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Adverse Outcome Pathway and Risks of Anticoagulant Rodenticides to Predatory Wildlife

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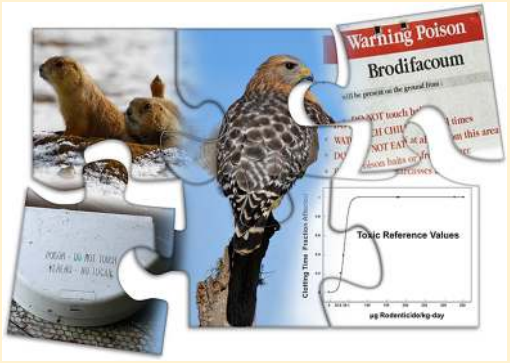
ABSTRACT: Despite a long history of successful use, routine application of some anticoagulant rodenticides (ARs) may be at a crossroad due to new regulatory guidelines intended to mitigate risk. An adverse outcome pathway for ARs was developed to identify information gaps and end points to assess the effectiveness of regulations. This framework describes chemical properties of ARs, established macromolecular interactions by inhibition of vitamin K epoxide reductase, cellular responses including altered clotting factor processing and coagulopathy, organ level effects such as hemorrhage, organism responses with linkages to reduced fitness and mortality, and potential consequences to predator populations. Risk assessments have led to restrictions affecting use of some second-generation ARs (SGARs) in North America. While the European regulatory community highlighted significant or unacceptable risk of ARs to nontarget wildlife, use of SGARs in most EU member states remains authorized due to public health concerns and the absence of safe alternatives. For purposes of conservation and restoration of island habitats, SGARs remain a mainstay for eradication of invasive species. There are significant data gaps related to exposure pathways, comparative species sensitivity, consequences of sublethal effects, potential hazards of greater AR residues in genetically resistant prey, effects of low-level exposure to multiple rodenticides, and quantitative data on the magnitude of nontarget wildlife mortality.



HISTORY AND USE OF ANTICOAGULANT RODENTICIDES

Anticoagulant rodenticides (ARs) are used worldwide for vertebrate pest control in urban and suburban settings, agriculture, and island restoration projects. These compounds block the vitamin K cycle and impede synthesis of active forms of several blood clotting factors (II, VII, IX, and X) necessary for hemostasis. Their discovery and development began with Karl Paul Link's investigations of "bleeding disease" in cattle consuming improperly cured sweet clover.[1] By 1940, Link had isolated, crystallized and synthesized dicumarol (similar in structure to vitamin K), that led to the synthesis of over 100 analogs with hemorrhagic properties, including the highly potent compound number 42, warfarin. By the early 1950s, warfarin was registered as a pesticide to control rats and mice, and its clinical application as the "blood thinner" Coumadin was approved for medicinal use, with U.S. President Dwight Eisenhower being a prominent treatment recipient in 1955.

In the opening sentence of their review, Hadler and Buckle[2] state, "Few modern pesticide groups have such a long history of successful use as the anticoagulant rodenticides", that continues to this very day. These compounds revolutionized vertebrate pest control. The first-generation anticoagulant rodenticides (FGARs; e.g., warfarin, chlorophacinone, diphacinone) require multiple feeds to cause death in rodents, but their use resulted in the emergence of genetic resistance in rats and house mice. The more potent and moderately persistent "superwarfarin" second-generation anticoagulants rodenticides (SGARs; e.g., brodifacoum, difethialone, bromadiolone, difenacoum, flocoumafen) were developed to overcome resistance and require only a single bait feeding to cause death in target rodent species. Although national and global AR market data are "confidential business information", estimates of AR use are illustrated by (i) a report indicating production or import of 1764 kg of active ingredient of four ARs in the U.S. in 1997,[3] (ii) a market analysis suggesting that U.S. homeowners spent $110 million on rodenticides in 2005,[4] (iii) the sale of 454 t of formulated product in California for agricultural purposes in 2007,[5] (iv) use of approximately 544 t of bait containing AR by local authorities in the UK in 2001,[6] and (v) application in over 700 of 1527 invasive species eradication projects worldwide.[7]

Despite their evident success in agriculture and conservation-based activities, continued use of some SGARs for control of commensal rodents in urban, suburban, rural and even agricultural settings may be at a crossroad. It is well-recognized

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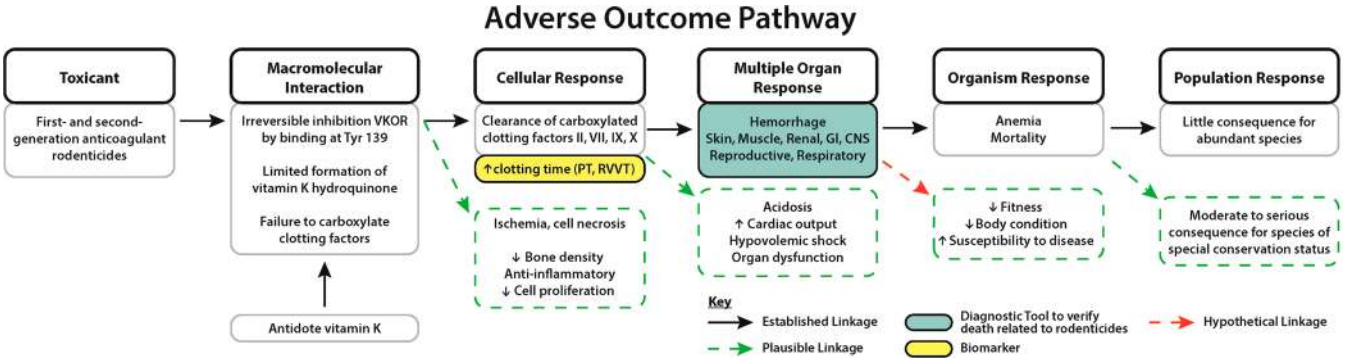

Figure 1. Proposed Adverse Outcome Pathway for anticoagulant rodenticides in nontarget predatory wildlife.

that AR application is the only current method for rapid and effective eradication of "*established*" rodent infestations.[8,9] Large scale applications of ARs have also been used to control population peaks of small mammals (e.g., rodent plagues) exhibiting demographic cycles.[10] However, it is also apparent that ARs are responsible for many unintentional exposures of children (mostly minor and asymptomatic), companion animals and nontarget wildlife, and a small fraction of such exposures result in fatalities.[11−15] New restrictions have been placed on the use of some AR baits to mitigate risk.[13,16] Herein, we present an AR adverse outcome pathway (AOP), and briefly review risk assessment data, recent regulatory changes on AR use in North America and elsewhere, risk mitigation, conservation uses of ARs, and unsolved issues on exposure and toxicity as they relate to predatory birds and mammals.

■ ADVERSE OUTCOME PATHWAY FOR ANTICOAGULANT RODENTICIDES

An AOP is a conceptual framework portraying existing knowledge as a logical sequence of processes linking a direct molecular initiating event to an adverse effect across multiple levels of biological organization, which is relevant in risk assessment.[17−19] In an ecological context, population-level responses are most germane for natural resource management, although for species of special conservation status (e.g., threatened or endangered, or highly valued to a particular stakeholder group), effects at the level of the individual may have important population consequences. The AOP framework has application in predictive and regulatory toxicology, particularly for well-studied chemicals like ARs (Figure 1).

Chemical Properties and Macromolecular Interactions. Anticoagulant rodenticides have low solubility in water and low volatility.[20] For all FGARs, and the SGARs bromadiolone and flocoumafen, octanol:water partition coefficients (log K_{ow}) are less than 5, and thus have low or moderate bioaccumulation potential. In contrast, the log K_{ow}'s for the SGARs difethialone, difenacoum and brodifacoum range from 5.17 to 8.50, and thus these compounds exhibit greater potential for bioaccumulation. Based upon studies examining the toxicity of 4-hydroxycoumarin and indandione ARs to sensitive and resistant strains of rats, bulky lipophilic extensions of the acetonyl side chain contribute to their increased affinity to the active site of vitamin K epoxide reductase, and compounds having tetrahydronaphthyl side-chains (e.g., difenacoum) are more resistant to biotransformation.[21] In contrast, FGARs are readily hydroxylated (notable exceptions include raptorial birds[22]) to inactive metabolites that are excreted.[23] Using solid-state structures of coumatetralyl and chlorophacinone as input geometries, computational chemistry efforts were conducted for 13 ARs.[24] Structure−activity relationship models suggest that toxicity is related to the length and hydrophobicity of the side chain at carbon 13, with the most active compounds having greater volume and bulky lipophilic groups in this activity domain (Figure 2).[21,24]

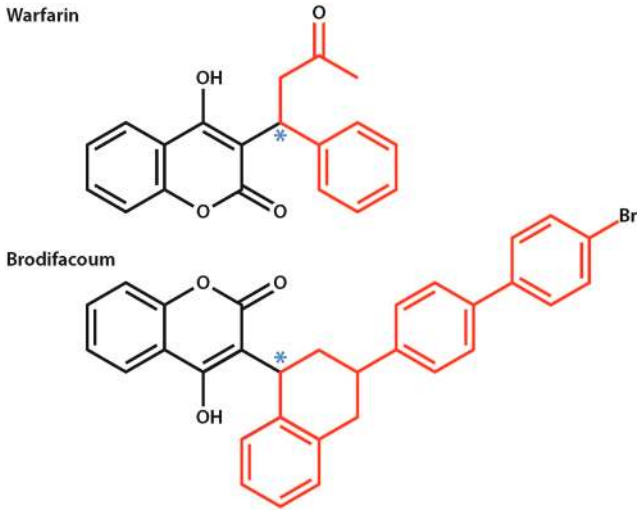

Figure 2. Structure of the first-generation anticoagulant rodenticide warfarin and the second- generation anticoagulant rodenticide brodifacoum, illustrating side chains (red) of the activity domain attached at carbon 13 (blue ∗).

Anticoagulant rodenticides bind tightly to and inactivate vitamin K epoxide reductase (VKOR), an integral membrane protein found on the rough endoplasmic reticulum in hepatocytes, and VKOR is also present in cells of other tissues.[25] Catalytic activity of VKOR is necessary for the reduction of both vitamin K epoxide and vitamin K to vitamin K hydroquinone, the biologically active form required for the γ-glutamyl carboxylation of glutamine residues (Figure 3) on clotting factors II (prothrombin), VII, IX, and X. The primary amino acid sequence and the gene encoding VKOR have been well-studied, and the membrane topology and active site (cysteine sulfhydryl groups at residues 132 and 135 and warfarin binding site at tyrosine 139) have been modeled (Figure 4).[25] Inhibition of VKOR activity by warfarin, and other anticoagulant rodenticides, limits the formation of vitamin K hydroquinone resulting in under-carboxylated clotting factors (e.g., des-γ-carboxy prothrombin)[26] that will not assemble on cell surfaces to form a clot (viz., molecular initiating/anchor event in AOP, Figures 1 and 3). Vitamin K_1 (phylloquinone) is an

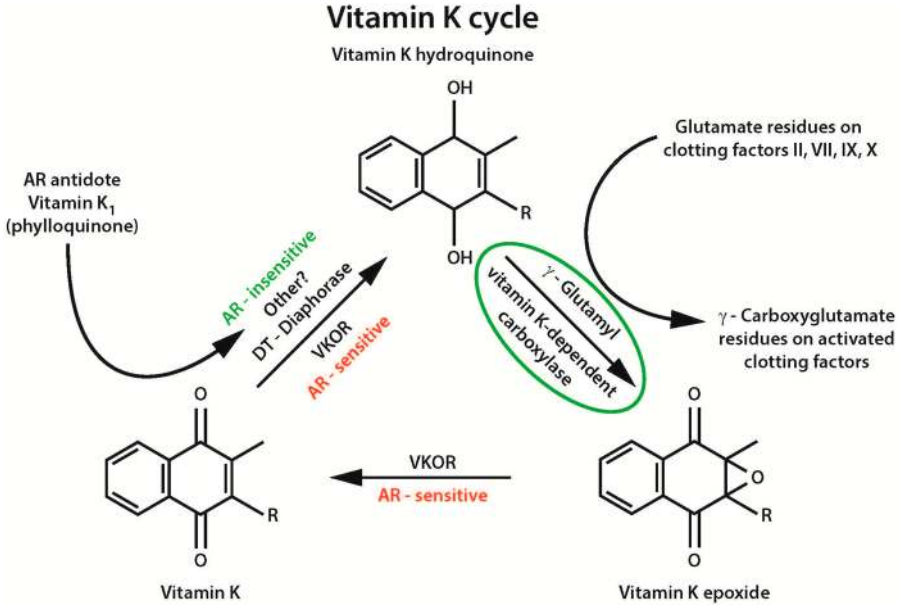

Figure 3. Diagram of the vitamin K cycle showing two anticoagulant rodenticide (AR) sensitive vitamin K epoxide reductase (VKOR) reactions and a warfarin-insensitive VKOR that reduces vitamin K to the biologically active vitamin K hydroquinone. Without adequate vitamin K hydroquinone, γ-glutamyl carboxylase lacks substrate to adequately carboxylate clotting factors II, VII, IX, and X (adapted from Tie and Stafford 2008).[25]

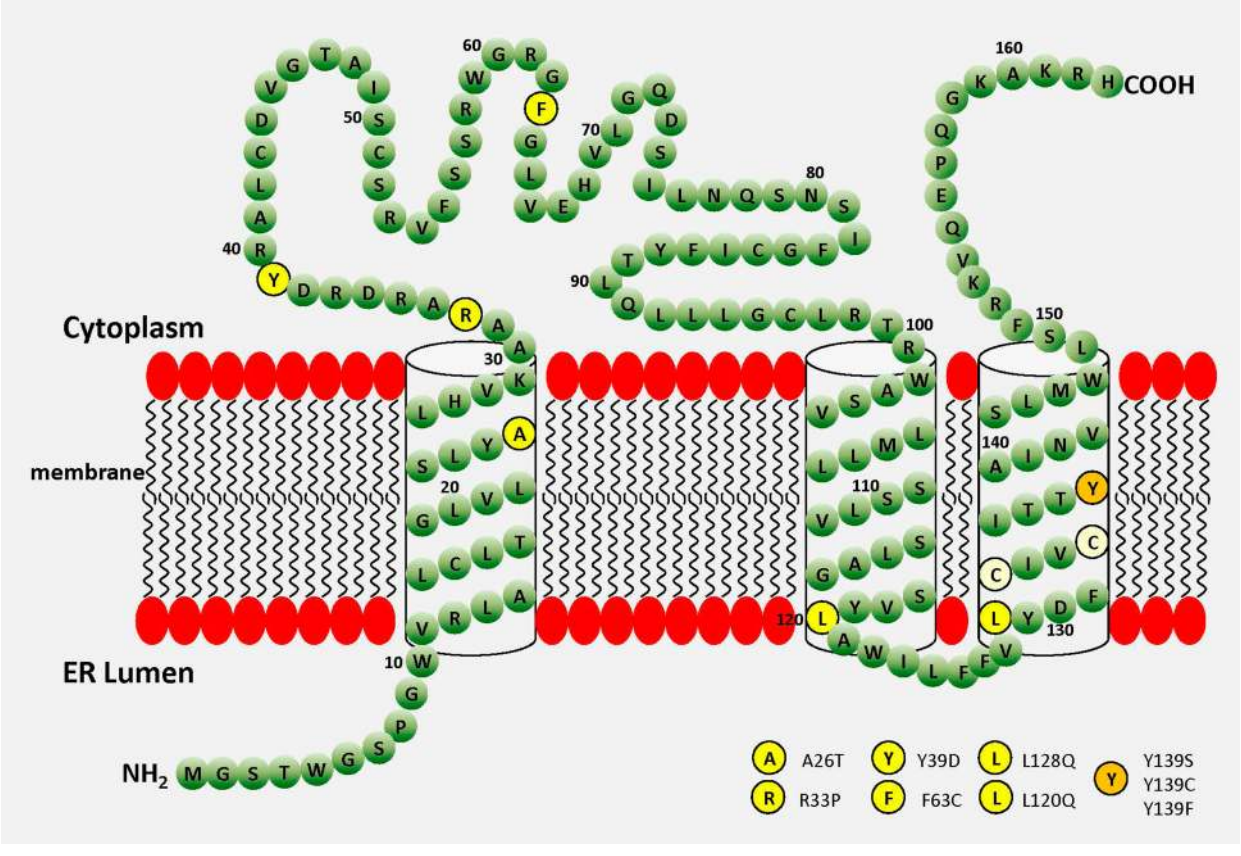

Figure 4. Primary structure and membrane topology of the anticoagulant rodenticide-sensitive vitamin K epoxide reductase (adapted from Tie and Stafford 2008).[25] All single letter amino acid abbreviations follow IUPAC nomenclature. The warfarin binding site is Y139 (orange) and the active redox sites are C132 and C135 (white). The most thoroughly studied mutation for warfarin resistance is at Y139; common mutations include substitutions of S, C, and F, for Y. Other common mutations that afford warfarin resistance are indicated in yellow.[9,30]

antidote to AR intoxication, and has long been used to treat people, companion animals, and occasionally wildlife.[27] Its administration results in the formation of the vitamin K hydroquinone by DT-diaphorase, a vitamin K cycle enzyme which is resistant to ARs,[25] thus restoring carboxylation of clotting factors.

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Widespread use of warfarin resulted in selection for warfarin-resistant rats associated with reduced or reversible binding to VKOR.[21] Mutation of the VKOR gene coding tyrosine 139 and amino acid substitutions at other locations can confer resistance to FGARs[28−30] and some SGARs.[9] There is also evidence that resistance can be conferred by other mechanisms including increased AR clearance associated with enhanced CYP3A2 expression.[31]

Cellular Responses. Blood coagulation is the central component of hemostasis.[32] At the cellular level, coagulation is initiated through an extrinsic pathway (tissue factor pathway) with the generation of tissue factor that complexes with carboxylated factor VII, which in turn activates factor X in the common pathway, and to a lesser degree in the intrinsic pathway (contact activation pathway), where factor IX is activated. Factors XI and XII of the intrinsic pathway are absent altogether in several avian species.[33] Through the common pathway, a number of reactions lead to the activation of prothrombin to form thrombin. Thrombin cleaves circulating fibrinogen into soluble fibrin monomers that polymerize, and it also activates factor XIII, which in the presence of calcium cross-links the polymer to form insoluble fibrin. In the classic cascade model, thrombin formation is markedly amplified through the intrinsic pathway. However, in vivo hemostasis is now better described by a cell-based model, in which stages overlap and are controlled by cellular components rather than protein levels and kinetics,[34] with alterations in factor IX having greatest effects on thrombin generation and clotting.[35]

Measurement of clotting time (e.g., prothrombin time, partial thromboplastin time, activated partial thromboplastin time) of citrated plasma has long been used as a routine diagnostic tool for AR intoxication in companion animals and people. Its application to diagnose AR intoxication in captive and free-ranging wildlife is rare.[36] Clotting time assays are sensitive, precise, inexpensive, linked to the pathogenesis of toxicity, and have applicability as biomarkers of exposure and effect in both controlled studies and field monitoring (Figure 1). In wildlife, lengthening of prothrombin time by more than 25%[37,38] or two standard deviations above baseline values[39] is suggestive of anticoagulant exposure, and best confirmed by analytical detection of AR residues in blood or tissue.

Following exposure to warfarin and other ARs, there is a lag period of one to several days before coagulopathy (detectable with biomarkers) becomes apparent. This is because fully carboxylated functional clotting factors, with half-lives ranging from 6 to 120 h[40] support hemostasis, but once cleared clotting is impaired (viz., key event in AOP, Figure 1). This lag period is well-documented in people, companion animals, laboratory rodents, and even raptorial birds.[41−43] Upon termination of AR exposure, coagulopathy can be resolved in a matter of days or weeks,[41,43] but VKOR activity may remain partially inhibited for weeks to months, reducing reserve capacity to synthesize vitamin K, and thus rendering animals highly sensitive to subsequent AR exposures.[44]

Multiple Organ System Responses. Animals can exhibit massive blood loss and succumb from fatal hemorrhage, but lethality can also result from small microscopic bleeds resulting in localized ischemia, hypoxia and cell death at vital sites (e.g., brain, heart, liver).[12,39,42] Aside from AR effects on hemostasis, there are many less well-established responses related to the impairment of the vitamin K cycle (viz., plausible linkage in AOP, Figure 1). For example, pediatric warfarin therapy can reduce bone density and increase incidence of fractures due to undercarboxylation of osteocalcin, the protein incorporating calcium into bone,[45] although in the single study conducted in SGAR-exposed predatory birds, no such effect was found.[46] Warfarin has been shown to exert anti-inflammatory effects,[47] possibly by altering signal transduction,[48] and also affect cell proliferation by inhibiting vitamin K-dependent growth factors.[49] In addition, the indandione rodenticides chlorophacinone and diphacinone may also affect cellular energy generation by uncoupling oxidative phosphorylation.[50]

Hemorrhage associated with coagulopathy can be spontaneous, but is often initiated and certainly exacerbated by trauma, which is not that unusual in free-ranging wildlife. A comprehensive review[11] provides 50 citations of affected sites and signs of hemorrhage in various organ systems (e.g., integument, musculoskeletal, respiratory, renal, gastrointestinal, reproductive, central nervous system) associated with sublethal and fatal AR poisoning in people. A similar tabulation of affected sites and signs has yet to be compiled for nontarget wildlife, although detailed results of necropsies do appear in some reports.[12,39,42,51,52] Overt signs often include bruising, bleeding from the mouth, nares, rectum, cloaca, and talons, and blood in droppings, scat and urine. Skin, mucus membranes, muscle and viscera can appear pale due to blood loss. At necropsy, affected sites often include skin, muscle, alimentary tract, peritoneal cavity, kidney, and heart pericardium. Assessment of such effects in animals found dead may be hampered due to deterioration of organs and tissues, and hemorrhage due to freezing of carcasses prior to necropsy.[53] There can be excessive bleeding from superficial wounds and hemorrhage from multiple sites. Blood loss accompanying AR exposure is a function of dose and frequency of exposure, and can range from mild to severe with classification of an individual as being anemic, and is easily quantified in vivo (e.g., reduced number of circulating red blood cells, increased reticulocyte counts from stimulation of hematopoiesis, and decreased hematocrit).[12,27,39,41,54] Blood loss can result in metabolic acidosis, tachycardia, and hypovolemic shock,[54] causing changes in tissue perfusion, organ dysfunction, and tissue necrosis.

Whole Animal Responses. At the organismal level, inter-individual variation seems to have a significant role in AR toxicosis.[12] Lethargy and abnormal posture are overt apical responses frequently observed in toxicity studies, and often described in AR-exposed wildlife undergoing rehabilitation. Body condition and weight loss are mentioned in many reports, and a significant negative relation between AR residues and body condition has been found in stoats (*Mustela ermine*) and weasels (*Mustela nivalis*).[55] Furthermore, an association between notoedric mange, mortality and AR exposure has been described in bobcats (*Lynx rufus*) residing in urban areas in southern California,[56] although such relationships may be correlative rather than causal. For example, animals suffering from mange may be forced to forage in poor habitat in closer proximity to people. Direct toxic effects of ARs on reproduction in laboratory mammals, livestock and free-ranging raptorial birds are somewhat equivocal,[57−60] although the European Chemicals Agency classifies some ARs as reproductive toxicants.[61] Clearly, such observations and data are difficult to translate into measurable consequences affecting the fitness (i.e., survival and reproduction) of free-ranging wildlife. Indirect effects, such as altering availability of rodent prey species, could certainly affect predator−prey dynamics.

Population Responses. Although rodenticides are widely used, effects of ARs at the population level of predatory birds

and mammals have not been established. Of the published reports that examine exposure and unintentional wildlife mortality,[12,51,52,62−68] definitive diagnosis of poisoning (i.e., post-mortem signs of hemorrhage, independent of trauma, coincident with the detection of rodenticide residues in liver) generally accounts for but a small fraction of exposures (perhaps <10%),[12,60] with exceptions.[51,67] As pointed out 15 years ago, there is no evidence that rodenticide use causes large-scale population declines of predatory and scavenging birds.[69] However, AR exposure does have the potential to cause additional mortality affecting populations "already experiencing critical limitations"[70] (viz., plausible linkage in AOP, Figure 1). Furthermore, for long-lived predators or scavengers with low reproductive rates (K-strategists), death of a few individuals could theoretically affect local populations on a temporary basis. In a contemporary effort to examine potential population consequences of ARs, hepatic residues and associated signs of intoxication were examined in a data set of 270 birds of prey from Canada.[71] Using an additive approach for SGAR residues (bromadiolone + brodifacoum + difethialone; viz., toxic units) and logistic regression plots to predict the probability of the death of a bird with a liver residue of any given magnitude, it was suggested that a minimum of 11% of the great horned owl (*Bubo virginianus*) population in Canada is at risk of being directly killed by SGARs. That assessment, however, was based on exposure levels of great horned owls in areas with high human population density and rodenticide use, and may not apply across broad areas of the Canadian landscape. Regardless, the prediction that 11% of the population of an abundant K-strategic species is at risk from a single stress factor should be carefully considered, and in some circumstances may not be acceptable to natural resource managers.

There have been some instances of label-recommended or permitted AR use that have resulted in mortality incidents involving species of special conservation status or those afforded special protection. For example, mortality incidents have been reported for weka (*Gallirallus australis*; vulnerable-IUCN Red List) in New Zealand,[72] red kites (*Milvus milvus*; near threatened-IUCN Red List) in Britain and France,[73,74] and bald eagles (*Haliaeetus leucocephalus*; Least Concern-IUCN Red List but safeguarded by The Bald and Golden Eagle Protection Act) in the U.S.[75] There are less definitive incidents involving the San Joaquin kit fox (*Vulpes macrotis mutica;* U.S. Federally endangered species) and northern spotted owl (*Strix occidentalis caurina;* near threatened-IUCN Red List).[76] The status of barn owl (*Tyto alba*) populations in southwestern British Columbia, Canada was recently up-listed to threatened due to many stressors including poisoning by rodenticides.[65,77] In such circumstances, an organismal response (i.e., death of an individual of a threatened or endangered species), rather than a population-level response, may be considered an anchoring event[17] in an AOP. Nonetheless, incidental take of a few individuals of a Federally listed species may be permitted under current regulations, as is the case for the black-footed ferret (*Mustela nigripes*), gray wolf (*Canis lupus*) and northern aplomado falcon (*Falco femoralis*) with Rozol Prairie Dog Bait (chlorophacinone) application.[78]

ANTICOAGULANT RODENTICIDE RISK ASSESSMENT FOR PREDATORS

Registration and Regulation. The use of pesticides requires detailed regulatory evaluations that ensure the compound does not pose an unacceptable risk to people or the environment. Such assessments take into account economic, social and environmental costs and benefits, and general requirements (e.g., new products, reregistrations, sale, distribution, use, etc.) of the vertebrate pesticide registration process.[79] Adverse reactions of nontarget species to pesticide active ingredients are predicted from toxic effects observed in surrogate species exposed in the laboratory. In the U.S., Canada and Europe, the required data have been generated on standard toxicological end points in traditionally used test species (e.g., bobwhite quail, *Colinus virginianus* and mallard, *Anas platyrhynchos*), and occasionally other species (historically, mustelids). In New Zealand, an array of introduced mammals has been included in registration studies for purposes of examining AR efficacy. These data, coupled with field observations, and residue and fate information, are used by regulatory agencies, industry, and other entities conducting ecological risk assessments.[79] Registered products undergo periodic review, which can be triggered by new findings and unexpected observations following their use.

In the U.S., the use profile (e.g., application site and method, formulation, pest species) of FGARs includes urban, suburban, and rural areas, and agricultural fields, with initial product registrations for warfarin dating back to 1950, followed by diphacinone in 1960, and chlorophacinone in 1971.[80] Registration of SGARs in the U.S. occurred much later (brodifacoum 1979, bromadiolone 1980, difethialone 1995),[80] and the use profile was far more restrictive and did not include agricultural fields (some SGARs are permitted for agricultural use in Europe). Product registrations for both FGARs and SGARs have been granted for conservation purposes, including eradication of invasive species on islands.[79,81]

Long after the initial registration of several FGARs and SGARs in the U.S., multiple nontarget wildlife mortality incident reports,[80] several peer-reviewed publications,[52,62,63,72,82] and public interest at the time of the Reregistration Eligibility Decision[83] were the impetus to undertake a comparative risk analysis of rodenticides.[80] Using a multiattribute rating technique (e.g., dietary risk quotient for primary exposure, percent mortality in secondary exposure, active ingredient retention time in blood and liver), the SGARs brodifacoum and difethialone were identified as posing the greatest potential risks to predatory and scavenging birds and mammals that feed on poisoned target and nontarget animals. Attempts to evaluate the risk of brodifacoum using probabilistic methods (i.e., dietary dose, uptake, and depuration models, probability of encountering contaminated prey) were hampered by data gaps and major uncertainties.[84] Deterministic evaluations led the U.S. EPA to request registrants to voluntarily withdraw certain ARs from the marketplace.[76]

In the U.S. EPA's comparative risk analysis, the FGARs seemed to be less hazardous to both target and nontarget species.[80] Some of this analysis relied on acute toxicity data. However, an acute exposure scenario is neither appropriate nor environmentally relevant (i.e., may underestimate environmental risk) as FGARs require multiple days of exposure to evoke toxicity.[85] Additionally, more FGAR bait is needed to achieve the same level of pest control as with SGARs, and thus the number of toxic units in the environment at the time of application is likely to be the same or greater. Furthermore, the development of FGAR resistance in commensal rodents may result in greater potential for exposure of and risk to predatory species.

Risk Mitigation Measures. In 2008, the U.S. EPA instituted measures to mitigate some nontarget risks of SGARs. These included new requirements on points of sale and distribution, and package size, to impede purchase by residential homeowners, and product labeling to permit use in and around agricultural buildings, but not human residences.[13] New bait station requirements were also instituted to minimize exposure of children, pets, and nontarget wildlife. Additional exposure modeling and quantitative risk assessments to evaluate direct bait ingestion (primary exposure) and consumption of prey containing AR residues (secondary exposure) were undertaken.[4] Based on toxicity and toxicokinetics, risk quotients for direct bait consumption indicated that under some exposure scenarios both SGARs (brodifacoum, difethialone) and FGARs (warfarin, chlorophacinone) exceeded levels of concern for nontarget birds and mammals. Consumption of SGAR-exposed prey also exceeded levels of concern for predatory birds and mammals. While consumption of FGAR-exposed prey posed a hazard for nontarget mammals, levels of concern were rarely exceeded for birds.[4] In some use scenarios (e.g., Rozol for control of prairie dogs, *Cynomys ludovicianus*), label requirements even state that applicators must make multiple follow-up visits after application to remove dead or dying target species to mitigate hazard to nontarget scavengers and predators.[86] Such practices to reduce potential AR exposure of predators may not always be followed.[86,87] At the Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel hearing in November 2011, some shortcomings of this screening-level risk assessment were identified, including data quality and interpretation, and overreliance on unrealistic worst-case scenarios.[88]

The U.S. EPA risk mitigation decision has resulted in actions to cancel consumer uses of some noncompliant rodenticides (some products containing warfarin, brodifacoum, and difethialone that failed to meet US EPA safety measures) though over 30 AR products remain available that meet protective standards.[89] Notably, the active ingredient of some replacement compounds (e.g., acute vertebrate pesticides such as bromethalin) lack diagnostic tests and antidotes. In the U.S., a few states conduct additional regulatory review of pesticides, and the State of California will be restricting the use of SGARs to certified pesticide applicators as of July 1, 2014.[90] The U.S. EPA and the Canadian Pesticide Management and Regulatory Agency collaborate to harmonize pesticide regulations in North America. Risk mitigation measures similar to those proposed by the U.S. EPA are now in effect in Canada, with some minor variances (e.g., bromadiolone can be applied by registered users along fence lines within 30 m of buildings).[16,91]

In Europe, a recent review (European Chemicals Agency) under the European Community Biocidal Products Directive (98/8/EC)[92] has highlighted significant or unacceptable risk of primary and/or secondary poisoning of birds and nontarget mammals from some SGARs used as biocides.[55,64,67,93−95] However, under this Directive the compounds were still authorized for use because they are deemed essential for human hygiene and public health, and appropriate alternatives are not at hand. In 2012, a new EU Biocidal Products Regulation (528/2012)[96] was adopted with similar criteria for authorization. Under this regulation, all SGAR use will be re-evaluated by the end of 2017. Requirement for any mitigation measures to reduce risk to nontarget exposure is at the discretion of individual EU member states. For example, in the United Kingdom, SGAR use has been widespread in both urban and rural environments.[6,97−99] Brodifacoum, flocoumafen and the more recently licensed difethialone have until now been restricted to indoor use because of their perceived risk of causing primary and secondary poisoning in nontarget species; other SGARs and FGARs have until now been licensed for indoor and outdoor use. There is prevalence of SGAR application in agricultural holdings[68] with concomitant widespread exposure in rural areas of a range of nontarget avian and mammalian predators.[68,70,99−101] A recent UK review related to the primary and secondary risks posed by all SGARs concluded that there was insufficient scientific evidence to distinguish between any of the SGARs in terms of their risk to nontarget species.[102] As a result, it is proposed that UK authorizations will change during 2014 or beyond, such that all SGARs may be used outdoors and there will be a stewardship program fostering practices to minimize exposure of nontarget species. Other EU member states may adopt alternate mitigation measures. For example, discussions on outdoor use of SGARs in The Netherlands are ongoing and it is proposed that SGARs may be used outdoors by certified personnel, in combination with certified Integrated Pest Management.

In New Zealand, brodifacoum typically has been the SGAR of choice for controlling rodents, all of which are invasive non-native species. However, repeated use of brodifacoum on the two main islands has been associated with substantial contamination of wildlife and game species, and secondary poisoning of nontarget species.[103] As a result, there has been use of low-residue alternatives (cholecalciferol) for control of possums and rodents, registration of para-aminopropiophenone for control of larger pest species (stoats and weasels), and exploration of some toxicant combinations (e.g., FGARs + cholecalciferol) for control of rodents.[104,105]

Special Considerations for Use in Conservation. Anticoagulant rodenticides have been used extensively for the control and eradication of introduced and invasive species,[7,106,107] particularly for island ecosystems. The use of these compounds in such settings is logistically complex and expensive, with the theoretical restoration benefit outweighing the risk of nontarget species mortality.[81] In contrast to standard use of ARs for commensal or agricultural rodent control, special regulatory attention is given to the application of these compounds for conservation purposes to restore habitat for native species. As an example of conservation use, a Special Local Needs pesticide registration for aerial broadcast of 0.005% diphacinone bait was undertaken in Hawaii to control rodents and wild pigs (*Sus scrofa*) in native ecosystems. Hazard was evaluated using both deterministic[108] and probabilistic[109] methods for the endemic Hawaiian hawk (*Buteo solitarius*), short-eared owl (*Asio flammeus sandwichensis*), and honeycreeper (*Melamprosops phaeosoma*). These evaluations found that the quantity of tissue that would have to be consumed by a predator in acute and subacute exposure scenarios was great (often exceeding the weight of the bird); thus, the risk to evoke lethality or prolonged clotting time was low. As previously mentioned, an acute exposure scenario is neither appropriate nor environmentally relevant for assessing risks of FGARs.[85] These assessments using data from traditional wildlife test species may have underestimated risk as recent studies have demonstrated that raptors are far more sensitive to diphacinone than previously thought.[39,42,43] Application of American kestrel (*Falco sparverius*) and Eastern screech-owl (*Megascops asio*) toxicity data for diphacinone in previous deterministic assessments,[4,108] and in probabilistic assessments,[39,110] suggest greater hazard to predatory birds than previously realized. Nonetheless, FGARs

are believed by some to be much less hazardous (perhaps by an order of magnitude)[72] than SGARs, presumably due to their shorter half-life in tissues and multiday exposure required to cause toxicity. These findings also demonstrate the importance of dose−response relationships, including use of toxic reference values,[43,110] to link a biomarker (clotting time) or tissue residues to an adverse effect. Such data are of value to natural resource managers. However, it is difficult to extrapolate the internal dose with effects across species and multiple studies. The AOP construct provides the basis to fill in these data gaps that may be used to help model and interpret dose−response relationships.[17]

■ UNSOLVED ISSUES

There are significant unknowns related to exposure and effects to predatory wildlife associated with use of ARs. Among these are basic and applied data needs to supplement risk assessments. Some of these data are best derived from controlled exposure trials using captive animals, while other information can only be generated from field observations and hypothesis-driven eco-epidemiological studies, and even a combination of these activities.

Exposure Pathways. While there are many conceptual models,[108,109] there are limited empirical field data detailing AR exposure pathways and compound transfer to predatory wildlife per se. This shortcoming was noted in the regulatory review of a probabilistic risk assessment for brodifacoum.[84] Many studies have focused on consumption of poisoned rodents. The exposure pathway starts with AR bait placement and its ingestion by target species. Secondary exposure of predatory and scavenging wildlife occurs exclusively through their diet, which at times can be quite variable. For example, a recent investigation identified the primary target organism, Norway rats (*Rattus norvegicus*), as the most important source of SGARs for several species of owls at farms in British Columbia, Canada.[91] Small mammals, songbirds, and invertebrates were also components of the exposure pathway for secondary consumers in this study.[91]

Exposure pathways can be complex, with nontarget predators encountering a combination of ARs. Notably, tissues analyzed from mortality incidents document exposure to multiple SGARs to varying degrees,[12,51,52,56,62,63,65,68,71,100] and occasionally even combinations of FGARs and SGARs.[51,56] That suggests some predators may reside and forage opportunistically at the interface of urban/suburban/rural and agricultural settings. For example, rats and nontarget small mammals (but not house mice) exposed to SGARs while indoors may move outdoors from unsealed buildings, and can travel considerable distances before becoming available to predators.[91,111] Likewise, the foraging range of many predators changes with season. For example, commensal rats seem to be a significant source of seasonal rodenticide exposure for polecats (*Mustela putorius*) that favor farmyards during fall and winter months.[100,112] Accordingly, estimating risk to nontarget predatory species by extrapolation of toxicity data from single-compound controlled laboratory and pen studies remains exceedingly difficult. As demonstrated in highly inbred laboratory rats, combined SGAR-FGAR exposures and their timing have marked effects on toxicity,[44] and deserve further attention from both an exposure pathway and potential effect standpoint.

Many investigations have documented AR exposure of invertebrates feeding on bait, and perhaps even small mammal feces, rodent carcasses and soil-bound AR residues. Their hazard to insectivorous birds and mammals has yielded mixed findings as only a small fraction of the invertebrate food base may be exposed in a treated area.[91,113−116] However, some suggest that ecological communities often contain both larger numbers of individuals and more species of insectivorous vertebrates compared to top-level vertebrate predators, and thus AR-contaminated invertebrates might actually pose a greater risk to this feeding guild than previously thought.[117] A significant data gap remains for insectivorous vertebrates, some of which may be ecologically vulnerable in island eradication projects.[118,119]

In contrast to the aforementioned terrestrial exposure pathway, there is now evidence that warfarin, at nanogram per liter quantities, is detectable in some wastewater effluents.[120] Its source is presumed to be of human origin. However, based on both its low concentration and log K_{ow} (2.37), it is highly unlikely that this is a significant source of exposure for predatory wildlife.

Macromolecular to Population-Level Effects. Remarkable differences in AR sensitivity have been reported in some omnivorous and predatory birds compared to commonly tested avian granivores.[39,42,72,110] Although interspecific variation in VKOR activity and AR metabolism may account for these observations,[22] there remains a need for additional comparative toxicity and metabolism data for predatory species. Furthermore, the relative in vitro potency of various ARs to VKOR,[121] and their use in additive toxicity models (e.g., toxic units or equivalents) should be further examined as it could serve as an alternative method reducing the need for some in vivo testing. It might be possible to screen for AR sensitivity of predatory wildlife by cross-species comparison of the primary structure of VKOR to that found in resistant target species, as has been done for the arylhydrocarbon and steroid hormone receptors, and other ligand binding sites.[122−124] However, such predictions do not account for interspecific differences in AR absorption, distribution, metabolism and elimination. While the role of vitamin K deficiency in hemorrhagic syndrome in chickens, and warfarin sensitivity and resistance in rats, has been studied in great detail,[125] vitamin K status has not been evaluated in predatory wildlife, and could be a major factor in AR susceptibility and tolerance.

Controlled AR exposure studies have principally focused on overt signs of toxicity and mortality, occasionally included measurement of AR residues and sublethal responses (e.g., behavior, condition, histopathology), and rarely quantification of blood clotting.[72,80,126] There are key issues and even deficiencies in such studies, including the use of artificial test conditions (e.g., no-choice continuous feed scenarios), and that spontaneous hemorrhage in AR-exposed animals is a "multicausative phenomena" affected by stress and other variables.[127] Many of these controlled studies failed to measure AR ingestion rate and concentration of residues in tissue that are needed to derive dietary- and tissue-based toxic reference values, and to estimate internal dose for modeling toxicokinetics.

A longstanding issue related to ARs, and environmental contaminants in general, is the significance of sublethal effects. As illustrated in the AOP (Figure 1), several responses may have hypothetical, plausible, or established linkages foreshadowing higher order organismal or even population-level effects. Based on existing data, predatory wildlife exposed to ARs either survive, with seemingly little or no direct long-term consequences, or they die. Alternatively, it is certainly possible that the proximate cause of death of an individual seemingly unrelated to poisoning might ultimately have been triggered by

AR residues and coagulopathy. This may be responsible for the absence of clear dose−response relationships. For example, a detailed analysis of birds of prey admitted to a veterinary clinic revealed that while 86% of 161 raptors contained AR residues, only 6% could be diagnosed as having succumbed from AR toxicosis.[12] No significant relation between liver brodifacoum residues and death was found, although the small number of individuals that died from causes other than trauma may have confounded this analysis.[12] Nonetheless, some contend that AR exposure is one of many chemical insults affecting "*condition*" (e.g., lethargy could impair hunting, loss of body mass could reduce energy stores during winter), susceptibility to disease, resilience (e.g., recovery from nonfatal collisions, accidents and trauma), tolerance to extreme weather, and even sensitivity to other toxicants (e.g., Pb that can result in anemia), and could exacerbate blood loss during molt. This impaired condition hypothesis remains challenging to test and resolve.

There is some evidence that SGARs are one of several factors (e.g., low food availability with the shift to intensive farming, road mortality, loss of roost sites) that may be responsible for declining populations of some species of predatory birds.[65,77] Based on extensive personal observations over a 21-year period (but not formal surveys), a decline in numbers of breeding pairs of raptors, and some circumstantial evidence of secondary AR poisoning, was noted with initiation of Klerat (active ingredient brodifacoum) use on sugar cane in Queensland, Australia.[57] Recent studies examined barn owl reproduction at oil palm plantations in Malaysia that were baited with warfarin or brodifacoum[58] and bromadiolone or chlorophacinone.[59] Over several breeding cycles, both owl hatching and fledging success in treated plots were significantly lower compared to the reference area. It was suggested that impaired reproductive performance was due to sublethal AR exposure of adults and nestlings, although confounding effects of reduced rat populations on reproductive parameters could not be discounted. Clearly, the direct and indirect consequences and uncertainties of ARs on reproduction and population responses in predatory species deserve further attention.

Exposure and Mortality Incidents. Some suggest that AR risk to predatory birds and mammals has been overestimated, with the proportion of mortality being quite low in comparison to actual use.[3,69] Anecdotal reports favor solitary events (e.g., death of a snowy owl, *Nyctea scandiaca*, which established residence near a correctional facility using 0.2% diphacinone tracking powder, with stomach contents full of rat remains).[52] Likewise, in agricultural settings, the risk to nontarget wildlife is generally perceived to be minimal[3,127] as the vast majority of applications involve FGARs on croplands and fields for grazing livestock. However, baits with the SGAR bromadiolone or the FGAR chlorophacinone have been responsible for some mortality of predatory and scavenging wildlife in France.[51,74,94] For eradication efforts involving introduced species on remote islands, practical experience has demonstrated that some projects create a surplus of readily available dead and dying rodents that can cause significant mortality of predatory birds (e.g., mortality of bald eagles and ravens with brodifacoum use on Langara Island, British Columbia;[106] carcasses and remains of 46 bald eagles associated with brodifacoum application on Rat Island, Alaska[75]). These findings demonstrate that patterns of AR use for control of commensal rodents and introduced species can result in a range of consequences.

Perhaps the greatest unknowns are quantitative estimates of the magnitude of nontarget predator mortality associated with AR use. Few rigorously designed field trials have focused on FGAR or SGAR exposure and effects on predators,[3,128] although two radiotelemetry studies generated some survival data which identified brodifacoum as a significant hazard to raptors in orchards.[129,130] In a more recent study, risk predictions suggested that bromadiolone application for control of the water vole (*Aricola terrestris*) posed a significant hazard to red kites.[94] While field surveys of the treated area detected three dead kites, and one moribund individual with clinical signs suggestive of AR exposure, residue concentrations did not confirm bromadiolone poisoning. Use of banding and radiotelemetry techniques with insectivorous and predatory birds during efforts to eradicate introduced species in New Zealand have documented mortality associated with some formulations of brodifacoum (e.g., insectivorous weka on Ulva Island;[72] morepork, *Ninox novaseelandiae* on Mokoia Island[131]). The vast majority of efforts to monitor AR effects on predators during field applications and eradication projects have entailed direct count observations, call counts, and carcass searches, all of which have varying degrees of inherent bias. While exposure of nontarget wildlife to ARs used for commensal rodent control is well-documented in urban and suburban settings,[12,56] overall effects on population dynamics have not been addressed. More rigorous efforts in monitoring of nontarget mortality should be routinely incorporated into pest control and eradication projects, assessing both short-term and long-term impacts to predatory species.

More extensive monitoring efforts on the magnitude of nontarget predator mortality could add to our ability to gauge the overall effects of new risk mitigation measures. Wildlife exposure and mortality incident schemes (e.g., Ecological Incident Information System of the U.S. EPA, the Predatory Bird Monitoring Scheme and the Wildlife Incident Investigation Scheme of the UK, and Wildlife Disease Surveillance System in France) have been the primary source of wildlife exposure data to date. However, the relationship among AR residues and their relative potencies, sublethal effects, and mortality are poorly defined and difficult to extrapolate between species.[12,60] Hepatic AR residues bound to high affinity and low affinity sites are not always a proxy of recent exposure or effect,[132,133] and in some instances pathological evaluations are incomplete, and potentially compromised by disease and postmortem storage conditions.[53]

Resistance. Genetic-based resistance to FGARs and SGARs in commensal rodents has been documented in numerous locations,[8,9,29,30] and it has been suggested to be a factor that could theoretically impact exposure of predatory wildlife.[60] There is no formally published evidence that resistant rodents accumulate greater body burdens of ARs compared to sensitive individuals.[31,134,135] However, compared to dead and often concealed rats,[73] the survival of AR-exposed resistant individuals for extended periods might enhance the likelihood of secondary poisoning of predators.[60,134,135] The role of resistance in mediating exposure, risk and even adaptation of nontarget species has not been adequately evaluated.

■ ALTERNATIVES

While not the intent of this review, it is worth noting that in addition to AR registration and label restrictions, there are multiple activities that attempt to minimize or prevent exposure and adverse effects to nontarget wildlife. Some large commercial users of rodenticides (e.g., Wal-Mart) have shown leadership in implementing such measures.[136] For large-scale applications and

eradication projects, these include carcass removal accompanied by appropriate disposal, raptor capture and hold/relocation, hazing, and in some situations seasonal timing of baiting to reduce exposure of migratory species. For smaller scale activities, education and outreach programs foster appropriate AR use (e.g., integrated pest management that includes habitat alteration, sanitation, exclusion of commensal pest species) and other practices (e.g., concealing bait to minimize nontarget exposure, carcass disposal, removing bait at end of treatment).[137,138] On a global scale, the number of registered vertebrate pesticides has actually "plummeted" over the last 50 years, with few newly registered compounds.[82] There are some acute vertebrate pesticides (e.g., bromethalin, cholecalciferol, zinc phosphide) for which secondary poisoning potential of nontarget wildlife is low, but these compounds show high acute toxicity, lack specific antidotes and may not be suitable for use in close proximity to man, while other compounds (e.g., sodium fluoroacetate, strychnine) lack effective antidotes and are considered inhumane. Recent research and development efforts have resulted in registration of para-aminopropiophenone in 2011[105] for control of larger pest species (stoats and weasels) in New Zealand. In addition, the combination of an FGAR and acute vertebrate pesticide (e.g., coumatetralyl + cholecalciferol) was at one time used in Germany[139] and is now undergoing trials for potential registration in New Zealand.[105] Other innovations include new delivery systems and bait coatings,[105] although their effectiveness has not been completely evaluated in the field. Biological controls, such as attracting raptors to predate rodents,[140,141] interaction of pathogens to reduce AR doses in baits,[142] and use of the highly pathogenic protozoan *Sarcocystis singaporensis* to debilitate rodents,[143] have been advocated by some, but do not result in rodent elimination.

CONCLUSIONS

Anticoagulant rodenticides are one of the principal vertebrate pesticides for the control of commensal rodents that damage crops and food stores, and cause health issues, as well as for the eradication of invasive species to restore biodiversity to oceanic islands. By constructing an AOP for ARs as they relate to nontarget predatory species, it is apparent that the "mechanism of action" from the molecular through cellular levels of organization is well-understood. However, our knowledge of the linkages and forecasting of responses at the level of the individual (behavioral, physiological, survival) through population (recruitment) is incomplete for this well-studied class of vertebrate pesticide agents. Effects of ARs on predatory birds and mammals at the population level have not been conclusively established. Our knowledge of the hazard associated with resistance development, that could potentially increase AR concentrations in target species, is inadequate. At these higher levels of biological organization, our understanding is less complete and characterized as "mode of action",[17] which is the case for many classes of pesticides and environmental contaminants. While we have identified numerous information needs, perhaps the most critical uncertainties related to AR risks to nontarget wildlife include (i) more complete understanding of exposure pathways, (ii) comparative sensitivity among predatory species, (iii) the relation among residues of multiple ARs, their relative potency, and combined effect at the level of the individual, (iv) quantitative estimates of mortality, particularly in light of new regulations that attempt to mitigate adverse effects, (v) identification of the occurrence of sublethal effects and their higher-tier population and long-term ecological consequences, and (vi) the effects of multiple low-level AR exposures.

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Notes

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