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Authors

Rattner, Barnett A.
Erickson, Richard A.
Lankton, Julia S.
et al.

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Brodifacoum Isomer Formulations with Potentially Lower Risk to Non-Target Wildlife

Barnett A. Rattner
U.S. Geological Survey, Eastern Ecological Science Center, Laurel, Maryland
Richard A. Erickson
Upper Midwest Environmental Science Center, La Crosse, Wisconsin
Julia S. Lankton
U.S. Geological Survey, National Wildlife Health Center, Madison, Wisconsin
Etienne Benoit
USC1233 RS2GP, VetAgro Sup, INRAe, University of Lyon, Marcy-l'Etoile, France
Virginie Lattard
USC1233 RS2GP, VetAgro Sup, INRAe, University of Lyon, Marcy-l'Etoile, France

ABSTRACT: Anticoagulant rodenticides (ARs) have a long history of successful use in controlling vertebrate pest and invasive species. Despite regulatory efforts to mitigate risk, non-target wildlife may be unintentionally exposed to ARs through various trophic pathways, and depending on dose, exposure can result in adverse effects and mortality. Second-generation ARs (SGARs) are mixtures of *cis*- and *trans*-diastereoisomers (each including two stereoisomers) that exhibit similar *in vitro* inhibitory potency for vitamin K epoxide reductase in rodent microsomal assay systems. Some diastereoisomers and hence some individual stereoisomers are preferentially metabolized *in vivo*, resulting in residue patterns in exposed target rodents that differ from the bait formulations. Use of less persistent but equally potent SGAR stereoisomers in baits results in lower tissue residues in target rodents, which in turn constitutes lower risk when consumed by non-target wildlife. The toxicity of two brodifacoum formulations with stereoisomers having markedly different elimination half-lives in rats (Formulation A containing the two least persistent stereoisomers, and Formulation B containing the most persistent stereoisomer) were tested in a 7-day dietary feeding trial with American kestrels. Based on previous kestrel studies using commercially available brodifacoum, Formulations A and B were each provided at three dietary concentrations (0.05, 0.1 and 0.5 µg/g diet, 4 kestrels/dose level) predicted to cause a range of toxicity. Compared to unexposed controls, all kestrels that ingested 0.5 µg/g diet of the longer-lived Formulation B exhibited extreme coagulopathy. In contrast, the 0.5 µg/g diet of the shorter-lived Formulation A yielded only a modest lengthening of clotting time in just 1 of the 4 exposed kestrels. These findings support the notion that SGAR baits enriched with less persistent stereoisomers may pose lower hazard and ultimately risk to non-target wildlife.

KEY WORDS: anticoagulant, birds, brodifacoum isomers, *Falco sparverius*, hazard, non-target effects, risk

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INTRODUCTION

The demand for vertebrate pest control supports a multi-billion-dollar annual sale of rodenticides worldwide (MarketsandMarkets™ Inc., 2022). For the past 75 years, anticoagulant rodenticides (ARs) have been in widespread use and are a highly effective method for control of commensal and invasive rodent pest species (Jacob and Buckle 2018). Their mechanism of action involves inhibition of vitamin K epoxide reductase (VKOR) that impairs activation of blood clotting factors, which can ultimately lead to hemorrhage and death (reviewed in Rattner et al. 2014, Feinstein et al. 2016, Rattner and Harvey 2021). Although ARs are effective in controlling target pest species, an ongoing challenge has been the presence and adaptive development of resistance mechanisms in targeted species (e.g., behavioral, *Vkorc1* gene, natural, pharmacokinetic; reviewed in McGee et al. 2020). Furthermore, under approved application methods, non-target organisms may be exposed and adversely affected by direct ingestion of AR bait (primary exposure) or indirectly by consuming AR-poisoned prey (secondary exposure). Such non-target AR exposure and poisoning have been documented in wildlife on nearly a global scale in numerous peer-reviewed publications (reviewed in López-Perea and Mateo 2018, Nakayama et al. 2019).

It is well-recognized that development of new, innovative, and safer tools to control vertebrate pests is costly and time consuming from a chemical synthesis, efficacy testing, safety evaluation and registration standpoint (Blackie et al. 2014, Hohenberger et al. 2022). However, recent findings from laboratory and field studies have demonstrated that bioaccumulation and retention of ARs in higher vertebrates may vary among isomer composition, with some second-generation AR (SGAR) diastereoisomers being rapidly and preferentially metabolized by target rodents (Damin-Pernik et al. 2017, Lattard and Benoit 2019, Lefebvre et al. 2020). Results of pharmacologic and toxicokinetic studies have shown: (i) *cis*-isomers of an SGAR are not interconverted *in vivo* into *trans*-isomers of that SGAR, (ii) inhibitory potencies of SGAR *cis*- and *trans*-forms for at least wild-type VKOR are relatively similar (i.e., equipotent in rodent *in vitro* microsomal assay

systems), and (iii) the initial half-life *cis*- and *trans*-SGARs for liver differ substantially in laboratory rats (Damin-Pernik et al. 2016, 2017).

For commercial formulations of the SGAR bromadiolone (70-90% trans-isomers, 10-30% cis-isomers), the *trans*-isomers are the predominant forms detected in laboratory rats (*Rattus norvegicus*), and in non-target red kites (*Milvus milvus*) and red fox (*Vulpes vulpes*), indicating elimination of *cis*-isomers by the double filter of target and non-target species (Damin-Pernik et al. 2017, Fourel et al. 2017, 2018). A recent large-scale analysis of 529 liver samples from 18 species of raptors demonstrated enantioselective bioaccumulation of just one of each of the four diastereoisomers for brodifacoum, bromadiolone, difenacoum, and difethialone which accounted for nearly 97% of the SGAR residues (Fourel et al. 2024). It was again suggested that formulations using the other less persistent and less bioaccumulative SGAR stereoiosomers could reduce exposure and tissue residues in non-target species and might constitute more "eco-friendly" AR baits.

We are currently investigating the hazard, accumulation, metabolism, and excretion of isomer formulations of the SGAR brodifacoum in American kestrels (*Falco sparverius*), a longstanding model species for raptors (Hohenberger et al. 2022) and some other raptors (e.g., Falconiformes) (Bardo and Bird 2009). Herein, we describe initial findings on the toxicity of two such formulations with differing half-lives in a 7-day feeding trial with captive American kestrels.

MATERIALS AND METHODS

Animals

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the U.S. Geological Survey (USGS) Eastern Ecological Science Center (EESC) as described in document 2020-3 P. Procedures used are similar to those of previous brodifacoum toxicity trials with American kestrels (Rattner et al. 2020). In the spring of 2023, adult two- to three-year-old male kestrels propagated at the EESC at the Patuxent Research Refuge, Laurel, Maryland, were transferred from flight pens to small outdoor cages (1.2 × 0.8 × 0.6 m) that had a shade roof, perches, food tray and water bowl. Individually housed kestrels were initially fed dead mice (previously frozen and thawed whole individuals) for 8 days, during which time a 0.9 mL jugular venipuncture sample was collected into 0.1 mL of 3.2% sodium citrate from each bird for determination of baseline clotting time. Microhematocrit was determined and then the remainder of each blood sample was centrifuged (2,000 × *g* for 5 min), citrated plasma harvested, and various volumes pipetted into cryotubes that were frozen and stored at -80ºC for clotting time assays. Kestrels were then shifted to a daily diet of two ~35 g Classic Bird of Prey diet (Nebraska Brand, North Platte, NE; hereafter NBP) formed into meatballs containing the vitamin supplement Vionate® (MiracleCorp Products, Dayton, OH) for at least 15 days prior to initiation of the brodifacoum exposure trial. The NBP diet contains menadione (i.e., inactive vitamin K_3 provitamin) at a nominal concentration of 0.3 mg/kg diet that approaches the National Research Council recommendation for laying poultry (National Research Council 1994; see Rattner and Mastrota 2018 for discussion of menadione inclusion in AR-exposure studies).

Brodifacoum Exposure Trial

Data from previous AR feeding trials with captive American kestrels using environmentally realistic concentrations of brodifacoum (LaVoie 1990, Rattner et al. 2020) guided dose selection for the present study. Specifically, following a 7-day dietary exposure of kestrels, dose-dependent hemorrhage, histopathological lesions and coagulopathy were observed at brodifacoum concentrations of 0.3, 1.0 and 3.0 µg/g diet wet weight (ww), and a dietary-based benchmark dose (i.e., toxicity reference value) at which anemia occurs (hematocrit <30%) in 10% of exposed kestrels was estimated to be 146 µg brodifacoum consumed/kg body weight per day (Rattner et al. 2020). We selected dietary concentrations that bracketed what we believed to be the onset of toxic signs (i.e., ~0.3 µg/g diet ww) in a 7-day exposure trial.

Brodifacoum (CAS 56073-10-0) was purchased from Sigma-Aldrich Chimie (St. Quetin Fallavier, FR) as 59% *cis*- and 41% *trans*-isomers, and was fractionated into its four stereoisomers, here referred to in elution order as E1 (a *trans* isomer), E2 (a *cis* isomer), E3 (a *cis* isomer), and E4 (a *trans* isomer). This was accomplished by high pressure liquid chromatography on a preparative Lux Cellulose-3 Chiral column (250 x 4.6 mm), comprised of a chiral stationary phase of tris(4-methylbenzoate) cellulose particles (Phenomenex, Le Pecq, FR), and using as liquid mobile phase a mixture formed by methanol (A), ethanol and formic acid in a proportion by volume of 0.1% in the ethanol (B) and water (C), with an A/B/C volume ratio of 95/0/5, for 23 min. This was followed by a gradient among A, B and C that reached in 5 min a final phase with an A/B/C volume ratio of 55/45/0 at a flow rate of 3 mL /min in the chromatography column. Each brodifacoum stereoisomer was tested separately by gavage (3 mg/kg) in both male and female Sprague-Dawley laboratory rats, with liver and blood samples collected at 24 to 216 hours post-exposure to estimate half-life during the initial phase of clearance. Elution fractions E1 and E2 exhibited an initial phase half-life in liver estimated to be three times shorter (initial $T_{1/2}$ ~ 2.3 to 3.4 hours) than the initial phase half-life of fraction E3 (i.e., more slowly cleared, initial $T_{1/2}$ ~ 6.9 to 10.5 hours) (V. Lattard, VetAgro Sup, INRAe, University of Lyon, unpublished data, April 26, 2023). Elution fractions E1 and E2 were combined to yield Formulation A (i.e., more quickly cleared), whereas Formulation B consisted of almost exclusively E3 (i.e., more slowly cleared) (Figure 1). Notably, the initial phase $T_{1/2}$ of elution fraction E4 differed considerably between male and female rats (1 versus 6 hours, respectively; V. Lattard, unpublished data, April 26, 2023), and was not included in the kestrel dietary exposure trial.

Several test diets were prepared and used in the exposure trial. Brodifacoum Formulations A and B prepared at the University of Lyon, Marcy-l'Etoile, FR were shipped to the EESC as 5 mg neat quantities in 5 mL volumetric flasks. The content of each flask was reconstituted with spectral grade acetone (HPLC grade; Sigma-Aldrich). Fractions of Formulation A and Formulation B were pipetted into volumetric flasks and brought to the mark by

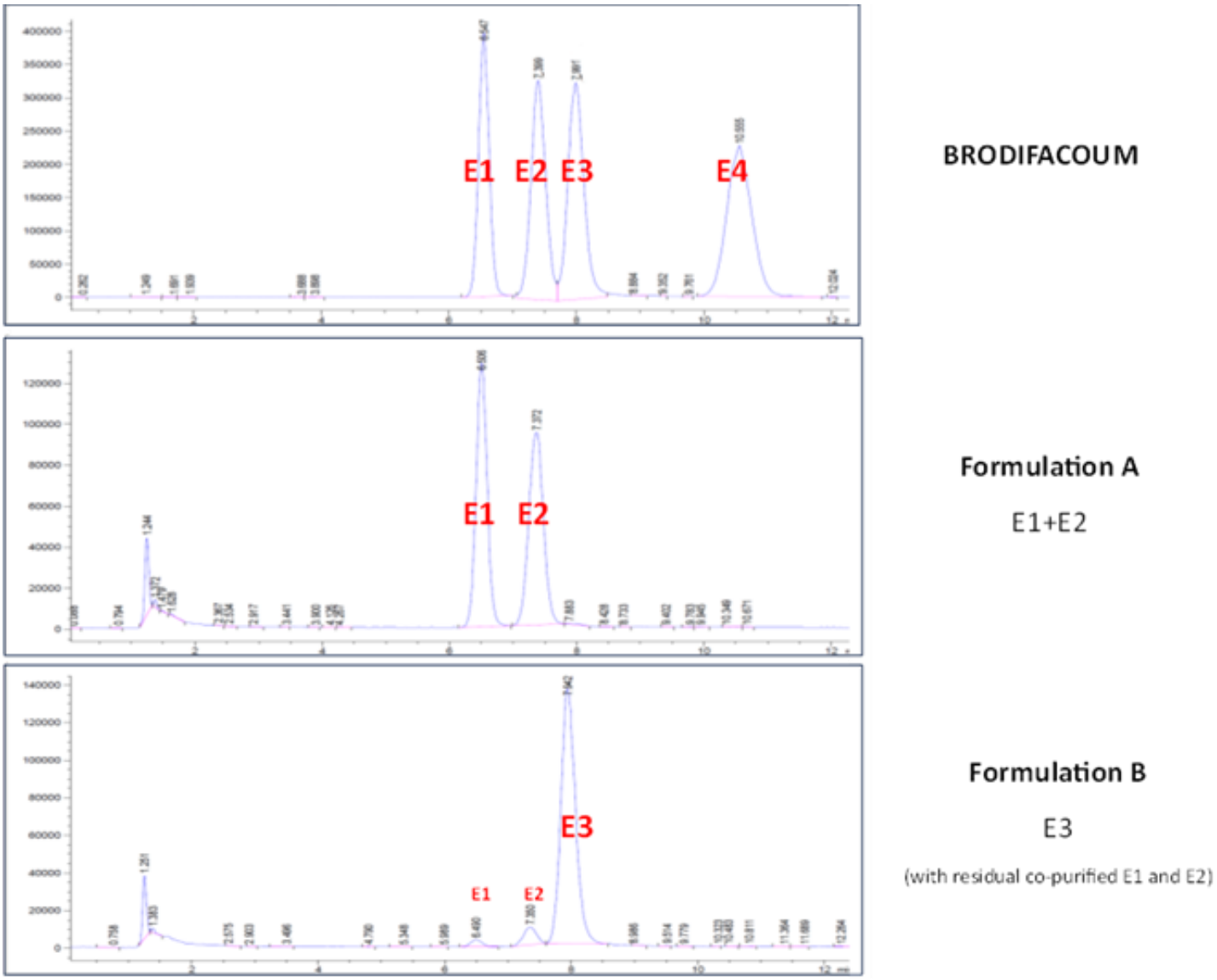

Figure 1. LC/MS analysis of Formulations A and B on a Lux Cellulose-3 Chiral column (150 × 2 mm, 3µm). E = elution fraction.

addition of acetone and corn oil (Mazola; ACH Food Companies, Inc., Chicago, IL) to a final ratio of 1:9. Either the vehicle (control group) or varying quantities of brodifacoum Formulation A or brodifacoum Formulation B were added to several kilograms of NBP (10 mL/kg), mixed by hand for 10 min, and then placed in a fume hood for 30 min to evaporate some acetone solvent. Vionate® supplement was then added (3.4 g/kg NBP), diet was remixed by hand for 5 min, and then placed again in a fume hood for another 30 min. Diets were formed into 25 ± 0.1 g meatballs, and batches of meatballs for each daily feeding were stored in plastic bags at -10°C. Samples of vehicle and brodifacoum meatballs were collected for chemical analysis and moisture determination. Nominal dietary concentrations of brodifacoum Formulation A (i.e., 0.05, 0.10 or 0.50 µg/g ww) or brodifacoum Formulation B (i.e., 0.05, 0.10 or 0.50 µg/g ww) were analytically verified to be close (70 to 100%) to target concentrations. For the exposure trial, kestrels (n=4/dietary dose) received either untreated NBP (controls), Formulation A at dietary concentrations of 0.05, 0.10 or 0.50 µg/g ww, or Formulation B at dietary concentrations of 0.05, 0.10 or 0.50 µg/g ww. Kestrels were briefly observed each day in the morning and afternoon for overt signs of intoxication. Birds received two 25 ± 0.1 g meatballs daily between 1100 and 1200 hours, and uneaten food scraps were carefully removed from each kraft paper lined pen the following day between 0800 and 1100 hours. The scraps were weighed, stored, desiccated in a drying oven and mass converted back to wet weight as previously described (Rattner et al. 2015, 2020). Kestrels were weighed and physically examined at the beginning of the trial (day 0), and again on days 3 and 5 of the exposure trial. At the end the study (day 7), each bird was examined, weighed, bled (0.9 mL jugular venipuncture blood drawn into 0.1 ml 3.2% sodium citrate), sacrificed using carbon dioxide, and then necropsied.

Histopathology

Formalin-fixed liver, kidney, pectoral muscle and portions of intestine were processed and embedded in paraffin (American HistoLabs, Gaithersburg, MD) using standard procedures (Luna 1968). Tissues were sectioned at approximately 6 μm, mounted on slides, and stained with Carazzi's hematoxylin and eosin. Histologic evaluation of samples was performed by a board-certified veterinary pathologist and score were assigned for hemorrhage severity on a semi-quantitative 0-4 scale (none, minimal, mild, moderate, and severe, respectively). Other microscopic abnormalities noted within the case set were scored as absent or present.

Prothrombin Time Assay

Prothrombin time of citrated kestrel plasma samples was used to evaluate effects of dietary exposure to brodifacoum on post-translational processing of clotting Factors II, VII, IX and X. The conduct, performance, and use of this assay in birds has been previously described in detail (Rattner et al. 2010, 2015, 2020). The mechanical clot endpoint was determined using a START4 Fibrometer (Diagnostica Stago Inc., Parsippany, NJ). The coefficient of variation for duplicate determination of baseline samples (N=27; 1 sample with clot in plasma excluded) and samples from the exposure trial (N=26; 2 venipuncture samples were incomplete and of poor quality) averaged 1.14%.

Statistical Methods

Initial body weight, weight change, average daily food and brodifacoum consumption, hematocrit and prothrombin time were compared among the seven groups (control, three dietary doses of Formulation A, three dietary doses of Formulation B) using an analysis of variance technique followed by Tukey's honestly significance difference (HSD) multiple comparison (Zar 2010). Clotting time and diet consumption were $\log_{10}$ transformed prior to analysis. A plot of residuals against fitted values, scale-location plot, normal Q-Q plot, and a plot of residuals against fitted leverages were used to assess fit, normality, and similar assumptions for each model (Dalgaard 2008). Prothrombin time 95% reference intervals, which provide the range of clotting time values in healthy unexposed individuals, were calculated with the baseline plasma sample values using the refLimit function in R (referenceInterval package version 1.3.0; Finnegan 2022). Because of the relatively small sample size (n=27 samples) for establishing the prothrombin time upper reference limit (Friedrichs et al. 2012), a 90% confidence interval around the upper reference limit was used as a guide to identify samples with seemingly prolonged clotting time (outliers). All analyses were conducted using R version 4.3.1 (R Core Team 2023).

RESULTS

Body weight of kestrels at the initiation of the exposure trial (Day 0) did not differ among treatment groups (one-way ANOVA $F_{6,21}$=1.001, P=0.451; Table 1). Relative to initial body weight, there was no effect of treatment on body weight (change in g/100 g initial weight) over the course of the trial, but there was slight weight loss (up to 4.35%) in all groups (repeated measures ANOVA: treatment $F_{6,21}$=0.994, P=0.455; day ANOVA $F_{3,63}$=20.63, P<0.0001; interaction of treatment and day $F_{18,63}$=0.951, P=0.524). Average daily food consumption (g consumed/kg initial body weight) did not differ among groups (one-way ANOVA $F_{6,21}$=2.534, P=0.053). Nominal brodifacoum consumption (μg/kg body weight-day) differed among the low, intermediate, and high dose groups of Formulations A and B (one-way ANOVA $F_{5,18}$=53.2, P<0.001), but not between comparable dietary doses of Formulations A and B (Tukey's HSD, P>0.95).

On the final day of treatment, a single male consuming the 0.50 μg brodifacoum Formulation B/g ww diet exhibited a small amount of blood in its oral cavity and had gurgling respiratory sounds. However, the other three birds receiving the high dose of Formulation B did not exhibit overt signs of intoxication, as was also the case for all the other brodifacoum-exposed and control kestrels in the study. Following jugular venipuncture (adequate blood samples for clotting time assay obtained from 26 of 28 individuals) and euthanasia, necropsy did not reveal macroscopic evidence of excessive hemorrhage in any individuals. Microscopic examination of tissues revealed mild to moderate hemorrhage present in 11 of 111 of tissues examined, including samples from the control group; hemorrhage was not related to dietary concentration of either brodifacoum formulation. Other observations included non-specific immune activation, suspect polyomavirus inclusions in the kidney, and minimal hepatocellular necrosis, but these were not considered likely to be clinically significant or related to brodifacoum exposure.

Hematocrit differed across groups (one-way ANOVA $F_{6,19}$=3.33, P=0.021), but post-hoc analysis could not differentiate any group differences (Tukey's HSD, P>0.05). Using 27 pre-study citrated plasma samples from the kestrels in this trial, we estimated prothrombin time reference intervals (Table 2) and used the upper reference limit and the upper bound of the 90% confidence interval to assist in identifying birds with prolonged clotting time. All four of the kestrels ingesting 0.50 μg/g ww of Formulation B had prolonged prothrombin time (Figure 2), with values exceeding the upper bound of the 90% confidence interval of the upper reference limit (i.e., 15.76 seconds, outlier threshold) by over 8-fold. In contrast, of the four kestrels ingesting 0.50 μg/g ww of Formulation A, none had prothrombin time exceeding the 15.76 second outlier threshold, although prothrombin time of one kestrel in this high dose group almost matched the outlier threshold. One-way analysis of variance revealed differences in prothrombin time among the seven groups ($F_{6,19}$=98.7, P<0.001), with values of 0.50 μg/g ww of Formulation B differing from all other groups (Tukey's HSD, P<0.001).

DISCUSSION

To the best of our knowledge, this is the first controlled exposure study in birds demonstrating that brodifacoum formulations composed of different isomers vary in their toxicity. Using prothrombin time as a sublethal indicator of toxicity, 7-day dietary exposure of American kestrels to 0.50 μg/g ww of brodifacoum Formulation A (fraction E1

Table 1. Body weight, food consumption, and brodifacoum consumption as mean (standard deviation).

Group	n	Initial body weight (g)	Body weight change day 0 to (g/100g)	Food consumption (g/kg body weight-day)	Nominal brodifacoum consumption (µg/kg body weight-day)
Control	4	116.1 (6.5)	-2.3 (1.6)	164.6 (21.1)	---
0.05 µg Fm A/g diet	4	109.5 (6.5)	-0.4 (2.5)	203.8 (11.9)	10.2^{C} (0.6)
0.10 µg Fm A/g diet	4	107.4 (6.7)	-2.4 (1.0)	189.6 (27.6)	19.0^{B} (2.8)
0.50 µg Fm A/g diet	4	116.2 (8.2)	-3.6 (3.1)	150.3 (30.4)	75.1^{A} (15.2)
0.05 µg Fm B/g diet	4	113.9 (6.3)	-2.2 (1.2)	176.7 (24.5)	8.8^{C} (1.2)
0.10 µg Fm B/g diet	4	118.0 (12.6)	-2.6 (0.5)	198.3 (27.0)	19.8^{B} (2.7)
0.50 µg Fm B/g diet	4	110.7 (6.9)	-4.4 (1.8)	160.5 (31.3)	80.2^{A} (15.6)

Abbreviation: formulation (Fm).
Mean brodifacoum consumption with different letter superscripts are different by Tukey's HSD method P<0.001).

Table 2. Reference intervals for prothrombin time in captive adult male American kestrels (*Falco sparverius*).[A]

Descriptive Statistic	Prothrombin Time(s)
Sample size	27
Extremes	11.6 - 15.2
Arithmetic mean	13.47
Median	13.6
Standard deviation	0.81
Lower reference limit	11.79
Lower 90% confidence interval	11.25 - 12.27
Upper reference limit	15.24
Upper 90% confidence interval	14.82 - 15.76

[A] 95% reference interval based on Gaussian distribution.

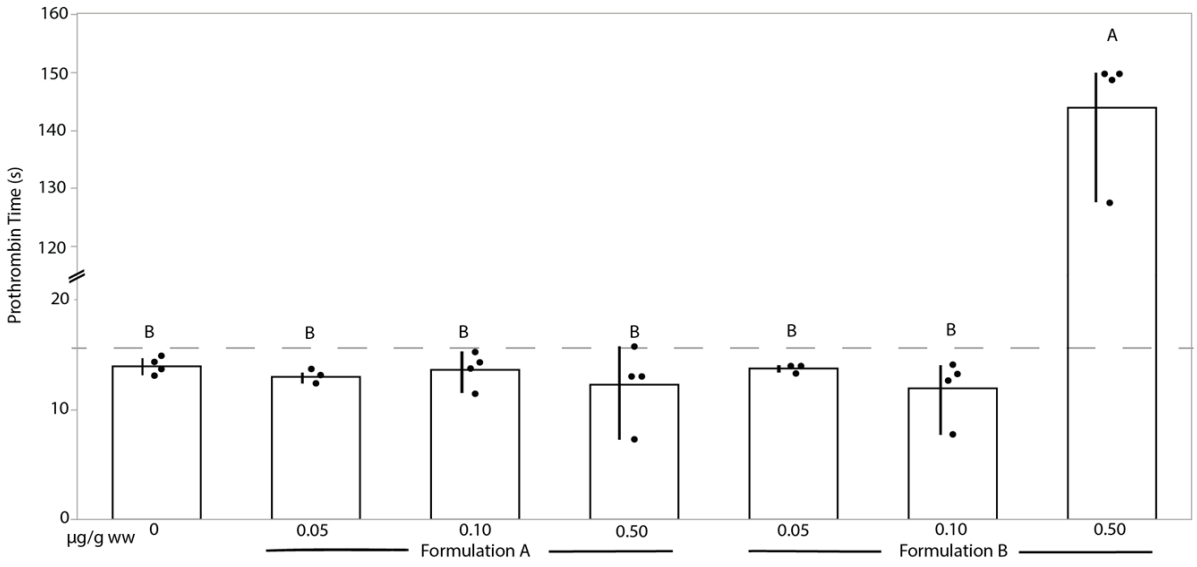

Figure 2. Prothrombin time (seconds; individual values and mean ± SD) of American kestrels following a 7-day dietary exposure to various concentrations of two brodifacoum formulations. Dashed line is the upper bound of the 90% confidence interval of the upper reference limit. Bars with different letters are significantly different by Tukey's HSD test (P<0.001).

and E2, shorter initial half-life isomers in rat) did not affect clotting time compared to the extreme coagulopathy observed at the same dietary dose of Formulation B (fraction E3, longer initial half-life isomer in rat; present study) or a commercial mixture containing all four isomers (trial 2 in Rattner et al. 2020; previous study). Both the *cis*- and *trans*-isomers of brodifacoum (Damin-Pernik et al. 2017) as well as other SGAR diastereoisomers (Damin-Pernik et al. 2017, Lattard and Benoit 2019, Lefebvre et al. 2020) have relatively similar inhibitory potency for VKOR in rodent microsomal *in vitro* assay systems. Similar to our findings with brodifacoum formulations in kestrels, studies by Damin-Pernik and co-workers (2016) demonstrated that *cis*-isomers of difenacoum administered to OFA-Sprague Dawley rats prolonged prothrombin time while shorter-lived *trans*-isomers did not, most likely due to the differential half-life of the diastereoisomers (i.e., longer-lived *cis*-isomer difenacoum causing greater toxicity). Liver, whole blood and fecal samples from the present kestrel study are being analyzed for brodifacoum isomers in Formulations A and B, and we hypothesize that there will be lower residues of Formulation A isomers in liver and blood, and likely greater residues of Formulation A isomers in fecal samples.

A recent study (Hohenberger et al. 2022) identified modifications in chemical structure of rodenticides to reduce ecotoxicity, bioaccumulation and persistence as critical short-, medium- and long-term strategies in the implementation of safe and sustainable-by-design principles for rodenticides. As described in that paper, one of the quickest ways to reduce adverse environmental effects while retaining rodenticide efficacy is the use of pure isomers that take advantage of shorter half-life and other pharmacokinetic properties. In fact, alpha-bromadiolone, principally composed of the short-lived *cis*-isomer (3-[(1RS,3RS)-3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxycoumarin) is undergoing consultation as a new candidate for substitution under the Biocidal Products Regulation (European Chemicals Agency 2020). As recently demonstrated for the SGAR difethialone (Lefebvre et al. 2020), once pharmacokinetic properties of individual isomers in target rodents are well-understood, it is possible to model, simulate and select combinations of *cis*- and *trans*-isomers that are efficacious with minimal ecotoxicity. An added benefit of such an *in silico* approach could be the reduction in animal use in support of the regulatory risk assessment for product review and potential approval.

In conclusion, our findings lend support to the notion that SGAR formulations composed of select shorter half-life stereoisomers pose lower risk to predatory and scavenging wildlife. Such data add to the weight of evidence supporting development and potential use of potent yet eco-friendly rodenticide formulations.

DATA AVAILABILITY STATEMENT

Much of the data described in this manuscript is publicly available: Rattner, B. A., R. A. Erickson, J. S. Lankton, and V. Lattard. 2024. Brodifacoum isomer formulation study: U.S. Geological Survey data release https://doi.org/10.5066/P14U4QQM, and J. S. Lankton, and B. A. Rattner. 2024. Histopathology of American kestrels (*Falco sparverius*) exposed to two brodifacoum isomer formulations with differing elimination half-lives: U.S. Geological Survey data release https://doi.org/10.5066/P1UMMZQV.

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