Environmental Toxicology and Chemistry—Volume 39, Number 2—pp. 468–481, 2020
Received: 5 August 2019 | Revised: 24 September 2019 | Accepted: 28 October 2019

Hazard/Risk Assessment

Brodifacoum Toxicity in American Kestrels (*Falco sparverius*) with Evidence of Increased Hazard on Subsequent Anticoagulant Rodenticide Exposure

Barnett A. Rattner,[a,*] Steven F. Volker,[b] Julia S. Lankton,[c] Thomas G. Bean,[d] Rebecca S. Lazarus,[a] and Katherine E. Horak[b]

[a]Patuxent Wildlife Research Center, US Geological Survey, Beltsville, Maryland, USA
[b]National Wildlife Research Center, Animal and Plant Health Inspection Service, US Department of Agriculture, Fort Collins, Colorado, USA
[c]National Wildlife Health Center, US Geological Survey, Madison, Wisconsin, USA
[d]Department of Environmental Science and Technology, University of Maryland, College Park, Maryland, USA

Abstract: A seminal question in ecotoxicology is the extent to which contaminant exposure evokes prolonged effects on physiological function and fitness. A series of studies were undertaken with American kestrels ingesting environmentally realistic concentrations of the second-generation anticoagulant rodenticide (SGAR) brodifacoum. Kestrels fed brodifacoum at 0.3, 1.0, or 3.0 μg/g diet wet weight for 7 d exhibited dose-dependent hemorrhage, histopathological lesions, and coagulopathy (prolonged prothrombin and Russell's viper venom times). Following termination of a 7-d exposure to 0.5 μg brodifacoum/g diet, prolonged blood clotting time returned to baseline values within 1 wk, but brodifacoum residues in liver and kidney persisted during the 28-d recovery period (terminal half-life estimates >50 d). To examine the hazard of sequential anticoagulant rodenticide (AR) exposure, kestrels were exposed to either the first-generation AR chlorophacinone (1.5 μg/g diet) or the SGAR brodifacoum (0.5 μg/g diet) for 7 d and, following a recovery period, challenged with a low dose of chlorophacinone (0.75 μg/g diet) for 7 d. In brodifacoum-exposed kestrels, the challenge exposure clearly prolonged prothrombin time compared to naive controls and kestrels previously exposed to chlorophacinone. These data provide evidence that the SGAR brodifacoum may have prolonged effects that increase the toxicity of subsequent AR exposure. Because free-ranging predatory and scavenging wildlife are often repeatedly exposed to ARs, such protracted toxicological effects need to be considered in hazard and risk assessments. *Environ Toxicol Chem* 2020;39:468–481. © 2019 SETAC

Keywords: Wildlife toxicology; Toxicokinetics; Rodenticide; Biomarkers; Species extrapolation

INTRODUCTION

Anticoagulant rodenticides (ARs) have a long history of successful use for the control of vertebrate pests and invasive species (Hadler and Buckle 1992; Jacob and Buckle 2018), and they remain one of the principal chemical groups in the near billion-dollar global rodenticide industry (Zion Market Research 2018). Although their risks to children, companion animals, and nontarget wildlife have resulted in some restrictions on the use of the more hazardous second-generation ARs (SGARs), application is permitted in many circumstances in the United States and Canada and is extensive in other parts of the world (Elliott et al. 2016; Buckle and Prescott 2018).

In the past 2 decades, over 40 reports have documented the presence of AR residues in tissues of nontarget predatory or scavenging birds and mammals. A summary of exposure data from North America and Europe indicates that >58% of the nearly 4200 specimens tested contain residues of one AR or more (López-Perea and Mateo 2018). Predators are often repeatedly exposed, as evidenced by detection of multiple ARs in individual liver samples. The exposure record of these samples is often predominated by brodifacoum and other SGARs because of the long tissue half-lives of these compounds. However, some specimens contain residues of both the shorter half-life first-generation ARs (FGARs) as well as SGARs (e.g., Lima and Salmon 2010; Murray 2011, 2017; Christensen et al. 2012; Serieys et al. 2015). Although the incidence of AR exposure of predators in some locations may be substantial, many individuals with AR residues are asymptomatic (e.g., Murray 2017).

A long-standing question in the fields of wildlife toxicology and forensics is the extent to which contaminant exposure and

This article includes online-only Supplemental Data.
* Address correspondence to brattner@usgs.gov
Published online 9 November 2019 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.4629

residue burdens evoke subtle prolonged effects on physiological function and overall fitness of the individual (Rattner et al. 2014). An often-cited study by Mosterd and Thijssen (1991) demonstrated such lasting effects of the SGAR brodifacoum in outbred Wistar laboratory rats (*Rattus norvegicus*). In their study, rats were given brodifacoum as a single oral dose (0.2 mg/kg body wt). Prothrombin complex activity was depressed (i.e., clotting time prolonged) by 65% within 1 d but returned to a normal level sometime between 2 and 7 d postexposure. However, activity of hepatic microsomal vitamin K epoxide reductase (VKOR; key enzyme in vitamin K recycling antagonized by ARs; Rattner et al. 2014) remained inhibited relative to controls for at least 30 d postexposure. On day 8 post–brodifacoum exposure, when prothrombin complex activity had returned to normal, rats were challenged with a single subcutaneous dose of the FGAR warfarin (0.1 mg/kg body wt). Although this warfarin dose caused only modest anticoagulant effects in naive (i.e., unexposed) control rats, pronounced coagulopathy developed within 24 h in rats previously exposed to brodifacoum. Another group of brodifacoum-exposed rats received the warfarin challenge dose some 25 d postexposure and still exhibited coagulopathy, albeit less than that observed in rats challenged with warfarin 8 d post–brodifacoum exposure. Overall findings from that pharmacologic study suggest that SGAR-exposed rats may more readily exhibit coagulopathy with subsequent AR exposure events compared to naive rats. Somewhat related studies in Japanese quail (*Coturnix japonica*) examined brodifacoum (0.2 and 0.4 mg/kg body wt) and difenacoum (0.2 and 1.0 mg/kg body wt) gavage exposure, with various SGAR dose combinations repeated after 26 d (Butler 2010). For quail initially exposed to brodifacoum, the magnitude and duration of coagulopathy were greater on repeat SGAR exposure compared to birds receiving a single SGAR dose. For difenacoum, the magnitude of anticoagulant effect increased on repeated SGAR exposure compared to single-dosed birds, but the response was short-lived. These studies provide evidence of lingering effects of SGAR exposure on blood clotting function, translating to effects on fitness and possibly even survival.

We report findings of a series of studies examining toxicity, liver residues, and recovery following dietary exposure to environmentally realistic concentrations of the SGAR brodifacoum and the response to sequential FGAR and SGAR exposure. These studies used the American kestrel (*Falco sparverius*) as an avian model species for hawks and some other predatory birds (Bardo and Bird 2009), which seem to be considerably more sensitive to some ARs than common avian test species (e.g., northern bobwhite, *Colinus virginianus*; mallard, *Anas platyrhynchos*; Rattner et al. 2010, 2011, 2012, 2015). These data have application for hazard and risk assessments of predatory birds that are exposed to ARs while foraging in urban, suburban, and agricultural settings.

MATERIALS AND METHODS

Animals

Adult American kestrels, propagated from the captive colony at the Patuxent Wildlife Research Center, were moved from flight pens to small outdoor cages (1.2 m long × 0.8 m wide × 0.6 m high) with a shade roof, perches, food tray, and water bowl (Porter and Wiemeyer 1970). Individually housed kestrels were acclimated for at least 2 wk, during which time they were fed dead laboratory mice (*Mus musculus*) or dead day old chicks (*Gallus gallus*). Kestrels were then shifted to a diet of Nebraska Classic Bird of Prey (Nebraska Brand; hereafter NBP) meatballs containing the vitamin supplement Vionate® (Gimborn) prior to exposure trials. All procedures were approved by the Institutional Animal Care and Use Committee of the US Geological Survey's Patuxent Wildlife Research Center and the US Department of Agriculture's National Wildlife Research Center.

Diets used in exposure trials

Several NBP diets were used in the exposure trials: 1) control diet containing 1% vehicle (1 part acetone, 9 parts corn oil); 2) various brodifacoum diets, nominally targeted to contain between 0.3 and 3.0 µg brodifacoum/g NBP wet weight, and 3) chlorophacinone diets, nominally targeted to contain either 0.75 or 1.5 µg chlorophacinone/g NBP wet weight. Stock solutions were prepared by dissolving mg quantities of brodifacoum (Chemical Abstracts Service [CAS] no. 56073-10-0; Sigma-Aldrich) or chlorophacinone (CAS no. 3691-35-8; Santa Cruz Biotechnology) in acetone. Fractions of these solutions were pipetted into volumetric flasks and brought to the mark by addition of acetone and corn oil to a final ratio of 1:9. Either the vehicle or AR stock solution was added to several kilograms of NBP (10 mL/kg), mixed by hand for 10 min, and then placed in a fume hood for 40 min to evaporate some acetone solvent. Vionate supplement was then added (3.4 g/kg NBP), and diet was remixed by hand for 5 min and then placed again in a fume hood for another 40 min. Diets were formed into 25 ± 0.1–g meatballs. Batches of meatballs for each daily feeding were stored in sealed plastic bags at –10 °C. Samples of vehicle, brodifacoum, and chlorophacinone meatballs were collected for chemical analysis and moisture determination. Nominal dietary concentrations of brodifacoum and chlorophacinone used in various trials were analytically verified to be close to target concentrations (75.5–101.0% of target).

Concentrations of brodifacoum and chlorophacinone in test diets are believed to be environmentally realistic for rodent prey foraging in proximity to AR control and eradication activities. For example, nominal concentrations of brodifacoum in test diets ranged from 0.3 to 3.0 µg/g wet weight; average carcass concentrations in target meadow voles (*Microtus pennsylvanicus*) and black rats (*Rattus rattus*) following field control and eradication activities have been reported to be 0.35 and 3.75 µg/g wet weight, respectively (Merson et al. 1984; Pitt et al. 2015), and estimated whole-body concentrations in several species of nontarget small mammals range from 0.3 to 3.6 µg/g wet weight (Shore and Coeurdassier 2018). Similarly, nominal concentrations of chlorophacinone in test diets were 0.75 and 1.5 µg/g wet weight, which fall within the range (0.131–1.59 µg/g wet wt) detected in carcasses of Belding's ground squirrels (*Spermophilus beldingi*), pocket gophers (*Thomomys bottae*), and voles (*Microtus* spp.) following field

baiting trials (Primus et al. 2001; Ramey et al 2007). A complicating factor is that some of the reported AR residue values (Primus et al. 2001; Ramey et al. 2007; Pitt et al. 2015) were derived from carcass samples from which liver had been removed. This may underestimate exposure because brodifacoum and chlorophacinone concentrations in liver of small mammal prey can be much greater than in the rest of the body (e.g., Vyas et al. 2012; Shore and Coeurdassier 2018). Moreover, hawk species for which the kestrel may be the most appropriate model do not ingest entire mammalian prey but feed selectively on liver, brain, muscle, and some organs. Thus, there is uncertainty in AR exposure based on the prey tissue preferentially consumed, and both the frequency and duration that AR-exposed prey would be consumed by nontarget raptors following a control or eradication activity.

Exposure trials

In a range-finding trial (September–October 2014, ambient temperature 7.22–23.9 °C), kestrels (8 females, 12 males; 2 yr old) were housed individually in kraft paper-lined pens and fed NBP meatballs for at least 2 wk. Birds (weighing 94.0–129.8 g) were randomly assigned to groups ($n = 5$/treatment) and provided two 25 ± 0.1–g meatballs containing vehicle or 0.3, 1.0, or 3.0 µg brodifacoum/g wet weight daily for 7 d. Each day, birds were fed between 1100 and 1200 h, and the following morning uneaten food scraps were collected and removed between 0900 and 1100 h before replenishment with 2 freshly thawed meatballs. Birds were observed twice daily and weighed and physically examined on days 0, 3, 5, and 7 of treatment. An estimate of average daily food intake and brodifacoum consumption was obtained for each bird by collecting all of its uneaten food scraps (determining dry wt mass and converting it to wet wt, dividing by 7 d, and multiplying by the analytically verified concentration of brodifacoum in feed). Following the exposure period, a 0.9-mL jugular venipuncture sample was collected into a syringe containing 0.1 mL 3.2% sodium citrate to prevent clotting. Hematocrit was immediately determined, citrated blood was centrifuged at 2000 *g* for 5 min, and the resultant plasma was divided and stored in separate vials for clotting time assays. Kestrels were humanely euthanized using carbon dioxide and necropsied, and portions of pectoral muscle, heart, intestine, kidney, and liver were fixed in 10% phosphate-buffered formalin for histopathological evaluation. A portion of the liver was stored in a cryovial at –80 °C for residue analysis.

A second trial was conducted to examine the time course of recovery of clotting function following brodifacoum exposure (April–May 2016, ambient temperature 3.3–31.7 °C). At least 2 wk prior to the study, a baseline 0.9-mL jugular venipuncture sample was collected for hematocrit and measures of clotting time from individually housed adult male kestrels (2–7 yr old) that had been fed dead chicks. Five to 10 d after venipuncture, birds were shifted to a diet of control NBP meatballs for 9 d. Kestrels (weighing 91.0–117.1 g) then received either two 25 ± 0.1–g meatballs daily containing vehicle ($n = 6$) or 0.5 µg brodifacoum/g wet weight diet for a 7-d exposure period ($n = 30$). During this exposure phase, food consumption was monitored ($n = 6$ vehicle controls and 12 brodifacoum-exposed kestrels), and birds were observed twice daily. At the end of the 7-d exposure phase, a single vehicle-treated kestrel (control) and 5 brodifacoum-treated kestrels (day 0 postexposure) were weighed, physically examined, blood-sampled, sacrificed, and then necropsied. All remaining birds were then shifted to a diet of NBP. On days 2, 4, 7, 14, and 28 postexposure (recovery period), a single vehicle-treated control kestrel and groups of 5 brodifacoum-treated kestrels were likewise weighed, examined, bled, euthanized, and then necropsied, with portions of liver and kidney stored at –80 °C for brodifacoum residue analysis.

The third trial examined clotting time and other measures of toxicity following sequential exposure to an FGAR or an SGAR followed by an FGAR (April–May 2018, ambient temperature –1.1 to 31.7 °C). As in trial 2, a baseline jugular venipuncture sample was collected for hematocrit and clotting time measurements from adult male kestrels (1–4 yr old) that had been fed mice. At 5 to 6 d after venipuncture, kestrels were shifted to a diet of NBP meatballs for 9 to 10 d. Kestrels (weighing 93.8–117.5 g) received two 25 ± 0.1–g NBP meatballs daily for a 7-d period containing either vehicle ($n = 6$ birds), 1.5 µg chlorophacinone/g wet weight diet ($n = 6$), or 0.5 µg brodifacoum/g wet weight ($n = 8$) during an initial exposure phase. Consumption of chlorophacinone or brodifacoum at these dietary concentrations for 7 d results in coagulopathy (Rattner et al. 2015, and trial 1 of the present study). These 20 kestrels were then shifted to the NBP vehicle control diet for a 7-d recovery phase, during which clotting time likely returned to baseline values. These kestrels were then fed 0.75 µg chlorophacinone/g wet weight diet for a 7-d challenge exposure phase, an exposure regimen that prolongs clotting time (Rattner et al. 2015). Hereafter, these groups are designated control-chlorophacinone challenge, chlorophacinone-chlorophacinone challenge, and brodifacoum-chlorophacinone challenge. In addition, a group of untreated kestrels ($n = 6$, untreated controls) were fed dead mice and monitored during this trial. All kestrels were observed daily and weighed and examined at the beginning and end of the initial exposure and chlorophacinone-challenge phases. Food consumption was monitored during the initial exposure and chlorophacinone-challenge exposure phases, after which a jugular venipuncture sample was drawn for hematocrit and clotting time assays. All AR-exposed kestrels and 4 of the 6 untreated controls were then euthanized and necropsied, and portions of pectoral muscle, heart, intestine, kidney, and liver were formalin-fixed, with a portion of the liver placed in a cryovial stored at –80 °C for residue analysis.

Determination of brodifacoum and chlorophacinone in diet and tissues

Chemical analysis of diets and kestrel tissues for brodifacoum and chlorophacinone were performed by several methods. The performance of each method, including accuracy, precision, and detection limits, is detailed in the Supplemental Data.

The concentration of brodifacoum and chlorophacinone mixed into NBP fed to kestrels was quantified in subsets of meatballs. In trial 1 (range-finding study), brodifacoum concentration (mean ± standard deviation; $n = 5$ meatballs/level; nominally 0.3, 1.0, and 3.0 μg/g NBP wet wt) averaged 90.3 ± 5.35, 99.4 ± 2.63, and 100.9 ± 4.23% of target concentration, respectively. In trial 2 (time course of recovery of clotting function), brodifacoum concentration ($n = 6$; nominally 0.5 μg/g NBP wet wt) averaged 93.9 ± 4.74% of target concentration. In trial 3 (sequential exposure study), brodifacoum concentration ($n = 4$; nominally 0.5 μg/g NBP wet wt) averaged 75.3 ± 4.28% of target concentration, and chlorophacinone concentration ($n = 5$; nominally 0.75 and 1.5 μg/g NBP wet wt) averaged 94.6 ± 4.41 and 91.1 ± 4.26% of target concentrations, respectively. Neither brodifacoum nor chlorophacinone were detected (less than the method limit of detection) in a subset of control NBP meatballs from trials 1, 2, and 3 ($n = 5$, 3, and 2, respectively). Concentrations of brodifacoum and chlorophacinone in liver and kidney are presented in *Results*.

Clotting time assays

Prothrombin time (PT) and Russell's viper venom time (RVVT) of citrated kestrel plasma samples were used to evaluate AR effects on posttranslational processing of clotting factors II, VII, IX, and X. Thrombin clotting time (TCT) was used as an indicator of fibrinogen concentration in plasma samples. Fibrinogen formation is insensitive to deficiency of vitamin K–dependent clotting factors, but its deficiency resulting from improper blood sample collection can prolong clotting time and confound AR toxicity studies. Thus, it is important to verify that fibrinogen concentration is adequate to promote clot formation. Reagents, conduct, and assay performance in kestrels have been previously described in detail (e.g., Rattner et al. 2011, 2015; Hindmarch et al. 2019). Additional background information and data on intra- and interassay precision of sample analyses in the present study are provided in the Supplemental Data.

Histopathology

Formalin-fixed tissues were processed and embedded in paraffin (American HistoLabs) using standard procedures (Luna 1968). Tissues were sectioned at approximately 4 μm, mounted on slides, and stained with Carazzi's hematoxylin and eosin. Histologic evaluation of samples collected in trials 1 and 3, including microscopic descriptions and morphologic diagnoses, was performed. Hemorrhage severity was scored on a semiquantitative 0–4 scale (none, minimal, mild, moderate, and severe, respectively).

Statistical analyses

Using SAS (Ver 9.3 and 9.4; SAS Institute) or R (Ver 3.5.1), body weight change relative to day 0, average daily food intake, estimated daily dose of brodifacoum and chlorophacinone consumption, tissue residues, hematocrit, PT, RVVT, and fibrinogen concentration were tested for homogeneity of variance (Levene's test) and normality (Shapiro-Wilk test, normal probability plot). Measurement endpoints were compared using analysis of variance (ANOVA) techniques and Tukey's honestly significant difference test, paired *t* test, or the Kruskal-Wallis nonparametric ANOVA with Dunn's multiple pairwise post hoc test. For trial 1, the quantity of ingested brodifacoum and hematocrit were used to estimate a diet-based toxicity reference value (Filipsson et al. 2003; US Environmental Protection Agency 2011a). Response parameters included brodifacoum consumption versus individuals classified as anemic (hematocrit <30%). Using the dose–response curve, benchmark doses at the 10, 20, 50, and 90% effect levels (BMD10, BMD50, and BMD90, respectively) and lower bound (BMDL) of the 95% confidence interval were calculated using 8 different model distributions that were compared with Akaike's information criterion (AIC) and AIC weights (Burnham and Anderson 2002; Supplemental Data). For trial 2, liver and kidney residue half-life estimates were conducted using brodifacoum concentration at the end of the dietary exposure phase (day 0 postexposure) through postexposure day 28 (overall half-life), and also between days 0 and 4 (initial phase half-life) and days 7 and 28 (terminal phase half-life; Bardal et al. 2011).

RESULTS

Trial 1: Range-finding trial

All kestrels survived the 7-d exposure trial. Inspection of daily food consumption data suggested a slight decrease at the intermediate and highest dietary brodifacoum concentrations (Table 1), although there were no statistical differences in food intake among groups (95% confidence intervals [CIs] overlapped, ANOVA $F_{3,16} = 2.69$, $p = 0.0810$). Relative to initial body weight (day 0), there were no differences in body weight change among treatments over the course of the trial (repeated measures ANOVA: treatment $F_{3,12} = 2.56$, $p = 0.1042$; day $F_{2,8} = 0.82$, $p = 0.4727$; interaction $F_{6,24} = 1.44$, $p = 0.2418$). Estimated daily dose of brodifacoum differed among groups (log-transformed values, ANOVA $F_{2,12} = 181.80$, $p < 0.0001$), with hepatic brodifacoum residue concentrations differing (ANOVA $F_{2,12} = 17.75$, $p = 0.0003$) and plateauing at dietary doses of 1.0 and 3.0 μg brodifacoum/g wet weight (Table 1).

All samples produced clots in the TCT assay indicative of reasonable quality of venipuncture samples, with fibrinogen concentration ranging from 40.1 to 230.2 mg/dL. Fibrinogen concentration differed among groups (ANOVA $F_{3,16} = 43.58$, $p < 0.0001$); at the intermediate and highest dietary brodifacoum levels, fibrinogen concentration was markedly greater (Tukey's honestly significant difference $p < 0.0001$) than the control and low-dose groups (Table 1), likely reflecting an up-regulation feedback mechanism (Kerins 1999; Rattner et al. 2015). The PT values of all brodifacoum-exposed kestrels exceeded those of controls (Figure 1). Notably, samples from 3 of 5 individuals in both the 1.0- and 3.0-μg brodifacoum/g wet weight diet groups failed to produce detectable clots after 200 s, and PT values for these groups were greater than those for controls (Kruskal-Wallis $\chi^2 = 16.5058$, $df = 3$, $p = 0.0009$ and

TABLE 1: Estimated daily exposure, sublethal responses, and liver residues following brodifacoum ingestion in American kestrels[a]

	Control	BROD (μg/g food wet wt)		
	0 <MLOD	0.3 0.271 ± 0.016	1.0 0.994 ± 0.026	3.0 3.03 ± 0.13
Food consumption (g wet wt/kg body wt/d)	254.2 ± 51.70	240.2 ± 42.89	208.9 ± 22.87	187.3 ± 41.68
Weight change days 0 to 7 (Δg/100 g body wt)	+1.88 ± 3.919	+4.64 ± 5.933	+0.34 ± 5.961	–3.40 ± 4.473
BROD consumed (μg/kg body wt/d)	—	65.1 ± 11.62C ~0.46	207.7 ± 22.73B ~1.45	567.6 ± 126.30A ~3.97
Cumulative 7-d BROD dose (mg/kg body wt)				
Liver BROD (μg/g wet wt)	<MLOD–0.015	1.39 ± 0.137B ~2.65	2.06 ± 0.268A ~3.92	2.10 ± 0.212A ~4.01
Liver BROD (nmol/g wet wt)				
Fibrinogen (mg/dL)	69.8 ± 17.93B	65.3 ± 12.57B	158.8 ± 26.37 A	195.8 ± 27.81 A
Overt signs of intoxication	0/5	0/5	1/5 Bruise on featherless tract on day 7	5/5 Blood in oral cavity on day 7 Subdued behavior on days 5 and 6 Tongue pale on day 5, subdued behavior on day 7 Blood on right foot on day 5 Tongue pale on day 5
Histological evidence of mild to severe hemorrhage	0/5	3/5	2/5	4/5
Gross abnormalities at necropsy	0/5	1/5 Bled profusely when dissected	1/5 Blood in oral cavity	4/5 Bruise on pectoral muscle, lungs pale Blood in oral cavity, pale tongue and viscera, bruise on pectoral muscle Blood in oral cavity, pale viscera Blood in oral cavity, pale viscera

[a]Values are mean ± standard deviation ($n = 5$ kestrels/group) or respondents/n. For continuously distributed endpoints, groups with different capital letters are different by Tukey's honestly significant difference method ($p < 0.05$). In column heads nominal concentrations are given first, followed by analytically verified concentrations. BROD = brodifacoum; MLOD = method limit of detection.

Dunn's tests with Bonferroni correction, both p values = 0.0043). Also, RVVT was prolonged in brodifacoum-exposed kestrels compared to controls (ANOVA $F_{3,16} = 22.14$, $p < 0.0001$ and Tukey's honestly significant difference $p \le 0.0045$; Figure 1). Hematocrit was reduced by ingestion of 1.0 and 3.0 μg brodifacoum/g wet weight (ANOVA $F_{3,16} = 24.15$, $p < 0.0001$ and Tukey's honestly significant difference $p \le 0.0069$; Figure 1), with some birds being classified as anemic (hematocrit <30%; intermediate brodifacoum dose, 1 of 5 anemic; highest brodifacoum dose, 4 of 5 anemic). Comparing daily brodifacoum consumption to the number of kestrels in a group with hematocrit <30% after the 7-d exposure (i.e., x respondents/5 individuals) yielded a BMD10 (BMDL) of 146.4 (39.2) μg brodifacoum consumed/kg body weight/day (Supplemental Data, Figure S1).

At the end of the trial (d 7) and prior to venipuncture, a bruise on the featherless tract over the jugular vein was observed on 1 kestrel ingesting 1.0 μg brodifacoum/g wet weight (Table 1). However, 4 of the 5 kestrels ingesting 3.0 μg brodifacoum/g wet weight exhibited overt signs of intoxication (e.g., subdued behavior, oral cavity pallor, blood on foot) by day 5 of the trial; at necropsy anticoagulant effects were most pronounced in this group (Table 1).

Histopathological evaluation of tissues revealed mild, moderate, or severe hemorrhage in all dose groups except controls (Table 1). Hemorrhage of mild or greater severity was most often found in the heart and kidney, with one area of severe hemorrhage in the pectoral muscle of a bird in the 3.0 μg brodifacoum/g wet weight group (Figure 2). No hemorrhage was noted in the liver or intestine in any bird. Minimal hemorrhage was found in kestrels in all groups, including controls, in the heart, kidney, and skeletal muscle; minimal erythrocyte extravasation can occur artifactually and may not represent true antemortem tissue hemorrhage. Other microscopic findings, considered incidental, included aortic and coronary arterial atherosclerosis, intramyocytic protozoal cysts, extramedullary hematopoiesis, mild pericarditis, mild lymphoplasmacytic interstitial nephritis, and mild renal tubular degeneration and regeneration.

Trial 2: Time course of recovery of clotting function

No overt signs of intoxication were observed in kestrels during the trial. Food consumption during the 7-d exposure phase of kestrels fed vehicle control and brodifacoum-treated diets did not differ (265.8 ± 52.85 vs 231.3 ± 30.1 g wet wt/kg body wt/d; ANOVA $F_{1,16} = 3.18$, $p = 0.0935$). Body weight change (relative to weight at the start of the trial) did not differ at the end of the exposure phase (day 0 postexposure) and during the remainder of the recovery period (days 2–28 postexposure) when compared to vehicle controls (ANOVA $F_{6,29} = 1.88$, $p = 0.1179$; Table 1).

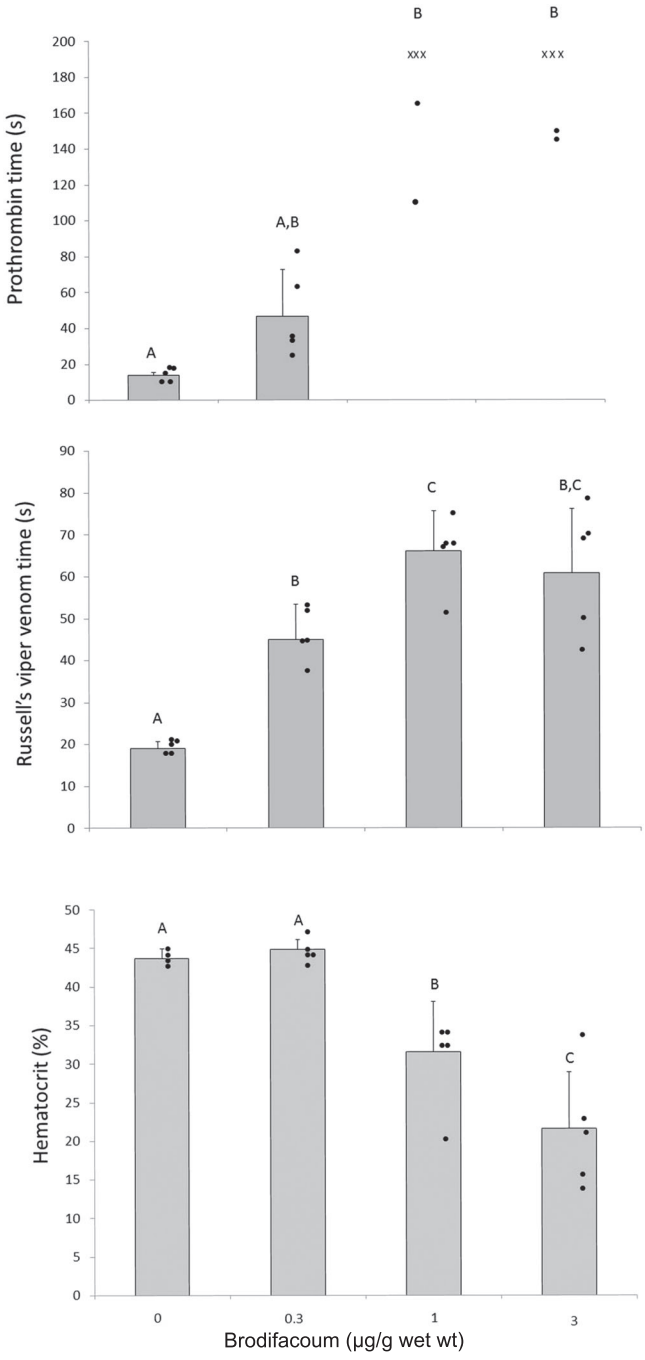

FIGURE 1: Prothrombin time, Russell's viper venom time, and hematocrit (individual values and mean ± standard deviation) of American kestrels following a 7-d dietary exposure to brodifacoum ($n = 5$ kestrels/group). X = sample that did not clot in the prothrombin assay. Bars with different capital letters are significantly different ($p < 0.05$).

Based on food consumption and the concentration of brodifacoum in the diet (analytically verified as 0.469 µg/g diet wet wt), the estimated daily dose of exposed birds was 108.5 ± 14.12 µg brodifacoum/kg body weight/day (cumulative 7-d dose ~0.760 mg/kg body wt). At the end of the 7-d brodifacoum exposure phase (day 0 postexposure), hepatic brodifacoum residues were in the same range as observed in kestrels consuming the low dietary brodifacoum dose in trial 1 (Table 2 vs Table 1, 95% CIs overlapped). Over the 28-d recovery period, brodifacoum concentration decreased markedly in liver (ANOVA $F_{5,24} = 13.37$, $p < 0.0001$, and Tukey's honestly significant difference for day 0 vs days 7, 14, and 28 p values ≤ 0.0026) and kidney (ANOVA $F_{5,24} = 11.45$, $p < 0.0001$, and Tukey's honestly significant difference for day 0 vs days 4, 7, 14 and 28 p values ≤ 0.0014; Table 2). The overall brodifacoum half-life estimate ($t_{1/2}$) in liver was 27.7 d ($y = 1270.3e^{-0.025x}$; initial $t_{1/2}$ from day 0 to 4 = 11.9 d and terminal phase $t_{1/2}$ from day 7 to 28 = 49.5 d), and the overall brodifacoum $t_{1/2}$ estimate in kidney was 34.6 d ($y = 330.02e^{-0.02x}$; initial $t_{1/2}$ from day 0 to 4 = 5.9 d; terminal phase $t_{1/2}$ from day 7 to 28 = 69.3 d; Figure 3). Notably, there was a positive association (slope 3.18, standard error 0.34) between liver and kidney residues in brodifacoum-exposed kestrels (linear regression ANOVA $F_{1,28} = 89.52$ $p < 0.0001$, adjusted $R^2 = 0.7532$).

Prior to the trial, baseline values ($n = 36$ kestrels) ranged from 38.4 to 129.9 mg/dL for fibrinogen, from 6.3 to 17.0 s for PT, from 11.3 to 22.3 s for RVVT, and from 37.7 to 51.1% for hematocrit. With the exception of one sample (problematic blood draw on day 7 post–brodifacoum exposure), all samples collected during the trial produced clots in the TCT assay, and fibrinogen concentrations did not differ among controls and various groups of brodifacoum-exposed kestrels (log-transformed values, ANOVA $F_{6,28} = 1.63$, $p = 0.1751$; Table 2). Following brodifacoum ingestion for 7 d, PT was prolonged (elevated in 4 of 5 birds relative to baseline) and statistically different compared to controls (log-transformed values, ANOVA $F_{6,27} = 4.54$, $p = 0.0026$, and Tukey's honestly significant difference $p = 0.0035$; Figure 4). By day 7 post–brodifacoum exposure, PT values had generally returned to the range of baseline values. Likewise, RVVT values were elevated at the end of the 7-d brodifacoum exposure phase compared to controls (log-transformed values, ANOVA $F_{6,28} = 5.63$, $p = 0.0006$, and Tukey's honestly significant difference $p = 0.0031$) and generally returned to the range of baseline values by day 7 post–brodifacoum exposure (Figure 4). Despite evidence of coagulopathy following brodifacoum exposure, no effects on hematocrit (log-transformed values, ANOVA $F_{6,29} = 0.81$, $p = 0.5702$; Table 2) were noted during the exposure phase and recovery period. Necropsies were generally unremarkable, although in a few instances during the post-exposure period (4 birds on day 4, 1 bird on day 7, and 1 bird on day 28) there seemed to be excessive extravasation (bleeding) during dissections.

Trial 3: Sequential exposure to an FGAR or an SGAR followed by an FGAR

All kestrels survived the initial exposure, recovery, and chlorophacinone challenge phases of the trial. During the initial exposure phase, a single kestrel fed 1.5 µg chlorophacinone/g wet weight had a small drop of blood on its cere; and during the chlorophacinone challenge phase, drops of blood were observed on the kraft paper lining the pen of 1 kestrel in the brodifacoum-chlorophacinone group. No overt signs of intoxication were observed in any of the other kestrels. Food consumption did not differ among the control-chlorophacinone, chlorophacinone-chlorophacinone, and the brodifacoum-chlorophacinone groups

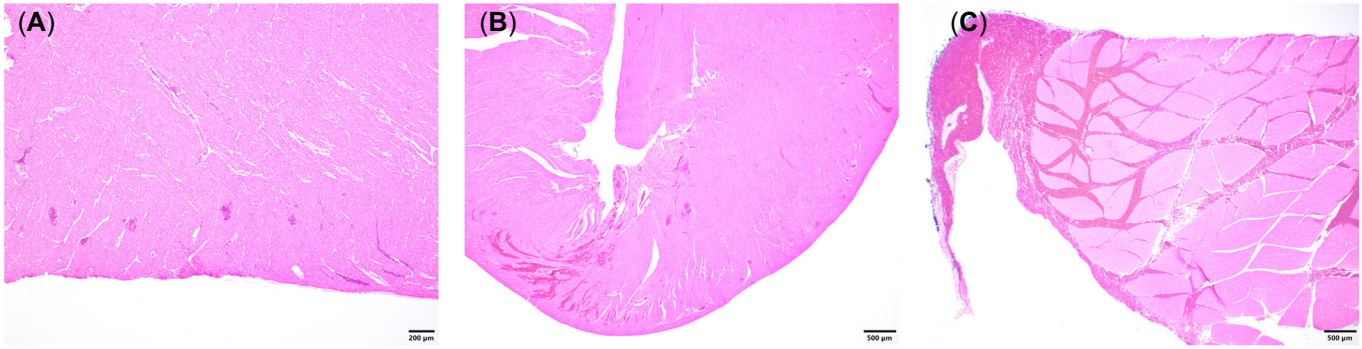

FIGURE 2: Mild (**A**), moderate (**B**), and severe (**C**) interstitial hemorrhage in heart or skeletal muscle of American kestrels following 7-d dietary exposure to brodifacoum. (**A**) Mild hemorrhage is characterized by few small discrete areas of hemorrhage in the myocardial interstitium (scale bar = 200 μm); (**B**) moderate hemorrhage in which myocardial interstitium is expanded by several coalescing areas of hemorrhage (scale bar = 500 μm); and (**C**) severe hemorrhage with abundant widespread interstitial and fascial hemorrhage in pectoral muscle (scale bar = 500 μm).

during the initial exposure phase (log-transformed values, ANOVA $F_{2,17} = 1.59$, $p = 0.2332$) or the challenge exposure phase (ANOVA $F_{2,17} = 0.11$, $p = 0.8921$) of the trial (Table 3). Consumption of chlorophacinone during the challenge phase was remarkably similar among groups (ANOVA $F_{2,17} = 0.11$, $p = 0.8921$). Weight change over the course of the trial (relative to initial body wt, day 0) was not affected by treatment (repeated measures ANOVA $F_{3,15} = 2.02$, $p = 0.1550$); however, there was a marginal interaction of treatment and day (repeated measures ANOVA $F_{6,30} = 2.15$, $p = 0.0763$) and an effect of day (i.e., exposure phase; repeated measures ANOVA $F_{2,14} = 16.33$, $p = 0.0002$). Although body weight in kestrels fed mice for the entire trial (untreated controls) was stable, birds receiving various NBP diets containing ARs exhibited incremental weight loss during the recovery and challenge phases (Table 3). Although chlorophacinone consumption did not differ among treatment groups during the challenge phase, hepatic chlorophacinone residues did (ANOVA $F_{2,17} = 14.86$, $p = 0.0002$). Compared with the brodifacoum-chlorophacinone exposure group, liver chlorophacinone concentrations were actually greater in both the control-chlorophacinone and chlorophacinone-chlorophacinone groups (Tukey's honestly significant difference $p = 0.0037$ and 0.0002, respectively). Some 14 d after brodifacoum consumption, hepatic brodifacoum residue concentrations were similar to those observed on day 14 postexposure in the recovery study (Table 3 vs Table 2).

Baseline fibrinogen values ($n = 26$ kestrels) ranged from 45.9 to 100.4 mg/dL, and at the end of the chlorophacinone challenge phase, values ranged from 43.4 to 139.9 mg/dL. There was no difference in fibrinogen concentration among groups (ANOVA $F_{2,23} = 1.09$, $p = 0.3742$) at the end of the trial (Table 3).

Baseline PT values of kestrels used in this trial ranged from 6.05 to 13.20 s. At the end of the trial, PT for 3 of the 6 untreated controls slightly exceeded the range of baseline values (Figure 5) but were still below the upper reference limit for PT (16.46 s) described for the American kestrel (Hindmarch et al. 2019). There were effects of the various treatments on PT (log-transformed values, ANOVA $F_{3,22} = 4.06$, $p = 0.0195$). Compared to untreated controls, PT at the end of the challenge phase was not greater in the control-chlorophacinone and chlorophacinone-chlorophacinone groups (Tukey's honestly significant difference $p = 0.9289$ and 0.9978, respectively). However, many of the kestrels initially exposed to the SGAR brodifacoum and then challenged with chlorophacinone (i.e., brodifacoum-chlorophacinone)

TABLE 2: Body weight change, sublethal responses, and tissue residues following brodifacoum ingestion in American kestrels[a]

		Days post-BROD exposure (0.5 μg/g food wet wt)[b]					
	Control	0	2	4	7	14	28
n	6	5	5	5	5	5	5
Weight change (Δg/100 g body wt)	−1.20 ± 2.442	−1.75 ± 3.771	+1.04 ± 5.247	−3.47 ± 1.230	−1.60 ± 2.603	−1.21 ± 6.060	−7.48 ± 3.409
Liver BROD (μg/g wet wt)	<MLOD	1.44 ± 0.240A	1.29 ± 0.212A,B	1.15 ± 0.200A,B	0.955 ± 0.149B,C	0.799 ± 0.129C	0.700 ± 0.074C
Liver BROD (nmol/g wet wt)		~2.75	~2.46	~2.19	~1.82	~1.53	~1.34
Kidney BROD (μg/g wet wt)	<MLOD	0.419 ± 0.050A	0.331 ± 0.047A,B	0.270 ± 0.086B,C	0.258 ± 0.039B,C	0.228 ± 0.030C	0.209 ± 0.035C
Fibrinogen (mg/dL)	87.0 ± 30.00	66.9 ± 14.33	105.5 ± 68.33	69.9 ± 13.84	66.8 ± 9.03 ($n = 4$)	81.8 ± 35.92	111.6 ± 26.13
Hematocrit (%)	41.6 ± 2.71	40.4 ± 3.76	39.9 ± 6.42	43.3 ± 1.56	42.5 ± 2.46	43.2 ± 1.94	42.4 ± 3.08

[a]Values are mean ± standard deviation.
[b]Analytically verified concentration was 0.469 ± 0.024 μg/g feed wet weight.
Means with different capital letters are different by Tukey's honestly significant difference method ($p < 0.05$).
BROD = brodifacoum; MLOD = method limit of detection.

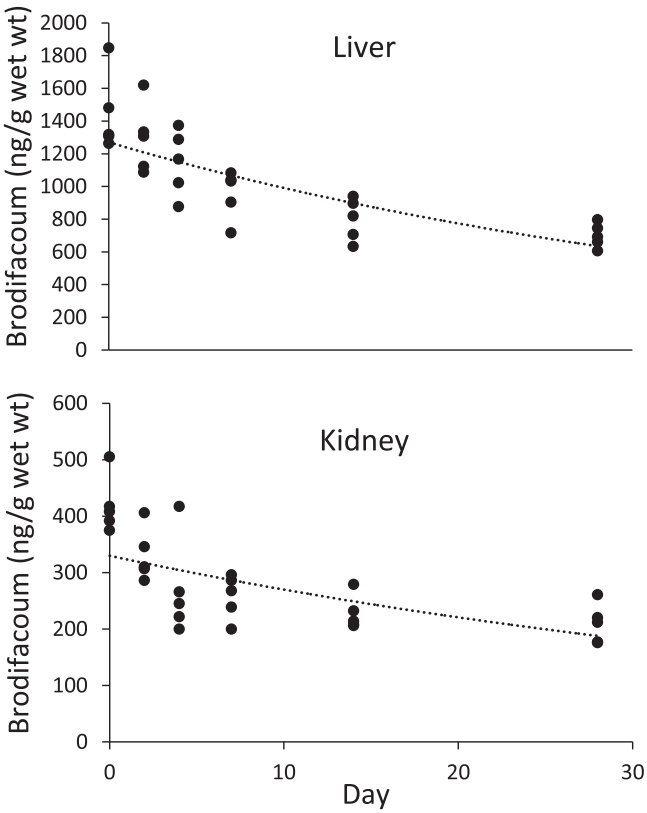

FIGURE 3: Concentrations of brodifacoum in liver and kidney of American kestrels at various postexposure times following a 7-d dietary exposure to 0.5 µg brodifacoum/g wet weight ($n = 5$ birds at each postexposure sampling time).

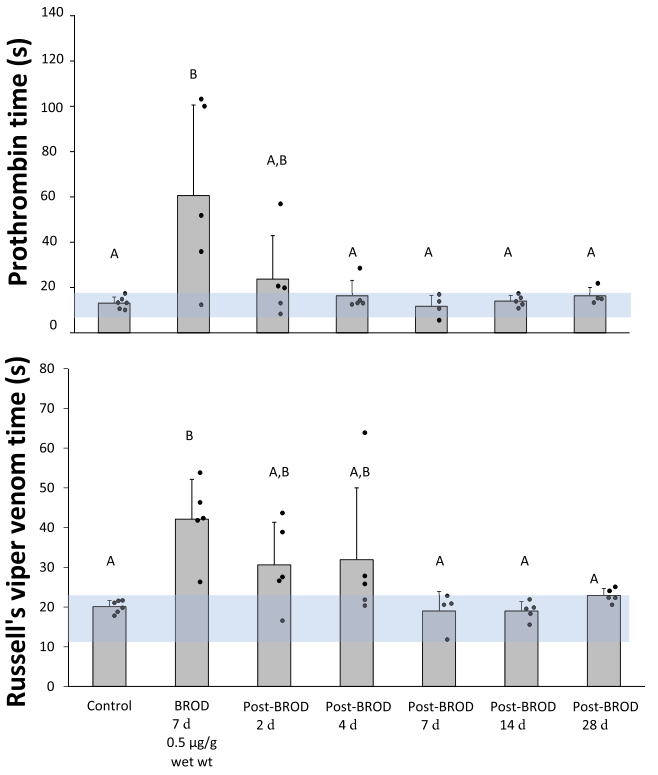

FIGURE 4: Prothrombin time and Russell's viper venom time (individual values and mean ± standard deviation) of American kestrels following a 7-d dietary exposure to 0.5 µg brodifacoum/g wet weight and at various postexposure times ($n = 4$–5 exposed birds at each sampling time and $n = 6$ controls). Horizontal shaded area encompasses the range of baseline values determined at least 2 wk before the trial. Bars with different letters are significantly different by Tukey's honestly significant difference test ($p < 0.05$). BROD = brodifacoum.

had PT values that were clearly prolonged (6 of 8 exceeded the upper reference limit, and 8 of 8 were >125% of their individual baseline value). The PT of the brodifacoum-chlorophacinone group was longer than that of the untreated control (Tukey's honestly significant difference $p = 0.0458$) and the chlorophacinone-chlorophacinone ($p = 0.0302$) groups. On an individual bird basis, baseline and end-of-trial PT values were examined using a paired t test. The PT increased in the brodifacoum-chlorophacinone group (log-transformed values, $t = 5.32$, $df = 7$, $p = 0.0011$), whereas no pronounced change was observed in any of the other groups (log-transformed values, $t = 0.78$–2.16, $df = 5$, $p = 0.0833$–0.4716; Figure 6).

As in trials 1 and 2, RVVT assays were initially run using a 3:5 dilution factor of citrated plasma in 8.3 mM phosphate buffer (pH 7.2). Surprisingly, at the end of the challenge phase, 2 untreated control samples had lengthy clotting times. After considerable investigation (see Supplemental Data), all samples were reassayed at a 4:5 dilution factor, which somewhat resolved this seemingly spurious observation. Baseline RVVT values of these reassayed samples ranged from 11.9 to 20.3 s. At the end of the exposure trial, there were differences in RVVT among groups (log-transformed values, ANOVA $F_{3,22} = 4.32$, $p = 0.0154$; Figure 5). The RVVT was prolonged in the brodifacoum-chlorophacinone group compared to untreated controls (Tukey's honestly significant difference $p = 0.0171$; Figure 5). Although the RVVT in the brodifacoum-chlorophacinone group did not differ from that of the chlorophacinone-chlorophacinone group (Tukey's honestly significant difference $p = 0.3469$), it was marginally greater than that in the control-chlorophacinone group ($p = 0.0524$).

Baseline hematocrit values ranged from 41.1 to 53.3%, and at the end of the trial values did not differ among groups (ANOVA $F_{3,22} = 1.20$, $p = 0.3343$; Table 3). At necropsy, there was no pronounced evidence of hemorrhage in any of the chlorophacinone-challenged groups or in untreated controls. Microscopically, as in the range-finding trial, minimal hemorrhage was occasionally present in skeletal muscle, heart, kidney, and liver of all groups. As noted, minimal extravasation can occur artifactually and may not represent antemortem hemorrhage. Other microscopic findings were similar to those described in the range-finding trial, with the addition of occasional mild hepatic glycogenosis and renal tubular cytokaryomegaly.

DISCUSSION

Our findings with American kestrels indicate that dietary exposure to environmentally realistic concentrations of the SGAR brodifacoum results in dose-dependent effects on hemostasis, as evidenced by bruising, frank or microscopic hemorrhage, prolonged clotting time, and anemia. On

TABLE 3: Estimated daily exposure, body weight change, and liver chlorophacinone and brodifacoum residues following sequential exposure of kestrels[a]

	Control	Initial exposure phase–challenge exposure phase		
	Untreated ($n = 6$)	CON-CPN ($n = 6$)	CPN-CPN ($n = 6$)	BROD-CPN ($n = 8$)
Initial exposure phase (days 0–7)	Dead mice	CON NBP diet	CPN NBP diet	BROD NBP diet
Nominal rodenticide concentration (µg CPN or BROD/g wet wt)		0	1.5	0.5
Analytically verified rodenticide concentration (µg CPN or BROD/g wet wt)	NA	<MLOD	1.37 ± 0.064	0.377 ± 0.021
Food consumption (g wet wt/kg body wt/d)	NA	215.3 ± 19.78	210.0 ± 11.16	235.8 ± 40.56
CPN or BROD consumed (µg/kg body wt/d)	0	0	286.7 ± 15.24	88.8 ± 15.28
Weight change (days 0–7: Δg/100 g body wt)	−0.85 ± 8.705	−0.24 ± 3.306	+0.02 ± 1.510	+0.78 ± 3.193
Recovery phase (days 8–14)	Dead mice	NBP diet	NBP diet	NBP diet
Weight change (days 0–14 Δg/100 g body wt)	+0.26 ± 8.598	−7.32 ± 1.258	−5.46 ± 3.184	−4.67 ± 4.428
Challenge exposure phase (days 15–21)	Dead mice	CPN NBP diet	CPN NBP diet	CPN NBP diet
Nominal rodenticide concentration (µg CPN or BROD/g wet wt)	NA	0.75	0.75	0.75
Analytically verified rodenticide concentration (µg CPN/g wet wt)	NA	0.709 ± 0.033	0.709 ± 0.033	0.709 ± 0.033
Food consumption (g wet wt/kg body wt/d)	NA	179.6 ± 34.73	178.1 ± 47.33	187.7 ± 39.76
CPN consumed (µg/kg body wt/d)	0	127.4 ± 24.63	126.4 ± 33.57	133.2 ± 28.20
Weight change (days 0–21: Δg/100 g body wt)	+1.05 ± 8.255	−9.05 ± 3.039	−7.33 ± 7.021	−6.34 ± 6.067
Liver CPN (µg/g wet wt)	<MLOD ($n = 4$)	0.748 ± 0.135A	0.827 ± 0.122A	0.517 ± 0.081B
Liver CPN (nmol/g wet wt)		~1.92	~2.21	~1.38
Liver BROD (µg/g wet wt)	<MLOD ($n = 4$)	<MLOD	<MLOD	0.710 ± 0.094
Liver BROD (nmol/g wet wt)				~1.36
Fibrinogen (mg/dL)	74.4 ± 17.77	60.3 ± 13.77	81.3 ± 17.11	77.3 ± 30.07
Hematocrit (%)	46.8 ± 2.77	44.8 ± 4.02	48.3 ± 2.28	47.2 ± 3.71

[a]Values are mean ± standard deviation.
Means with different capital letters are different by Tukey's honestly significant difference method ($p < 0.05$).
BROD = brodifacoum; CON = control; CPN = chlorophacinone; MLOD = method limit of detection; NA = not analyzed; NBP = Nebraska bird of prey.

termination of brodifacoum exposure, overt signs of toxicity and coagulopathy were resolved within 1 wk. Following the 7-d recovery period, subsequent exposure to an environmentally realistic dietary concentration of chlorophacinone prolonged PT in kestrels initially exposed to brodifacoum, suggestive of lingering and potentially long-term differences in the sensitivity of SGAR-exposed individuals.

Comparison of brodifacoum toxicity in kestrels with other species of birds

With the notable exception of brodifacoum, acute toxicity data indicate that ARs are 1 to 2 orders of magnitude more potent in rodents than commonly tested avian species (Rattner and Mastrota 2018). Based on northern bobwhite and mallard acute oral and dietary toxicity data (median lethal dose = 0.25–11.6 mg/kg body wt, median lethal concentration = 1.33–2.75 mg/kg diet or ppm; Rattner and Mastrota 2018), brodifacoum is categorized as being "very highly toxic" to birds (US Environmental Protection Agency 2004, 2011b) and is the most potent AR registered in the United States, much of Europe, and elsewhere. Although data are available on the liver residue concentrations of brodifacoum and other SGARs that are associated with mortality in raptors, robust empirical data of the dietary dose of brodifacoum causing lethality in seemingly more sensitive predatory and scavenging birds are lacking (Rattner and Mastrota 2018). Focusing on secondary exposure, several studies have been conducted in barn owls (*Tyto alba*) fed brodifacoum-exposed rodents (*Rattus* sp., *Mus* sp. fed brodifacoum 0.002–0.005% active ingredient baits) for as long as 15 d. Owls exhibited overt signs of intoxication and qualitative measures indicative of impaired blood clotting function, with many individuals succumbing (Mendenhall and Pank 1980; Newton et al. 1990; Gray et al. 1994; Lee 1994; Wyllie 1995). Signs of intoxication were often protracted, with mortality occurring days to weeks postexposure. The cumulative dietary dose of brodifacoum associated with mortality in barn owls was highly variable, ranging from 0.15 to 5.4 mg/kg body weight (Newton et al. 1990; Gray et al. 1994; Lee 1994), and was likely even less because of regurgitation of varying quantities of the administered dose (Newton et al. 1990, 1994).

Secondary exposure studies in Falconiformes have documented that consumption of brodifacoum-poisoned rodents (0.005% bait) for 4 d resulted in death of red-tailed and red-shouldered hawks (*Buteo jamaicensis* and *B. lineatus*), whereas golden eagles (*Aquila chrysactos*) survived but exhibited external bleeding (Marsh and Howard 1978, ICI Americas, Goldsboro, NC, USA, unpublished report). Somewhat more definitive are the results of a 5-d dietary study in which American kestrels were fed vole (*Microtus* spp.) tissue containing brodifacoum (0.3, 0.8, 1.6, 3.2, or 6.0 ppm; Lavoie 1990). The lowest lethal dietary concentration causing mortality was 0.8 ppm, with 1 of 8 kestrels succumbing (~1 mg/kg brodifacoum "potentially" consumed/kg body wt), whereas at 6.0 ppm 4 of 8 kestrels died (~7.3 mg brodifacoum consumed/kg body wt kestrel over 5 d; Lavoie 1990). The lowest nominal dietary brodifacoum concentration in our range-finding study was 0.3 ppm, and based on food consumption measurements, the cumulative 7-d dose was

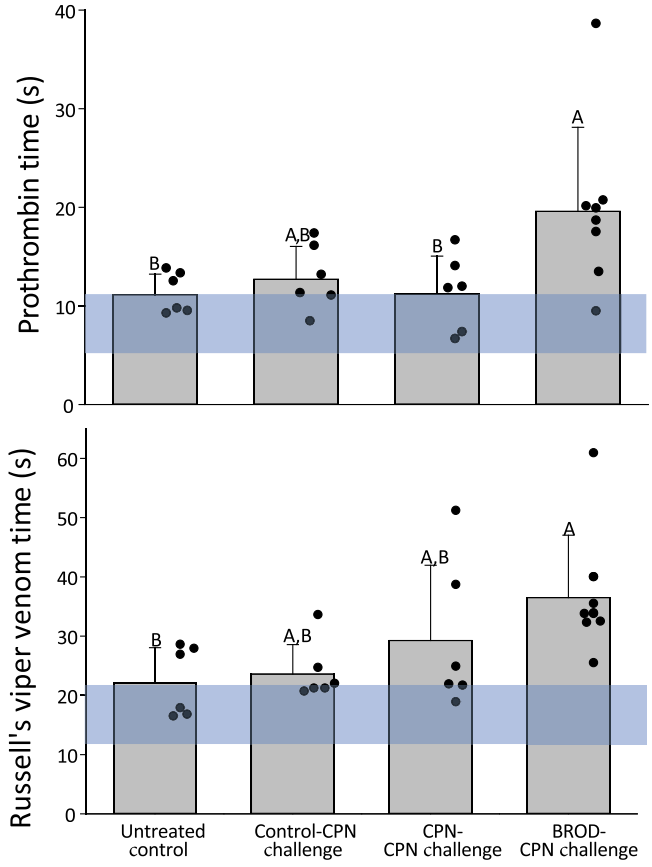

FIGURE 5: Prothrombin time and Russell's viper venom time (individual values and mean ± standard deviation) of American kestrels following various first- and second-generation anticoagulant rodenticide dietary exposure regimens ($n = 6–8$ kestrels per group). Horizontal shaded area encompasses the range of baseline values determined at least 2 wk before the trial. Bars with different letters are significantly different by Tukey's honestly significant difference test ($p < 0.05$). BROD = brodifacoum; CPN = chlorophacinone.

estimated to be 0.46 mg/kg body weight (i.e., 0.271 µg brodifacoum/g food wet wt × 240.2 g food wet wt consumed/kg body wt × 7 d = 455.7 µg/kg body wt, ~5% of the dietary concentration causing 50% mortality; Lavoie 1990). This low dose was found to prolong clotting time and produced gross and microscopic evidence of hemorrhage. Using the benchmark dose method, the cumulative 7-d dietary brodifacoum dose associated with anemia in 10% of the exposed kestrels is approximately 1 mg/kg body weight (Supplemental Data, Figure S1; 146.4 µg brodifacoum consumed/kg body wt/d × 7 d = 1025 µg/kg body wt). In our second trial examining recovery of clotting function following a 7-d exposure to approximately 0.5 ppm brodifacoum (cumulative dose ~0.76 mg/kg body wt; i.e., 108.5 µg brodifacoum consumed/kg body wt/d × 7 d), no mortality was observed over the 4-wk postexposure period. Thus, data from the present study suggest that a 7-d dietary exposure at ≤0.5 ppm brodifacoum constitutes a sublethal dose in kestrels, whereas greater dietary brodifacoum concentrations (1 and 3 ppm for 7 d in trial 1, ≥0.8 ppm for 5 d in Lavoie 1990) are associated with more profound coagulopathy and hemorrhage, as well as lethality. Taken together, captive American kestrels are seemingly more tolerant

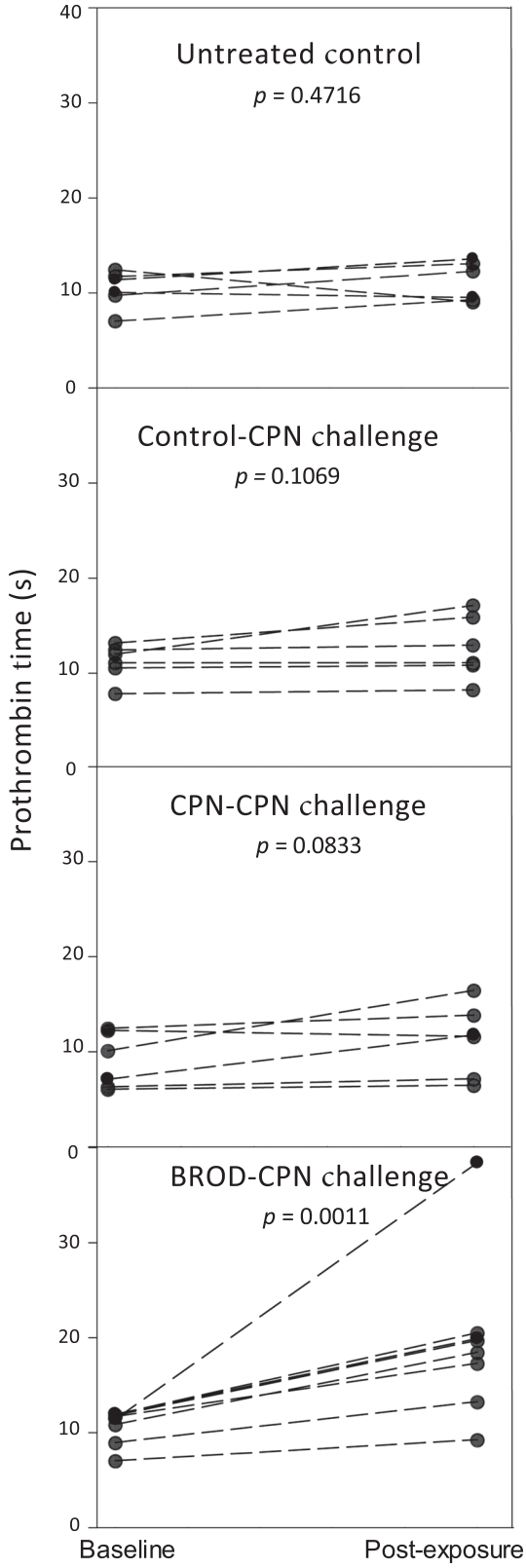

FIGURE 6: Individual baseline and postexposure prothrombin time values of American kestrels following various first- and second-generation anticoagulant rodenticide dietary exposure regimens ($n = 6–8$ kestrels per group). p values for each group are by paired t test. BROD = brodifacoum; CPN = chlorophacinone.

to brodifacoum (lowest lethal dose ~1 mg/kg body wt) than captive barn owls (lowest lethal dose 0.15 mg/kg; Newton et al. 1990). Because ARs have steep dose–response characteristics (e.g., Rattner et al. 2015), it is not surprising that a rigorous brodifacoum dietary exposure threshold (lowest-observed–adverse effect level or toxicity reference value) for sublethal effects has yet to be established for predatory and scavenging birds.

Liver residues and half-life of brodifacoum in kestrels and other species of birds

Several brodifacoum dietary exposure trials with captive birds describe residue concentrations in liver of individuals exhibiting hemorrhage, coagulopathy, or mortality. Liver brodifacoum residues in 4 of 6 barn owls that died 6 to 17 d after being fed poisoned mice for a single day (cumulative brodifacoum dose to barn owls ~0.150–0.182 mg/kg body wt) ranged from 0.63 to 1.25 μg/g wet weight (1.20–2.38 nmol/g; Newton et al 1990). In another study barn owls were fed brodifacoum-poisoned mice for up to 15 d (cumulative brodifacoum dose to barn owls ~1.9–5.4 mg/kg; Gray et al. 1994), and liver residues in owls that were sacrificed 15 d after the final dose ($n = 3$) or succumbed ($n = 1$ on d 14 of treatment) were similar (0.55–1.67 μg/g wet wt, 1.05–3.19 nmol/g) to those in the aforementioned study (Newton et al. 1990) despite the dose being 10 to 36 times greater. In the present kestrel study, the cumulative 7-d dietary exposure doses (trials 1 and 2: 0.46–3.97 mg/kg body wt) and hepatic brodifacoum residues (1.39–2.10 μg/g wet wt, 2.65–4.01 nmol/g) were not unlike doses and residue values in studies with barn owls that were associated with anticoagulant toxicity (Newton et al. 1990; Gray et al. 1994).

A noteworthy finding at the end of the chlorophacinone challenge phase of our sequential exposure study was that hepatic chlorophacinone residues in kestrels initially exposed to brodifacoum were lower compared to residue concentrations observed in the other challenge groups (control-chlorophacinone, chlorophacinone-chlorophacinone; Table 3). Because consumption of chlorophacinone during the challenge phase did not differ among treated groups, it seems likely that brodifacoum residues in kestrel liver may have limited chlorophacinone accumulation during the challenge phase. This phenomenon has been previously noted in other repeat-dose SGAR studies in rats and quail. For example, in rats dosed once per week with the SGAR flocoumafen (0.1 mg/kg body wt), liver residues plateaued by week 4; and together with other low-exposure dose (0.02 mg/kg body wt), the data demonstrated that accumulation was biphasic, likely involving 2 accumulation sites (Huckle et al. 1988). The high-affinity flocoumafen binding site was saturated at approximately 2.2 nmol/g liver, a level associated with transient anticoagulant toxicity (Huckle et al. 1988). Similarly, Japanese quail repetitively fed a flocoumafen diet (5, 15, or 50 ppm) 1 d/wk had relatively constant liver flocoumafen residues between 4 and 20 wk of exposure (0.38–0.75 μg/g wet wt), suggestive of a capacity-limited high-affinity binding site (saturated at ~1 nmol flocoumafen/g liver), as noted in the rat (Huckle et al. 1988, 1989). The presence of a common binding site for ARs in rat liver has been described, with SGARs including brodifacoum accumulating at 2 to 3 nmol/g liver and having a greater affinity than the FGAR coumatetralyl (Parmar et al. 1987). Not unlike our observation of limited liver chlorophacinone accumulation in brodifacoum-exposed kestrels are repeat SGAR dose exposures in Japanese quail. Specifically, initial exposure to brodifacoum (0.2 and 0.4 mg/kg body wt) seemed to limit (viz., actually reduced) accumulation of difenacoum administered at 0.2 mg/kg body weight some 26 d later in a dose-dependent manner when compared with quail that received only difenacoum (Butler 2010).

An often-cited residue toxicity threshold (i.e., potentially lethal range) for brodifacoum and other SGARs in liver is >0.1 to 0.2 μg/g wet weight (Newton et al. 1999a, 1999b). This threshold was derived from studies with captive barn owls exposed to SGARs and from postmortem examination of wild barn owls that had liver SGAR concentrations >0.1 μg/g wet weight with classic signs of AR toxicosis including hemorrhage. A more recent probabilistic analysis using summed hepatic SGAR residues in 270 owl and red-tailed hawk environmentally exposed specimens described a significant likelihood of toxicosis in 5% of the individuals with liver concentrations of 0.02 μg/g wet weight and in 20% of the individuals with concentrations at 0.08 μg/g wet weight (Thomas et al. 2011). For brodifacoum, these toxicosis threshold values (e.g., 0.08 μg/g wet wt, 0.15 nmol/g wet wt) are well below liver residue values associated with anticoagulant toxicity in laboratory studies with birds (0.55–2.10 μg/g wet wt, 1.05–4.01 nmol/g wet wt; Newton et al. 1990; Gray et al. 1994; present study) and rodents (1.2 μg/g wet wt, 2.2 nmol/g wet wt; Huckle et al. 1988). It is noteworthy that in trial 2 of the present kestrel study, brodifacoum residues in liver were much greater (1.44 μg/g wet wt, 2.75 nmol/g after 7-d brodifacoum exposure to 0.700 μg/g wet wt, 1.34 nmol/g on day 28 postexposure) than values in these environmentally exposed raptors (Thomas et al. 2011), and although coagulopathy was initially observed in kestrels (day 0–7 post–brodifacoum exposure), there was no mortality over the course of the trial. This discrepancy would suggest that kestrels and other avian species tested in captivity are likely far more tolerant to brodifacoum, and other SGARs, than free-ranging birds with more complex daily activities (e.g., movement, foraging, behavioral interactions) and encounters with a myriad of environmental stressors (e.g., extreme weather, disease, other contaminants), and highlights the challenges and limitations of laboratory to field extrapolations. It is not clear if liver AR residues are uniformly diagnostic of a potentially toxic dose evoking anticoagulant effects; such a cause–effect relationship may not be appropriate (US Environmental Protection Agency 2004). Furthermore, there are genetic and biochemical variants of VKOR that afford resistance (VKORC1, VKORC1L1; Pelz et al. 2005; Hammed et al. 2013), as well as other pharmacokinetic (e.g., cytochrome P450–mediated, AR binding; Watanabe et al. 2010, 2015) and possibly diet-based (Thijssen 1995) mechanisms that could result in AR tolerance.

Several brodifacoum single–oral dose trials have been conducted in domesticated granivorous birds that examine toxicity and residue kinetics. For example, Japanese quail gavaged with 0.2 to 2.5 mg brodifacoum/kg body weight had relatively similar

liver brodifacoum residues (~0.40–0.45 μg/g wet wt [Butler 2010]; 0.23–0.51 μg/g wet wt [Webster et al. 2015]) even though the dose differed by an order of magnitude and post-exposure sample collection ranged from 1 to 26 d. Likewise, a single 0.5 mg brodifacoum/kg body weight oral dose administered to domestic chickens yielded liver residue values averaging 0.62 to 0.70 μg/g wet weight over a 14-d post-exposure period (Fisher 2009). These data demonstrate brodifacoum persistence in the liver of birds. Various studies in laboratory rodents indicate that the half-life of brodifacoum in liver may be as long as 350 d (reviewed in Horak et al. 2018), and a year-long sampling study in birds (Japanese quail gavaged at 0.4 mg brodifacoum/kg body wt) predicted the half-life to be 297 d using a single-phase decay model (Butler 2010). Using this same single-phase model, estimated brodifacoum half-life in liver and kidney of American kestrels (cumulative 7-d brodifacoum dose ~0.76 mg/kg body wt) over a 4-wk period was only 27.7 and 34.6 d, respectively. However, between days 7 and 28 postexposure, the terminal half-life estimate approached 50 d in liver and 70 d in kidney and would likely have been greater had sampling during the elimination phase been extended (beyond the scope and purpose of the present recovery study). Although the half-life of brodifacoum in kestrel liver is shorter than that reported in rodents and quail, it is lengthy compared with that of the FGAR diphacinone that has also been examined in kestrels (i.e., overall liver half-life of 0.9 d, terminal phase half-life of 2.5 d; Rattner et al. 2011).

Response to sequential AR exposure

The extent to which multiple contaminant exposures and residue burdens affect biota is a seminal question in the fields of wildlife toxicology and risk assessment. This is particularly true for nontarget predatory and scavenging birds that are often exposed to and carry burdens of various pesticides, industrial compounds, and metals that could potentially affect physiological function and overall fitness. The present study with captive American kestrels demonstrates that dietary exposure to environmentally realistic concentrations of the SGAR brodifacoum causes coagulopathy that is resolved within 1 wk, yet subsequent challenge exposure to the FGAR chlorophacinone caused pronounced anticoagulant effects reflected by prolonged PT and RVVT, far greater than observed in naive and previously FGAR-exposed kestrels. Results of similar large-scale single–oral dose studies in Japanese quail reported that brodifacoum exposure followed by difenacoum exposure weeks later enhanced both the magnitude and duration of the anticoagulant effect (Butler 2010). Together, these controlled exposure studies provide further evidence that multiple AR exposure in free-ranging predatory and scavenging wildlife, a seemingly common occurrence (López-Perea and Mateo 2018), could have lasting and potentially cumulative effects. However, the challenge dose in laboratory studies, or the frequency and magnitude of repeated AR exposure in free-ranging animals carrying SGAR residues, is likely critical to this lingering effect on sensitivity (Butler 2010). Notably, in a companion study that used an experimental design identical to trial 3, a lower 7-d challenge dietary exposure (i.e., 0.25 μg chlorophacinone/g wet wt diet) failed to prolong clotting time in brodifacoum-exposed kestrels (Rattner et al. 2018).

It is commonplace for AR field monitoring and forensic investigations to report summed residue concentrations of ARs on a unit basis (μg/g, mg/kg, or ppm) when describing exposure, attempting to determine cause (AR exposure) of mortality, and even predicting SGAR toxicity thresholds. In our sequential exposure study (trial 3), the hepatic concentration of brodifacoum in such exposed kestrels at the end of the trial was 0.710 μg/g (~1.36 nmol/g; Table 3), a level not associated with coagulopathy in trial 2 (Table 2). The chlorophacinone concentration in brodifacoum-exposed kestrels was 0.517 μg/g (~1.38 nmol/g) and, when added to the brodifacoum value (~1.36 nmol/g) on a molar basis, totaled 2.74 nmol/g, a hepatic brodifacoum concentration that was coincident with coagulopathy in trials 1 and 2 (i.e., >2.65 and 2.19–2.75 nmol/g, respectively) and associated with increased PT and RVVT in trial 3. Ignoring differences in inhibition potency of brodifacoum (median inhibitory concentration ~0.22 μM) and chlorophacinone (5.1 μM) for kestrel hepatic microsomal VKOR activity (J.-K. Tie and X. Chen, University of North Carolina, Chapel Hill, NC, USA, unpublished data), hepatic residues of kestrels only exposed to chlorophacinone were lower (0.748 and 0.827 μg chlorophacinone/g), and on a nmol/g basis (~1.92 and ~2.21 nmol chlorophacinone/g) below that observed in birds exhibiting coagulopathy in trial 2 (~2.75 nmol brodifacoum/g). These data suggest that it may be more appropriate for controlled exposure trials and field monitoring studies to sum AR residues on a nmol/g basis rather than on a μg/g, mg/kg or ppm basis. Moreover, although VKOR inhibition by ARs is clearly linked to anticoagulant effects, the mere presence of AR residues in liver does not demonstrate inhibition of VKOR. Some sites of AR sequestration in liver may prevent AR toxicity, or alternatively coagulation function is maintained until accumulation sites are saturated and a high proportion of VKOR binding sites are blocked by free AR (Huckle et al. 1989). A better understanding of the relation between AR residues (perhaps on a nanomolar basis incorporating inhibitory potency for VKOR) with thresholds for toxic effects in wildlife, companion and domestic animals, and even humans is warranted.

Although our sequential exposure study was focused on anticoagulant effects that could potentially affect individuals and even local populations of nontarget wildlife, there is recent evidence that AR exposure can affect disease susceptibility, immune function, and other endpoints in mammals (Rattner et al. 2014; Serieys et al. 2018; Fraser et al. 2018). In view of the likelihood and environmental relevance of repeated exposure to multiple ARs, investigation of other sublethal effects is appropriate. Such information would enhance the evaluation of hazard and the assessment of ecological risk to nontarget wildlife.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4629.

Acknowledgment—The authors thank W.C. Bauer, B. Clauss, B.A. Clauss, D.D. Day, R.C. Doyle, C.S. Hulse, J.P. Male, M.E. Maxey, R.M. Mickley, S. Peregoy, and C.C. Shafer for assistance with animal care and food preparation; B. Abbo and D.A. Goldade for diet and tissue rodenticide analyses; M.E. Barton Bohannon, C. Cray, R.A. Eberius, M.K. Karouna-Renier, J.J. López-Perea, and S.L. Schultz for help with sample collection or analysis; J.-K. Tie and X. Chen for initial work on American kestrel hepatic microsomal vitamin K epoxide reductase; and R.F. Shore and R.A. Erickson for providing valuable comments on a draft of the manuscript. The present study was supported in part by the California Department of Food and Agriculture Vertebrate Pest Control Research Advisory Committee (grant no. 14-0583-SA), the US Department of Agriculture/Animal and Plant Health Inspection Service/Wildlife Services National Wildlife Research Center, and the Contaminant Biology Program of the US Geological Survey's Environmental Health Mission Area.

Disclaimer—Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US government.

This article has earned an Open Data/Materials badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data are available at [https://doi.org/10.5066/P9707L0Q; https://doi.org/10.5066/P9503Y4X; https://doi.org/10.5066/P9K6U6R4]. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

Data Availability Statement—The data are publicly available (IP-110895, "Brodifacoum toxicity in American kestrels (*Falco sparverius*) with evidence of increased hazard upon subsequent anticoagulant rodenticide exposure," US Geological Survey data release, https://doi.org/10.5066/P9707L0Q; IP-109867, "Histopathology of American kestrels (*Falco sparverius*) exposed to brodifacoum," US Geological Survey data release, https://doi.org/10.5066/P9503Y4X; and IP-102045, "Histopathology of American kestrels (*Falco sparverius*) sequentially exposed to first and second generation anticoagulant rodenticides," US Geological Survey data release, https://doi.org/10.5066/P9K6U6R4).

REFERENCES

Bardal S, Waechter J, Martin D. 2011. *Applied Pharmacology*. Elsevier, St. Louis, MO, USA.

Bardo L, Bird DM. 2009. The use of captive American kestrels (*Falco sparverius*) as wildlife models: A review. *J Raptor Res* 43:345–364.

Buckle A, Prescott C. 2018. Anticoagulants and risk mitigation. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 319–355.

Burnham KP, Anderson DR. 2002. *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*, 2nd ed. Springer-Verlag, New York, NY, USA.

Butler SE. 2010. The sub-lethal effect of second generation anticoagulant rodenticides on birds. PhD thesis. University of Leicester, Leicester, UK.

Christensen TK, Lassen P, Elmeros M. 2012. High exposure rates of anticoagulant rodenticides in predatory bird species in intensively managed landscapes in Denmark. *Arch Environ Contam Toxicol* 63:437–444.

Elliott JE, Rattner BA, Shore RF, van den Brink N. 2016. Paying the pipers: Mitigating the impact of anticoagulant rodenticides on predators and scavengers. *BioScience* 66:401–407.

Filipsson AF, Sand S, Nilsson J, Victorin K. 2003. The benchmark dose method—Review of available models, and recommendations for application in health risk assessment. *Crit Rev Toxicol* 33:505–542.

Fisher P. 2009. Residual concentrations and persistence of the anticoagulant rodenticides brodifacoum and diphacinone in fauna. PhD thesis, Lincoln University, Lincoln, New Zealand.

Fraser D, Mouton A, Serieys LEK, Cole S, Carver S, Vandewoude S, Lappin M, Riley SDD, Wayne R. 2018. Genome-wide expression reveals multiple systemic effects associated with detection of anticoagulant poisons in bobcats (*Lynx rufus*). *Mol Ecol* 27:1170–1187.

Gray A, Eadsforth CV, Dutton AJ, Vaughan JA. 1994. The toxicity of three second-generation rodenticides to barn owls. *Pest Sci* 42:179–184.

Hadler MR, Buckle AP. 1992. Forty-five years of anticoagulant rodenticides—Past, present and future trends. In *Proceedings of the Fifteenth Vertebrate Pest Conference*. University of California, Davis, CA, USA, pp 149–155.

Hammed A, Matagrin B, Spohn G, Prouillac C, Benoit E, Lattard V. 2013. VKORC1L1, an enzyme rescuing the vitamin K 2,3-epoxide reductase activity in some extrahepatic tissues during anticoagulant therapy. *J Biol Chem* 288:28733–28742.

Hindmarch S, Rattner BA, Elliott JE. 2019. Use of blood clotting assays to assess potential anticoagulant rodenticide exposure and effects in free-ranging birds of prey. *Sci Total Environ* 657:1205–1216.

Horak KE, Fisher PM, Hopkins B. 2018. Pharmacokinetics of anticoagulant rodenticides in target and non-target organisms. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 87–108.

Huckle KR, Hutson DH, Warburton PA. 1988. Elimination and accumulation of the rodenticide flocoumafen in rats following repeated oral administration. *Xenobiotica* 18:1465–1479.

Huckle KR, Warburton PA, Forbes S, Logan CJ. 1989. Studies on the fate of flocoumafen in the Japanese quail (*Coturnix coturnix japonica*). *Xenobiotica* 19:51–62.

Jacob J, Buckle A. 2018. Use of anticoagulant rodenticides in different applications around the world. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 11–43.

Kerins GM. 1999. Plasma fibrinogen concentration is increased following depletion of vitamin K–dependent clotting factors by the indirect anticoagulant difenacoum in Norway rats (*Rattus norvegicus*). *Comparative Haematology International* 9:76–82.

Lavoie GK. 1990. A study of the anticoagulant brodifacoum to American kestrels (*Falco sparverius*). *Proceedings*, 3rd International Conference on Plant Protection in the Tropics, Genting Highlands, Pahang, Malaysia, March 20–23, pp 27–29.

Lee CH. 1994. Secondary toxicity of some rodenticides to barn owls. *Proceedings*, 4th International Conference on Plant Protection in the Tropics, Kuala Lumpur, Malaysia, March 28–31, pp 161–163.

Lima LL, Salmon TP. 2010. Assessing some potential environmental impacts from agricultural anticoagulant uses. In Timm RM, Fagerstone KA, eds, *Proceedings of the Twenty-Fourth Vertebrate Pest Conference*, University of California, Davis, CA, USA, pp 199–203.

López-Perea JJ, Mateo R. 2018. Secondary exposure of anticoagulant rodenticides and effects in predators. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 159–193.

Luna LG, ed. 1968. *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology*, 3rd ed. McGraw Hill, New York, NY, USA.

Mendenhall VM, Pank LF. 1980. Secondary poisoning of owls by anticoagulant rodenticides. *Wildl Soc Bull* 8:311–315.

Merson MH, Byers RE, Kaukeinen DE. 1984. Residues of the rodenticide brodifacoum in voles and raptors after orchard treatment. *J Wildl Manage* 48:212–216.

Mosterd JJ, Thijssen HHW. 1991. The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Br J Pharmacol* 104:531–535.

Murray M. 2011. Anticoagulant rodenticide exposure and toxicosis in four species of birds of prey presented to a wildlife clinic in Massachusetts, 2006–2010. *J Zoo Wildl Med* 42:88–97.

Murray M. 2017. Anticoagulant rodenticide exposure and toxicosis in four species of birds of prey in Massachusetts, USA, 2012–2016, in relation to use of rodenticides by pest management professionals. *Ecotoxicology* 26:1041–1050.

Newton I, Dale L, Finnie JK, Freestone P, Wright J, Wyllie I. 1999a. Wildlife and pollution: 1997/98 Annual report. Report 285. Joint Nature Conservation Committee, Peterborough, UK. [cited 2019 April 30]. Available from: http://jncc.defra.gov.uk/pdf/jncc285.pdf

Newton I, Shore RF, Wyllie I, Birks JDS, Dale L. 1999b. Empirical evidence of side-effects of rodenticides on some predatory birds and mammals. In Cowan DP, Feare CJ, eds, *Advances in Vertebrate Pest Management*. Filander Verlag, Furth, Germany, pp 347–367.

Newton I, Wyllie I, Freestone P. 1990. Rodenticides in British barn owls. *Environ Pollut* 68:101–117.

Newton I, Wyllie I, Gray A, Eadsforth CV. 1994. The toxicity of the rodenticide flocoumafen to barn owls and its elimination via pellets. *Pestic Sci* 41:187–193.

Parmar G, Bratt H, Moore R, Batten PL. 1987. Evidence for a common binding site in vivo for the retention of anticoagulants in rat liver. *Hum Toxicol* 6:431–432.

Pelz H-J, Rost S, Hünerberg M, Fregin A, Heiberg A-C, Baert K, MacNicoll AD, Prescott CV, Walker A-S, Oldenburg J, Müller CR. 2005. The genetic basis of resistance to anticoagulant rodenticides. *Genetics* 170:1839–1847.

Pitt WC, Berentsen AR, Shiels AB, Volker SF, Eisemann JD, Wegmann AS, Howald GR. 2015. Non-target species mortality and the measurement of brodifacoum rodenticide after rat (*Rattus rattus*) eradication on Palmyra Atoll, tropical Pacific. *Biol Conserv* 185:36–46.

Porter RD, Wiemeyer SN. 1970. Propagation of American kestrels. *J Wildl Manage* 34:594–604.

Primus TM, Eisemann JD, Matschke GH, Ramey C, Johnston JJ. 2001. Chlorophacinone residues in rangeland rodents: An assessment of the potential risk of secondary toxicity to scavengers. In Johnston JJ, ed, *Pesticides and Wildlife*, Vol 771—ACS Symposium Series. American Chemical Society, Washington, DC, pp 164–180.

Ramey CA, Matschke GH, Engeman RM. 2007. Chlorophacinone baiting for Belding's ground squirrels. *Proceedings*, 12th Wildlife Damage Management Conference, Corpus Christi, TX, USA, April 9–12, pp 526–537.

Rattner BA, Horak KE, Lazarus RS, Schultz SL, Knowles S, Abbo BG, Volker SF. 2015. Toxicity reference values for chlorophacinone and their application for assessing anticoagulant rodenticide risk to raptors. *Ecotoxicology* 24:720–734.

Rattner BA, Horak KE, Warner SE, Day DD, Meteyer CU, Volker SF, Eisemann JD, Johnston JJ. 2011. Acute toxicity, histopathology, and coagulopathy in American kestrels (*Falco sparverius*) following administration of the rodenticide diphacinone. *Environ Toxicol Chem* 30:1213–1222.

Rattner BA, Horak KE, Warner SE, Johnston JJ. 2010. Acute toxicity of diphacinone in northern bobwhite: Effects on survival and blood clotting. *Ecotoxicol Environ Saf* 73:1159–1164.

Rattner BA, Lazarus RS, Bean TG, Horak KE, Volker SF, Lankton J. 2018. Is sensitivity to anticoagulant rodenticides affected by repeated exposure in hawks? In Woods DM, ed, *Proceedings of the Twenty-Eighth Vertebrate Pest Conference*, University of California, Davis, CA, USA, pp 247–253.

Rattner BA, Lazarus RS, Eisenreich KM, Horak KE, Volker SF, Campton CM, Eisemann JD, Meteyer CU, Johnston JJ. 2012. Comparative risk assessment of the first-generation anticoagulant rodenticide diphacinone to raptors. In Timm RM, ed, *Proceedings of the Twenty-Fifth Vertebrate Pest Conference*, University of California, Davis, CA, USA, pp 124–130.

Rattner BA, Lazarus RS, Elliott JE, Shore RF, van den Brink N. 2014. Adverse outcome pathway and risks of anticoagulant rodenticides to predatory wildlife. *Environ Sci Technol* 48:8433–8445.

Rattner BA, Mastrota FN. 2018. Anticoagulant rodenticide toxicity to nontarget wildlife under controlled exposure conditions. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 45–86.

Serieys LEK, Armenta TC, Moriarty JG, Boydston EE, Lyren LM, Poppenga RH, Crooks KR, Wayne RK, Riley SPD. 2015. Anticoagulant rodenticides in urban bobcats: Exposure, risk factors and potential effects based on a 16-year study. *Ecotoxicology* 24:844–862.

Serieys LEK, Lea AJ, Epeldegui M, Armenta TC, Moriarty J, VandeWoude S, Carver S, Foley J, Wayne RK, Riley SPD, Uittenbogaart CU. 2018. Urbanization and anticoagulant poisons promote immune dysfunction in bobcats. *Proc Biol Sci* 285:20172533.

Shore RF, Coeurdassier M. 2018. Primary exposure and effects in non-target animals. In van den Brink N, Elliott JE, Shore RF, Rattner BA, eds, *Anticoagulant Rodenticides and Wildlife*. Springer Nature, Cham, Switzerland, pp 135–157.

Thijssen HHW. 1995. Warfarin-based rodenticides: Mode of action and mechanism of resistance. *Pestic Sci* 43:73–78.

Thomas PJ, Mineau P, Shore RF, Champoux L, Martin PA, Wilson LK, Fitzgerald G, Elliott JE. 2011. Second generation anticoagulant rodenticides in predatory birds: Probabilistic characterization of toxic liver concentrations and implications for predatory bird populations in Canada. *Environ Int* 37:914–920; corrigendum 40:256.

US Environmental Protection Agency. 2004. Potential risks of nine rodenticides to birds and nontarget mammals: A comparative approach. EPA P.2004.27 A. Washington, DC. [cited 2016 August 26]. Available from: http://www.fwspubs.org/doi/suppl/10.3996/052012-JFWM-042/suppl_file/10.3996_052012-jfwm-042.s4.pdf

US Environmental Protection Agency. 2011a. Benchmark Dose Software (BMDS) Ver 2.2 R65 [Build: 04/13/2011]. National Center for Environmental Assessment, Washington, DC. [cited 2014 September 12]. Available from: https://www.epa.gov/bmds

US Environmental Protection Agency. 2011b. Risks of non-compliant rodenticides to nontarget wildlife—Background paper for scientific advisory panel notice of intent to cancel non-RDM compliant rodenticide products. EPA-HQ-OPP-2011-0718-0006. [cited 2016 August 26]. Available from: https://www.regulations.gov/document?D=EPA-HQ-OPP-2011-0718-0006

Vyas NB, Hulse CS, Rice CP. 2012. Chlorophacinone residues in mammalian prey at a black-tailed prairie dog colony. *Environ Toxicol Chem* 25:2513–2516.

Watanabe KP, Kawata M, Ikenaka Y, Nakayama SMM, Ishii C, Darwish WS, Saengtienchai A, Mizukawa H, Ishizuka M. 2015. Cytochrome P450–mediated warfarin metabolic activity is not a critical determinant of warfarin sensitivity in avian species: In vitro assays in several birds and in vivo assays in chickens. *Environ Toxicol Chem* 34:2328–2334.

Watanabe KP, Saengtienchai A, Tanaka KD, Ikenaka YM, Ishizuka M. 2010. Comparison of warfarin sensitivity between rat and bird species. *Comp Biochem Physiol C Toxicol Pharmacol* 152:114–119.

Webster KH, Harr KE, Bennett DC, Williams TD, Cheng KM, Maisonneuve F, Elliott JE. 2015. Assessment of toxicity and coagulopathy of brodifacoum in Japanese quail and testing in wild owls. *Ecotoxicology* 24:1087–1101.

Wyllie I. 1995. Potential secondary poisoning of barn owls by rodenticides. *Pesticide Outlook* 6:19–25.

Zion Market Research. 2018. Rodenticides market to proper with CAGR of 5.6% in the future period. [cited 2018 August 31]. Available from: https://www.zionmarketresearch.com/news/global-rodenticides-market

