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*Environmental Toxicology*TOXICOKINETICS AND COAGULOPATHY THRESHOLD OF THE RODENTICIDE
DIPHACINONE IN EASTERN SCREECH-OWLS (*MEGASCOPS ASIO*)BARNETT A. RATTNER,*† KATHERINE E. HORAK,‡ REBECCA S. LAZARUS,† DAVID A. GOLDADE,‡
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Abstract: In the United States, new regulations on second-generation anticoagulant rodenticides will likely be offset by expanded use of first-generation anticoagulant rodenticides. In the present study, eastern screech-owls (*Megascops asio*) were fed 10 µg diphacinone/g wet weight food for 7 d, and recovery was monitored over a 21-d postexposure period. By day 3 of exposure, diphacinone (DPN) was detected in liver (1.63 µg/g wet wt) and kidney (5.83 µg/g) and coagulopathy was apparent. By day 7, prothrombin time (PT) and Russell's viper venom time (RVVT) were prolonged, and some individuals were anemic. Upon termination of exposure, coagulopathy and anemia were resolved within 4 d, and residues decreased to <0.3 µg/g by day 7. Liver and kidney DPN elimination occurred in 2 phases (initial rapid loss, followed by slower loss rate), with overall half-lives of 11.7 d and 2.1 d, respectively. Prolonged PT and RVVT occurred in 10% of the exposed owls with liver DPN concentrations of 0.122 µg/g and 0.282 µg/g and in 90% of the owls with liver concentrations of 0.638 µg/g and 0.361 µg/g. These liver residue levels associated with coagulopathy fall in the range of values reported in raptor mortality incidents involving DPN. These tissue-based toxicity reference values for coagulopathy in adult screech-owls have application for interpreting nontarget mortality and assessing the hazard of DPN in rodent-control operations. Diphacinone exposure evokes toxicity in raptors within a matter of days; but once exposure is terminated, recovery of hemostasis occurs rapidly. *Environ Toxicol Chem* 2014;33:74–81. © 2013 SETAC. This article is a US Government work and is in the public domain in the USA.

Keywords: Anticoagulant rodenticide Clotting time Half-life Secondary poisoning Toxicity reference value

INTRODUCTION

Anticoagulant rodenticides are used for the control of vertebrate pest species in urban and suburban settings, agriculture, and island restoration projects. Despite widespread use, there are growing concerns about their risk to children, companion and domestic animals, and nontarget wildlife. In the United States, new restrictions have been placed on the use of some second-generation anticoagulant rodenticide baits in and around buildings [1,2]. This regulatory action will probably result in expanded use of some first-generation anticoagulant rodenticides that are considered to be less hazardous than second-generation anticoagulant rodenticides [3].

Although first-generation anticoagulant rodenticides (e.g., chlorophacinone, diphacinone, warfarin) are considered to be less hazardous, they have nonetheless been implicated in nontarget wildlife mortality incidents. For example, diphacinone (DPN) has been identified as a potential cause of unintentional wildlife mortality involving owls (barn owl [*Tyto alba*], snowy owl [*Nyctea scandiaca*]), hawks (Cooper's hawk [*Accipiter cooperii*], red-tailed hawk [*Buteo jamaicensis*]), and scavenging birds (turkey vulture [*Cathartes aura*], bald eagle [*Haliaeetus leucocephalus*]) [4–7]. Recent studies have demonstrated that DPN is approximately 20 to 30 times more acutely toxic to American kestrels (*Falco sparverius*) than reported for traditionally used wildlife test species (mallard [*Anas platyrhynchos*] and northern bobwhite [*Colinus virginianus*]) [8]. Sublethal exposure of kestrels and eastern screech-owls

(*Megascops asio*) resulted in prolonged clotting time, reduced hematocrit, and/or gross and histological evidence of hemorrhage at daily doses as low as 0.24 mg DPN/kg body weight (cumulative dose 1.68 mg/kg) [8,9]. Incorporating these kestrel and owl data into a deterministic risk assessment conducted by the US Environmental Protection Agency [6] and into a probabilistic risk assessment indicated that adverse effects associated with DPN exposure occur at very low levels and that its hazard has been underestimated by risk assessments that rely on acute toxicity test scenarios and on data generated in bobwhites and mallards [10,11].

The present study in eastern screech-owls examined clotting time and tissue residues during dietary exposure to DPN and the time course of recovery from coagulopathy and residue clearance following termination of exposure. Using these data, estimates of liver DPN residues associated with adverse effects were undertaken to more fully evaluate the hazard of first-generation anticoagulant rodenticides to raptorial birds.

MATERIALS AND METHODS

Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committees of the Patuxent Wildlife Research Center and the National Wildlife Research Center. Eastern screech-owls, propagated in flight pens in a captive colony at Patuxent [12], were sexed by gene amplification (CHD1-Z and CHD1-W) using real-time polymerase chain reaction analysis [13]. A total of 40 owls ($n = 24$ males and 16 females) were used in the present study. Birds were moved from flight pens to small, shaded outdoor cages (1.2 m long × 0.8 m wide × 0.6 m high) containing 2

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perches, a food tray, and a water bowl. Owls were offered dead laboratory mice (*Mus musculus*; Charles River Laboratories, fed 5L79 rodent chow containing 3.4 ppm dry wt vitamin K) for 2 wk, at which time a jugular venipuncture sample (0.9 mL in a 1-mL syringe containing 0.1 mL of 3.2% sodium citrate; Sigma-Aldrich) was collected from each bird to determine baseline clotting time. Microhematocrit (50 μ L) was determined on each sample. The remainder of the blood sample was centrifuged (2000 g for 5 min), citrated plasma was harvested, divided into 3 vials that were stored at -80°C , and subsequently used to establish baseline clotting times. Owls were then fed Classic Bird of Prey diet (containing 2.63 ppm dry wt vitamin K; Nebraska Brand) supplemented with Vionate (Gimborn US) for 2 wk. Individuals that readily consumed the Bird of Prey diet during this acclimation period ($n = 37$; 33 fed DPN, 4 controls), along with 3 owls (additional controls) that would only consume mice, were included in the DPN study. The dosing trial was conducted between 24 October and 21 November 2011 (ambient temperature -1.1°C to 20.6°C), and initial body weight (mean \pm standard deviation [SD]) of owls was 171.8 ± 21.71 g.

Diphacinone toxicity and recovery study

Neat DPN was dissolved in acetone, and then corn oil was added (1:20) to yield nominal concentrations of 1.0 mg/mL. The acetone-corn oil vehicle (control) and the DPN solution were mixed into the Bird of Prey diet at a ratio of 1:100, followed by the evaporation of acetone [9]. The control and DPN diets containing nominal concentrations of 0 μ g and 10 μ g DPN/g wet weight (analytically verified, see Diphacinone purity and concentrations in diet and tissues) were formed into meatballs weighing 25.0 ± 0.1 g on a wet weight basis and stored at -20°C . The 10 μ g/g wet weight dietary concentration falls within the range of residues detected in the liver of small mammals (less than the method limit of detection to 12 μ g/g) collected following aerial broadcast application of DPN (Ramik Green, 0.005% DPN; Hacco) for rodent control in Mauna Loa, Hawaii, USA (E.B. Spurr, Pacific Island Ecosystem Research Center, US Geological Survey, Honolulu, HI, unpublished data).

Owls were provided two 25-g meatballs daily for up to 7 d and then shifted to an untreated diet (dead mice) for up to 21 d. Birds were observed 3 times daily (7:00–8:00 AM, 12:00–1:00 PM, 4:00–5:00 PM) during the trial. Just before daily feeding (4:00–5:00 PM), uneaten meatball scraps were collected from the kraft paper-lined pens and saved to estimate DPN consumption (i.e., difference between quantity fed and not eaten, including conversion of dry wt mass back to wet wt) [9]. Groups of DPN-treated owls were weighed, bled, killed, and necropsied on day 3 and day 7 of the exposure period ($n = 5/\text{d}$, sexes divided nearly equal) and on day 1, day 2, day 4, day 7, and day 21 during the postexposure period ($n = 5$ owls/d except on day 21, when $n = 3$; sexes nearly equal). Controls ($n = 4$) received a similar but untreated diet containing the acetone-corn oil vehicle, and additional controls ($n = 3$) were fed mice throughout the trial. A single control owl was weighed and bled each time DPN-treated birds were killed during the course of the exposure and postexposure periods; however, only 4 of 7 controls were killed and necropsied. Microhematocrit values were determined, and plasma samples for clotting time assays and liver and kidney samples for quantification of DPN residues were stored at -80°C .

Clotting time assays

Prothrombin time (PT) and Russell's viper venom time (RVVT) of plasma samples were used to evaluate DPN effects

on posttranslational processing of clotting factors II, VII, IX, and X; and thrombin clotting time (TCT) was used to insure sample quality (i.e., adequate fibrinogen concentration to promote clotting). The assay of PT, RVVT, and TCT in eastern screech-owl samples has been described in detail [9]. The coefficients of variation for duplicate determinations of all PT and RVVT samples (mean \pm SD) were $8.8 \pm 8.38\%$ and $4.2 \pm 5.15\%$, respectively. As previously noted [9], precision deteriorated in samples with clotting time >100 s (extreme coagulopathy). For the TCT assay, the fibrinogen concentration of all DPN-exposed owls exceeded 50 mg/dL, with the precision of duplicate determinations being $2.4 \pm 2.87\%$.

Diphacinone purity and concentrations in diet and tissues

The purity of neat DPN was determined by reversed-phase, ion-paired high-performance liquid chromatography (HPLC) with ultraviolet detection at 285 nm and found to be 99.2% (% w/w) [8]. The Bird of Prey diet and liver and kidney samples were homogenized, solvent extracted, prepped using solid-phase extraction cartridges, and DPN was quantified by reversed-phase ion-paired HPLC [9]. The recovery of DPN from spiked control samples (mean \pm SD) was $99.1 \pm 10.29\%$ for liver ($n = 11$) and $100.6 \pm 7.80\%$ for kidney ($n = 8$). Recovery of the DPN (nominal concentration 10 μ g/g wet wt) from fortified meatballs ($n = 3$ meatballs from each of 4 batches) was $98.3 \pm 9.08\%$, and no DPN was detected in the control meatballs ($n = 3$). For liver and kidney samples, the method limit of detection and method limit of quantification were 0.049 μ g/g and 0.16 μ g/g wet weight for liver and 0.12 μ g/g and 0.41 μ g/g wet weight for kidney, respectively.

Statistical analyses

Body weight change, hematocrit, PT, RVVT, TCT, and DPN consumption and residues were tested for homogeneity of variances (F_{max} test) and normality (Shapiro-Wilk test, normal probability plot), and transformed when appropriate. Diphacinone concentrations below the detection limit were assigned a value of one-half the method limit of detection. Measurement end points were compared by one-way analysis of variance and Tukey's honestly significant difference test. The relations between DPN residues (liver, kidney) and clotting time (PT, RVVT) were examined using linear regression.

Liver and kidney residue half-life estimates were conducted by using DPN concentrations at the end of the exposure period (day 7) through day 21 of the postexposure period. The elimination of DPN appeared to occur in 2 phases; therefore, initial, terminal, and overall half-life were calculated. These half-life calculations were conducted using the slope of the log concentration versus time plot and the following equations: slope = $k_e / -2.303$ and $t_{1/2} = 0.693/k_e$, where k_e is the elimination rate constant.

Assessing the potential hazard of chemicals in toxicology has often depended on the use of a no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL). These estimates in risk assessment have been criticized because they do not adequately describe the exposure-response relationship. In the present study, the concentration of DPN in the liver at which clotting time was prolonged in 10% of the study owls (i.e., tissue-based toxicity reference value) was estimated. The distribution of liver DPN concentrations was divided into quintiles, and the mean DPN residue in a quintile served as the dose. The response parameters included clotting time (continuous, PT or RVVT values in seconds) and the number of individuals with prolonged clotting time

(dichotomous, number of owls in each liver DPN quintile with PT or RVVT exceeding mean baseline clotting time value by 2 SDs). Using the dose–response curve, a benchmark dose at the 10% effect level and a benchmark dose low (lower bound of the 95% confidence interval of benchmark dose 10%) was calculated with different models (gamma, multistage, Weibull, quantal-linear, logistic, log-logistic, probit, and log-probit) for continuous and dichotomous data (Benchmark Dose software, Ver 2.2 [14]). In addition, a benchmark dose was calculated for threshold liver DPN concentrations at which 20%, 50%, and 90% of the study owls exhibited prolonged clotting time. Models were evaluated based on Akaike's information criterion and *p* values for model fit. The benchmark dose was estimated in a similar manner for kidney DPN residues and clotting time.

RESULTS

Toxicity of diphacinone

Cumulative DPN consumption averaged 4.74 mg/kg body weight for owls provided the DPN-treated diet for 3 d (*n* = 5) and, 7.98 mg/kg body weight for individuals that had been exposed for 7 d (*n* = 28; 5 from exposure period + 23 from postexposure period; Table 1). The daily DPN dose during the exposure period averaged 1.21 ± 0.281 mg/kg body weight-d. Small, nonsignificant changes in body weight (−6 to +5 g/100 g body wt) occurred during the trial, with the exception of a marked increase in weight on day 21 postexposure (+24 g/100 g body wt). This weight gain is attributed to greater food consumption accompanying cooler weather and changing seasons toward the end of the trial.

On day 7 of DPN exposure and on day 1, day 2, and day 4 of the postexposure period, several owls exhibited external signs of bruising and internal evidence of hemorrhage (Table 1). The incidence of these signs subsided after day 4 of the postexposure period. Hematocrit values ≤30, presumably indicative of blood loss anemia [15], were observed in 2 of 5 owls killed on day 7 of DPN exposure and in 2 of 5 owls killed on day 1 of the postexposure period.

Before the toxicity study, the baseline value (mean ± SD) for PT was 9.3 ± 1.87 s (range, 4.3–13.9 s) and that for RVVT was 19.0 ± 3.08 s (range, 13.4–25.5 s). On day 3 of exposure, PT was not significantly different from controls (*n* = 7 bled over the course of the study), but all day 3 PT values of exposed owls (range, 13.6–21.9 s) exceeded both the mean of the controls and the prestudy baseline mean by more than 2 SDs (Figure 1). However, RVVT had increased significantly (*p* < 0.0001) on day 3 of exposure compared with controls (Figure 2). Both PT and RVVT values were significantly greater (*p* < 0.0001) than controls by day 7. Upon termination of DPN exposure, coagulopathy and anemia were resolved to baseline values within 1 d to 4 d (Table 1).

Diphacinone residues and tissue half-life

Diphacinone residues in liver and kidney were greatest during the exposure period, notably with average concentrations in kidney exceeding those in liver (nonoverlapping ranges) by over 3-fold (Table 1). Postexposure concentrations decreased rapidly within 24 h; within 1 wk, liver and kidney values were <0.3 µg/g wet weight, and by 3 wk, values were <0.1 µg/g (liver, DPN detected in 2 of 3 owls; kidney, DPN detected in only 1 of 3 owls). The elimination of DPN from liver seemed to occur in 2 phases: an initial phase between day 7 of exposure and day 2 postexposure with a half-life (*t*_{1/2}) of 0.88 d, and then a

Table 1. DPN consumption, body weight change, evidence of hemorrhage, and tissue residues during the exposure and postexposure periods^a

	Controls	DPN exposure					Postexposure				
		3 d	7 d	1 d	2 d	4 d	7 d	21 d			
<i>n</i>	7	5	5	5	5	5	3				
Total DPN consumed during exposure period (mg/kg body wt)	—	4.74 ± 0.902 B	8.03 ± 0.744 A	7.38 ± 1.728 A	7.15 ± 1.185 A	8.49 ± 1.497 A	7.61 ± 0.747 A				
Body weight change (Δg/100 g body wt)	+1.08 ± 5.521 B,C	+4.55 ± 5.921 B	−5.89 ± 4.012 C	+2.60 ± 4.258 B,C	−0.73 ± 3.978 B,C	+3.49 ± 4.463 B,C	−0.16 ± 3.403 B,C				
Individuals showing gross signs at necropsy	0/7	0/5	3/5	4/5	1/5	1/5	0/3				
External or gross signs shown at necropsy	—	—	Frank blood, bruising, pale viscera	Bruising, pale viscera	Bruising	Pooled blood in muscle	—				
Hematocrit (%)	44.4 ± 3.42 A,B	44.0 ± 2.86 A,B	34.4 ± 9.70 B	36.4 ± 9.062 A,B	38.6 ± 4.81 A,B	43.1 ± 1.43 A,B	46.2 ± 1.25 A				
Hematocrit ≤30	0/7	0/5	2/5	2/5	0/5	0/5	0/3				
Liver (µg DPN/g wet wt)	<MLOD ^b	1.628 A (0.966–2.93)	0.955 A,B (0.653–1.65)	0.427 B,C (0.247–0.699)	0.273 C (0.091–0.448)	0.270 C (0.109–0.449)	0.176 C,D (0.113–0.211)				
Kidney (µg DPN/g wet wt)	<MLOD ^b	5.834 A (4.97–5.65)	5.523 A (4.03–7.07)	1.550 B (0.795–2.11)	1.156 B,C (0.689–2.01)	0.691 C (0.495–1.060)	0.285 D (0.145–0.629)				

^a Values presented are mean ± SD for DPN consumption, body weight change, and hematocrit; respondents/*n* for overt or gross signs at necropsy and hematocrit ≤30; and geometric mean (range in parentheses) for diphacinone residues. Values in a row with different capitalized letters are significantly different by Tukey's HSD method (*p* < 0.05).

^b *n* = 4 owls that were sacrificed; all values <MLOD, not included in statistical analysis.

^c 2 of 3 values <MLOD; no mean calculated, not included in statistical analysis.

DPN = diphacinone; MLOD = method detection limit.

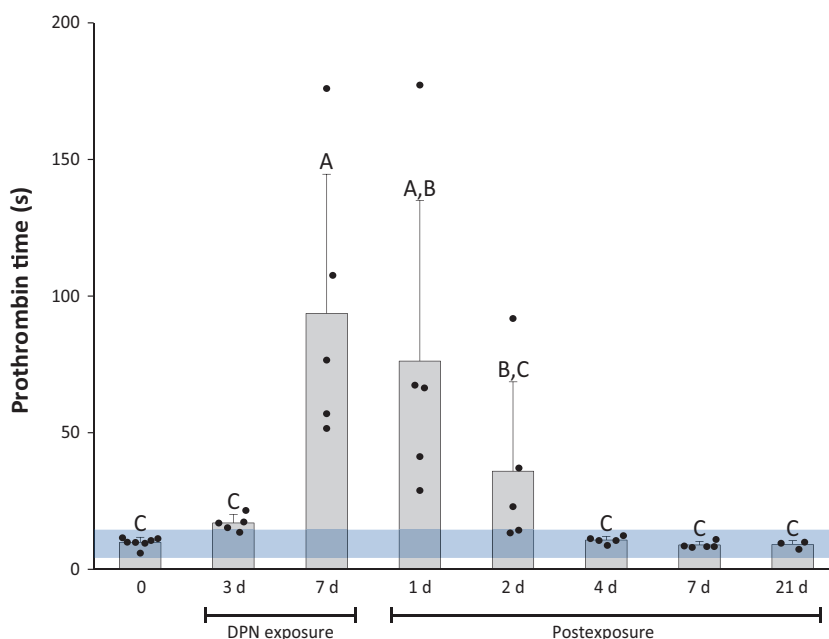


Figure 1. Prothrombin time (individual values in seconds, mean \pm standard deviation) of eastern screech-owls fed diphacinone (DPN; 10 $\mu\text{g/g}$ wet wt) for up to 7 d and then shifted to a diet of untreated mice for up to 21 d. Horizontal shaded line encompasses the range of baseline values determined 2 wk before the trial. Untreated controls (0) included 4 owls fed Bird of Prey diet (days 1–7) and 3 owls fed only mice. Bars with different letters are significantly different ($p < 0.05$).

slower loss rate between day 4 and day 21 postexposure with a $t_{1/2}$ of 29.2 d. The overall $t_{1/2}$ was 11.7 d. Although the initial concentration of DPN in kidney was greater than that in liver, $t_{1/2}$ values indicate that it was cleared more rapidly than in liver. Kidney DPN elimination was characterized by a rapid initial phase $t_{1/2}$ of 0.14 d and a slower terminal phase $t_{1/2}$ of 10.2 d, with an overall $t_{1/2}$ of 2.1 d.

There was an association between liver and kidney DPN residues in exposed owls ($p < 0.0001$, $R^2 = 0.886$). More importantly, visual inspection of these data suggested there

might be a relation between prolonged clotting time and tissue DPN residues. This was investigated by regression analysis ($p < 0.001$; PT and liver DPN $R^2 = 0.270$, PT and kidney DPN $R^2 = 0.424$; RVVT and liver DPN $R^2 = 0.529$, RVVT and kidney DPN $R^2 = 0.722$), and with the exception of RVVT and kidney DPN, predictive relationships were weak.

Tissue-based toxicity reference values

The concentration of DPN in the liver of the 37 owls that were killed ranged from the method limit of detection to 2.93 $\mu\text{g/g}$ wet wt

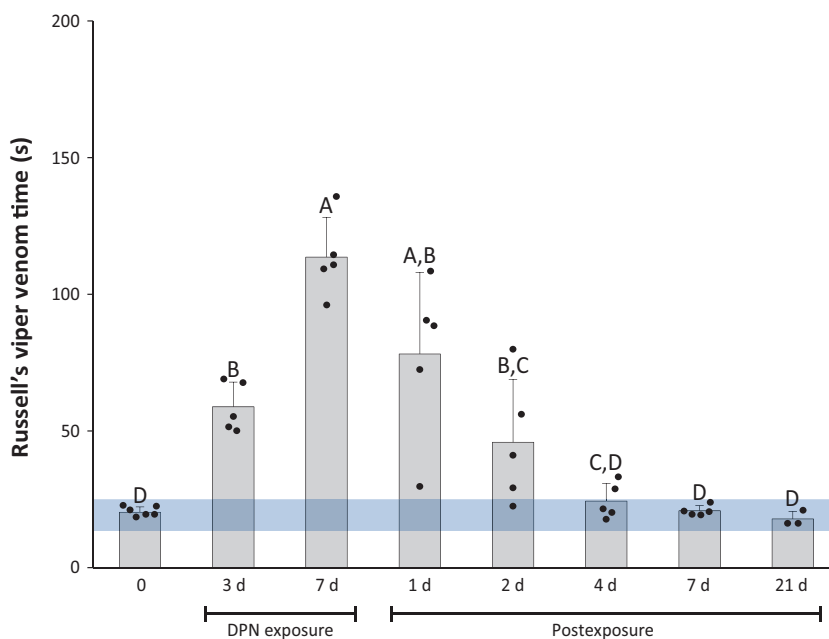


Figure 2. Russell's viper venom time (individual values in seconds, mean \pm standard deviation) of eastern screech-owls fed diphacinone (DPN; 10 $\mu\text{g/g}$ wet wt) for up to 7 d and then shifted to a diet of untreated mice for up to 21 d. Horizontal shaded line encompasses the range of baseline values determined 2 wk before the trial. Untreated controls (0) included 4 owls fed Bird of Prey diet (days 1–7) and 3 owls fed only mice. Bars with different letters are significantly different ($p < 0.05$).

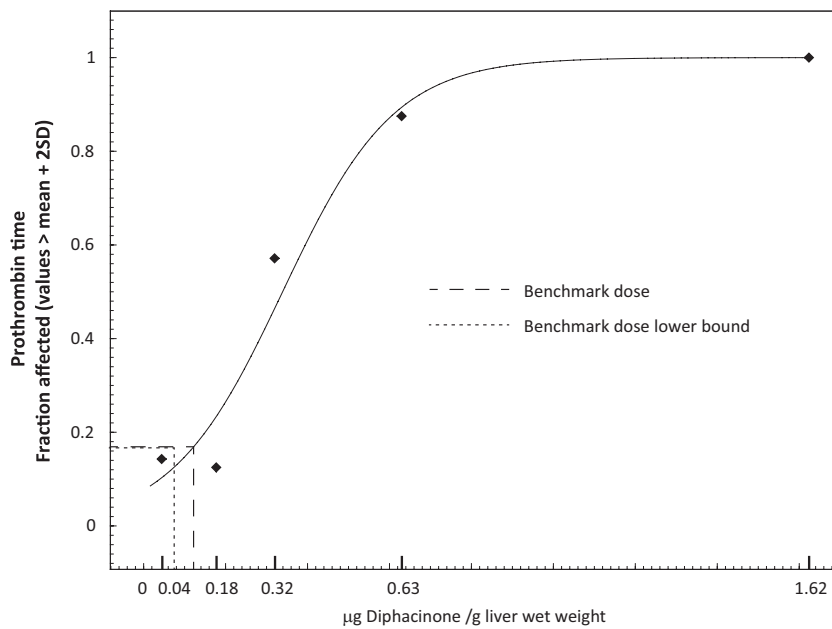


Figure 3. Dose–response relation of liver diphacinone concentration and prothrombin time in eastern screech-owls. Using the benchmark dose method, a tissue-based toxicity reference value at which prothrombin time in 10% of the test population exceeded the baseline mean by 2 standard deviations occurred at 0.122 $\mu\text{g/g}$ wet weight (benchmark dose lower bound 10% = 0.075 $\mu\text{g/g}$).

weight. Quintile means of 0.044 $\mu\text{g/g}$, 0.178 $\mu\text{g/g}$, 0.320 $\mu\text{g/g}$, 0.630 $\mu\text{g/g}$, and 1.62 $\mu\text{g/g}$ liver were used as the concentration (internal dose) in the benchmark dose 10% models. Based on Akaike's information criterion and visual inspection of the plots, these model fits for continuously distributed PT and RVVT values were not robust. Far better benchmark dose model fits were achieved with dichotomous data, when the incidence of prolonged PT and RVVT exceeding baseline (i.e., prestudy mean + 2 SDs for PT = 13.6 s and for RVVT = 25.2 s) was analyzed with quintile dose. For PT, a logistic model yielded a tissue-based toxicity reference value derived from benchmark

dose 10% calculations of 0.122 $\mu\text{g DPN/g}$ liver (benchmark dose lower bound 10% = 0.075 $\mu\text{g/g}$) (Figure 3). For PT, the DPN concentrations at which 20%, 50%, and 90% of the study owls exhibited coagulopathy were estimated to be 0.197 $\mu\text{g DPN/g}$ liver, 0.345 $\mu\text{g DPN/g}$ liver, and 0.638 $\mu\text{g DPN/g}$ liver, respectively. For Russell's viper venom time, a log-logit model yielded a better fit based on Akaike's information criterion, with a toxicity reference value of 0.282 $\mu\text{g DPN/g}$ liver (benchmark dose lower bound 10% = 0.131 $\mu\text{g/g}$; Figure 4). For RVVT, the DPN concentrations at which 20%, 50%, and 90% of the study owls exhibited coagulopathy were estimated to be 0.296 μg

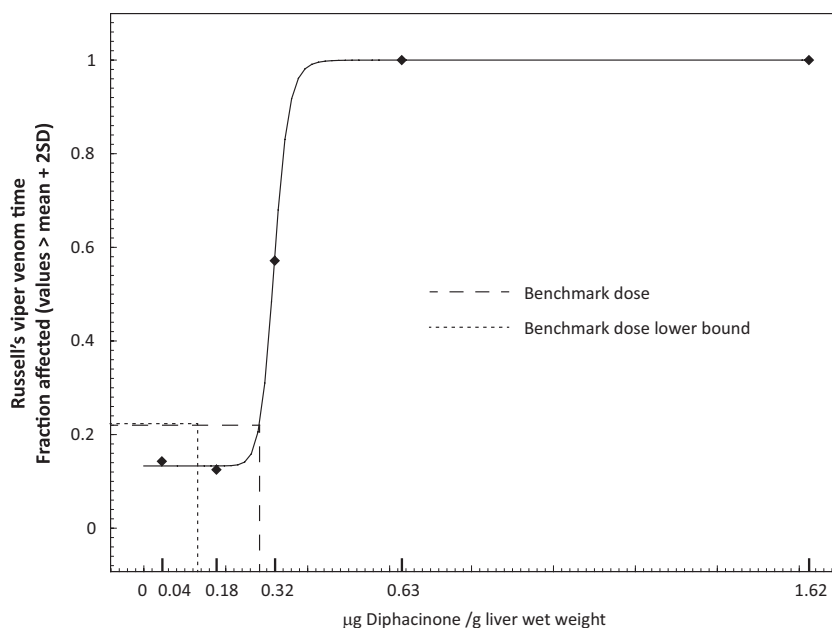


Figure 4. Dose–response relation of liver diphacinone concentration and Russell's viper venom time in eastern screech-owls. Using the benchmark dose method, a tissue-based toxicity reference value at which Russell's viper venom time in 10% of the test population exceeded the baseline mean by 2 standard deviations occurred at 0.282 $\mu\text{g/g}$ wet weight (benchmark dose lower bound 10% = 0.131 $\mu\text{g/g}$).

DPN/g liver, 0.319 μg DPN/g liver, and 0.361 μg DPN/g liver, respectively. When this analysis was conducted using kidney DPN residues, the dichotomous grouping of clotting time in a log-logistic model yielded the best fit. Because the proportion of owls with prolonged PT and RVVT was identical for each quintile dose, the toxicity reference value for both of these end points was 0.800 μg DPN/g kidney (benchmark dose lower bound 10% = 0.351 $\mu\text{g}/\text{g}$).

DISCUSSION

Ingestion of DPN by eastern screech-owls (10 $\mu\text{g}/\text{g}$ wet wt in food) prolonged clotting time within 3 d of exposure (3-d cumulative dose = 4.74 mg/kg body wt), with more pronounced effects on clotting time and anemia apparent on day 7 of exposure (7-d cumulative dose = 7.98 mg/kg body wt). This time course of DPN toxicity expands on our previous observations in eastern screech-owls [9] in which coagulopathy was apparent at similar (7-d cumulative dose = 7.0 mg/kg body wt) and even lower (LOAEL 7-d cumulative dose = 1.68 mg/kg body wt) dietary exposure levels. The onset of coagulopathy was not unlike that observed in a study of mixed-breed dogs (*Canis lupus familiaris*) orally administered a total of 2.5 mg DPN/kg body weight (divided dose administered over 3 consecutive d) [16]. The mechanism of action of DPN involves irreversible inhibition of an epoxide reductase that is responsible for the recycling of oxidized vitamin K to its active reduced form. Vitamin K is necessary for the posttranslational carboxylation of coagulation factors II, VII, IX, and X, which are required for the formation of active clotting complexes on cell surfaces [17]. The lag time between exposure and coagulopathy reflects the decreased rates of carboxylation of these clotting factors and the clearance of carboxylated clotting factors from blood [16,17]. A similar time course for coagulopathy was also observed in American kestrels following administration of a much larger acute DPN dose (50 mg/kg body wt) [8]. However, a large acute dose does not mimic the multiple-day, low-level field exposures that result in mortality of rodent pest species [18,19] and nontarget wildlife [9–11].

Clotting times and hematocrit of eastern screech-owls were restored to baseline values within 2 d to 4 d following termination of DPN exposure. This is in contrast to a 5-d dietary exposure of golden eagles (*Aquila chrysaetos*) to DPN (2.7 $\mu\text{g}/\text{g}$ wet wt in meat from dosed-sheep, *Ovis aries*; cumulative dose to most sensitive individual = 0.55 mg/kg body wt) that also included daily bouts of physical exercise [20]. This exposure-exercise regimen resulted in prolonged PT on day 5, with maximal effects on PT occurring on day 10 (i.e., 5 d after termination of DPN exposure) and recovery to initial values by day 15 (i.e., 10 d postexposure). Likewise, coagulopathy and reduced hematocrit persisted for more than 4 wk postexposure in dogs orally administered DPN for 3 d [16]. The reasons for the rapid recovery in owls compared with the more protracted coagulopathy in golden eagles and dogs are unclear. Cumulative exposure doses were relatively comparable between owls (7.98 mg/kg body wt) and dogs (2.5 mg/kg body wt) and as much as an order of magnitude lower for golden eagles (0.55 mg/kg body wt). Half-life estimates of DPN in liver of mammals ($t_{1/2} = 3$ d in laboratory rats, *Rattus norvegicus*; $t_{1/2} = 5.43$ d in domestic pig, *Sus scrofa*) [21,22] are somewhat less than that of screech-owls (overall $t_{1/2} = 11.7$ d). Hydroxylation rates of the first-generation anticoagulant rodenticide warfarin (coumarin compound) in owls (great-horned owl [*Bubo virginianus*] and snowy owl [*Bubo scandiacus*]) are much lower than

that of the laboratory rat [23]; if a similar tendency exists for DPN (indandione compound), recovery from coagulopathy would be prolonged in DPN-exposed screech-owls, which was not the case. Finally, owls were fed diets that contained synthetic vitamin K (menadione sodium bisulfate complex), while golden eagles received an unsupplemented diet of mutton. It is certainly possible that the vitamin K in the formulated Bird of Prey diet or in dead mice may have enhanced the recovery rate of screech-owls compared to golden eagles. Thus, there is no simple explanation of the more rapid recovery of hemostasis in screech-owls compared with that in other species.

Tissue residues

Diagnosis of anticoagulant rodenticide poisoning in free-ranging wildlife is difficult. The most definitive cases entail the presence of postmortem signs of hemorrhage (independent of trauma) coincident with the detection of rodenticide residues in liver [4,24–27]. In some instances, hemorrhage may be apparent only microscopically (e.g., Vyas and Rattner [8]), and often only a limited suite of anticoagulant compounds is quantified. Such determinations are strengthened by a detailed exposure history, clinical signs including measurement of coagulation time, and response to vitamin K treatment [25]. Hepatic concentrations of anticoagulant rodenticides associated with adverse effects in raptors have yet to be definitively established, but raptors found dead have shown signs of coagulopathy at concentrations ranging from >0.1 $\mu\text{g}/\text{g}$ to >0.4 $\mu\text{g}/\text{g}$ wet weight in liver [4,5,24,27]. Diagnostic concentrations may be affected by exposure dose and frequency of exposure. Liver residues of free-ranging raptors diagnosed as having potentially or definitively succumbed from DPN exposure ($n = 12$ incidents) range from trace quantities to 3.5 $\mu\text{g}/\text{g}$ wet weight [4–7]. These values fall within the range of liver DPN concentrations observed in screech-owls in the present study (Table 1) and thus support the environmental relevance of the 10 $\mu\text{g}/\text{g}$ dietary exposure scenario. The observation of kidney DPN residues exceeding concentrations in liver during exposure and through much of the postexposure period was surprising. Furthermore, the relation between DPN residues and prolonged clotting time in exposed screech-owls was seemingly stronger for kidney than liver. Such a relationship was not observed in kestrels following acute oral exposure to DPN (B.A. Rattner, unpublished data) and is not the case in numerous first- and second-generation anticoagulant rodenticide tissue distribution studies in mammals (e.g., Vandembroucke et al. [28] and Johnston et al. [29]). To the best of our knowledge, diagnostic investigations of poisoned raptors have not included first- or second-generation anticoagulant rodenticide residue measurements in kidney. While the liver is considered to be the principal target organ of these rodenticides, the kidney does contain vitamin K epoxide reductase activity, albeit at much lower levels than in the liver [30]. Thus, it is not clear if the seemingly greater residues in the kidney are commonplace in raptors, unique to owls, or possibly an artifact of the exposure regimen employed in the present study. Measurement of first- and second-generation anticoagulant rodenticides in kidney tissue of poisoned raptors and other nontarget wildlife deserves further attention and could potentially aid in the diagnosis of mortality events.

Due to their greater affinity to binding sites [29] and long half-life in the liver (e.g., 80–307 d for brodifacoum) [1,31], second-generation anticoagulant rodenticides are more toxic than first-generation anticoagulant rodenticides and thus account for the vast majority of nontarget mortality incidents involving rodenticides [4,5,24,27]. The short half-life of first-generation

anticoagulant rodenticides requires multiday exposure to elicit toxicity in target rodents [18]. For free-ranging nontarget raptors, empirical DPN toxicity data, tissue half-life estimates, and predictions from risk assessments suggest that multiday exposure to poisoned rodents is required to elicit adverse effects [8–10,32,33]. In fact, there have been relatively few reported cases of DPN poisoning in nontarget wildlife, although its short tissue half-life may impede identification of its role in some mortality incidents. It is noteworthy that some eradication projects with second-generation anticoagulant rodenticides have used DPN as a secondary bait [34], and laboratory studies have demonstrated that exposure to the second-generation anticoagulant rodenticide brodifacoum increases the sensitivity of laboratory rats exposed weeks later to the first-generation anticoagulant rodenticide warfarin [35]. Residues of both first- and second-generation anticoagulant rodenticides are occasionally found in a single liver sample in a nontarget wildlife incident, and in such circumstances it is certainly possible that mortality due to first-generation anticoagulant rodenticides could be underdiagnosed.

Potential application of toxicity reference values

The hazard of first- and second-generation anticoagulant rodenticides to nontarget wildlife has principally been evaluated by deterministic risk assessments using NOAELs and LOAELs in a hazard quotient [6,33]. This approach has been critiqued and criticized by many, because NOAEL and LOAEL values are highly dependent on study design (e.g., dose interval, sample size, method of dose administration) and often ignore the shape of the dose–response relationships [36,37]. Notably, first-generation anticoagulant rodenticide nontarget wildlife assessments have begun to use more robust probabilistic approaches to address hazard [9,10,32]. For example, using data derived from a study in eastern screech-owls [9,10], model predictions allowed estimation of the distribution of risk (e.g., coagulopathy, mortality) associated with low-level dietary DPN exposure of 2 endangered raptors in Hawaii (USA). Furthermore, a dose-based toxicity reference value associated with anemia in adult screech-owls was estimated to be 0.17 mg DPN consumed/kg body weight-d [10].

In the present study, a range of internal DPN doses in the liver and the fraction of individuals exhibiting prolonged clotting time were modeled using a benchmark dose approach to derive tissue-based toxicity reference values at which 10% of the study population exhibited prolonged clotting time. The toxicity reference values derived from the benchmark dose 10% in adult screech-owls (0.122 μg DPN/g liver for PT and 0.282 μg DPN/g liver for Russell's viper venom time) are at the low end of DPN concentrations associated with mortality events in raptors [4–7] and may be appropriate for threatened and endangered species. For abundant species, however, these diagnostic thresholds are not particularly useful because 90% of the birds would not be affected. In such circumstances, a benchmark dose encompassing a larger fraction of the population may be applied (e.g., benchmark dose 50% or 90%). The threshold at which 90% of exposed individuals exhibit coagulopathy would occur at 0.638 μg DPN/g liver for PT and 0.361 μg DPN/g liver for RVVT. Since the dose–response curve is steep, these benchmark dose 90% concentrations still fall in the range of DPN liver values in raptor mortality events. In combination with uncertainty factors, these toxicity reference values (benchmark dose 10% and 50% or 90%) may have application for interpreting the significance of hepatic DPN residues in suspect raptor mortality incidents. It is important to note that prolonged

clotting time is a sublethal effect and is not equivalent to death. Because the present study utilized a 7-d no-choice dietary exposure regime, these toxicity reference values would be most applicable to island eradication projects or large-scale agricultural settings where raptors would have more limited access to unexposed rodent prey.

CONCLUSIONS

Diphacinone is widely used in urban and agricultural settings and has been used as primary or secondary bait in over 50 eradication projects [34]. Our findings demonstrate that continuous dietary exposure to seemingly environmentally realistic concentrations of this first-generation anticoagulant rodenticide can evoke toxicity (overt signs of intoxication, prolonged clotting time, and anemia) in eastern screech-owls in a matter of days. Coagulopathy is associated with liver DPN concentrations exceeding approximately 0.1 $\mu\text{g}/\text{g}$ wet weight; because of its short half-life in tissues, however, once exposure is terminated, recovery occurs rapidly. In order to translate these findings from captive owls to environmentally relevant exposure scenarios, natural resource managers must consider the duration of access to contaminated prey species to predict potential adverse effects on nontarget raptors.

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