examina la relación entre el PT y las concentraciones sanguíneas de RAs como biomarcador de efecto.

Resultados y discusión

Se recolectaron un total de 136 muestras de sangre de lechuzas adultas (*n*=12), pollos de lechuza (*n*=54) y pollos de cernícalo (*n*=70) en 42 territorios reproductivos en 2021–2022. Mientras que los FGARs se detectaron con poca frecuencia y en bajas concentraciones, los SGARs fueron prevalentes. En las lechuzas, el 50% de los pollos y el 100% de los adultos dieron positivo para SGARs, observándose concentraciones más altas en los adultos (mediana = 1.21 frente a 0.18 ng mL^{-1}). En los pollos de cernícalo, el 68.6% dio positivo para SGARs, con una mediana de 0.25 ng mL^{-1} y un valor máximo de 11.26 ng mL^{-1} para flocumafen. Brodifacum y flocumafen fueron los SGARs más frecuentemente detectados en ambas especies. El 16% de los pollos de lechuza y el 33% de los de cernícalo exhibieron múltiples SGARs, indicando un riesgo de exposición repetida y acumulativa.

Las variables ambientales influyeron de manera diferente en la exposición a RAs entre las dos especies. En los cernícalos, la prevalencia de SGARs se correlacionó con el porcentaje de superficies artificiales dentro de los territorios de cría, mientras que no se observaron diferencias significativas entre los sitios de estudio. Por el contrario, la exposición en las lechuzas estuvo afectada principalmente por la densidad humana, el porcentaje de áreas artificiales y los cultivos mixtos, que fueron particularmente frecuentes en paisajes urbano-agrícolas. La mayor prevalencia (78.6%) se registró en la zona que rodea la ciudad de Murcia, donde los cultivos mixtos y los asentamientos humanos probablemente se asocian a un mayor uso de RAs.

Los patrones de alimentación y las características ecológicas también influyeron en la probabilidad de exposición. La dieta diversificada de los cernícalos y su proximidad a la actividad humana pueden aumentar su exposición, mientras que la dieta especializada de las lechuzas varió según el sitio, con una mayor presencia de presas del género *Rattus spp*. en el sitio de estudio urbano-agrícola. La prevalencia de SGARs en las lechuzas se correlacionó con la presencia de *Rattus spp.* en su dieta, ya que estos roedores son altamente susceptibles a la contaminación por RAs y están asociados con áreas urbanas.

El PT se prolongó significativamente en individuos con mayores concentraciones de SGARs, lo que sugiere efectos adversos subletales en curso.

Conclusiones principales

1. Tanto las lechuzas comunes como los cernícalos que habitan en regiones agrícolas mediterráneas estuvieron ampliamente expuestos a SGARs. Aunque las concentraciones fueron generalmente bajas, la alta prevalencia y la detección frecuente de múltiples compuestos sugieren una exposición repetida en el tiempo.
2. El riesgo de exposición varió entre las especies según sus características ecológicas. Los cernícalos, como rapaces generalistas que se alimentan en áreas urbanas, mostraron una probabilidad ligeramente mayor de exposición, potencialmente relacionada con rutas de contaminación que implican especies no objetivo. Las lechuzas, que cazan principalmente en espacios abiertos y naturales, estuvieron en mayor riesgo cerca de centros urbanos, donde la presencia de presas objetivo puede amplificar la exposición.
3. La medición de la función de coagulación ofrece un método prometedor para evaluar los efectos adversos de los RAs en aves rapaces silvestres.
4. El estudio destaca la necesidad de integrar factores ecológicos, como la biología reproductiva y el comportamiento de búsqueda de alimento, con evaluaciones de exposición para desarrollar estrategias de conservación y gestión orientadas a mitigar el impacto de los contaminantes ambientales en las poblaciones de rapaces.

Capítulo III. Explorando la exposición y los efectos de los rodenticidas anticoagulantes en pollos de búho real (*Bubo bubo*) en una región mediterránea semiárida

Introducción

El búho real euroasiático, un superdepredador con plasticidad ecológica, es una especie valiosa para la biomonitorización debido a su diversidad alimentaria y su uso consolidado en estudios de toxicología ambiental (Espín et al., 2014; Gómez-Ramírez et al., 2021). En la Región de Murcia, de clima semiárido, los búhos reales habitan en una variedad de paisajes donde la disponibilidad de presas y las actividades humanas se superponen.

Además, los ecosistemas ribereños y los embalses de riego cercanos a los territorios de los búhos reales pueden actuar como vías alternativas de contaminación por RAs. De hecho, en las regiones semiáridas, las escorrentías urbanas y agrícolas que transportan pesticidas y RAs a menudo se acumulan en cauces de ríos, creando puntos concentrados de exposición (Arenas-Sánchez et al., 2016). Estas áreas, que retienen agua y vegetación incluso durante períodos secos, pueden actuar como puntos críticos de interacción entre los contaminantes y la fauna silvestre (Steward et al., 2012). Los búhos reales frecuentemente crían en roquedos y a lo largo de los cauces de ríos, donde sus presas son más abundantes. Esta selección de hábitat, combinada con la dependencia de la fauna silvestre de estas fuentes de agua, amplifica el potencial de biomagnificación de los RAs.

Este estudio se centra en evaluar la exposición a RAs en pollos de búho real y su relación con factores ecológicos y antropogénicos, incluidos el uso del suelo, la proximidad a cauces de ríos, granjas ganaderas y vertederos. Además, se evaluaron la función de coagulación y los parámetros bioquímicos plasmáticos para explorar posibles efectos subletales de los RAs. Estos hallazgos tienen como objetivo proporcionar información crítica sobre las vías de contaminación por RAs y contribuir al desarrollo de estrategias de conservación dirigidas a reducir los riesgos para las aves depredadoras en paisajes mediterráneos semiáridos.

Resultados y discusión

Se recolectaron un total de 106 muestras de sangre de pollos de búho real en 34 territorios de cría en 2021 y 2022. Los RAs se detectaron en el 91.5% de las muestras, siendo los SGARs significativamente más prevalentes (91.5%) que los FGARs (11.3%). Flocumafen, difenacum y brodifacum fueron los compuestos más detectados, y el flocumafen alcanzó también la concentración más alta (57.43 ng mL^{-1}). Múltiples RAs se encontraron en el 70.8% de los pollos, y las concentraciones totales de RAs (**Σ**RAs) oscilaron entre 0.03 y 57.81 ng mL^{-1}.

No se encontró una relación significativa entre la prevalencia de RAs y las variables ambientales seleccionadas, excepto para el brodifacum y el difenacum, que se asociaron con la proximidad a cauces de río. Los RAs pueden llegar a los cauces de río no permanentes a través de la escorrentía superficial, particularmente después de eventos

de lluvia o riego. Depredadores como el búho real se sienten atraídos por estas áreas debido a la disponibilidad de presas y al acceso al agua, lo que aumenta su riesgo de exposición secundaria a RAs. Además, los RAs pueden persistir en materia orgánica y sedimentos, acumulándose en organismos acuáticos (Regnery et al., 2018). Estudios han confirmado la presencia de RAs en mamíferos vinculados a ecosistemas ribereños en Murcia (Andrés-Esteso et al., 2023), así como en depredadores acuáticos en Europa (Fournier-Chambrillon et al., 2004; Regnery et al., 2024), lo que indica que los cauces de agua juegan un papel importante en la exposición a RAs.

Las concentraciones más altas de ΣRAs se detectaron en el área de las badlands del centro-este, donde la urbanización y el uso inadecuado de RAs probablemente contribuyen a la exposición. La proximidad a vertederos también se correlacionó con concentraciones elevadas, probablemente debido al aumento de las poblaciones de roedores y la aplicación de RAs. Estos patrones coinciden con los hallazgos de otros estudios que asocian los entornos antropizados con mayores riesgos de exposición a RAs en depredadores no objetivo (Geduhn et al., 2015; López-Perea et al., 2019).

Aunque la presencia de RAs en sangre indica una exposición reciente, el estudio no reveló una relación significativa entre ΣARs y el PT, y no se observaron signos de coagulopatía. Los parámetros bioquímicos plasmáticos estuvieron dentro de los rangos esperados para pollos de búho real sanos, similares a los reportados por Gómez-Ramírez et al. (2016) y amplían el panel con parámetros adicionales valiosos para la investigación y la evaluación de la salud de la fauna en centros de recuperación.

<u>Conclusiones principales</u>

1. El estudio reveló una exposición generalizada y significativa a RAs en los búhos reales y muchos individuos mostraron contaminación por múltiples compuestos. Estos resultados subrayan la vulnerabilidad de este depredador superior a los RAs y las posibles implicaciones para su dinámica poblacional y estado de conservación en paisajes mediterráneos.
2. Las evidencias apuntan a la acumulación de RAs en las redes hidrológicas, particularmente en regiones semiáridas donde la escasez de agua puede llevar a la concentración de estos compuestos. Estos hallazgos sugieren que los cauces de

agua y sus alrededores actúan como puntos críticos de contaminación por RAs, enfatizando la necesidad de estrategias de mitigación específicas en estos entornos.

3. Aunque no se observaron signos de intoxicación aguda, la alta prevalencia de RAs encontrada en la sangre plantea preocupaciones sobre la exposición crónica. Dicha exposición podría comprometer su salud, induciendo efectos subletales como la alteración de la coagulación y la reducción de la aptitud, lo que podría afectar negativamente su supervivencia y reproducción a largo plazo.
4. Son necesarios estudios adicionales a largo plazo para monitorizar los impactos de los RAs en los búhos reales y en otras especies de rapaces en peligro que comparten hábitats y presas.

Capítulo IV. Biomarcadores moleculares en ecotoxicología de la fauna silvestre: cambios en la expresión génica de *vkorc1* y *vkorc1l1* en respuesta a rodenticidas anticoagulantes

Introducción

Los mecanismos subyacentes a la sensibilidad de las rapaces a los RAs siguen siendo poco comprendidos. Estudios sugieren que las aves de presa presentan una actividad reducida de la enzima hepática VKOR y un metabolismo menor de los RAs mediado por el citocromo P450 en comparación con aves domésticas y mamíferos (Khidkhan et al., 2024; Nakayama et al., 2020; Watanabe et al., 2010). El complejo enzimático VKOR, crítico para la coagulación sanguínea, está compuesto por dos enzimas parálogas: VKORC1 y VKORC1-like 1 (VKORC1L1), que difieren en su distribución tisular y función (Caspers et al., 2015; Lacombe et al., 2017). VKORC1 es la diana principal de los RAs, y las variaciones genéticas en *vkorc1* se asocian con resistencia en especies objetivo de los RAs (Bermejo-Nogales et al., 2022; Goulois et al., 2017). VKORC1L1 parece desempeñar un papel compensatorio en tejidos extrahepáticos (Lacombe y Ferron, 2018). Las diferencias en la expresión génica de *vkorc1* y *vkorc1l1* entre especies de aves podrían explicar la mayor sensibilidad a los RAs observada en rapaces (Nakayama et al., 2020).

En este estudio, se evaluó la expresión génica de *vkorc1* y *vkorc1l1* como biomarcadores de exposición y efecto de RAs en una población silvestre de búho real euroasiático en el

sureste de España. Los objetivos específicos fueron: (1) evaluar la expresión génica en muestras de sangre de pollos de búho de 26 territorios reproductivos y (2) determinar si factores ambientales y antropogénicos (uso del suelo, explotaciones ganaderas, vertederos, cauces fluviales) influyen en la expresión génica. Se esperaba un aumento en la expresión de los genes *vkor* tras la exposición a RAs como un mecanismo compensatorio o una respuesta adaptativa (Gioeli et al., 2011; Lopez et al., 1998; Lu et al., 2015). Además, se planteó la hipótesis de que ciertos factores antropogénicos, como las áreas urbanas y los vertederos, influirían significativamente en la expresión génica, dada su asociación con la exposición a RAs en estudios previos (Spadetto et al., 2024a, 2025). Comprender estas dinámicas podría aportar información valiosa sobre la respuesta de las rapaces a la exposición a RAs y los factores ambientales que modelan estas respuestas.

Resultados y discusión

Investigaciones previas han estudiado ampliamente la expresión del gen *vkor* en tejidos de mamíferos como el hígado, cerebro y pulmones (Oldenburg et al., 2015). Sin embargo, la sangre entera de aves es una matriz valiosa para estudios de expresión génica debido a la presencia de eritrocitos nucleados, una característica compartida por aves, peces y reptiles, lo que convierte la sangre en un tejido importante para estudios ambientales y toxicológicos (Farag y Alagawany, 2018; Martos-Sitcha et al., 2017).

Los resultados indican que *vkorc1* es el gen predominantemente expresado en la sangre del búho real (mediana = 5.16 RU), mientras que su parálogo *vkorc1l1* presenta niveles de expresión más bajos (mediana = 2.49 RU), con un ratio medio *vkorc1*:*vkorc1l1* de 1.79. En consecuencia, la expresión total del complejo *vkor* en sangre entera está principalmente impulsada por *vkorc1*, con *vkorc1l1* desempeñando un papel secundario. Sin embargo, en el 31.9% de las muestras analizadas, se observó un ratio inferior a 1, lo que sugiere que *vkorc1l1* predomina en un subconjunto de pollos.

Estudios previos en mamíferos y aves han resaltado diferencias interespecíficas en las proporciones de expresión de *vkorc1* y *vkorc1l1*. En gallina, por ejemplo, el ratio de expresión hepática *vkorc1*:*vkorc1l1* es aproximadamente 2:3, lo que indica un papel significativo de *vkorc1l1* en el metabolismo de la vitamina K (Nakayama et al., 2020). La disponibilidad limitada de secuencias génicas aviares de *vkor*, especialmente en rapaces, resalta la necesidad de investigaciones adicionales sobre las diferencias evolutivas y

funcionales de estos genes. No obstante, según hallazgos previos (Hammed et al., 2013; Lacombe y Ferron, 2018), se planteó la hipótesis de que la sobreexpresión de *vkorc1l1* representa una respuesta compensatoria a la inhibición de VKORC1, ofreciendo una protección a corto plazo frente a la exposición a RAs sin necesariamente mejorar la aptitud a largo plazo.

El análisis estadístico reveló que la expresión de *vkorc1l1* fue significativamente mayor en áreas urbanizadas, lo que respalda hallazgos previos de que los entornos urbanos representan un riesgo importante de exposición a RAs debido a aplicaciones frecuentes (López-Perea et al., 2019; Spadetto et al., 2024a). En contraste, no se observó asociación entre el uso agrícola del suelo y la expresión de *vkor*, lo que es coherente con el marco regulador en España que restringe la aplicación de RAs en espacios abiertos (Ministerio de Sanidad, 2023). Cuando se agruparon las categorías de uso del suelo, se encontró una relación negativa entre la expresión de *vkorc1* y la proporción de hábitats naturales, lo que sugiere una menor exposición a RAs en estas áreas. La presencia de cursos de agua naturales también surgió como un factor asociado con la aumentada expresión de *vkorc1*, lo que podría indicar una exposición repetida a RAs a lo largo del tiempo. Los cursos de agua efímeros podrían servir como vías de exposición a RAs para la fauna, como se sugirió anteriormente (Spadetto et al., 2025). De manera similar, la presencia de granjas de ganado se vinculó a la expresión de *vkorc1*, mientras que no se detectó ninguna asociación con *vkorc1l1*, lo que indica que no hay una respuesta compensatoria inmediata. El aumento de la expresión de *vkorc1* en presencia de factores asociados al riesgo de exposición a RAs podría ser consecuencia de una respuesta adaptativa heredada desarrollada a lo largo del tiempo. Finalmente, los vertederos fueron nuevamente identificados como una fuente potencial de exposición a RAs, con una asociación significativa a la expresión de *vkorc1l1* . La falta de regulaciones estandarizadas sobre el uso de RAs en vertederos antes de 2024 podría haber exacerbado este problema.

<u>Conclusiones principales</u>

1. Este estudio proporciona nuevos conocimientos sobre la expresión génica de *vkorc1* y *vkorc1l1* en la sangre de una especie de rapaz.

2. Los genes *vkorc1* y *vkorc1l1* desempeñan un papel clave en la respuesta bioquímica a la exposición a RAs, sugiriendo mecanismos compensatorios y adaptativos en especies no objetivo como el búho real.
3. La expresión de *vkorc1* y *vkorc1l1* en pollos de búho real está significativamente influenciada por variables ambientales y actividades humanas.
4. Este enfoque integrado aporta información valiosa sobre las dinámicas de exposición a RAs en depredadores apicales y respalda la hipótesis de que los genes *vkorc1* y *vkorc1l1* pueden servir como biomarcadores para evaluar el impacto de los RAs en la fauna silvestre.

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