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Hazard / Risk Assessment

Responses to sequential anticoagulant rodenticide exposure

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

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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 (BROD). Kestrels fed BROD at 0.3, 1.0 or 3.0 µg/g diet wet wt 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 BROD/g diet, prolonged blood clotting time returned to baseline values within a week, but BROD residues in liver and kidney (terminal half-life estimates >50 d) persisted during the 28 d recovery period. In order to examine the hazard of sequential AR exposure, kestrels were exposed to either the first-generation AR chlorophacinone (CPN; 1.5 µg/g diet) or the SGAR BROD (0.5 µg/g diet) for 7 d, and following a recovery period, were challenged with a low dose of CPN (0.75 µg/g diet) for 7 d. In BROD-exposed kestrels, the challenge exposure clearly prolonged prothrombin time compared to naïve controls and kestrels previously exposed to CPN. These data provide evidence that the SGAR BROD may have prolonged effects that increase toxicity of subsequent AR exposure. As 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.

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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 remain one of the principal chemical groups in the near billion-dollar global rodenticide industry (Zion Market Research 2016). While their risks to children, companion animals and non-target 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 two decades, over 40 reports have documented the presence of AR residues in tissues of non-target predatory or scavenging birds and mammals. A summary of exposure data from North America and Europe indicates that over 58% of the nearly 4200 specimens tested contain residues of one or more ARs (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 (BROD) and other SGARs, due to the long tissue half-lives of these compounds. However, some specimens contain residues of both the shorter half-life first-generation anticoagulant rodenticides (FGARs) as well as SGARs (e.g., Lima and Salmon 2010; Murray 2011; Christensen et al. 2012; Serieys et al. 2015; Murray 2017). While 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 longstanding question in the fields of wildlife toxicology and forensics is the extent to which contaminant exposure and 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 BROD in outbred Wistar laboratory rats (*Rattus norvegicus*). In this study, rats were given BROD 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 post-exposure. However, activity of hepatic microsomal vitamin K epoxide hydroxylase (VKOR; key enzyme in vitamin K recycling antagonized by ARs; Rattner et al. 2014) remained inhibited relative to controls for at least 30 d post-exposure. On day 8 post-BROD 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). While this warfarin dose caused only modest anticoagulant effects in naïve (i.e., unexposed) control rats, pronounced coagulopathy developed within 24 h in rats previously exposed to BROD. Another group of BROD-

exposed rats received the warfarin challenge dose some 25 d post-exposure, and still exhibited coagulopathy, albeit less than that observed in rats challenged with warfarin 8 d post-BROD exposure. Overall findings from this pharmacologic study suggest that SGAR-exposed rats may more readily exhibit coagulopathy with subsequent AR exposure events compared to naïve rats. Somewhat related studies in Japanese quail (*Coturnix japonica*) examined BROD (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 BROD, the magnitude and duration of coagulopathy was greater upon repeat SGAR exposure compared to birds receiving a single SGAR dose. For difenacoum, the magnitude of anticoagulant effect increased upon 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.

Herein we report findings of a series of studies examining toxicity, liver residues and recovery following dietary exposure to environmentally realistic concentrations of the SGAR BROD, and the response to sequential FGAR and SGAR exposure. These studies used the American kestrel (*Falco sparverius*) as an avian wildlife 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 Patuxent, were moved from flight pens to small outdoor cages (1.2 m long x 0.8 m wide x 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 two wk, during which time they were fed dead lab mice (*Mus musculus*) or dead day old chicks (*Gallus gallus*). Kestrels were then shifted to a diet of two ~35 g Nebraska Classic Bird of Prey diet meatballs (Nebraska Brand; hereafter NBP) containing the vitamin supplement Vionate® (Gimborn) prior to exposure trials. All procedures were approved by the Institutional Animal Care and Use Committee of the USGS-Patuxent Wildlife Research Center and the USDA National Wildlife Research Center.

Diets Used in Exposure Trials

Several NBP diets were used in the exposure trials. These include (i) control diet containing 1% vehicle (1 part acetone, 9 parts corn oil), (ii) various brodifacoum (BROD) diets, nominally targeted to contain between 0.3 to 3.0 µg BROD/g wet wt, and (iii) chlorophacinone (CPN) diets, nominally targeted to contain either 0.75 or 1.5 µg CPN/g wet wt. Stock solutions were prepared by dissolving mg quantities of BROD (CAS 56073-10-0; Sigma-Aldrich) or CPN (CAS 3691-35-8; Santa Cruz Biotehnology, Inc.) 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 NPB), 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, BROD and CPN meatballs were collected for chemical analysis and moisture determination. Nominal dietary concentrations of BROD and CPN used in various trials were analytically verified to be close to target concentrations (75.5 to 101.0% of target).

Concentrations of BROD and CPN in test diets are believed to be environmentally realistic for rodent prey in foraging proximity to anticoagulant rodenticide control and eradication activities. For example, nominal concentrations of BROD in test diets ranged from 0.3 to 3.0 µg/g wet wt; average carcass concentrations in target meadow voles (*Microtus pinorum*) and black rats (*Rattus rattus*) following field control and eradication activities have been reported to be 0.35 and 3.75 µg/g wet wt, respectively (Merson et al. 1984, Pitt et al. 2015), and estimated whole body concentrations in several species of non-target small mammals range from 0.3 to 3.6 µg/g wet wt (Shore and Coeurdassier 2018). Similarly, nominal concentrations of CPN in test diets were 0.75 and 1.5 µg/g wet wt, which fall within the range (0.131 to 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 CPN 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 as BROD and CPN concentrations in liver of small mammal prey can be much greater than in the rest of their 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. There is also 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 non-target raptors following a control or eradication activity.

Exposure trials

In a range finding trial (September-October 2014, ambient temperature 7.22 to 23.9°C), kestrels (8 females, 12 males; 2 years old) were housed individually in kraft paper lined pens and fed NBP meatballs for at least 2 wk. Birds (weighing 94.0 to 129.8 g) were randomly assigned to groups (n = 5/treatment) and provided two 25 ± 0.1 g meatballs daily containing vehicle or 0.3, 1.0 or 3.0 µg BROD/g wet wt 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 d 0, 3, 5 and 7 of treatment. An estimate of average daily food intake and BROD consumption was obtained for each bird by collecting all 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 BROD 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 x *g* for 5 min, and resultant plasma was divided and stored in separate vials for clotting time assays. Kestrels were humanely euthanized using carbon dioxide, 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 BROD exposure (April to May 2016, ambient temperature 3.3 to 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 to 7 years 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 BROD/g wet wt diet for a 7-day exposure period (n = 30). During this exposure phase, food consumption was monitored (n = 6 vehicle controls and 12 BROD-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 BROD-treated kestrels (d 0 post-exposure) were weighed, physically examined, blood sampled, sacrificed and then

necropsied. All remaining birds were then shifted to a diet of NBP. On d 2, 4, 7, 14 and 28 post-exposure (recovery period), a single vehicle-treated control kestrel and groups of 5 BROD-treated kestrels were likewise weighed, examined, bled, euthanized and then necropsied, with portions of liver and kidney stored at -80°C for BROD residue analysis.

The third trial examined clotting time and other measures of toxicity following sequential exposure to an FGAR or an SGAR followed by a FGAR (April to 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 to 4 years old) that had been fed mice. Five to 6 d after venipuncture, kestrels were shifted to a diet of NBP meatballs for 9-10 d. Kestrels (weighing 93.8 to 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 CPN/g wet wt diet (n = 6) or 0.5 µg BROD/g wet wt (n = 8) during an initial exposure phase. Consumption of CPN or BROD at these dietary concentrations for 7 d results in coagulopathy (Rattner et al. 2015, and trial 1 of this study). These 20 kestrels were then shifted to NBP vehicle control diet for a 7-d recovery phase, during which time clotting time likely returns to baseline values. These kestrels were then fed 0.75 µg CPN/g wet wt 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 (CON-CPN), chlorophacinone-chlorophacinone challenge (CPN-CPN), and brodifacoum-chlorophacinone challenge (BROD-CPN). 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 CPN-challenge phases. Food consumption was monitored during the initial exposure and CPN-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, necropsied and portions of pectoral muscle, heart, intestine, kidney and liver were formalin-fixed and a portion of the liver was 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 BROD and CPN were performed by several analytical methods. Performance of each method including accuracy, precision, and detection limits are detailed in the Supplemental Data section.

The concentration of BROD and CPN mixed into NBP fed to kestrels was quantified in subsets of meatballs. In trial 1 (range finding study), BROD concentration (mean ± SD; 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), BROD 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), BROD concentration (n = 4; nominally 0.5 µg/g NBP wet wt) averaged 75.3 ± 4.28% of the target concentration and CPN 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 BROD nor CPN were detected (<MLOD) in a subset of control NBP meatballs from trials 1, 2 and 3 (n =

5, 3 and 2, respectively). Concentrations of BROD and CPN in liver and kidney are presented in the Results section.

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 post-translational 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 rodenticide toxicity studies. Thus, it is important to verify that fibrinogen concentration is adequate to promote clot formation. Reagents, conduct and assay performance in kestrels has been previously described in detail (e.g., Rattner et al. 2011, 2015; Hindmarch et al. 2019). Additional background information and data on intra- and inter-assay precision of sample analyses in this study are provided in the Supplemental Data section. *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 semi-quantitative 0-4 scale (none, minimal, mild, moderate, and severe, respectively).

Statistical analyses

Using SAS (version 9.3 and 9.4, SAS Institute) or R (version 3.5.1), body weight change relative to d 0, average daily food intake, estimated daily dose of BROD and CPN consumption and 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 HSD test, Student's t-test or the Kruskal-Wallis non-parametric ANOVA with Dunn's multiple pairwise post-hoc test. For trial 1, the quantity of ingested BROD and hematocrit were used to estimate a dietary-based toxicity reference value (Filipsson et al. 2003; US EPA 2011a). Response parameters included BROD 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) and lower bound (BMDL) of the 95% confidence interval were calculated using eight 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 BROD concentration at the end of the dietary exposure phase (d 0 post-exposure) through post-exposure d 28 (overall half-life), and also between d 0 to 4 (initial phase half-life) and d 7 to 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 seemed to suggest a slight decrease at the intermediate and highest dietary BROD concentrations (Table 1), although there were no statistical differences in food intake among groups (95% CIs overlapped, ANOVA $F_{3,16} = 2.69$, $p = 0.0810$). Relative to initial body weight (d 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 BROD differed among groups (log transformed values, ANOVA $F_{2,12} = 181.80$, $p < 0.0001$), with hepatic BROD residue concentrations differing (ANOVA $F_{2,12} = 17.75$, $p = 0.0003$) and plateauing at dietary doses of 1.0 and 3.0 µg BROD/g wet wt (Table 1).

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

p = 0.0009 and Dunn's tests with Bonferroni correction both p values = 0.0043). Russell's viper venom time was also prolonged in BROD-exposed kestrels compared to controls (ANOVA $F_{3,16}$ = 22.14, $p < 0.0001$ and Tukey's HSD $p \leq 0.0045$; Figure 1). Hematocrit was reduced by ingestion of 1.0 and 3.0 µg BROD/g wet wt (ANOVA $F_{3,16}$ = 24.15, $p < 0.0001$ and Tukey's HSD $p \leq 0.0069$, Figure 1), with some birds being classified as anemic (hematocrit <30%; intermediate BROD dose: 1 of 5 anemic, highest BROD dose: 4 of 5 anemic). Comparing daily BROD consumption to the number of kestrels in a group with hematocrit <30% after a 7-d exposure (i.e., x respondents/5 individuals) yielded a BMD10 (BMDL) of 146.4 (39.2) µg BROD consumed/kg body wt-d (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 BROD/g wet wt (Table 1). However, 4 of the 5 kestrels ingesting 3.0 µg BROD/g wet wt exhibited overt signs of intoxication (e.g., subdued behavior, oral cavity pallor, blood on foot) by d 5 of the trial (d 5); 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 BROD/g wet wt 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 protozal 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 BROD-treated diets did not differ (265.8 ± 52.85 versus 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 start of the trial) did not differ at the end of the exposure phase (d 0 post-exposure) and during the remainder of the recovery period (d 2-28 post-exposure) when compared to vehicle controls (ANOVA $F_{6,29} = 1.88$, $p = 0.1179$) (Table 1). Based upon food consumption and the concentration of BROD 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 BROD/kg body wt-day (cumulative 7 d dose ~0.760 mg/kg body wt). At the end of the 7-d BROD exposure phase (d 0 post-exposure), hepatic BROD residues were in the same range as observed kestrels consuming the low dietary BROD dose in trial 1 (Table 2 versus Table 1, 95% CIs overlapped). Over the 28-d recovery period, BROD concentration decreased markedly in liver (ANOVA $F_{5,24} = 13.37$, $p = 0.0001$ and Tukey's HSD for d 0 versus d 7, d 14 and d 28 p values ≤ 0.0026) and kidney (ANOVA $F_{5,24} = 11.45$, $p = 0.0001$ and Tukey's HSD for d 0 versus d 4, d 7, d 14 and d 28 p values ≤ 0.0014) (Table 2). The

overall BROD half-life estimate ($t_{1/2}$) in liver was 27.7 d ($y = 1270.3e^{-0.025x}$; initial $t_{1/2}$ from d 0 to 4 = 11.9 d and terminal phase $t_{1/2}$ from d 7 to 28 = 49.5 d) and the overall BROD $t_{1/2}$ estimate in kidney was 34.6 d ($y = 330.02e^{-0.02x}$; initial $t_{1/2}$ from d 0 to 4 = 5.9 d; terminal phase $t_{1/2}$ from d 7 to 28 = 69.3 d) (Figure 3). Notably, there was a positive association (slope 3.18, SE 0.34) between liver and kidney residues in BROD-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, 6.3 to 17.0 s for PT, 11.3 to 22.3 s for RVVT, and 37.7 to 51.1% for hematocrit. With the exception of one sample (problematic blood draw on d 7 post-BROD exposure), all samples collected during the trial produced clots in TCT assay, and fibrinogen concentrations did not differ among controls and various groups of BROD-exposed kestrels (log transformed values, ANOVA $F_{6,28} = 1.63$, $p = 0.1751$) (Table 2). Following BROD 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 HSD $p = 0.0035$) (Figure 4). By d 7 post-BROD exposure, PT values had generally returned to the range of baseline values. Likewise, RVVT values were elevated at the end of the 7 d BROD exposure phase compared to controls (log transformed values, ANOVA $F_{6,28} = 5.63$, $p = 0.0006$ and Tukey's HSD $p = 0.0031$), and generally returned to the range of baseline values by d 7 post-BROD exposure (Figure 4). Despite evidence of coagulopathy following BROD-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 d 4, 1 bird on d 7, and 1 bird on d 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 CPN challenge phases of the trial.

During the initial exposure phase, a single kestrel fed 1.5 µg CPN/g wet wt had a small drop of blood on its cere, and during the CPN challenge phase drops of blood were observed on the kraft paper lining the pen of 1 kestrel in the BROD-CPN group. No overt signs of intoxication were observed in any of the other kestrels. Food consumption did not differ among the CON-CPN, CPN-CPN and the BROD-CPN groups 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). Chlorophacinone consumption 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, d 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 $F_{2,14} = 16.33$, $p = 0.0002$). While body wt in kestrels fed mice for the entire trial (untreated controls) was stable, birds receiving various NBP diets containing ARs exhibited incremental wt loss during the recovery and challenge phases (Table 3). While CPN consumption did not differ among treatment

groups during the challenge phase, hepatic CPN residues did (ANOVA $F_{2,17} = 14.86$, $p = 0.0002$). Compared to the BROD-CPN exposure group, liver CPN concentrations were actually greater in both the CON-CPN and CPN-CPN groups (Tukey's HSD $p = 0.0037$ and 0.0002, respectively). Some 14 d after BROD consumption, hepatic BROD residue concentrations were similar to those observed on d 14 post-exposure in the recovery study (Table 3 versus Table 2).

Baseline fibrinogen values ($n = 26$ kestrels) ranged from 45.9 to 100.4 mg/dL, and at the end of the CPN 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 CON-CPN and CPN-CPN groups (Tukey's HSD $p = 0.9289$ and 0.9978, respectively). However, many of kestrels initially exposed to the SGAR BROD and then challenged with CPN (i.e., BROD-CPN) had PT values that were clearly prolonged (6 of 8 exceeded upper reference limit, and 8 of 8 were >125% of their individual baseline value). Prothrombin time of the BROD-CPN group was longer than that of the untreated control (Tukey's HSD $p = 0.0458$) and CPN-CPN ($p = 0.0302$) groups. On an individual

bird basis, baseline and end of the trial PT values were examined using a paired *t*-test. Prothrombin time increased in the BROD-CPN group (log transformed values, $t = 5.32$, df = 7, $p = 0.0011$), while no pronounced change was observed in any of the other groups (log transformed values, $t = 0.78$ to 2.16, df =5, $p = 0.0833$ to 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 section), all samples were re-assayed at a 4:5 dilution factor which somewhat resolved this seemingly spurious observation. Baseline RVVT values of these re-assayed 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). Russell's viper venom time was prolonged in the BROD-CPN group compared to untreated controls (Tukey's HSD $p = 0.0171$) (Figure 5). While RVVT in the BROD-CPN group did not differ from the CPN-CPN group (Tukey's HSD $p = 0.3469$), it was marginally greater than in the CON-CPN 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 CPN 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 dose groups. As noted above, 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 BROD results in dose-dependent effects on hemostasis, as evidenced by bruising, frank or microscopic hemorrhage, prolonged clotting time, and anemia. Upon termination of BROD exposure, overt signs of toxicity and coagulopathy were resolved within a week. Following the 7 d recovery period, subsequent exposure to an environmentally realistic dietary concentration of CPN prolonged PT in kestrels initially exposed to BROD suggestive of lingering and potentially long-term differences in sensitivity of SGAR-exposed individuals.

Comparison of BROD toxicity in kestrels with other species of birds

With the notable exception of BROD, acute toxicity data indicate that ARs are one to two orders of magnitude more potent in rodents than commonly tested avian species (Rattner and Mastrota 2018). Based upon Northern bobwhite and mallard acute oral and dietary toxicity data (LD50 = 0.25-11.6 mg/kg body wt, LC50 = 1.33-2.75 mg/kg diet or ppm; Rattner and Mastrota 2018), BROD is categorized as being “very highly toxic” to birds (US EPA 2004, 2011b) and is the most potent AR registered in the US, much of Europe and elsewhere. Although data are available on the liver residue concentrations of BROD and other SGARs that are associated with mortality in raptors,

robust empirical data of the dietary dose of BROD causing lethality in seemingly more sensitive predatory and scavenging birds (Rattner and Mastrota 2018) are lacking. Focusing on secondary exposure, several studies have been conducted in barn owls (*Tyto alba*) fed BROD-exposed rodents (*Rattus sp., Mus sp.* fed BROD 0.002 to 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 post-exposure. The cumulative dietary dose of BROD associated with mortality in barn owls was highly variable, ranging from 0.15 to 5.4 mg/kg body wt (Newton et al. 1990; Gray et al. 1994; Lee 1994), and was likely even less due to regurgitation of varying quantities of the administered dose (Newton et al. 1990, 1994).

Secondary exposure studies in Falconiformes have documented that consumption of BROD-poisoned rodents (0.005% bait) for 4 d resulted in death of red-tailed and red-shouldered hawks (*Buteo jamaicensis* and *B. lineatus*), while golden eagles (*Aquila chrysactos*) survived, but exhibited external bleeding (Marsh and Howard 1978). Somewhat more definitive are the results of a 5 d dietary study in which American kestrels were fed ground vole (*Microtus* spp.) tissue containing BROD (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 BROD “potentially” consumed/kg body wt), while at 6.0 ppm, 4 of 8 kestrels died (~7.3 mg BROD consumed/kg body wt kestrel over 5 d) (Lavoie 1990). The lowest nominal dietary BROD concentration in our

range finding study was 0.3 ppm, and based on food consumption measurements, the cumulative 7 d dose was estimated to be 0.46 mg/kg body wt (i.e., 0.271 ug BROD/g food wet wt x 240.2 g food wet wt consumed/kg body wt x 7 d = 455.7 ug/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 BROD dose associated with anemia in 10% of the exposed kestrels is ~1 mg/kg body wt (Supplemental Data, Figure S1; 146.4 µg BROD consumed/kg body wt-d x 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 BROD (cumulative dose ~0.76 mg/kg body wt; i.e., 108.5 µg BROD consumed/kg body wt-d x 7 d), no mortality was observed over the 4 wk post-exposure period. Thus, data from the present study suggest that a 7-d dietary exposure at ≤0.5 ppm BROD constitutes a sublethal dose in kestrels, while greater dietary BROD 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, and lethality. Taken together, captive American kestrels are seemingly more tolerant to BROD (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 BROD 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 BROD in kestrels and other species of birds

Several BROD dietary exposure trials with captive birds describe residue concentrations in liver of individuals exhibiting hemorrhage, coagulopathy, or mortality. Liver BROD residues in 4 of 6 barn owls that died 6 to 17 d after being fed poisoned mice for a single day (cumulative BROD dose to barn owls ~0.150 to 0.182 mg/kg body wt) ranged from 0.63 to 1.25 µg/g wet wt (1.20 to 2.38 nmol/g) (Newton et al 1990). In another study barn owls were fed BROD-poisoned mice for up to 15 d (cumulative BROD dose to barn owls ~1.9 to 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 to 1.67 µg/g wet wt, 1.05 to 3.19 nmol/g) to 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 to 3.97 mg/kg body wt) and hepatic BROD residues (1.39 to 2.10 µg/g wet wt, 2.65 to 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 CPN challenge phase of our sequential exposure study was that hepatic CPN residues in kestrels initially exposed to BROD were lower compared to residue concentrations observed in the other challenge groups (CON-CPN, CPN-CPN) (Table 3). As consumption of CPN during the challenge phase did not differ among treated groups, it seems likely that BROD residues in kestrel liver may have limited CPN 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) data demonstrated that accumulation was biphasic, likely involving two accumulation sites (Huckle et al. 1988). The high affinity flocoumafen binding site was saturated at about 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) one day per week had relatively constant liver flocoumafen residues between 4 and 20 weeks of exposure (0.38 to 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 BROD accumulating at 2-3 nmol/g liver and having a greater affinity than the FGAR coumatetralyl (Parmar et al. 1987). Not unlike our observation of limited liver CPN accumulation in BROD-exposed kestrels are repeat SGAR dose exposures in quail. Specifically, initial exposure to BROD (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 wt some 26 days later in a dose-dependent manner when compared to quail that received only difenacoum (Butler 2010).

An often-cited residue toxicity threshold (i.e., potentially lethal range) for BROD and other SGARs in liver is >0.1 to 0.2 µg/g wet wt (Newton et al. 1999a, 1999b). This threshold was derived from studies with captive barn owls exposed to SGARs and from post-mortem examination of wild barn owls that had liver SGAR concentration >0.1 µg/g wet wt with classic signs of 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 wt and in 20% of the individuals with concentrations at 0.08 µg/g wet wt (Thomas et al. 2011). For BROD, 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 to 2.10 µg/g wet wt, 1.05 to 4.01 nmol/g wet wt, Newton et al. 1990, Gray et al. 1994, and 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 our kestrel study, BROD residues in liver were much greater (1.44 µg/g wet wt, 2.75 nmol/g after 7 d BROD exposure to 0.700 µg/g wet wt, 1.34 nmol/g on d 28 post-exposure) than values in these environmentally-exposed raptors (Thomas et al. 2011), and while coagulopathy was initially observed in kestrels (d 0 to d 7 post-BROD exposure), there was no mortality whatsoever 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 BROD, 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 EPA 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, Wantanabe et al. 2010, 2015) and possibly diet-based (Thijssen 1995) mechanisms that could result in AR tolerance.

Several BROD 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 BROD/kg body wt had relatively similar liver BROD residues (~0.40 to 0.45 µg/g wet wt, Butler 2010; 0.23-0.51 µg/g wet wt; Webster et al. 2015) even though the exposure dose differed by an order of magnitude and post-exposure sample collection ranged from 1 to 26 d. Likewise, a single 0.5 mg BROD/kg body wt oral dose administered to domestic chickens yielded liver residue values averaging 0.62 to 0.70 µg/g wet wt over a 14 d post-exposure period (Fisher 2009). These data demonstrate BROD persistence in liver of birds. Various studies in laboratory rodents indicate that the half-life of BROD 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 BROD/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 BROD half-life in liver and kidney of American kestrels (cumulative 7 d BROD dose ~0.76 mg/kg body wt) over a 4-week period was only 27.7 d and 34.6 d, respectively. However, between d 7 and 28 post-exposure, 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 scope and purpose of this recovery study). While the half-life of BROD in kestrel liver is shorter than reported in rodents and quail, it is lengthy compared

to 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 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 non-target 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 environmental realistic concentrations of the SGAR BROD causes coagulopathy that is resolved within a week, yet subsequent challenge exposure to the FGAR CPN 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 BROD exposure, followed by difenacoum exposure weeks later enhanced both the magnitude and duration of anticoagulant effect (Butler 2010). Together, these controlled exposure studies birds 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 CPN/g wet wt diet) failed to prolong clotting time in BROD-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/g 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 BROD 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 CPN concentration in BROD-exposed kestrels was 0.517 µg/g (~1.38 nmol/g), and when added to the BROD value (~1.36 nmol/g) on a molar basis totaled 2.74 nmol/g, a hepatic BROD concentration that was coincident with coagulopathy in trials 1 and 2 (i.e., >2.65 and 2.19 to 2.75 nmol/g, respectively) and associated with increased PT and RVVT in trial 3. Ignoring differences in inhibition potency of BROD (IC50 ~0.22 µM) and CPN (5.1 µM) for kestrel hepatic microsomal VKOR activity (unpublished data, J-K. Tie and X. Chen, University of North Carolina), hepatic residues of kestrels only exposed to CPN were lower (0.748 and 0.827 µg CPN/g), and on a nmol/g basis (~1.92 and ~2.21 nmol CPN/g), below that observed in birds exhibiting coagulopathy in trial 2 (~2.75 nmol BROD/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/g or ppm basis. Moreover, while 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.

While our sequential exposure study was focused on anticoagulant effects that could potentially affect individuals and even local populations of non-target 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 non-target wildlife.

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

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https://www.sciencebase.gov/catalog/item/5d279019e4b0941bde650f17; and IP-102045: Histopathology of American kestrels (*Falco sparverius*) sequentially exposed to first and second generation anticoagulant rodenticides. U.S. Geological Survey data release,

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Figures

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

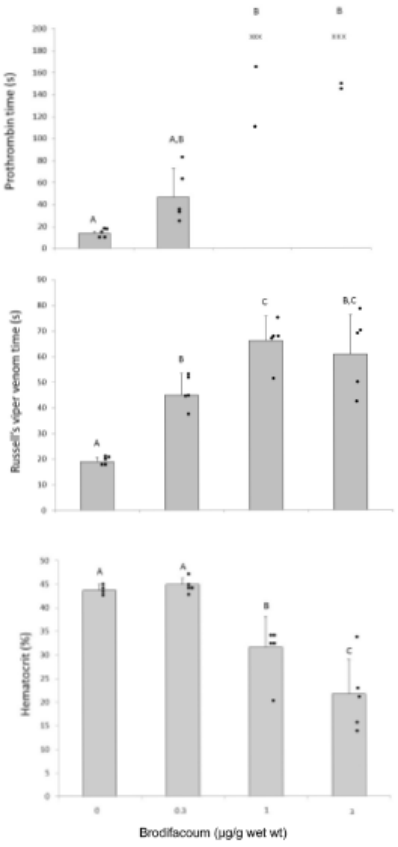

Figure 2. Mild (A), moderate (B) and severe (C) interstitial hemorrhage in heart or skeletal muscle of American kestrels following 7 d dietary exposure to BROD. (A) Mild hemorrhage is characterized by few small discrete areas of hemorrhage in the myocardial interstitium (scale bar = 200 µm); (B) moderate 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).

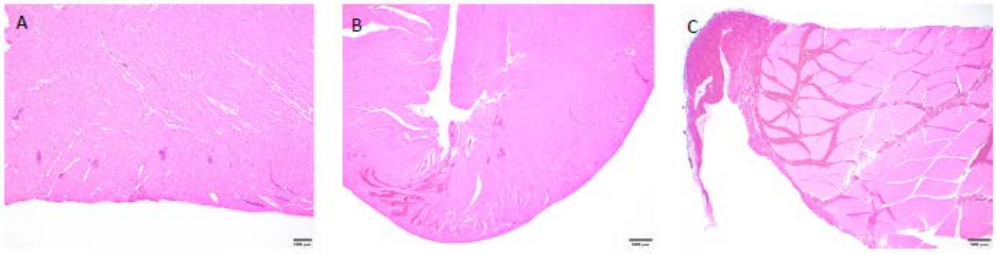

Figure 3. Concentrations of BROD in liver and kidney of American kestrels at various post- exposure times following a 7 d dietary exposure to 0.5 µg BROD/g wet wt (n = 5 birds at each post-exposure sampling time).

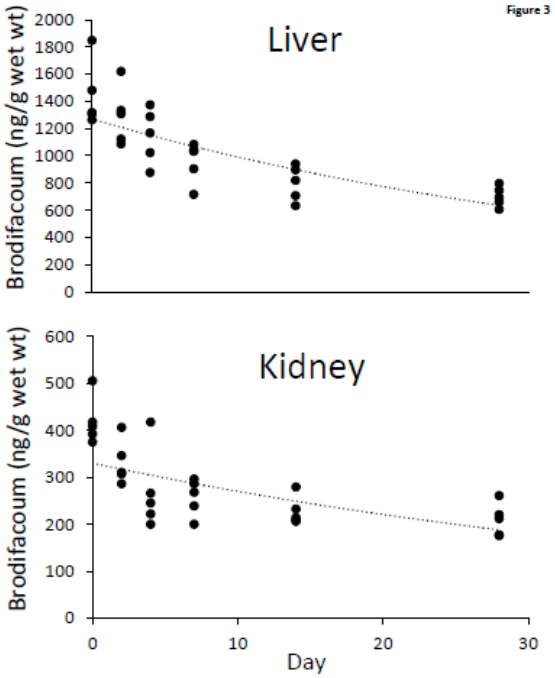

Figure 4. Prothrombin time and Russell's viper venom time (individual values and mean ± SD) of American kestrels following a 7 d dietary exposure to 0.5 µg BROD/g wet wt and at various post-exposure times (n = 4-5 exposed birds at each sampling time and n = 6 controls). Horizontal shaded area encompasses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

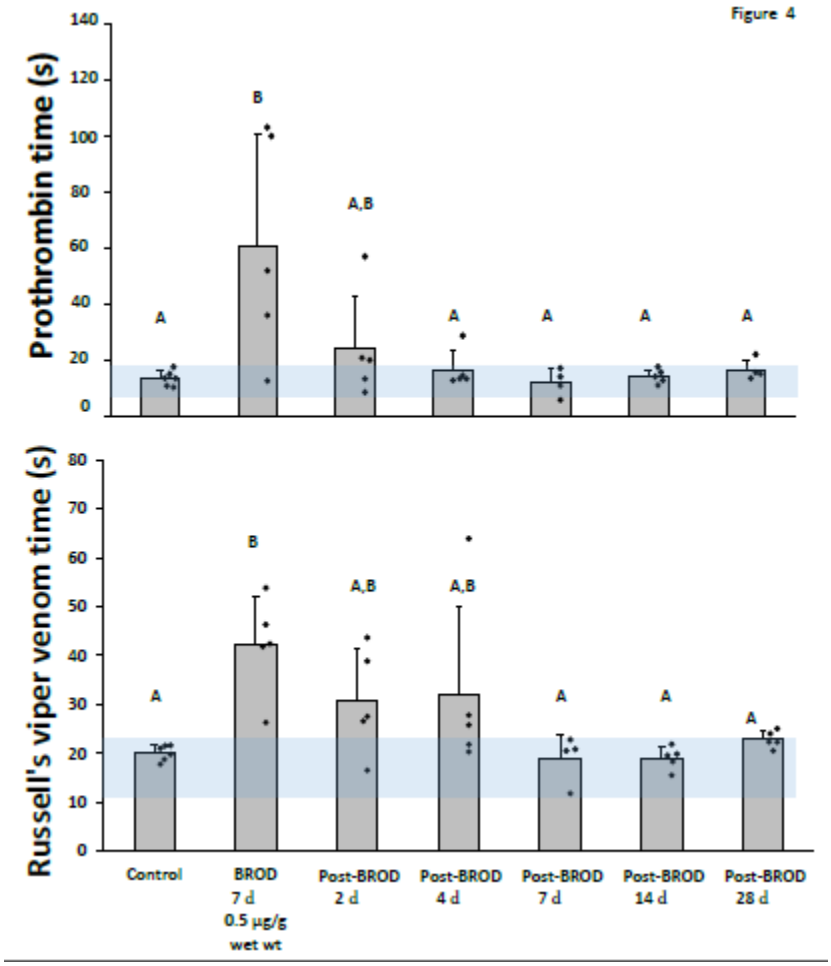

Figure 5. Prothrombin time and Russell's viper venom time (individual values and mean ± SD) of American kestrels following various first- and second-generation anticoagulant rodenticide dietary exposure regimens (n = 6-8 kestrels per group). Horizontal shaded area encompasses the range of baseline values determined at least 2 wk before the trial. Bars with different letters are significantly different by Tukey's HSD test ($p < 0.05$).

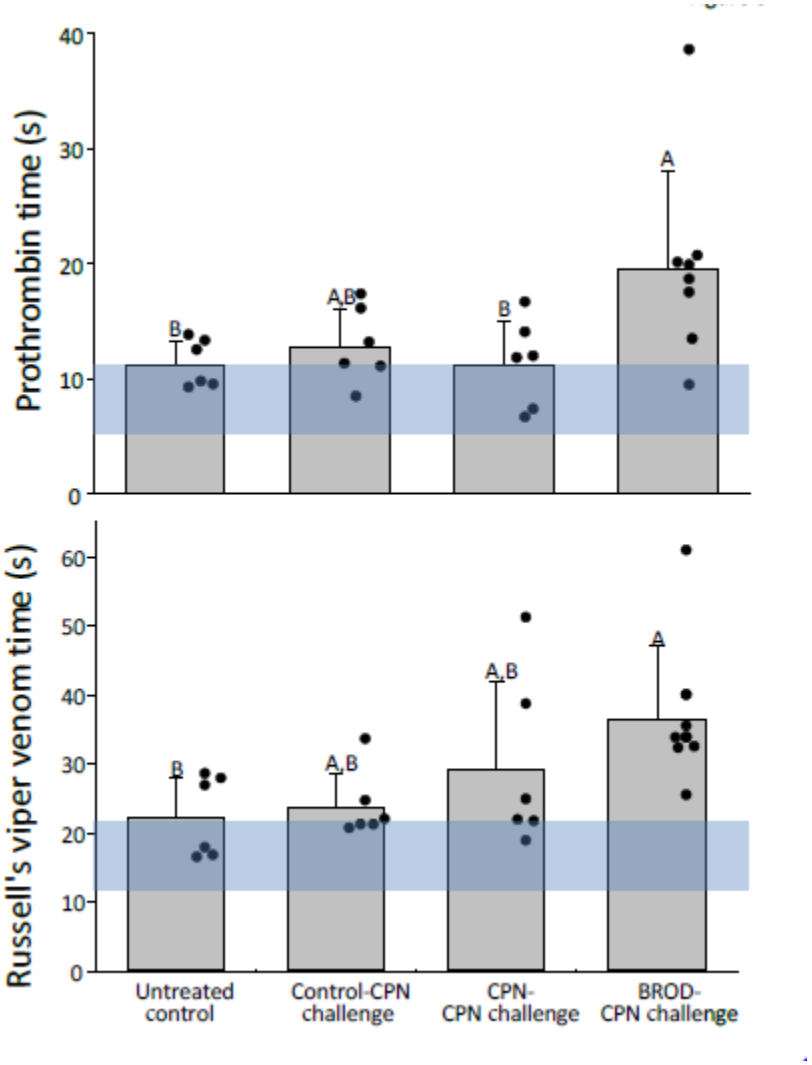

Figure 6. Individual baseline and post-exposure prothrombin time values of American kestrels following various first- and second-generation anticoagulant rodenticide dietary exposure regimens (n = 6-8 kestrels per group) p values for each group are by paired t-test.

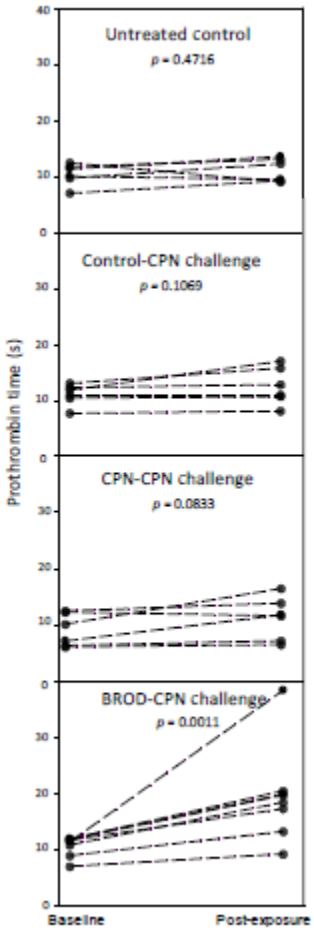

Table 1 Estimated daily exposure, sublethal responses and liver residues following brodifacoum ingestion in America kestrels[a]

	Control	Brodifacoum (µg/g food wet wt)		
Nominal concentration	0	0.3	1.0	3.0
Analtyically-verified concentration	<MLOD	0.271 ± 0.016	0.994 ± 0.026	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 ± 11.62[C]	207.7 ± 22.73[B]	567.6 ± 126.30[A]
Cumulative 7 day BROD dose (mg/kg		~0.46	~1.45	~3.97

body wt)				
Liver BROD (µg/g wet wt)	<MLOD-0.015	1.39 ± 0.137[B]	2.06 ± 0.268[A]	2.10 ± 0.212[A]
Liver BROD (nmol/g wet wt)		~2.65	~ 3.92	~4.01
Fibrinogen (mg/dL)	69.8 ± 17.93[B]	65.3 ± 12.57[B]	158.8 ± 26.37[A]	195.8 ± 27.81[A]
Overt signs of intoxication	0/5	0/5	1/5	5/5
			Bruise on featherless tract on d 7	Blood in oral cavity on d 7
				Subdued behavior d 5 and 6
				Tongue pale on d 5, subdued behavior on d 7
				Blood on right foot

				d 5
				Tongue pale on d 5
Histological evidence of mild to severe hemorrhage	0/5	3/5	2/5	4/5
Gross abnormalities at necropsy	0/5	1/5	1/5	4/5
		Bled profusely when dissected	Blood in oral cavity	Bruise on pectral 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 ± SD (n = 5 kestrels/group) or respondents/n. For continuously distributed endpoints, groups with different capital letter superscripts are different by Tukey's HSD method ($p < 0.05$).

BROD = brodifacoum; MLOD = method limit of detection.

Table 2. Body weight change, sublethal responses and tissue residues following brodifacoum ingestion in America kestrels[a]

	Control	Days post-brodifacoum exposure (0.5 µg/g food wet wt)[b]					
		d 0	d 2	d 4	d 7	d 14	d 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 ± 0.240^{A}	1.29 ± 0.212^{A,B}	1.15 ± 0.200^{A,B}	0.955 ± 0.149^{B,C}	0.799 ± 0.129^{C}	0.700 ± 0.074^{C}
Liver BROD (nmol/g wet		~2.75	~2.46	~2.19	~1.82	~1.53	~1.34

wt)

Kidney BROD (µg/g wet wt)	<MLOD	0.419 ± 0.050 A	0.331 ± 0.047 A,B	0.270 ± 0.086 B,C	0.258 ± 0.039 B,C	0.228 ± 0.030 C	0.209 ± 0.035 C
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 ± SD.

[b] Analytically verified concentration was 0.469 ± 0.024 µg/g feed wet wt.

Means with different capital letter superscripts are different by Tukey's HSD method ($p < 0.05$).

BROD = brodifacoum; MLOD = method limit of detection.

Table 3. Estimated daily exposure, body weight change, and liver chlorophacinone and brodifacoum residues following sequentical exposure of kestrels[a]

Treatment	Control	Initial Exposure Phase - Challenge Exposure Phase		
	Untreat	CON-	CPN-	BROD-

	ed	CPN	CPN	CPN
n	6	6	6	8
Initial Exposure Phase (d 0 to 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-verifed 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 bwt-d)	NA	215.3 ± 19.78	210.0 ± 11.16	235.8 ± 40.56
CPN or BROD consumed (ug/kg body wt-d)	0	0	286.7 ± 15.24	88.8 ± 15.28
Weight change (d 0 to 7: Δg/100 g body wt)	−0.85 ± 8.705	−0.24 ± 3.306	+0.02 ± 1.510	+0.78 ± 3.193
Recovery Phase (d 8 to 14)	dead mice	NBP diet	NBP diet	NBP diet
Weight change (d 0 to 14: Δg/100 g	+0.26	−7.32 ±	−5.46 ±	−4.67 ±

body wt)	± 8.598	1.258	3.184	4.428
Challenge Exposure Phase (d 15 to 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-verifed 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 bwt-d)	NA	179.6 ± 34.73	178.1 ± 47.33	187.7 ± 39.76
CPN consumed (ug/kg body wt-d)	0	127.4 ± 24.63	126.4 ± 33.57	133.2 ±28.20
Weight change (d 0 to 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 ± 0.135^{A}	0.827 ± 0.122^{A}	0.517 ± 0.081^{B}
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 ± SD.

Means with different capital letter superscripts are different by Tukey's HSD method ($p < 0.05$).

BROD = brodifacoum; CON = control; CPN = chlorophacinone; MLOD = method limit of detection; NA = not analyzed; NBP = Nebraska bird of prey.