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Chapter 3
Anticoagulant Rodenticide Toxicity to Non-target Wildlife Under Controlled Exposure Conditions

Barnett A. Rattner and F. Nicholas Mastrota

1 Introduction

Our knowledge of the toxicity of anticoagulant rodenticides (ARs) can be traced to investigations of Karl Paul Link and colleagues on "bleeding disease" in cattle, the eventual isolation of dicoumarol from moldy sweet clover, synthesis of this causative agent, and its application as a therapeutic anticoagulant in clinical medicine in 1941 (Link 1959). The notion of a coumarin-based rodenticide as a better "mouse-trap" occurred to Link in 1945 while reviewing laboratory chemical and bioassay data. By 1948, the highly potent compound number 42, warfarin, was promoted as a rodenticide (Link 1959; Last 2002). Through laboratory studies and clinical use of warfarin (Coumadin), a detailed understanding of the mechanism of action and toxicity of warfarin and related ARs (Fig. 3.1) unfolded in the decades that followed.

Our understanding of AR toxicity has been principally derived from an array of biochemical through whole animal studies. Structure-activity relationship models indicate that AR potency (i.e., toxicity in rodents) is related to the length and hydrophobicity of the side chain in the vicinity of carbon 13 (Fig. 3.2), with the most active compounds having greater volume and bulky lipophilic groups in this activity domain (Thijssen 1995; Domella et al. 1999). At the molecular level, both coumadin- and indandione-based ARs inactivate vitamin K epoxide reductase (VKOR), a membrane protein present in the endoplasmic reticulum of liver and other tissues. Catalytic activity of VKOR is required for the reduction of vitamin K epoxide and vitamin K to form vitamin K hydroquinone (Fig. 3.3). This biologically-active

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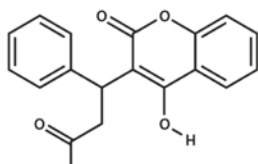
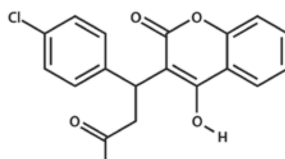
Emerging Topics in Ecotoxicology 5, DOI 10.1007/978-3-319-64377-9_3

First-generation hydroxycoumarins

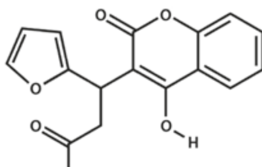
Warfarin
81-81-2



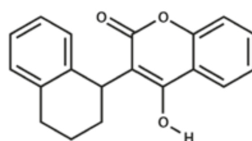
Coumachlor
81-82-3



Coumafuryl
117-52-2

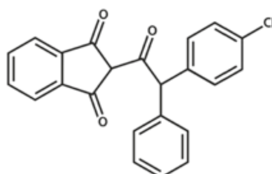


Coumatetralyl
5836-29-3

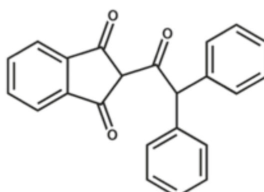


Intermediate-generation indandiones

Chlorophacinone
3691-35-8



Diphacinone
82-66-6



Pindone
83-26-1

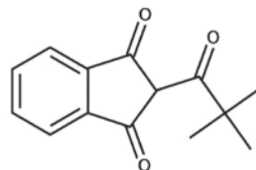


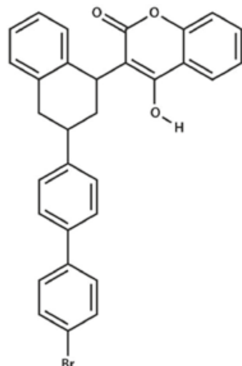
Fig. 3.1 Class, compound, Chemical Abstracts Service Number and structure of 12 anticoagulant rodenticides (From: https://www.ncbi.nlm.nih.gov/pccompound)

hydroquinone is required for γ-glutamyl carboxylation of clotting factors. Inhibition of VKOR by ARs limits the formation of vitamin K hydroquinone resulting in under-carboxylation of clotting factors II, VII, IX and X (Furie et al. 1999) that do not assemble on cell surfaces to form a clot. It is believed that ARs bind tightly to the proposed warfarin-binding site of VKOR at tyrosine residue 135 in close proximity to the active site (cysteines 132 and 135) of this 163 amino acid enzyme (Tie and Stafford 2008). Notably, some point mutations can impede AR binding and thus confer resistance in target pest species (Boyle 1960; Pelz et al. 2005).

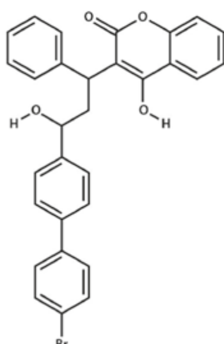
Once the fully-functional clotting factors are cleared from the blood, the des-γ carboxyl dysfunctional clotting factors no longer support hemostasis. Hemorrhage may ensue spontaneously or can be triggered by traumatic events. Coagulopathy may be accompanied by anemia, hypovolemic shock, altered tissue perfusion, organ

Second-generation hydroxycoumarins

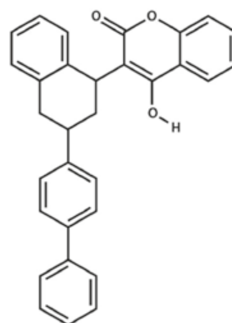
Brodifacoum
56073-10-0



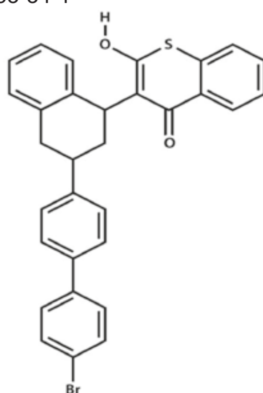
Bromadiolone
28772-56-7



Difenacoum
56073-07-5



Difethialone
104653-34-1



Flocoumafen
90035-08-8

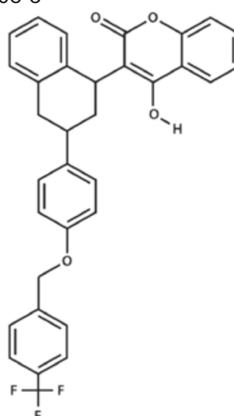


Fig. 3.1 (continued)

dysfunction, and necrosis. Overt signs of intoxication include bruising, bleeding, blood in droppings and urine, pallor, and other signs not specific to coagulopathy (e.g., asthenia, ataraxia, labored breathing, immobility). The proximate cause of death may seemingly be unrelated to AR poisoning, but in fact ultimately triggered by AR-residues and coagulopathy. In addition to impaired blood clotting, some ARs have been shown to increase membrane permeability, affect other vitamin K-dependent proteins, growth factors, and signal transduction (reviewed in Rattner et al. 2014a). Notably, large doses of indandiones can cause toxicity and result in death independent of coagulopathy (Kabat et al. 1944), probably by impairing cellular energy generation through the uncoupling of oxidative phosphorylation

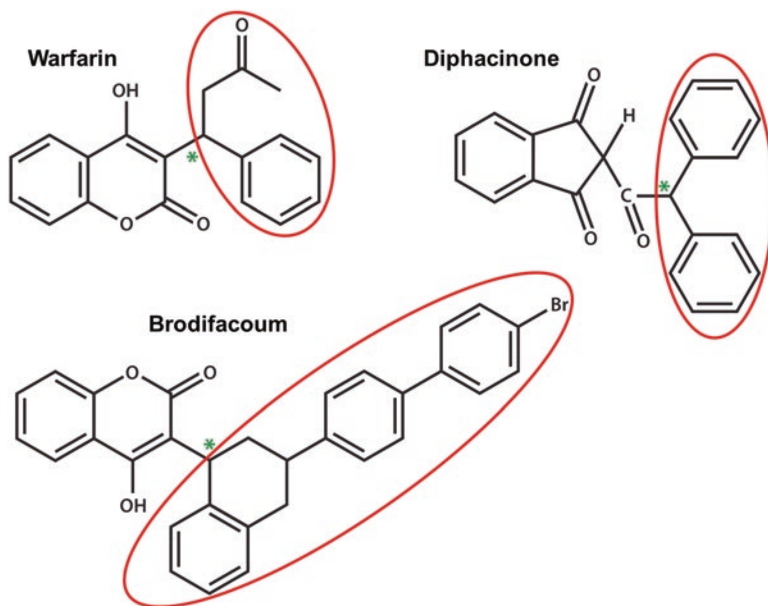


Fig. 3.2 Structure of the first-generation anticoagulant rodenticides warfarin and diphacinone, and the second- generation anticoagulant rodenticide brodifacoum, illustrating side chains (*red*) of the activity domain (*) in vicinity of carbon 13 (Modified with permission from Rattner et al. 2014a, Copyright 2015 American Chemical Society) (Color figure online)

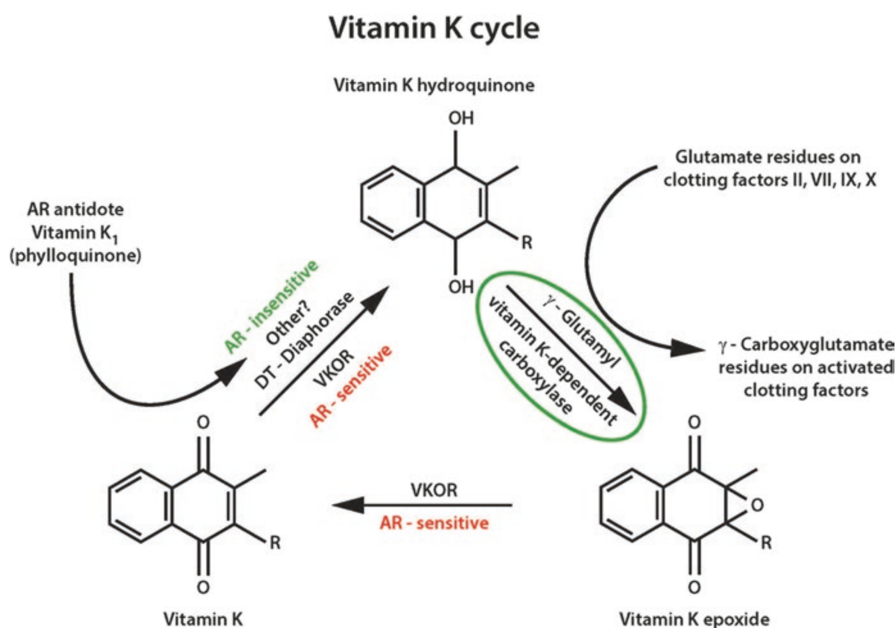


Fig. 3.3 Vitamin K cycle illustrating anticoagulant rodenticide (AR) sensitive vitamin K epoxide reductase (VKOR) reactions and a warfarin-insensitive VKOR that reduces vitamin K to the biologically-active vitamin K hydroquinone. Without adequate vitamin K hydroquinone, γ-glutamyl carboxylase (critical reaction circled in green) lacks substrate to adequately carboxylate clotting factors II, VII, IX and X (Reprinted with permission from Rattner et al. 2014a, Copyright 2015 American Chemical Society)

(van den Berg and Nauta 1975). Numerous controlled exposure studies have documented *in vitro* biochemical effects, and *in vivo* physiological, pharmacological and whole organism responses in domesticated species and to a lesser degree captive wildlife (reviewed in IPCS 1995; Joermann 1998; Rattner et al. 2014a), and much is known from clinical use and accidental poisoning incidents in humans (Watt et al. 2005). Recently, a proposed adverse outcome pathway, identifying the molecular initiating/anchoring event, and established and plausible linkages associated with toxicity through individual and even population levels, has been developed for non-target predatory birds and mammals (Fig. 3.4) (Rattner et al. 2014a)

The use of vertebrate pesticides, and specifically ARs, requires detailed toxicological knowledge and regulatory evaluation to ensure a compound does not pose an unacceptable risk to non-target biota and the environment (Eason et al. 2010). The review and approval process takes into account economic, social and environmental costs and benefits (Eason et al. 2010). An integral component of this process is the generation of toxicity data for non-target wildlife. These data are used to examine the potential hazard and risk associated with direct bait ingestion and consumption of AR-exposed prey by non-target species. For purposes of AR registration, much of these data are generated using standardized toxicity testing methods. However, additional research on AR absorption, distribution, metabolism, pharmacokinetics and underlying mechanism of action is often undertaken to more fully evaluate and explain interspecific differences in toxicity. The generation of these data usually entails *in vivo* testing in species maintained in captivity using various exposure scenarios. This chapter will principally focus on data generated from such studies in terrestrial wildlife (mammals, birds and reptiles) or domesticated surrogate species used to predict effects in non-target wildlife.

1.1 Standardized Tests, Their Limitations and Implications

As terrestrial wildlife may be exposed by direct consumption of AR-containing bait and/or by predation or scavenging on exposed or poisoned rodents, standardized tests have focused on the dietary route of exposure. Notably, exposure pathways have yet to be clearly elucidated for aquatic species. Standardized testing protocols allow regulators to compare the toxicities of various chemicals to terrestrial wildlife and examine the range of species sensitivities to a particular chemical. In the wildlife-pesticide regulatory arena, the most commonly used endpoint for toxicity is mortality because of its definitive nature. The two most commonly conducted standardized tests for lethality are the single-dose acute oral toxicity test that is used to generate a median lethal dose (LD50) and the 5-day subacute dietary toxicity test that generates a median lethal dietary concentration (LC50). Other endpoints may be monitored during such tests (overt signs of intoxication, food consumption, body weight change, and evidence of pathological lesions). The fixed dose procedure, acute toxic class method, up–and-down procedure, and sequential testing schemes have been developed that

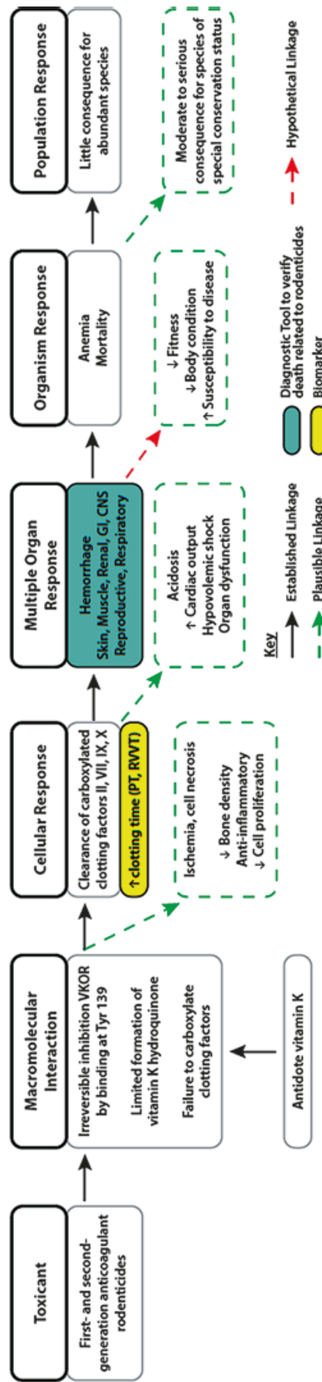


Fig. 3.4 Proposed adverse outcome pathway for anticoagulant rodenticides in non-target predatory wildlife (Reprinted with permission from Rattner et al. 2014a, Copyright 2015 American Chemical Society)

permit estimation of various parameters (e.g., limit dose, median lethal dose, slope of dose-response curve) with greatly reduced numbers of test subjects (reviewed in Andrew 2014; OECD 2010). The most commonly tested species include the mallard (*Anas platyrhynchos*) and bobwhite (*Colinus virginianus*) that are available from game farms as a model avian species (note: used out of convenience rather than likelihood of AR exposure), and common strains of domesticated laboratory mice (*Mus musculus*) and rats (*Ratus norvegicus*) that are used as surrogates for wild mammals. While the focus of this chapter is on non-target species, it is worth noting that a great deal of effort has been devoted to the development of tests and data addressing the efficacy of ARs on target species (e.g., Prescott and Johnson 2015).

The acute oral toxicity test generally entails administration of a single dose of the test compound by gavage or in a capsule, followed by a 14-day observation period. Acute oral exposure may also involve repeated dosing within a 24-h period to achieve sufficient exposure for compounds of low acute toxicity. The 5-day subacute dietary toxicity test entails *ad libitum* exposure of animals to feed amended with the test compound for 5 days followed by an untreated diet for 3 days. In both the acute oral and subacute dietary tests, the post-exposure observation period can be lengthened (e.g., 21, 25 or 30 days post-exposure) to detect latent effects, which are often seen with ARs. The LD50 and LC50 values, their 95% confidence intervals, and slopes are used as indices to compare toxicity among different compounds and species (Klaassen 1986; Hill 1994). These measures of toxicity have application for assessing the hazards of direct ingestion of AR bait, and when tissue residue data are available or modeled, they can be used to assess the potential hazard of ingestion of poisoned prey by non-target predatory wildlife. While standardized test methods are used in chemical registration and in research, their application in risk assessments without regard to the exposure regimen and mode of action can sometimes be misleading, and even result in a false sense of safety. This is especially true when examining and comparing acute oral toxicity data for the first-generation and indandione ARs to second-generation compounds, and will be discussed in detail later in this chapter. Another challenging aspect of AR toxicity studies is that mortality may be delayed for days to weeks after termination of AR exposure, seemingly because VKOR activity may remain partially inhibited, and thus render an individual more vulnerable to the effects of subsequent exposure and/or trauma for an extended period of time (e.g., Mosterd and Thijssen 1991).

Less standardized are secondary exposure tests in which predatory or scavenging birds and mammals are provided whole or ground carcasses of AR-exposed rats or mice, or meat amended with ARs. As reviewed by Joermann (1998) and the USEPA (2004), numerous studies have examined the hazard of first-generation ARs (FGARs; e.g., warfarin, coumachlor, coumafuryl, coumatetralyl), intermediate-generation indandione ARs (e.g., chlorophacinone, diphacinone, pindone; often classified as FGARs), and second-generation ARs (SGARs; e.g., brodifacoum, bromadiolone, difenacoum, difethialone, flocoumafen) in predators. It is noteworthy that the exposure duration in such studies has been highly variable, ranging from 1 to 90 days; mortality, rather than sublethal effects, has been the principal measurement endpoint.

It has long been acknowledged that there are inherent differences among domesticated and captive species compared to free-ranging animals that encounter nutritional, temperature, disease, injury and other chemical stressors that may alter sensitivity to ARs (Vyas et al. 2006). There are key issues and even deficiencies in controlled exposure studies which may limit the extrapolation of results to wild animals. For example, test conditions are artificial, generally entailing no-choice *ad libitum* feeding scenarios, which limit social interactions and physical trauma associated with foraging in free-ranging animals that could trigger bleeding events. Such factors may affect the spontaneous nature of hemorrhage in AR-exposed animals, which seems to be a "multi-causative phenomena" affected by stress and other variables (Kaukeinen 1982). While such interacting factors are acknowledged, the extrapolation of toxicity thresholds and lethality estimates derived from controlled exposure studies have been of value in both forensic evaluations and in assessing risk of ARs to non-target wildlife.

2 Acute Oral Toxicity Studies

Table 3.1 is a compilation of the reported acute oral LD50 estimates (single dose studies) for 12 ARs in common laboratory test species (mammal: laboratory rat in column 2; bird: bobwhite, Japanese quail (*Coturnix japonica*) and mallard in column 4). Data for these species were principally derived from pesticide registration or re-registration submissions that have been compiled and summarized in formal reviews by the U.S. Environmental Protection Agency (USEPA 1998, 2004, 2011), or described in other criteria documents (International Programme on Chemical Safety 1995; McLoed and Saunders 2013). For most of these compounds, the test methods have been critiqued and the results often statistically re-evaluated by regulatory agencies to insure quality and comparability for use in ecological risk assessments. Data sets for some of the older compounds were generated with fewer test subjects and dose levels than currently required. Furthermore, many compounds exhibit shallow dose-response curves making estimates of LD50s challenging, with 95% confidence interval and slope estimates not being available or robust for many ARs. With all of these caveats in mind, here are some of the highlights from common test species that include the laboratory rat, bobwhite, Japanese quail and mallard:

1. Using an acute oral (single or multiple doses within a 24 h period) exposure regimen, SGARs are more toxic (potent) than FGARs.
2. It is noteworthy that the range (i.e., extremes) of LD50 estimates for an AR in a given species can be substantial. Notably, there are differences in AR sensitivity among outbred strains of commonly-tested laboratory rats, with LD50 estimates varying by twofold (Ashton et al. 1986) to orders of magnitude (Jackson and Ashton 1992; USEPA 2004). For example, the reported LD50s in laboratory rats for warfarin range from 2.5 to 680 mg/kg body weight (USEPA 2004).

Table 3.1 Anticoagulant rodenticide acute oral toxicity values for mammals and birds

Class				
Compound	Laboratory, Brown and Norway Rat	Other Mammals	Bobwhite, Japanese Quail, Mallard	Other Birds
	LD50 estimate (mg/kg body weight)	LD50 estimate (mg/kg body weight)	LD50 estimate (mg/kg body weight)	LD50 estimate (mg/kg body weight)
First-generation hydroxycoumarins				
Warfarin	2.5–680	Mouse 374, Rabbit 800, Cat 2.5–40, Dog 20–300,	525 to >2150	chicken 942–1000
		Pig 1–15		
Coumachlor	900		>4.3	
Coumafuryl	0.4			
Coumatetralyl	16.5	Mouse >1000, Rabbit >500	2000	
Intermediate-generation indandiones				
Chlorophacinone	3.1–11.0	House mouse 1.0–6, Pine vole 14.2, Deer mouse 1.0–3.75	258–495	Ring-necked pheasant >100,
		Black-tailed prairie dog 1.94, Rabbit 50, Dog 50–100		Red-winged blackbird 430
Diphacinone	1.9–20	House mouse 141–340, Meadow vole 14.0, Pine vole 57–67.7, Rabbit 35,	1630–3158	American kestrel 96.8
		Mongoose 0.18, Dog 0.88–7.5, Coyote 0.6, Cat 5–15, Pig >150, Cattle >5		
Pindone	50	Rabbit 25, Brushtail possum >100, Dog 75–100, Sheep >74, Pig >10,	241	
Second-generation hydroxycoumarins				
Brodifacoum	0.39–0.56	Richardson's ground squirrel 0.13, Meadow vole 0.72, Pine vole 0.36,	0.25–11.6	Canada goose <0.75, Black-backed gull <0.75,
		Mouse 0.4, Rabbit 0.2–0.3, Mink 9.2, Dog 0.25–3.5, Cat ~25,		Pukeko (purple gallinule) 0.95, California quail 3.3,

(continued)

Table 3.1 (continued)

		Pig 0.5–2, Sheep ~10, Brushtail possum 0.17, Red-necked wallaby 1.3		Black-billed gull <5, Ring-necked pheasant 0.54 to 10, Australasian harrier 10,
				Black bird >3, Hedge sparrow >3, House sparrow >6
Bromadiolone	0.56–1.8	Mouse 0.86–1.75, Pine vole 3.9, Rabbit 0.3–1,	138–170	
		Dog 6 to 11, Cat >25, Pig 0.5 to 3		
Difenacoum	1.8–3.5	Rabbit 2.0, Pig 80–100, Cat >100, Dog >50,	56–4273	Chicken >50
Difethialone	0.4–0.56	Roof rat 0.38, House mouse 0.47, Mouse 1.29, Norway rat 0.29–0.51,	0.26–23.4	
		Hare 0.75, Dog 4–11.8, Cat > 16, Pig 2 to 3		
Flocoumafen	0.25–0.56	Mouse 0.79–2.9, Black rat 1.0–1.8, Rabbit 0.2–0.7, Cat >10,	24 to >300	Chicken >100
		Dog 0.075–0.25, Pig ~60		

Source documents: DEFRA 1987, IPCS 1995, McLoed and Saunders 2013, USEPA 1998, 2004, 2011

Important Note: As described in Sect. 2 of the text (Acute Oral Toxicity Studies; Single-day versus multi-day exposure on AR toxicity), first-generation hydroxycoumarins and intermediate-generation indandiones require multiple feedings to evoke toxicity and the LD50 for these FGARs may not be the appropriate toxicity index to compare to the LD50 for single feed SGARs

Mammals: Black rat, *Rattus rattus*; Black-tailed prarie dog, *Cynomys ludovicianus;* Brushtail possum, *Trichosurus vulpecula*; Cat, *Felis catus*; Cattle, *Bos primigenius*; Coyote, *Canis latrans*; Deer mouse, *Peromyscus maniculatus*; Dog, *Canis lupus familiaris*; Hare, *Lepus capensis*; House mouse, *Mus musculus*; Laboratory, Brown or Norway rat, Rattus norvegicus; Meadow vole, *Microtus pennsylvanicus*; Mongoose, *Herpestes auropunctatus*; Mouse, *Mus musculus*; Pig, *Sus scrofa*; Pine vole, *Microtus pinetorum*; Rabbit, *Oryctolagus cuniculus*; Red-necked wallaby, *Macropus rufogriseus*; Richardson's ground squirrel, *Spermophilus richardsonii*; Roof rat, *Rattus rattus*; Sheep, *Ovis aries*

Birds: American kestrel, *Falco sparverius*; Australasian harrier, *Circus approximans*; Black-backed gull, *Larus dominicans*; Black billed gull, *Larus bulleri*; Blackbird, *Turdus philomelos*; Bobwhite, *Colinus viginianus*; California quail, *Calllipepa californica*; Canada goose, *Branta canadensis*; Chicken, *Gallus gallus domesticus*; Hedge sparrow, *Prunella modularis*; House sparrow, *Passer domesticus*; Japanese quail, *Coturnix japonica*; Mallard, *Anas platyrhynchos*; Pukeko (purple gallinule), *Porphyrio porphyrio*; Red-winged blackbird, *Agelaius phoeniceus*; Ring-necked pheasant, *Phasianus colchicus*

3. Median lethal dose estimates for most ARs appear to be one to two orders of magnitude lower (i.e., more toxic or potent) in laboratory rats than in commonly tested avian species. Notable exceptions include the SGARs brodifacoum and difethialone for which LD50 values are quite comparable between these mammalian and avian species.

In addition, Table 3.1 also contains LD50 estimates, range and limit data for other domesticated and wild birds and mammals. Here are some conclusions that can be drawn from comparisons of toxicity findings among species:

1. For a given AR, order of magnitude differences are apparent among various species of mammals.
2. Apparent differences in species sensitivity are inconsistent among compounds. For example, domestic cats seem to be more sensitive to warfarin than domestic dogs, while the opposite seems to be the case for brodifacoum, difenacoum and flocoumafen.
3. There are limited data on the acute oral toxicity of ARs for avian species other than bobwhite, Japanese quail and mallards. While brodifacoum has been tested in many other species of birds (Godfrey 1985; McLoed and Saunders 2013), the toxicity estimates may not be robust as most of the species were wild-caught, no control birds were used, some species had high levels of lead in liver, and some individuals were reported as not eating and sustaining injuries (capture stress).
4. There is some evidence that the American kestrel is 15–20 times more sensitive to diphacinone than common avian test species, as rigorous studies were conducted for these species by the same group of investigators (Rattner et al. 2010, 2011).

Remarkable gender differences in the LD50 dose of warfarin have been noted, with female rats being nearly an order of magnitude more sensitive than males (Hagan and Radmonski 1953). This observation is not uniform among ARs (USEPA 1998), and some studies suggest that male rats and mice seem to be more sensitive to difenacoum than females (Winn et al. 1987). While not the objective of this chapter, a major research effort entailing controlled exposure studies has characterized the development, magnitude and genetic basis of resistance to first- and second-generation ARs in "target" Norway rats and other rodents (e.g., Greaves and Cullen-Aryes 1988; Thijssen 1995; Pelz et al. 2005; Prescott et al. 2007; Buckle 2013). The issue of differential sensitivity between sexes extends into the realm of genetically-based AR resistance (Pelz et al. 2005; Berny 2011), with resistant-female rodents often exhibiting greater tolerance than resistant-males (e.g., Wallace and MacSwiney 1976; Thijssen 1995). In contrast to rodents, potential effects of sex and resistance on AR toxicity have not been thoroughly examined in birds and lower vertebrates.

While the potential hazards of ARs to reptiles have been discussed in many reviews (e.g., Pauli et al. 2010), to our knowledge only three studies have generated acute toxicity data from which a median lethal dose could be estimated. The acute oral toxicity of warfarin and diphacinone was studied in the brown tree snake (*Boiga irregularis*) (Brooks et al. 1998). From the data presented, the LD50 for diphacinone

is estimated to be 32.2 mg/kg, while the limited dataset for warfarin suggested that lethality occurred at 20 and 40 mg/kg when ethanol was used as a vehicle. Notably, neither warfarin nor diphacinone evoked hemorrhage or other signs of intoxication in the brown tree snakes that is typically found in AR-intoxicated higher vertebrates. Incidentally, dermal exposure to diphacinone (40 mg/kg) using ethanol as a vehicle did not evoke toxicity. In highly detailed studies, Weir et al. (2015) reported that brodifacoum administered orally (capsule) or applied dermally (neat material placed on dorsal surface and covered with an occlusive bandage) to Western fence lizards (*Sceloporus occidentalis*) did not evoke signs of intoxication at doses ranging up to 1750 mg/kg. Using similar methods, the acute oral median lethal dose for coumatetralyl exceeded 1750 mg/kg, for diphacinone the LD50 was ~1750 mg/kg, and for pindone the LD50 was estimated to be 550 mg/kg (Weir et al. 2016). Thus, the LD50s for coumatetralyl, diphacinone, pindone and brodifacoum in Western fence lizards exceeded laboratory rodent values by one to two orders of magnitude.

2.1 Single-Day Versus Multi-Day Exposures on AR Toxicity

The older first-generation hydroxycoumarin and indandione ARs require multiple daily bait feedings to kill target pest species, while the newer SGARs, with longer tissue half-lives, may only require a single feeding to kill pest species. However, the toxicity of FGARs may be greatly enhanced when administered repeatedly. Specifically, FGARs administered for 5 consecutive days to laboratory rats yield lower cumulative dose LD50 estimates (i.e., more toxic or potent) than LD50 estimates derived from single-day oral dose trials (Ashton et al. 1986; Jackson and Ashton 1992). This trend is apparent when one uses the lowest LD50 estimates for laboratory rats presented in Table 3.1. For example, the 5-day cumulative exposure LD50 estimate for warfarin is 1.65 mg/kg (Ashton et al. 1986; Jackson and Ashton 1992), while the single-day oral dose LD50 estimate is 2.5 mg/kg (Table 3.1). Likewise, the 5-day cumulative LD50 estimate for chlorophacinone is 0.95 mg/kg (Ashton et al. 1986; Jackson and Ashton 1992), while the single-day oral dose LD50 estimate is 6.26 mg/kg (Table 3.1). Some comparisons even suggest order of magnitude differences in lethality between cumulative dose from consecutive day exposures versus a single oral dose exposure. For example, when diphacinone was administered in the diet of Eastern screech-owls (*Megascops asio*) for 7 days, the lowest lethal cumulative dose was 5.75 mg/kg, yet when administered as a single-day oral dose, the lowest lethal dose during the week long observation period was 171.2 mg/kg (Rattner et al. 2012a, b). Toxicity tests employing multiple day exposures may be more appropriate and environmentally relevant (i.e., multiple daily bait feedings are necessary to evoke death in target rodents) for assessing the risk of FGARs in non-target species (Vyas and Rattner 2012). Furthermore, comparatively greater potency of SGARs compared to FGARs is diminished when FGARs are administered on multiple days.

3 Subacute Dietary Toxicity Studies

Table 3.2 presents the range of reported dietary LC50 estimates for 5 ARs in laboratory rats and 8 ARs in common avian test species (bobwhite and Japanese quail, mallard). There are apparently no standardized subacute dietary toxicity test data available for the FGARs coumachlor, coumafuryl, coumatetralyl or pindone. For the SGARs difenacoum, difethialone, and flocoumafen, only dietary exposure test data are available for avian species. For the FGARs warfarin, chlorophacinone and diphacinone, and the SGAR bromadiolone, LC50 estimates in birds are more than an order of magnitude greater (i.e., less toxic) than in laboratory rats. However, for the SGAR brodifacoum, LC50 estimates are rather comparable between birds and mammals, which is similar to the trend noted in the acute oral toxicity tests (Table 3.1).

Innumerable dietary exposure trials have assessed AR efficacy in target rodents and some other mammalian pest species. However, far fewer studies have examined AR lethality in non-target animals from direct consumption of AR bait. Although these non-target studies have not generated LC50 estimates, they do utilize realistic exposure scenarios with practical application. In a review by Mount and coauthors (1986), the minimum quantity of several ARs fed or administered over several days that could potentially be lethal in dogs was evaluated. For warfarin, 1–5 mg warfarin/kg body weight for 5–15 days was found to be lethal. However, in a study with fox terriers, dogs succumb by ingesting warfarin bait at daily doses as low as 0.19 mg/kg for 12 days (Prier and Derse 1962), which is considerably lower that reported by Mount and coworkers (1986). For indandiones, Mount et al. (1986) estimated lowest lethal doses of 0.05 mg chlorophacinone/kg body weight administered for 10 days, 0.16 mg diphacinone/kg body weight administered for 3 days, and 15–35 mg pindone/kg body weight administered over several days. For SGARs, most studies in dogs have examined hazard by considering bait ingestion for a single day. Lethality of brodifacoum in dogs was estimated to be as low as 0.25 mg/kg (Mount et al. 1986), which corresponds to 68 g of brodifacoum bait (0.005% ai) and others have suggested as few as 8 brodifacoum (Talon®) pellets (Mackintosh et al. 1988). Bromadiolone lethality in dogs was estimated to be about 11–15 mg/kg body weight (Mount et al. 1986), although a lowest-observed-adverse-effect-level has been estimated for 90 day exposures (i.e., 0.008 mg bromadiolone/kg body weight/day; Bromadiolone Assessment Report 2010). The maximum tolerated dose of difethialone bait (0.0025% ai) is estimated at 400 g of bait (Lechevin and Poche 1988), although it would appear that these data are for an acute exposure scenario.

The dietary toxicity of warfarin was assessed in a 28-day feeding trial in mink (*Mustela vison*), and yielded an LC50 estimate of 11.7 mg/kg feed, with overt signs of intoxication apparent at a dietary concentration of 7 mg/kg feed (Aulerich et al. 1987). Oral sub-chronic toxicity studies in pigs (*Sus scrofa*) have documented mortality in as few as 8 days for warfarin (5 mg/pig/day) and for brodifacoum in as few as 14 days (1 mg/pig/day), although details of the body weight of the pigs are not

readily available (Lechevin and Poché 1988). Toxicity data for warfarin for feral pigs fed warfarin baits for 3 days are in the order of 20 mg/kg body weight (Hone and Kleba 1984). Although not truly a dietary study, daily oral dosing with pindone for 3 days (decreasing from 10 to 3 to 2 mg/kg body weight) to simulate bait ingestion by merino sheep (*Ovis aries*), and even repeating this exposure regimen 8 days later, did not evoke mortality (Robinson et al. 2005). However, a subsequent trial found 11% mortality of pindone-exposed sheep due to excessive bleeding following shearing. A 5-day dietary difethialone exposure trial with European ferrets (*Mustela putoris furo*) found signs of intoxication, but dose-response characteristics did not permit estimation of an LC50 (>112 mg/kg food; USEPA 2004). A detailed study of behavioral toxicity of brodifacoum in the destructive vertebrate pest, the brushtail possum (*Trichosurus vulpecula*), revealed that ingestion of about 0.88 mg over a 3 day period resulted in mortality of 16 of 18 individuals by day 50, with an average time to death of 20.7 days (Littin et al. 2002).

Dietary studies in which various species of birds (chukar, *Alectoris chukar*; Japanese quail; Leghorn chicks, *Gallus domesticus*) were fed warfarin for up to 30 days at concentrations ranging up to those in bait products found little or no evidence of toxicity (Crabtree and Robison 1952; Christopher et al. 1984). Lund (1981) provided laying domestic leghorn hens a choice of untreated diet or diet containing various ARs at concentrations corresponding to those found in some bait products (0.025% warfarin; 0.03% coumatetralyl; 0.005% brodifacoum, bromadiolone or difenacoum) for up to 15 days. While ingestion of warfarin did not affect hens, the FGAR coumatetralyl and all three SGARs evoked signs of intoxication or death within 6–14 days (Lund 1981). Notably, with chronic dietary exposure (up to 20 weeks), mortality and other signs of intoxication have been reported in chickens fed warfarin at dietary concentrations of 25–100 ppm, which is less than levels in some

Table 3.2 Subacute dietary toxicity values for commonly tested mammals and birds

Class	Mammal	Bird
Compound	LC50 (mg/kg diet) for Laboratory Rat	LC50 (mg/kg diet) for Bobwhite, Japanese Quail or Mallard
First-generation hydroxycoumarins		
Warfarin	4.41–6.03	428–5000
Intermediate-generation indandiones		
Chlorophacinone	1.13–1.27	55.8–426
Diphacinone	2.08–2.55	906–10,000
Second-generation hydroxycoumarins		
Brodifacoum	0.53–0.84	1.33–2.75
Bromadiolone	0.92–1.98	37.6–464
Difenacoum	NA	18.9–989
Difethialone	NA	0.56–1.96
Flocoumafen	NA	1.7

For some compounds, estimates are not available (NA) (Source documents: DEFRA 1987, USEPA 1998, 2004, 2011)

rodenticide bait products (Veltmann et al. 1981). When brodifacoum or bromadiolone baits diets were fed on alternate days to 3-week-old chicks for up to 21 days, signs of intoxication were observed in only 2 of 18 chicks fed bromadiolone, whereas brodifacoum evoked mortality in 12 of 18 exposed chicks (Christopher et al. 1984).

4 Toxicity Studies Involving Secondary Exposure

Two significant reviews have summarized controlled studies that examine AR exposure and effects in predators and scavengers (i.e., secondary consumers) (Joermann 1998; USEPA 2004), but few secondary exposure studies have been conducted since 2000. In the feeding trials described in these reviews and other documents, whole AR-exposed target rodents, tissue derived from exposed prey, or tissue from a prey surrogate were offered to predators using various exposure regimens. The prey used in most studies were target rodents (rats or mice), but some studies used other small mammals or birds. The studies generally exposed the prey to food containing one or more concentrations of an AR that is registered for use in commercial rodent bait products. In most feeding trials, AR residues in whole prey or prey tissue were estimated (but rarely analytically verified), thus making it challenging to fully characterize secondary exposure and risk to non-target birds and mammals. The most commonly used endpoints of exposed predators or scavengers include death and survival, overt signs of intoxication (e.g., hemorrhage, pallor, changes in behavior, debilitation), gross pathology of animals which succumb (a few studies have examined histopathological lesions), and changes in blood clotting time as indicators of adverse effects. Abbreviated findings of the secondary exposure studies are described in the sections that follow. A generalized summary of prey exposure and secondary consumer mortality is presented in Table 3.3.

4.1 Secondary Exposure Studies in Mammals

First-generation ARs As described by Erickson and Urban (USEPA 2004), secondary exposure studies in mammals consuming warfarin exposed prey indicate that it is less hazardous than indandiones and SGARs, although secondary poisoning is possible. In the earliest study, all 5 dogs survived for 8 weeks while consuming 4–10 mice per day that had ingested either 0.025% or 0.05% warfarin bait for variable durations (Prier and Derse 1962). These investigators concluded that secondary poisoning in dogs was “outside the realm of practical possibility”. However, in another small trial, dogs consumed nutria (coypu; *Myocastor coypus*) poisoned with 0.025% warfarin bait for up to 10 days, one dog succumbed in 8 days and two others exhibited coagulopathy and other signs of intoxication (Evans and Ward 1967). Mink (*Neovison vison*) were tested using a similar protocol, and all three subjects

exhibited hemorrhage and coagulopathy, succumbing within 16 days (Evans and Ward 1967). In a trial in which least weasels (*Mustela nivalis*) were fed mice exposed to either 0.001%, 0.005% or 0.02% warfarin baits for 3 days, intoxication in weasels was related to bait potency and residues in prey (0.42, 1.58 and 2.95 μg warfarin/g mouse) (Townsend et al. 1984). Weasels that consumed mice on a daily basis that had been fed a 0.001% warfarin bait exhibited coagulopathy but survived the 90 day exposure period; 1 of 2 weasels ingesting mice fed a 0.005% warfarin bait succumbed after 29 days and the survivor exhibited coagulopathy at 90 days; both weasels died (days 12 and 57) when given mice fed a 0.02% warfarin bait. It was suggested that such prolonged exposure is unlikely, but nonetheless free-ranging weasels "could be at risk" to this AR (Townsend et al. 1984). Signs of hemorrhage, but not mortality, were apparent in mink fed tissues from rabbits that had consumed baits mixed with tissues from untreated rabbits to yield diets containing 2.2–22.5 ppm warfarin (Aulerich et al. 1987). In other studies, European ferrets and raccoons fed rodents that had ingested 0.025% and 0.05% warfarin baits for up to 15 days survived with no signs of intoxication (Poché and Mach 2001; Mach 1998 and USEPA 1982 as cited in USEPA 2004).

Secondary exposure studies with other FGARs suggest their risk is generally similar to that of warfarin. In a small trial, mongoose (*Herpestes auropunctatus*) were daily fed a single rat that had ingested 0.025% coumafuryl bait for 1, 3, 6 or 7 days; mongoose consuming rats for 6 or 7 consecutive days succumbed with evidence of prolonged clotting time, while those ingesting rats for 1 or 3 days survived without signs of intoxication (Pank and Hirata 1976). In studies with coumatetralyl, weasels ingesting mice that had consumed 0.0375% bait *ad libitum* died between days 11 and 68 during the exposure period (anonymous study cited in Joermann 1998), and a cat consuming Cape sparrows (*Passer melanrus*) that had ingested bread saturated with coumatetralyl (equivalent to 0.053% bait) succumbed, and exhibited internal bleeding, after consuming 79 birds over a 14 day period (Heÿl 1986). On 3 consecutive days, ferrets were offered a dead rat that had been fed 0.0375% coumatetralyl bait for 3 days, and then returned to a diet of unexposed rats for up to 30 days (O'Connor et al. 2003). Of the 10 coumatetralyl-exposed ferrets, 2 died within 7 days; however those that survived did not exhibit adverse effects that could be directly linked to the rodenticide.

Secondary exposure studies with indandione ARs indicate that chlorophacinone, diphacinone and pindone may also pose a hazard to mammalian predators and scavengers (Evans and Ward 1967; Pank and Hirata 1976; Fisher and Timm 1987 and studies derived from unpublished reports summarized by Joermann 1998 and USEPA 2004). In nine studies utilizing predators and scavengers that had consumed chlorophacinone exposed prey (e.g., baits ranging from 0.005% to 0.1% fed to mice, rats, voles, prairie dogs and ground squirrels for varying durations), 32 of 55 individuals succumbed (7 of 8 mongoose; 3 of 7 coyotes; 1 of 1 red fox *Vulpes vulpes*; 18 of 35 European ferrets; 3 of 4 weasels), and clotting time, when measured, was often prolonged (USEPA 2004).

While predator or scavenger exposure to chlorophacinone exposed prey was as long as 90 days, much of the mortality occurred within days to weeks of exposure.

Table 3.3 Summary of secondary exposure studies in birds and mammals

Class	Birds				Mammals			
Compound								
	Exposure of prey	Predator mortality	Signs of intoxication in surviving predators	Total number of studies	Exposure of prey	Predator mortality	Signs of intoxication in survivng predators	Total number of studies
First-generation hydroxycoumarins								
Warfarin	Orally dosed, formulated diet, or 0.005–0.05% bait	2/25	–	4	Acute oral dose, formulated diets, 0.001–0.025% bait	9/100	+	8
Coumafuryl	0.025% bait	0/2	–	1	0.025% bait	2/4	–	1
Coumatetralyl	0.002–0.075% bait	0/19	–	4	0.0375% to 0.053% bait	6/15	+	3
Intermediate-generation indandiones								
Chlorophacinone	Formulated diet, 0.005–0.01% bait	0/126	+	8	0.0025–0.01% bait	32/55	+	9
Diphacinone	Formulated diet, 0.005–0.01% bait	5/50	++	4	Formulated diet, liver from dead predator, 0.005–0.01% baits	19/33	+	3
Pindone					0.01–0.025% bait	8/10	–	2
Second-generation hydroxycoumarins								
Brodifacoum	Formulated diet, 0.002–0.005% bait	63/149	++	13	Orally dosed, 0.002% bait	8/26	++	5
Bromadiolone	0.005–0.01% bait	11/110	+	6	0.005% to 0.01% bait	6/26	++	4
Difenacoum	0.005% bait	7/22	+	4	0.005% bait	4/8	++	3
Difethialone	Acute oral LD50 dose	3/3	No survivors	1				
Flocoumafen	0.005% bait	6/18	++	4	Orally dosed	0/2	+	1

Gross evidence of external or internal bleeding, hematoma, microscopic observations suggestive of AR effects, or prolonged clotting time observed in most (++) or some (+) survivors, or not apparent (–) in survivors

Three reports describe findings of studies in which predatory and scavenging mammals were either fed rodents baited with 0.005% to 0.1% diphacinone, liver from diphacinone-poisoned owls or meat containing 0.5 ppm diphacinone, for periods up to 18 days (Evans and Ward 1967; Pank and Hirata 1976; Savarie et al. 1979). Of the 33 predator or scavenger test subjects, 19 died (3 of 3 mink; 7 of 8 mongoose; 1 of 2 ermine *Mustela ermine*; 0 of 5 striped skunks *Mephitis mephitis*; 3 of 3 dogs; 4 of 8 rats; 1 of 4 deer mice), and blood clotting time, when measured, was often prolonged. In secondary exposure studies in which nutria that had ingested 0.01% pindone bait for up to 10 days were fed to dogs and mink, mortality, and signs of hemorrhage and coagulopathy, were observed within 6–15 days of exposure (Evans and Ward 1967). An interesting conclusion of Evans and Ward (1967) was "the demand for nutria as mink and pet food, coupled with the secondary poisoning hazards, rules out the use of these rodenticides for nutria control in coastal areas of the United States". Results of a pindone study in mongoose were more variable. Death and prolonged clotting were observed in mongoose following consumption of rats baited with 0.025% baits for 1 or 6 days, but surprisingly no mortality or coagulopathy was observed in mongoose following consumption of pindone-exposed rats for 3 or 10 days (Pank and Hirata 1976).

Second-generation ARs Exposure studies with SGARs generally indicate that ingestion of prey containing these compounds poses a high risk to predatory and scavenging mammals. Studies have been conducted in which rodents, orally dosed with 15 mg brodifacoum/kg body weight or rodents that consumed 0.002% baits, were fed to predators or scavengers for 1–52 days (Pank and Hirata 1976; Godfrey 1985, unpublished reports summarized in Joermann 1998 and USEPA 2004). Mortality was observed in 8 of 26 individuals (2 of 5 red fox and gray fox *Urocyon cinereoargenteus*; 1 of 5 mongoose, 4 of 4 weasels; 1 of 6 domestic dogs), with signs of intoxication, including coagulopathy, observed in many of the survivors. In bromadiolone studies, rodents fed 0.005% to 0.01% baits for varying durations, were offered to predatory or scavenging mammalian predators for up to 6 days (Pank and Hirata 1976; Lund and Rasmussen 1986; Grolleau et al. 1989, and unpublished reports summarized in Joermann 1998 and USEPA 2004). Of the 26 bromadiolone-exposed subjects, 6 succumbed (3 of 4 mongoose; 2 of 7 coyotes; 1 of 11 ermine; 0 of 4 stone marten *Martes foina*), having been exposed to prey for 3 or more days. Notably, signs of intoxication including hemorrhage were reported in all 10 surviving ermine, modest changes in all 4 stone martens (increased fragility of small vessels in musculature on top of skull; Lund and Rasmussen 1986), but no observed effects in the 5 surviving coyotes.

During an exposure period ranging up to 10 days, 4 mongoose fed rats that had ingested 0.005% difenacoum bait did not succumb, but there was some evidence of prolonged coagulation time (Pank and Hirata 1976). In contrast, all 4 weasels consuming mice that had ingested 0.005% difenacoum bait died over a 9–33 day exposure period, while another study reported that an exposed weasel survived a 12 day exposure period, but exhibited impaired blood clotting (reports summarized in Joermann 1998). In a small study in which 2 ferrets consumed mice orally dosed

with flocoumafen (10 mg/kg) for 5 days, both survived but exhibited some effects on blood coagulation (Bachhuber and Beck 1988 cited in Joermann 1998).

4.2 Secondary Exposure Studies in Birds

First-generation ARs Several feeding studies have been conducted in which warfarin-exposed rodents were fed to predatory birds. Formulated diets or 0.005–0.05% warfarin baits have been fed to tawny owls (*Strix aluco*), barn owls (*Tyto alba*), black-billed magpies (*Pica pica*), and an Eurasian buzzard (*Buteo buteo*) for durations ranging from 5 days to as long as 90 days (Townsend et al. 1981, Lee 1994; Telle 1955 and March 1997 as reviewed in USEPA 2004). Whole body warfarin residues in mice fed to the tawny owls ranged from 27.4 to 344 μg/mouse, the lower concentration corresponding to levels found in mice poisoned with 0.005% bait (Townsend et al. 1981, 1984). In this detailed study, warfarin consumption by tawny owls was about 0.175 mg/kg body weight per day in a 90 day trial and 0.78 mg/kg body weight per day in a 28 day trial. While signs of intoxication (e.g., internal hemorrhage, prolonged clotting time) were reported in some of these studies, it is notable that of the 23 warfarin-exposed birds, only two barn owls died (Lee 1994). In this latter study, rats received a 0.025% warfarin bait formulation, while even more potent formulations (up to 0.054%) have been registered in the US (USEPA 2011). Based on bait consumption by rats, Lee (1994) estimated the cumulative warfarin dose of the two owls that succumbed to be 86 mg warfarin per kg body weight owl (corresponding to about 12 mg warfarin per kg body weight owl per day for 5–7 days), with mortality occurring some 2–3 weeks following termination of warfarin exposure (Lee 1994). However, this estimate is likely a flawed overestimation, as the concentration of the AR in exposed rats was not analytically quantified, and warfarin metabolism and excretion by rats, which can be substantial, was not considered.

Secondary exposure studies with other FGARs seldom found mortality. Studies in which over 50 coumatetralyl-killed Cape sparrows were fed to a spotted eagle owl (*Bubo africanus*) or to a steppe buzzard (*Buteo buteo*) for 18 days (Hëyl 1986), coumatetralyl-poisoned rats were fed to the omnivorous weka (*Gallirallus aurtralis*) for 3 days (O'Connor et al. 2003), and coumatetralyl-exposed mice were fed to buzzards for 2 week (described in Joermann 1998) or to kestrels (*Falco tinnunculus*) for 22 days (Galanos 1991 described in Madden 2002), did not cause mortality of these secondary consumers. However, kestrels fed mice that had been poisoned at two to four times the label rate did exhibit signs of anticoagulant intoxication at necropsy (Galanos 1991 described in Madden 2002). In a small trial, two barn owls fed coumafuryl-exposed rats for 10 days survived without apparent intoxication (Mendenhall and Pank 1980).

A number of investigations have examined the toxicity of chlorophacinone- and diphacinone-exposed rodents (fed 0.005–0.01% bait) offered as food to captive raptors or scavenging birds for durations ranging up to 61 days (e.g., barn owl, great-

horned owl *Bubo virginianus*, saw-whet owl *Aegolius acadicus*, Mendenhall and Pank 1980; American kestrel, Radvanyi et al. 1988; tawny owl, Eurasian buzzard, Riedel et al. 1988; great-horned owl, red-tailed hawk *Buteo jamaicensis*, Askham and Poché 1992; American crow *Corvus brachyrhynchos*, Massey et al. 1997; black-billed magpie, carrion crow *Corvus corone* and white stork *Ciconia ciconia* as reviewed in USEPA 2004; barn owl, Salim et al. 2014; American kestrel, Rattner et al. 2015). Signs of intoxication (lethargy, behavioral aberrations, wing droop, gross and histological evidence of hemorrhage, prolonged clotting time) were observed in some of these studies (e.g., Radvanyi et al. 1988; Riedel et al. 1988; Massey et al. 1997; Salim et al. 2014; Rattner et al. 2015), but not in others (Mendenhall and Pank 1980; Askham and Poché 1992, reviewed in USEPA 2004). Rattner et al. (2015) actually derived a dietary-based toxicity reference value (TRV; ~40 μg chlorophacinone/kg kestrel per day) for which 50% of the exposed non-target raptors would exhibit coagulopathy. It is remarkable that of these eight reports describing indandione secondary exposure trials utilizing nearly 100 predatory or scavenging birds, mortality was limited to two great-horned owls and a saw-whet owl that consumed diphacinone-poisoned mice for 5 days, and these three owls did not exhibit overt signs of intoxication (Mendenhall and Pank 1980).

In an often cited study by Savarie and coworkers (1979), golden eagles (*Aquila chrysaetos*) were fed muscle from diphacinone-treated sheep (*Ovis aries*) (2.7 mg diphacinone/kg muscle) for 5–10 days. While these diphacinone concentrations in sheep muscle are quite unlikely for livestock and wild game ingesting bait in a field setting (e.g., see data for wild pigs in Eisemann and Swift 2006), adverse effects (e.g., extreme weakness, ataxia, hemorrhage, prolonged prothrombin time, reduced hematocrit, but not mortality) were observed in golden eagles that received an estimated cumulative dose of 1.08 mg/kg body weight. Likewise, dietary exposure of eastern screech-owls (*Megascops asio*) to graded concentrations of diphacinone mixed into bird of prey diet for 7 days evoked similar effects at a cumulative dose of 1.68 mg/kg body weight (Rattner et al. 2012a). From these diphacinone data sets, the lowest-observed-adverse-effect level (LOAEL) for prolonged clotting time was estimated to be 110 μg/kg body weight per day in golden eagles and 160 μg/kg body weight per day in screech-owls, and these values have been used in some predatory bird risk assessments (Eisemann and Swift 2006, Rattner et al. 2012a, b). Using a statistically more robust approach than the LOAEL, Rattner and coworkers (2012b) derived a dietary-based TRV of 170 μg diphacinone/kg owl per day for a week at which 10% of the non-target raptors would exhibit reduced hematocrit (i.e., classified as anemic) associated with coagulopathy (Rattner et al. 2012b). Notably, using data for diphacinone-poisoned rats from Hawaii (extreme value of 12 μg diphacinone/g liver, E.B. Spurr, USGS as cited in Rattner et al. 2012a), and assuming that owls fed exclusively on these rats, the dietary dose would be ~145 μg diphacinone/kg owl per day, which approaches this TRV (i.e., 170 μg diphacinone/kg owl per day for a week) for AR-induced anemia.

Second-generation ARs The most detailed avian studies have been conducted with brodifacoum, which is the most potent of the group (Joermann 1998; US EPA

2004). These trials generally found high levels of mortality with secondary exposure. Several studies have been conducted in which barn owls were fed rodents (*Rattus sp., Mus sp.*) that had been exposed to environmentally realistic concentrations of brodifacoum baits (baits containing 0.002–0.005% active ingredient) (Mendenhall and Pank 1980; Newton et al. 1990; Gray et al. 1994; Lee 1994; Wyllie 1995). Many of the owls fed rodents for as long as 15 days exhibited signs of intoxication (coagulopathy, hemorrhage, pale viscera) and a substantial number of birds died during these trials (5 of 6 owls succumbed – Mendenhall and Pank 1980; 4 of 6 died – Newton et al. 1990, 3 of 4 died – Lee 1994, and 1 of 4 died – Gray et al. 1994, 4 of 10 died – Wyllie 1995). Some of these studies attempted to describe the cumulative concentration of brodifacoum in consumed rodents that would result in barn owl mortality; estimates are quite variable ranging from 0.15 to 0.18 mg/kg owl (Newton et al. 1990) to 5.4 mg/kg owl (Gray et al. 1994). Lee (1994) estimated a high cumulative concentration of 9.68 mg brodifacoum/kg owl, but since this value assumed no rodenticide metabolism or elimination by the rats, it should be discounted. Furthermore, even those estimates that quantified brodifacoum in rodents that were ingested (~15.36 μg/mouse) are compromised by the regurgitation of pellets by owls that contain rodenticide (perhaps as much as 27% of the exposure dose; Newton et al. 1990, 1994). American kestrels fed tissue from brodifacoum-exposed voles containing varying concentrations of this AR for 4 days exhibited mortality principally at the greatest dose (6.0 μg brodifacoum/kg vole) (LaVoie 1990). It was estimated that kestrels that succumbed could have consumed up to 7.3 mg brodifacoum/kg body weight (LaVoie 1990). Godfrey (1985) fed brodifacoum-dosed rabbits to harrier hawks (*Cirus approximans*) and suggested that the dose evoking mortality in hawks was about 6.5 mg/kg, but in contrast to the aforementioned reports, downplayed its risk. There are several other unpublished studies documenting mortality and overt signs of intoxication in red-tailed hawks, red-shouldered hawks (*Buteo lineatus*), American kestrels, Eurasian buzzard and laughing gulls (*Larus atricilla*) (Howard and Marsh 1978, Savarie and LaVoie 1979, Lutz 1987, ICI Americas, Inc. 1979 all of which are summarized in Joermann 1998 and USEPA 2004).

Secondary exposure studies with raptors indicate that bromadiolone may be slightly less toxic than brodifacoum. For barn owls fed bromadiolone-exposed rodents (baits containing 0.005% active ingredient) for as long as 10 days (Mendenhall and Pank 1980; Lee 1994; Wyllie 1995), some birds exhibited signs of intoxication and hemorrhage, but when compared to the aforementioned brodifacoum trials, mortality was more limited (1 of 6 owls succumbed – Mendenhall and Pank 1980; 3 of 4 owls died – Lee 1994, 0 of 18 owls died – Wyllie 1995). An estimate of the cumulative bromadiolone dose based on concentration in ingested prey that would result in death of captive barn owls is not available. These types of studies with other raptors (e.g., red-tailed hawk and great horned owl, *Bubo virginianus*, Poché 1988; buzzard, Grolleau et al. 1986 and Lutz 1986 described in Joermann 1998 and USEPA 2004) yielded similar findings, namely that rodents that have ingested commonly used bromadiolone baits (0.0025–0.01% active ingredient) for

several days evoked less pronounced signs of toxicity in captive raptors than observed in raptors consuming brodifacoum-exposed rodents.

Ingestion of rodents exposed to difenacoum baits (0.005% active ingredient) by barn owls for durations ranging from 1 to 15 days resulted in limited mortality (all 6 survived – Mendenhall and Pank 1980, all 6 survived – Newton et al. 1990, 1 of 4 died, Gray et al. 1994). The single owl that succumbed was estimated to have consumed 3.7 mg difenacoum/kg over a 13 day period. There was evidence of impaired blood clotting and hemorrhage in many of the owls receiving treated rodents for 3 or more days. In contrast to findings in barn owls, ingestion of difenacoum-exposed mice by tawny owls (*Strix aluco*) for an extended period resulted in mortality of all 6 owls between days 8 and 41 of exposure (anonymous 1981 cited in Joermann 1998).

Limited data are available for controlled secondary exposure studies with difethialone. Barn owls were fed bandicoot rats (*Bandicota bengalensis;* orally dosed with the median lethal dose of difethialone) for 1 day, followed by a 20 day recovery period, then offered exposed rats for 3 consecutive days, followed by another recovery period, and then offered difethialone-dosed rats a third time (Saravanan and Kanakasabai 2004). In the third exposure phase of this study, during which the owls were to be offered difethialone-dosed rats for 6 consecutive days, all owls succumbed before the target exposure duration and exhibited either overt or internal hemorrhage.

In secondary exposure studies with flocoumafen, barn owls fed exposed rodents (baits containing 0.005% active ingredient) for up to 15 days exhibited varying mortality among studies (1 of 4 died – Gray et al. 1994, 3 of 4 died – Lee 1994, 1 of 5 died – Newton et al. 1994). Many of the owls exhibited overt signs of intoxication. Notably, molting in one owl during the course of its exposure may have exacerbated hemorrhage and contributed to mortality (Newton et al. 1994). Estimates of the cumulative dose of flocoumafen associated with mortality ranged from 0.93 to 2.2 mg/kg owl (Gray et al. 1994; Newton et al. 1994). Limited work in other species includes mortality of 2 of 5 buzzards (*Buteo buteo*) fed flocoumafen baited mice for 5 days (Ueckermann and Lutz 1986 cited in Joermann 1998).

4.3 Some Conclusions from Secondary Exposure Studies

While acute toxicity data are available for a dozen ARs (Table 3.1), only 10 compounds have been examined in secondary exposure studies with birds and mammals. To the best of our knowledge, only one secondary exposure study has been conducted in reptiles (5 rattlesnakes, *Crotalus viridis,* exposed to bromadiolone-baited mice survived without signs of intoxication; Poché 1988). Because there are no formally harmonized protocols or test species for secondary exposure studies, comparisons among ARs and species remain challenging. Most of the studies are fixed-dose, with a variable exposure period, and use death as the principal endpoint, but note signs of intoxication (e.g., bruising, hematomas, hemorrhage and

prolonged clotting time). The percent active ingredient of the bait or food used to expose prey varies considerably. The duration of prey exposure to bait formulation is usually in the order of days, while exposure of the secondary consumer is far more variable, ranging up to 3 months. Such data sets are not particularly amenable for examining dose-response relationships, and estimating effect thresholds and dietary- or tissue-based toxicity reference values.

Despite fewer and smaller secondary exposure studies being conducted with mammalian predators and scavengers compared to raptorial and scavenging birds, nearly all ARs caused some mortality (exceptions coumafuryl, flocoumafen). To the best of our knowledge, no difethialone secondary exposure studies have been conducted in mammals. Nonetheless, in the comparative risk analysis of the USEPA (2004, 2011), the "opportunity for secondary exposure to exceed the median lethal dose was estimated to be greater for brodifacoum and difethialone" (USEPA 2011). In contrast to birds, substantial secondary mortality was even noted in mammalian studies with the FGARs chlorophacinone and diphacinone.

In studies comparing mortality rate in predators fed prey that had consumed either realistic bait formulations or AR-containing tissue diets thought to mimic realistic exposures, brodifacoum appears to pose the greatest risk among ARs to predatory and scavenging birds (Joermann 1998; USEPA 2004, 2011). The data from the described exposure scenarios suggest that bromadiolone, difenacoum, flocoumafen and difethialone are slightly less hazardous than brodifacoum, but pose greater risk to predatory and scavenging birds than the FGARs warfarin, coumatetralyl, coumafuryl, chlorophacinone and diphacinone. Some, but not all, of these ARs have been examined in comparative risk models that incorporate pharmacokinetic accumulation and elimination data. These studies have also found that SGARs pose the greatest risk, with brodifacoum (Fisher et al. 2004; USEPA 2004, 2011) and difethialone (USEPA 2004, 2011) identified as posing the greatest risk to predatory and scavenging birds (USEPA 2004, 2011).

5 Chronic Toxicity Studies

Traditionally, chronic studies entail ≥ 90-day oral, dermal or inhalation exposures in rodent and occasionally other test species (e.g., OECD 1998, 2009). It has been suggested that such testing with ARs is inherently difficult to conduct, necessitating some dose levels below the analytical limit of detection for some compounds (IPCS 1995, ECHA 2003). Based upon the nature of AR use (i.e., non-food application), no chronic ecotoxicity studies are required for registration, and thus only limited data are available (e.g., USEPA 1998; European Union 2010). For example, a 90-day dietary exposure to warfarin in laboratory rats yielded an LD50 (based on measured food consumption) of 0.077 mg/kg/day (about 1/20th of the 1-day acute oral LD50: 1.6 mg/kg), with a "safe" concentration (no mortality to 300 days) estimated to be 0.02 mg/kg-day (Hayes 1967). As previously described (Sect. 3 Subacute Dietary Toxicity Studies), broiler chicks fed vitamin K deficient diets

containing warfarin (25–100 ppm) for up to 20 weeks exhibited dose-dependent increases in mortality, prolonged prothrombin time, hemorrhage and impaired growth compared to controls, although no attempt was made to estimate a safe level of exposure (Veltmann et al. 1981). However, the objectives and design of the rat study (Hayes 1967 focusing on generating a median lethal dose) and chicken study (Veltmann et al. 1981, focusing on warfarin-induced endocardial lesions as affected by a vitamin K deficient diet) were quite different and do not permit a direct comparison of warfarin toxicity between these species. Chronic oral toxicity studies with chlorophacinone in rats revealed mortality at doses ≥ 0.020 mg/kg body weight/day, with evidence of hemorrhage and prolonged clotting time at ≥ 0.010 mg/kg body weight/day (ECHA 2014a). In a 90-day feeding trial with diphacinone, 2 of 72 rats succumbed (dietary doses of 0.065 and 0.25 mg/kg diet), with signs of subdural hemorrhage; remarkably none of the survivors had prolonged prothrombin time, but 10 treated rats did exhibit a pinkish eye discharge that was not dose-related (Elias and Johns 1981). In a chronic study in which dogs were orally dosed with bromadiolone, mortality and signs of intoxication (hemorrhage, prolonged clotting time) were apparent at doses ≥ 20 mg/kg/day, while no effects were apparent at 8 mg/kg/day (ECHA 2014b). In contrast, evidence of hemorrhage and prolonged clotting time were apparent in a chronic oral toxicity study with New Zealand white rabbits receiving ≥ 0.001 mg/kg/day (ECHA 2014b). Several repeated dose studies have been conducted with difenacoum, with dietary concentration ≥ 0.1 mg/kg body weight/day causing mortality in rats (ECHA 2014c). While it is certainly possible, and even probable, that non-target wildlife might encounter chronic low-level AR exposure (continuous or sporadic), the toxicity associated with long-term AR exposure in non-target wildlife has not been examined in a controlled exposure setting.

6 Sublethal Effects

As described in the Introduction and illustrated in Figs. 3.2, 3.3 and 3.4, the principal mechanism of action of ARs entails inhibition of the vitamin K cycle resulting in under-carboxylation of the clotting factors II, VII, IX and X yielding dysfunctional clotting and prolonged clotting time or failure of blood to clot altogether. Based upon decades of warfarin (Coumadin) use in humans, and extensive studies in other mammals, the dose-response relationship for warfarin (and related compounds) is known to be very steep. There are established procedures and nomograms for determining the appropriate Coumadin maintenance dose for patients of varying genotypes, and these are largely dependent on measurement of prothrombin time to titrate the desired "therapeutic effects" (e.g., Anderson et al. 2007). Prolonged clotting time, bruising, frank bleeding, microscopic evidence of bleeding, blood in droppings and urine, anemia and pallor are frequently noted "toxicological effects" in AR exposure studies in wildlife. These sublethal responses are part of the sequelae

of AR intoxication in higher vertebrates that are linked to physiological condition and mortality (see Fig. 3.3, Rattner et al. 2014a).

6.1 Prolonged Clotting Time

Due to greater societal concerns for humans, domesticated and companion animals, the effects of ARs on hemostasis in mammals are far better characterized than in birds and lower vertebrates. In addition, there are some notable inter-specific differences in clotting mechanisms between mammals and birds (e.g., less functional intrinsic clotting pathway in birds, Belleville et al. 1982, James et al. 1998, Thomson et al. 2002, Ponczek et al. 2008, Harr 2012; mammals have platelets while birds have thrombocytes that spread less efficiently and do not aggregate as readily, Schmaier et al. 2011), and some suggestion that avian whole blood clotting time is slower than that of mammals (Belleville et al. 1982; Scanes 2015). A variety of clotting assays have been used to document coagulopathy following AR exposure in captive wild birds and mammals. Clotting tests range from timed visual observations of whole blood (e.g., fibrin formation in sample detected in a sequentially broken capillary tube or with a hooked needle, Evans and Ward 1967, Mendenhall and Pank 1980; cessation of movement of a metal rod in a rocked capillary tube blood sample, Newton et al. 1990; time for clot formation in a serum collection tube, James et al. 1998; activated clotting time by assessing microclot formation of a sample drawn into a tube containing diatomaceous earth, Webster et al. 2015) to fibrometer-based assays of citrated plasma using purified reagents to measure prothrombin time and Russell's viper venom time (e.g., Savarie et al. 1979; Littin et al. 2002, Bailey et al. 2005; Rattner et al. 2010; Webster et al. 2015). More detailed information on blood sample collection requirements, and the selection and conduct of assays to assess AR effects, is outside the scope of this chapter (see Triplett and Harms 1981; Brooks and De Laforcade 2012). However, it is worth noting that generation and standardization of clotting time data for birds has been hampered because commercially available mammalian clotting assay kits do not perform very well in tests of citrated bird plasma (e.g., Guddorf et al. 2014). Furthermore, there are currently no commercial sources of avian thromboplastin, which necessitates preparation and characterization of crude avian thromboplastin extracts to conduct prothrombin time assays (e.g., Rattner et al. 2010; Webster et al. 2015). As clotting time in birds exposed to ARs may be many times longer than basal values in healthy unexposed individuals, even crude clotting tests may be helpful in diagnosing AR exposure (James et al. 1998).

In studies with captive wildlife, clotting time has been used to detect coagulopathy (adverse effect) as a toxicological endpoint associated with exposure to a particular AR (concentration known or estimated) for varying durations (see Sect. 4. Toxicity Studies Involving Secondary Exposure). In some studies involving severe AR exposure, a simple descriptive statement is made implying that blood did not

clot (e.g., 30+ min, Pank and Hirata 1976; 27 min to clot, James et al. 1998), while other studies utilizing sublethal exposures report clotting time values and make statistical comparisons to unexposed animals or baseline values generated prior to AR exposure (Savarie et al. 1979) to estimate a LOAEL (Eiseman and Swift 2006). Some toxicokinetic studies have demonstrated the well-known lag time between AR exposure and the onset of prolonged clotting time associated with clearance of active carboxylated clotting factors during the onset of VKOR inhibition (e.g., Littin et al. 2002; Rattner et al. 2011, 2014b). The time course to restore clotting time to baseline following termination of AR exposure has also been monitored in a few studies (Savarie et al. 1979; Rattner et al. 2014b). It has been suggested that an increased prothrombin time by more than 25% is suggestive of AR exposure (Shlosberg and Booth 2006). Rattner and coworkers (2012a, 2014b, 2015) have defined the threshold for AR-induced coagulopathy as values exceeding control or baseline clotting time by more than two standard deviations; the number of affected individuals at a given dietary dose or tissue residue level was used to generate dietary-based and tissue residue-based toxicity reference values for coagulopathy. It is surprising that only a few studies have attempted to relate changes in clotting time to AR tissue residues (e.g., Rattner et al. 2014b, 2015). The authors are not aware of reports that describe a level of AR-induced coagulopathy that results in death. However, AR-exposed animals with coagulopathy are clearly compromised, and the proximate cause of death may be a physical injury exacerbated by coagulopathy, which may be considered the ultimate cause of death.

6.2 Decreased Hematocrit and Anemia

While it is well-known that companion animals and non-target wildlife suffering from AR toxicosis commonly exhibit decreased hematocrit (packed cell volume) due to blood loss and may be clinically classified as anemic (e.g., Mount et al. 1986; Murray 2011), it is surprising that few AR exposure studies with wildlife monitor and report hematocrit in test subjects. In various trials involving golden eagles (Savarie et al. 1979), eastern screech-owls (Rattner et al. 2012a, 2014b), American kestrels (Rattner et al. 2015) and Japanese quail (Webster et al. 2015) exposed to environmentally realistic concentrations of ARs, some individuals had hematocrit values of ≤30 and could be classified as anemic (i.e., >25% decrease in hematocrit compared to controls; e.g., Goodwin et al. 1992). In studies with diphacinone, the LOAEL for anemia in owls was 0.36 mg diphacinone consumed/kg body weight-day for a week and toxicity reference value estimates for which 10% of exposed owls would exhibit anemia was 0.17 mg diphacinone consumed/kg body weight-day for a week (Rattner et al. 2012b). Although many disease states and other toxicants (most notably lead) may cause anemia, measurement of hematocrit should be more frequently incorporated into AR exposure studies in non-target wildlife.

6.3 Gross and Microscopic Evidence of Hemorrhage

Many of the aforementioned AR exposure studies with captive wildlife (see Sect. 4. Toxicity Studies Involving Secondary Exposure) describe bruising, frank bleeding from the oral cavity, nares, rectum, cloaca, and talons, and blood in droppings, scat and urine. Evidence of pallor of mucous membranes may be apparent. Even with sublethal AR exposure, euthanized animals may exhibit hemorrhage (e.g., skin, alimentary tract, peritoneal cavity, kidney, liver) and excessive bleeding during necropsy. The severity of these overt signs is generally dose-dependent, although their appearance may be delayed for days to even weeks following the onset of AR exposure (USEPA 2004). There is some concern that hemorrhage and excessive bleeding can be initiated by self-trauma (cage sustained injuries), and even capture and handling in more active captive species.

Surprisingly few controlled exposure studies in wildlife have undertaken histopathological evaluations to identify cellular lesions and hemorrhage associated with AR exposure. While microscopic evidence of hemorrhage in heart, lung, kidney, liver and skeletal muscle, and tissue necrosis have been reported, their prevalence in these tissues is not always dose-related (perhaps a function of examining too few sections per tissue and even artifacts related to tissue trimming and processing) (e.g., Rattner et al. 2011, 2012a, 2015). Histological observations alone are not considered diagnostic of AR intoxication (DuVall et al. 1989).

6.4 Other Sublethal Responses

Aside from signs of intoxication related to coagulopathy, there are limited data on other sublethal effects associated with AR exposure. Other effects noted in AR acute oral LD50 studies include lethargy, piloerection, diarrhea, and anorexia (USEPA 2011). Behavioral responses of AR-exposed target species have been described in both laboratory studies and field trials (e.g., ranging from no apparent response to lethargy, reduced grooming, escape response and thigmotactic behavior, and uncoordinated and staggering gate; Cox and Smith 1992, Hooker and Innes 1995, Littin et al. 2002, Brakes and Smith 2005). However, behavioral changes in AR-exposed captive wildlife are principally descriptive in nature (e.g., anorexia, lethargy, reduced agility, wing droop) and are commonly apparent with many other types of contaminant intoxication (i.e., not AR-specific). Studies have yet to examine changes in complex behavioral processes (e.g., prey capture efficiency) that could affect survival of free-ranging wildlife.

Laboratory studies provide little information on how sublethal effects of ARs might alter fitness of free-ranging wildlife. There is some evidence of impaired growth in layer and broiler chickens chronically ingesting feed containing warfarin at concentrations at and below those found in some rodent bait products (Veltmann et al. 1981). Indices of body condition have been reported to be negatively related to

AR residues in free-ranging stoats (Elmeros et al. 2011), although no such relation has been reported in AR exposure studies with captive wildlife. Nonetheless, in a study of captive red-tailed hawks fed chlorophacinone-poisoned prairie dogs during winter, ptiloerection (fluffing of feathers) was observed in several hawks upon release (notably birds with prolonged clotting time), suggestive of compromised physiological condition (Vyas et al. 2014).

7 Differences in AR Sensitivity Among Taxa

Based upon the toxicity data generated from controlled exposure studies in birds and mammals, it is not possible to make a general statement of the relative sensitivity of these two vertebrate classes to ARs. Using mortality as an endpoint, examination of acute, subacute dietary and secondary exposure data from the present summary and others (Joermann 1998; USEPA 2004, 2011) suggests that mammals are more sensitive to ARs than birds, particularly for first-generation hydroxycoumarins and intermediate-generation indandiones. Notably, Watanabe and coworkers (2010) demonstrated that while hepatic VKOR activity of laboratory rats is about seven times greater than chickens (i.e., V_{max} of 514.5 pmol/min/mg protein for rat vs. 71.7 pmol/min/mg protein for chicken), the inhibitory constant for rats is 40 times lower than for chickens (i.e., K_i of 0.28 μM for rats vs. 11.3 μM for chickens), and the ability to hydroxylate warfarin is about eight times lower in rats than in chickens (i.e., 196 pmol/min/nmol P450 for rat vs. 1660 pmol/min/nmol P450 for chicken). In a recent pharmacokinetic analysis, Watanabe et al. (2015) suggested that while warfarin is more readily metabolized by chickens than rats, its half-life in chickens is relatively long compared to mammals, and thus factors other than metabolism may be critical determinants of differences in sensitivity among vertebrate classes. It was postulated that blood albumin in chickens may have greater warfarin binding capacity (i.e., limiting opportunity for free warfarin to interact and bind to VKOR), resulting in longer half-life and less toxicity (Watanabe et al. 2015). There is also the possibility that differences in FGAR sensitivity between vertebrate classes could be due to differences in blood clotting mechanism (see Sect. 6). The limited data for reptiles (Brooks et al. 1998; Weir et al. 2015, 2016) does not permit a generalized sensitivity comparison to higher vertebrates.

As noted by others (e.g., Joermann 1998), the potential hazard of individual substances seems to differ markedly among avian species, but less so for mammals. Remarkable differences in AR sensitivity have been reported among some omnivorous and predatory birds compared to commonly tested avian granivores (e.g., LD50 for diphacinone in American kestrels is 20–30 times less than for granivores; see Eason et al. 2002; Rattner et al. 2011, 2012a, b). Studies with warfarin in chicken, ostrich (*Stuthio camelus*), mallard, crow (*Corvus macrorhynchos*) and snowy owl (*Bubo scandiacus*) describe inter-specific differences in warfarin metabolism that could account for inter-specific differences in sensitivity (Watanabe et al. 2010, 2015). As previously suggested (Rattner et al. 2014a), it might be possible that AR

tolerance in some non-target species may be related to differences in the primary structure of VKOR that has been reported in genetically resistant rodents (Pelz et al. 2005). Preliminary investigation of the primary structure of VKORC1L1 in 14 species of birds using GenBank suggests that the primary structure of VKORC1L1 is highly conserved in the region of the active site (N. Karouna-Renier, personal communication).

While the data presented in this chapter describes the toxicity of ARs in controlled exposure trials, it does not address exposure pathways and risk to non-target birds and mammals. The USEPA has devoted considerable effort assessing the risk associated with direct bait ingestion (primary exposure) and consumption of prey containing residues (secondary exposure) of current use rodenticide formulations in the United States (USEPA 2011). Based on toxicity, toxicokinetics and exposure modelling, under some scenarios warfarin, chlorophacinone, brodifacoum and difethialone exceeded levels of concern for non-target birds and mammals. Consumption of SGAR-exposed prey, and to lesser degree FGAR-exposed prey, also exceeded levels of concern for predatory birds and mammals. These and other findings have resulted in a range of risk mitigation measures (e.g., changes in product labeling, permitted uses and points of sale; required use of bait stations to minimize non-target exposure) in the United States, with some harmonization in Canada (Health Canada 2012). The European Community Biocidal Products Directive (98/8/EC) also highlights significant or unacceptable risk of some SGARs to non-target wildlife, with SGAR use and mitigation measures at the discretion of EU member states to be re-evaluated by the end of 2017 (European Union 2012).

8 Critical Information Gaps and Research Needs

Although there are extensive AR toxicity data on acute, subacute and secondary AR exposure scenarios in non-target wildlife, there are some critical information needs that might ideally be derived under controlled exposure conditions in non-target wildlife that would ultimately enhance ecological risk assessments. These include (i) comparative toxicity data, particularly for understudied taxa, (ii) sublethal effects seemingly unrelated to coagulopathy, (iii) response to AR mixtures and sequential exposure, and (iv) vitamin K status and provitamin diet supplementation in toxicity studies.

8.1 Comparative Toxicity and Understudied Taxa

From a phylogenetic perspective, our knowledge of lethality (LD50, LC50) for a dozen ARs is relatively complete for commonly used test species. However, interspecific differences within the vertebrate class Aves (and perhaps Mammalia) may be substantial, and deserve further attention so that species sensitivity distributions

might be constructed and used in risk assessments. Perhaps the most critical question is the relatively sensitivity of predatory and scavenging birds compared to commonly tested mallard and bobwhite, as limited comparative data indicates that the former may be significantly more sensitive than the latter (Eason et al. 2002; Rattner et al. 2011; 2012a, b). Clearly, further genetic, metabolic and pharmacokinetic studies in non-target wildlife might reveal the causes of inter-specific differences in sensitivity. The paucity of toxicity data for reptiles, and its absence altogether for amphibians, warrant generation of such data for these vertebrate classes, particularly in view of potential AR hazards associated with eradication of rodents on remote islands in temperate zones. Such data for reptiles and amphibians should employ contemporary oral toxicity testing schemes (and possibly dermal toxicity for amphibians) that minimize numbers of test subjects (e.g., OECD 2008), and for FGARs, a multi-day rather than single day acute oral dosing scheme (see Sect. 2. Single-day versus multi-day exposures on AR toxicity). It is noteworthy that there is considerable interest in potential exposure and toxicity to fish and other aquatic species at sites of AR use (e.g., Primus et al. 2005; Pitt et al. 2015; Riegerix and Tillitt 2015) and also their use in AR mechanistic studies (Weigt et al. 2012). While substantial data are available on some aquatic species (e.g., IPCS 1995; USEPA 2015), their review and discussion are outside of the scope (i.e., "wildlife") of this chapter.

8.2 Sublethal Effects of ARs Unrelated to Coagulopathy

With the exception of coagulopathy, limited data are available on other responses that could be associated with AR exposure in non-target wildlife. Vitamin K and its antagonists (including ARs) are now known to affect many biochemical and cellular processes beyond coagulation (Benzakour 2008). Vitamin K hydroxyquinone serves as a cofactor for γ-glutamyl carboxylation of specific glutamic acid residues (Fig. 3.3) of certain proteins (Gla-proteins), and this along with other non-cofactor functions appear to affect cellular metabolism and signaling, inflammation, oxidative damage and sphingolipid synthesis (van den Berg and Nauta 1975; Kater et al. 2002; Shearer and Newman 2008).

In women, the hazards of oral AR use during pregnancy to the developing fetus are well-documented (e.g., impaired fetal development, mental retardation, life-threatening hemorrhage, fetal warfarin syndrome; Hall et al. 1980; Stevenson et al. 1980), and teratogenic responses have been studied using rodent and zebra fish *(Danio rerio)* as animal models (Howe and Webster 1992; Weigt et al. 2012). Somewhat related are findings in barn owls that suggest potential adverse effects on egg hatching and fledging rates (Naim et al. 2011; Salim et al. 2013), although the toxicity pathway is not clear (i.e., coagulopathy or other effects of vitamin K antagonism), and it is certainly possible that these responses could be indirect nutritional effects. Clinical observations in children receiving long-term warfarin therapy have documented reduced bone density related to vitamin K-antagonism (Barnes et al. 2005), although changes in bone density and breaking strength

were not apparent in a study of raptors from the United Kingdom (Knopper et al. 2007) and have yet to be investigated in studies with captive wildlife. An association between notoedric mange and AR exposure has been described in bobcats (*Lynx rufus*) residing in urban areas in southern California (Riley et al. 2007). While this relationship may be correlative rather than causal, incidence of mange could be related to impaired immune function, potentially caused by AR exposure (Eichbaum et al. 1979; Kater et al. 2002; Popov et al. 2011). These findings suggest that chronic AR exposure might evoke some sublethal effects in non-target birds and mammals (e.g., impaired reproduction, teratogenesis including skeletal defects, altered immune function) which would be of concern to risk assessors and natural resource managers. These hypotheses have yet to be tested, and might be best resolved by studies with captive wildlife. Considering the prevalence of accumulated AR residues in a large proportion of many populations of predatory and scavenging wildlife, the significance of sublethal exposure and its long-term consequences at the level of the individual or population remain an important unanswered question.

8.3 Responses to AR Mixtures and Sequential Exposures

Studies documenting exposure in free-ranging wildlife frequently report the presence of multiple SGARs, and occasionally a combination of FGARs and SGARs in a single individual (reviewed in Rattner et al. 2014a). Given the similar mode of action of all FGARs and SGARs, the toxicity of multiple ARs are expected to be more or less additive. In one highly cited report (Thomas et al. 2011), the summed hepatic concentrations of various SGARs and incidence of signs of intoxication were examined using logistic regression to predict the likelihood of death of a bird of prey with a given residue of SGARs. However, the relative potency of various ARs for inhibiting VKOR is highly variable, and the validity of their use in additive toxicity models (e.g., toxic units or equivalents) most certainly deserves further examination, potentially by *in vivo* or *in vitro* testing.

One of the key challenges in AR toxicity data in risk assessments is that test protocols routinely utilize no-choice continuous exposure conditions. While these test conditions may be somewhat more realistic for non-target wildlife during remote island eradication projects that yield an abundance of AR-exposed rodent prey, they are likely unrealistic of exposure patterns routinely encountered in rural, suburban and urban settings. Notably, combined SGAR-FGAR exposures and their timing have marked effects on AR toxicity in laboratory rats (e.g., Mosterd and Thijssen 1991), and effects of sequential AR exposure are currently being investigated in American kestrels (Rattner, unpublished data). Testing or modelling effects of various combinations of ARs, and intermittent and sequential AR exposure seems warranted.

The combined effect of simultaneous exposure in wildlife to ARs and other common bioaccumulating environmental contaminants, such as lead, deserves further investigation. For non-target scavenging birds, ingestion of spent lead ammunition is a well-known hazard in the United States and elsewhere (Golden et al. 2016). While the mechanisms of toxicity of ARs and lead differ vastly, both of these toxicants can cause anemia. It is certainly possible that sequential exposure (AR-lead, lead-AR) could occur and exacerbate overall toxicity.

8.4 Vitamin K Status and Provitamin Diet Supplementation

Research is needed to better understand the interaction of dietary vitamin K intake in wildlife and AR toxicity. Impaired blood coagulation was in fact the basis of the discovery of vitamin K. A hemorrhagic syndrome in chickens dating back to the 1930's led to the discovery of an antihemorrhagic lipid soluble component found in vegetable and animal sources (Vitamin K, Dam 1935). Subsequently, it was demonstrated that prothrombin activity (Factor II) decreased in vitamin K deficient chicks (Dam et al. 1936), and by the 1950's it was recognized that the synthesis of other clotting factors (VII, IX, X) were also depressed by diets deficient in vitamin K (reviewed in McDowell 2000). In mammals, it is known that a combination of sources, namely diet [vitamin K_1 (phylloquinone) and perhaps other forms of vitamin K] and gut flora [vitamin K_2 (various menaquinones)], are needed to meet the vitamin K requirement to prevent deficiency (reviewed in McDowell 2000; Shearer and Newman 2008). Despite intestinal gut flora sources of vitamin K, animals may become deficient if placed on a vitamin K-free diet (McDowell 2000). In domestic poultry, and presumably companion and wild birds, digestive tract synthesis of vitamin K is less than half of that found in domestic mammals and man (McDowell 2000). Because of the relatively shorter digestive tract of bird, synthesis of vitamin K occurs near the distal end of the tract which limits absorption, and transit time of food passage is rapid (McDowell 2000). Thus, poultry (and presumably other avian species) are seemingly more dependent on dietary sources of vitamin K than mammals. Furthermore, hepatic VKOR activity in 4–5 week old poultry chicks is about 10% of that found in laboratory rats, which minimizes recycling of vitamin K and likely accounts for their greater dietary requirement than rats and other mammals (Will et al. 1992; McDowell 2000).

Vitamin K dietary requirements have been established for man, and domestic and companion animals (e.g., McDowell 2000). However, vitamin K status in man is not assessed by simple plasma measurements of phylloquinone alone, but rather as a combination of measures of clotting time, proteins induced by vitamin K absence (PIVKA-II), undercarboxylated serum osteocalcin, and urinary γ-carboxyglutamic acid excretion (Bach et al. 1996; Sokoll and Sadowski 1996; Shearer and Newman 2008; Shearer et al. 2012). To the best of our knowledge, there have been no detailed investigations measuring vitamin K_1 or K_2 in combination with clotting time,

PIVKA-II, osteocalcin or other factors to systematically assess vitamin K status of captive or free-ranging wildlife. Such data might in fact help address the differential sensitivity of some non-target species (e.g., predatory birds) to ARs.

Dietary vitamin K is a significant factor in the management of oral anticoagulant therapy in man (Holmes et al. 2012). However, from a pest management perspective, providing a diet of forage rich in vitamin K_1 to various target rodents had little effect on the toxicity of various AR baits (Witmer and Burke 2009; Witmer et al. 2013). Based on the present review of AR toxicity studies in non-target wildlife, vitamin K content of maintenance and/or exposure diets has been inconsistent, and often unspecified. Some studies report that diet rations are supplemented with vitamin K in the form of menadione (i.e., vitamin K_3, an inactive provitamin; Buitenhuis et al. 1990) (e.g., Rattner et al. 2014b, 2015), and others purposefully used vitamin K deficient diets (e.g., Greaves and Ayres 1973; Veltman et al. 1981; important note: vitamin K deficient diets are not necessarily devoid of vitamin K). While it might seem that inclusion of dietary menadione in AR studies is akin to administering antidote with the test compound, this may, or may not, be the case. Specifically, menadione is much less potent than vitamin K_1 in restoring prothrombin time following therapeutic anticoagulant administration in man (Hanson et al. 1951) and in treating AR intoxication in dogs (Mount et al. 1986), and it does not reverse prolonged clotting time in chickens receiving coumarin-type ARs (Griminger 1965). The provitamin menadione must undergo enzymatic prenylation (rate limited) to form menaquinone-4 (MK-4, member of the K_2 family) prior to VKOR metabolism to the biologically active form (i.e., vitamin K hydroquinone) required for γ-glutamyl vitamin K-dependent carboxylation (Hirota et al. 2013). Even if there are more than adequate quantities of menaquinone-4 and some other forms of vitamin K, they will not be optimally recycled if VKOR is inhibited by an AR. Furthermore, as described above, vitamin K deficient diets can result in prolonged blood clotting, and might actually exacerbate AR toxicity. In secondary exposure studies, the vitamin K status of AR-exposed prey fed to predators would seemingly be low. Clearly, vitamin K content of test diets should be carefully considered when designing AR toxicity studies, particularly those that examine toxicokinetics of recovery following termination of AR exposure (e.g., Rattner et al. 2014b).

9 Conclusion

Anticoagulant rodenticides continue to be one of the principal vertebrate pesticides used for the control of commensal rodents that damage crops and food stores, and cause health issues, and for the eradication of invasive species to restore biodiversity to oceanic islands. Their use will likely continue into the foreseeable future. There remain some significant knowledge gaps and uncertainties that should be addressed to more completely assess AR risk to non-target wildlife. Despite the availability of extensive acute toxicity data to support ecological risk assessments, the breadth of species (laboratory rats, mice and rabbits, quail, mallards,

passerines) is limited, with data generally lacking for predatory and scavenging wildlife which have the greatest likelihood of exposure. Additional data on the risks associated with repeated exposure, exposure to multiple compounds, and sublethal effects would be beneficial. Furthermore, it remains difficult to link body burdens with coagulopathy and other adverse effects. The effect of vitamin K status on sensitivity to ARs deserves further attention, particularly in predatory and scavenging birds. While some of these critical data gaps would be best derived through whole animal research studies, others might be addressed by use of scaling and extrapolation factors (e.g., Mineau et al. 2001; Awkerman et al. 2008), alternative toxicology testing methods and *in silico* modeling methods (Committee on Toxicity Testing and Assessment of Environmental Agents 2007; Allen and Water 2013). Obtaining more complete knowledge on the toxicity of ARs to wildlife would enable pesticide regulators and natural resource managers to better predict and even mitigate risk, particularly in forecasting effects in wildlife from the individual though population levels.

Disclaimer This manuscript was subjected to review by USEPA's Office of Pesticide Programs and was approved for submission. Approval does not signify that the contents reflect the views of USEPA. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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