Comparative Toxicity of Diphacinone to Northern Bobwhite (*Colinus virginianus*) and American Kestrels (*Falco sparverius*)

Barnett A. Rattner
U.S. Geological Survey, Patuxent Wildlife Research Center, Beltsville, Maryland
Katherine E. Horak
USDA APHIS Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado
Sarah E. Warner and **Daniel D. Day**
U.S. Geological Survey, Patuxent Wildlife Research Center, Beltsville, Maryland
John J. Johnston
USDA APHIS Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado

ABSTRACT: The acute oral toxicity of the anticoagulant rodenticide diphacinone was found to be about 20 times greater to American kestrels (LD_{50}=97 mg/kg) than to northern bobwhite (LD_{50}=2,014 mg/kg). Several precise and sensitive clotting assays (prothrombin time, Russell's Viper venom time, thrombin clotting time) were adapted for use in these species, and this combination of assays is recommended to detect effects of diphacinone and other rodenticides on coagulation. Oral administration of diphacinone over a range of doses (sublethal to the extrapolated LD_{15}) prolonged prothrombin time and Russell's Viper venom time within 24 to 48 hrs post-exposure. Prolongation of *in vitro* clotting time reflects impaired coagulation complex activity and was detected before or at the onset of overt signs of toxicity and lethality. These data will assist in the development of a pharmacodynamic model to assess and predict rodenticide toxicity to non-target avian species.

KEY WORDS: anticoagulant, birds, clotting time, diphacinone, fibrinogen, non-target effects, prothrombin time, Russell's Viper venom time, secondary poisoning, thrombin clotting time

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INTRODUCTION

In the past 15 years, several current use anticoagulant rodenticides have been identified as potential hazards to predatory and scavenging birds, and adverse effects have been reported in many countries (e.g., Newton et al. 1990, Eason and Spurr 1995, Howald et al. 1999, Stone et al. 1999, 2003; Lambert et al. 2007, Walker et al. 2008, Albert et al. 2009). For example, in the state of New York between 1971 and 1997 there were at least 51 confirmed cases of death by hemorrhage with detection of rodenticides in tissues of wildlife (Stone et al. 1999). A surveillance program (1998 to 2001) reported that nearly half of the 265 raptors examined had detectable quantities of anticoagulant rodenticides in liver tissue, and these compounds were considered the cause of death in about 15% of these cases (Stone et al. 2003). Many of the incidents involved birds of prey, particularly great horned owls (*Bubo virginianus*) and red-tailed hawks (*Buteo jamaicensis*), that consume exposed or poisoned rodents. The global magnitude of secondary poisoning by rodenticides in birds is unknown, as most events are probably unnoticed or not reported.

A risk assessment by the U.S. Environmental Protection Agency (US EPA) identified several rodenticides that pose a significant risk to birds and non-target mammals (Erickson and Urban 2004), and subsequently some restrictions were placed on the sale, distribution, and packaging of brodifacoum, difethialone, bromadiolone, and difenacoum (US EPA 2008). This action may be offset by expanded use of other anticoagulant rodenticides, including diphacinone. The hazard of diphacinone to non-target organisms is inadequately characterized. Accordingly, sublethal responses (blood clotting time) and lethality were determined in northern bobwhite (*Colinus virginianus*), a species traditionally used in wildlife pesticide risk assessments, and also in the American kestrel (*Falco sparverius*), a well-studied toxicological model for raptorial species (Bardo and Bird 2009). Rather than using *ad libitum* dietary exposure in which food consumption can be highly variable, a controlled oral dosing regimen was employed to more accurately estimate dose-related sublethal and lethal effect thresholds. These and other data will ultimately assist in the development of a pharmacodynamic model of diphacinone in birds, and also in selection of efficacious baiting strategies that may mitigate risk to non-target species.

METHODS

Animals

Adult northern bobwhite were obtained from R & R Game Birds (Lamar, CO), housed individually in indoor pens (61 cm × 46 cm × 33 cm high) at the USDA National Wildlife Research Center, Fort Collins, CO (NWRC), maintained in a 12 hr light:12 hr dark photoperiod at 18-21ºC, and provided food (Purina Game Bird Maintenance Chow® Product 5440, 12.5% protein, Denver, CO) and water *ad libitum*. Body weight of quail ranged from 149-224 g. American kestrels were propagated in the captive colony of the USGS Patuxent Wildlife Research Center, Laurel, MD (PWRC) (Porter

and Wiemeyer 1970), where they were maintained in outdoor flight pens (6.1 m × 2.4 m × 2.1 m high), and provided daily rations of either Classic Bird of Prey diet (Nebraska Brand, North Platte, NE) supplemented with Vionate® (Gimborn US, Atlanta, GA), dead mice (*Mus musculus*), or dead hatchling chickens (*Gallus gallus*), and water. Body weight of kestrels ranged from 98-132 g.

Acute Toxicity of Diphacinone

An acute oral toxicity test was conducted in which quail were gavaged with technical grade diphacinone (99% active ingredient) (Hacco, Inc. Randolph, WI) suspended in vegetable oil (Crisco®, Orville, OH). Doses ranging from 917 to 3,666 mg/kg body weight (n = 9-10 quail/dose; about equal sex distribution/dose) were selected through a stepwise process. Due to the low solubility of diphacinone, birds in 4 of the treatment groups received multiple doses within a 24-hr period (total amount administered: 1,033 and 2,065 mg/kg – 2 doses/day; 2,868 mg/kg – 3 doses/day; and 3,666 mg/kg – 4 doses/day). The heaviest bird in a dosage group received 1 ml of the suspension, and the remaining quail in that group received a fraction of the volume determined by their weight. Vegetable oil (vehicle) was administered 1-3 times to 9 quail that served as controls. Birds were observed twice daily for signs of toxicity for 14 days.

Using the results of the bobwhite acute toxicity test, range finding trials with kestrels were undertaken. Serious problems were encountered due to regurgitation of diphacinone. Through an iterative process, a dosing procedure was developed that minimized regurgitation. In September and October 2009, kestrels were moved from their flight pens to small outdoor cages (1.2 m × 0.8 m × 0.6 m high containing a rope perch, food tray, and water bowl) where they were housed individually and fed Classic Bird of Prey diet for at least a 10-day acclimation period. Following an overnight fast, small quantities of neat diphacinone (<200 mg/kg), freeze-dried bird of prey diet, and 5 µl FD&C Blue #1 food dye (to better detect regurgitation; McCormick & Co., Inc., Baltimore, MD) were loaded into a number 4 gelatin capsule (E. Lilly and Co., Indianapolis, IN) that was administered to the level of the proventriculus using a pilling device (modified pet piller, Jorgetsen Laboratories, Inc., Loveland, CO) and a plastic probe. The capsule was chased with 0.2 ml of distilled water by gavage, and the dosed kestrel was returned to its pen and immediately presented with a highly desired food item (i.e., dead chicken hatchling). By repeating this procedure 4 times within a 24-hr period, total daily dosages ranging from 35.1 to 675 mg/kg body weight were achieved (n=19 kestrels, with sexes divided near evenly among dosages, plus 4 controls that received capsules containing only the freeze-dried diet and blue dye). Pens were lined with kraft paper to monitor regurgitation, and kestrels were observed for overt signs of intoxication several times each day for a week.

Effects of Diphacinone on Clotting Time

Based upon the results of the acute toxicity test in quail, another study was conducted in which bobwhite were gavaged with either vegetable oil (control, n=6), 434 mg diphacinone/kg body weight (n=16), or 783 mg diphacinone/kg (n=16). Quail were euthanized and immediately bled by cardiac puncture at 6, 12, 24, and 48 hrs post-dose, and controls were sacrificed and bled at 48 hrs post-dose. Blood samples (~0.5 ml) were collected into syringes containing 50 µl of 0.5 M EDTA, a suitable alternative to sodium citrate (Cerón et al. 2008). The samples were centrifuged, plasma was harvested and frozen at -80ºC, and subsequently shipped to PWRC for analysis.

Using the aforementioned protocol from the kestrel acute toxicity trial, another kestrel diphacinone study was conducted in which a total of 50 mg/kg body weight was administered as divided doses over a 24-hr period. Controls were treated similarly except capsules did not contain diphacinone. At 48 hrs after administration of the final capsule, a 0.9-ml jugular venipuncture sample was drawn into a syringe containing 0.1 ml of 3.2% sodium citrate (n=3 diphacinone-treated and n=3 control kestrels), and blood samples were collected from the remaining birds (n=3 diphacinone-treated and n=2 controls) after 168 hrs. Blood samples were centrifuged and citrated plasma was frozen at -80ºC for clotting time assays.

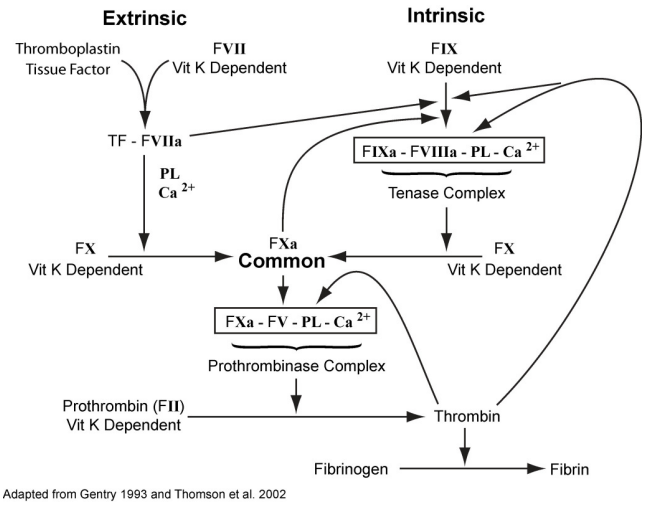

Figure 1. Blood coagulation pathway in birds (adapted from Gentry 1993 and Thomson et al. 2002; F = Factor, PL = phospholipid).

One-Stage Prothrombin Time Assay

An excess of tissue factor and phospholipid (thromboplastin) interacts with plasma Factor VII to form an active complex, and through a cascade of reactions fibrinogen is eventually converted to fibrin which forms a clot (Figure 1). Crude chick hatchling thromboplastin (CHT) was prepared by the method of Quick as modified by Griminger et al. 1970 and Doerr et al. 1975. The CHT (50 mg) was suspended in 2,500 µl of 25 mM $CaCl_2$, and incubated at 42ºC for 15 min with intermittent vortexing. Following centrifugation of the suspension (1,500 × g for 20 min), the supernatant was diluted (1:1) with 25 mM $CaCl_2$ (~220 µg protein/ml). Clotting time was determined using a BBL fibrometer (Becton Dickson & Co., Baltimore, MD). Plasma (100 µl) was incubated at 37ºC

for 2 min, and the reaction was initiated by the addition of 200 µl of diluted CHT. Intra-assay precision (mean coefficient of variation ± standard deviation) for duplicate determinations of quail and kestrel plasma was 4.6 ± 4.5% (n=30) and 2.0 ± 2.2% (n=11), respectively. Inter-assay precision over the course of a year for human reference samples using Simplastin® (rabbit brain thromboplastin; Trinity Biotech, Berkeley Heights, NJ) was 2.9 ± 2.2% (n=13). When a quail or a kestrel plasma pool was diluted with 8.3 mM Na/K phosphate buffer (pH 7.2), clotting time was relatively stable at dilutions containing as little as 50% plasma, but increased at greater dilution.

Russell's Viper Venom Time (RVVT)

Russell's Viper venom (RVV) directly activates Factor X (but not Factor VII) in the common pathway of the clotting cascade (Figure 1). Reconstituted RVV Factor X activator (American Diagnostica, Stamford, CT) was diluted 1:10 with imidazole buffered saline (IBS; 0.0125 M imidazole 0.109 M NaCl, pH7.4) and maintained at room temperature. Plasma (100 µl) was incubated at 37ºC in a sample cup for 2 min, and 100 µl of diluted RVV was added and incubated for 15 sec. The reaction was initiated with 100 µl 25 mM $CaCl_2$, and clotting time was determined (Triplett and Harms 1981a). Intra-assay precision for duplicate determinations of quail plasma was 6.5 ± 13.5% (n=19) and kestrel plasma was 3.4 ± 4.1% (n=9). Clotting time remained relatively stable when quail or kestrel plasma was diluted by as much as 60% with phosphate buffer, but RVVT increased dramatically at greater dilutions.

Thrombin Clotting Time (TCT)

This assay measures the time for conversion of fibrinogen to fibrin (Figure 1) using a standard thrombin solution (Triplett and Harms 1981b). The assay is an indicator of the amount of fibrinogen in the plasma sample, and insensitive to deficiency of vitamin K-dependent clotting factors. We used the AMAX Fibrinogen kit (Trinity Biotech) which includes bovine thrombin reagent and human fibrinogen reference material. A fibrinogen standard curve was prepared (65 to 520 mg/dL), and quail or kestrel plasma samples were diluted 1:10 with IBS. Diluted plasma (200 µl) was incubated at 37ºC in a sample cup for 2 min, and the reaction initiated by the addition of 100 µl of thrombin reagent. Clotting time of the test sample was transformed to fibrinogen concentration (mg/dL) from the standard curve. Intra-assay precision for duplicate determinations of quail plasma was 5.7 ± 6.8% (n=32) and kestrel plasma was 1.8 ± 2.2% (n=9).

Statistical Methods

For the acute toxicity trial, the median lethal dose (LD_{50}) of diphacinone in bobwhite and kestrels was estimated using probit analysis (SAS Institute, Carey, NC). For sublethal dosing studies, prothrombin time, RVVT, and TCT were tested for homogeneity of variance (Fmax test) and normality (Shapiro-Wilk test, normal probability plot and descriptive statistics). The measurement endpoints were then compared by one-way analysis of variance (ANOVA) in the quail study and by a 2 × 2 factorial ANOVA (dosage × time) in the kestrel study. Tukey's HSD test was used as a mean separation procedure.

RESULTS

Acute Toxicity Studies

Survival of northern bobwhite was significantly related ($P < 0.0001$) to dose of diphacinone (survivors: 9 of 10 at 917 mg/kg; 8 of 9 at 965 mg/kg; 10 of 10 at 1,033 mg/kg; 7 of 10 at 2,065 mg/kg; 1 of 10 at 2,868 mg/kg; 0 of 10 at 3,666 mg/kg). Bobwhite receiving the greatest doses (2,868 and 3,666 mg/kg) succumbed within 1-3 days of exposure. Some dosed quail exhibited subcutaneous bruises on the breast and back regions which could reflect coagulopathy; however, there was no evidence of frank internal or external bleeding. All vehicle-dosed controls survived the 14-day trial. The LD_{50} estimate was 2,014 mg/kg (95% confidence interval 1,620-2,475 mg/kg), and the slope of the dose-response curve was steep (probit/$\log_{10}$ ± SE = 9.92 ± 2.27).

Survival of American kestrels was also significantly related ($P < 0.023$) to dose of diphacinone (survivors: 2 of 2 at both 35.1 and 52.7 mg/kg, 2 of 3 at 79.0 mg/kg, 0 of 3 at 118.6 mg/kg, 0 of 2 at 177.8 mg/kg, 0 of 1 at 200 mg/kg, 1 of 1 at 266.7 mg/kg, 0 of 2 at 300 mg/kg, 0 of 1 at both 450 and 675 mg/kg). Sample size for two intermediate doses was only 1, as a bird scheduled to receive 266.7 mg/kg died before the final capsule containing its divided dose could be administered. Birds scheduled to receive 600 and 900 mg/kg died before the final divided dose could be given, so the actual administered doses were 450 and 675 mg/kg. Kestrels that succumbed appeared to exhibit a progression of toxic signs (loss of balance on perch, standing on floor of pen rather than perch, non-reactive when approached, subdued behavior, appearance of tan urate deposits) and died between 10 to 48 hrs after receiving the initial capsule of the divided dose. Two kestrels (266.7 and 79.0 mg/kg doses) exhibited toxic signs, but appeared to recover by the third day of the trial. All controls survived the 7-day trial. The LD_{50} of diphacinone for kestrels was estimated to be 97 mg/kg (95% confidence interval 38-219 mg/kg), and the slope of the dose-response curve was 6.69 ± 2.94 probit/$\log_{10}$.

Sublethal Exposure and Effects on Clotting Time

In the quail study, the 434 mg/kg dose was slightly greater than the lower 95% confidence limit of the $LD_{01,}$ while the 783 mg/kg dose fell between the estimated LD_{01} and LD_{02}. Both of these doses prolonged clotting time when compared to controls, but the temporal response was highly variable (e.g., see scatter of prothrombin times in Figure 2). However, fibrinogen concentration in 5 samples as determined in the TCT assay was undetectable, and the volume of another sample was too small to determine fibrinogen concentration. The absence of fibrinogen in these samples suggest that they may have been collected improperly (viz., cardiac puncture of euthanized birds, partially clotted blood). Fibrinogen is generally in great excess, and its conversion to fibrin in the TCT assay is not influenced by vitamin K antagonists.

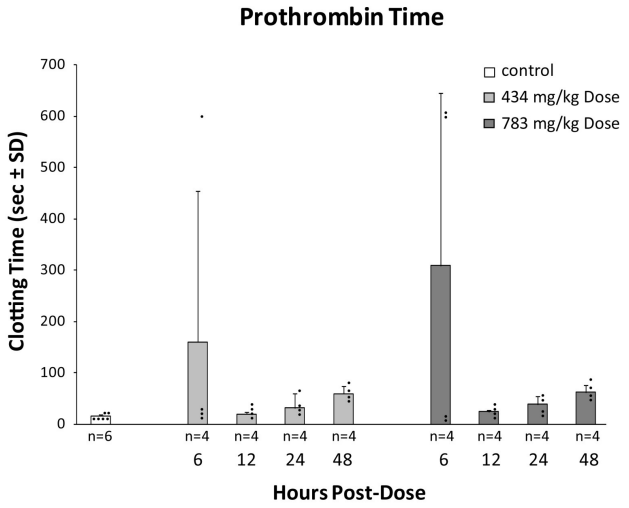

Figure 2. Prothrombin time (mean ± standard deviation; · = data point) of all quail gavaged with vehicle (control) or at 6, 12, 24, and 48 hrs following administration of diphacinone.

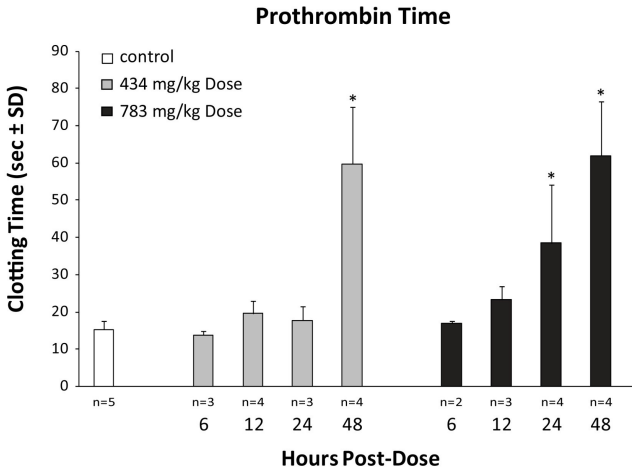

Figure 3. Prothrombin time of quail gavaged with vehicle (control) or diphacinone (6, 12, 24, and 48 hrs post-dose) with plasma fibrinogen concentration >60 mg /dL.
* = significantly different (P < 0.05) than control.

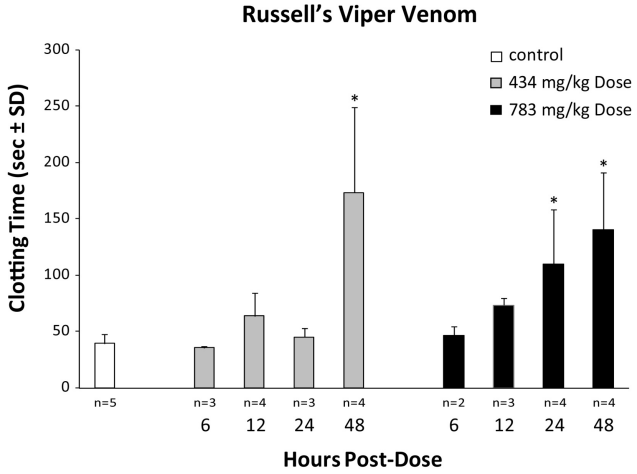

Figure 4. Russell's Viper venom time of quail gavaged with vehicle or diphacinone (6, 12, 24, and 48 hrs post-dose) with plasma fibrinogen concentration >60 mg /dL.
* = significantly different (P < 0.05) than control.

When these 6 samples were excluded, the remaining samples contained more than 60 mg fibrinogen/dL (range: 63 to 254 mg/dL, n=32). For these remaining samples, prothrombin time and RVVT (Figures 3 and 4) were prolonged about three- to four-fold ($P < 0.05$) at 48 hrs after administration of 434 mg/kg, and similarly prolonged by 783 mg/kg at both 24 hrs and 48 hrs ($P < 0.05$), when compared to the control group.

In the kestrel study, the 50 mg/kg dose fell between the estimated LD_{10} and LD_{15}, and all birds survived the trial. However, one diphacinone-dosed kestrel was subdued on days 2 and 3, and before it was bled a subcutaneous hematoma was observed on its neck. A significant dose × time interaction was detected by ANOVA for both prothrombin time ($P < 0.023$) and RVVT ($P < 0.027$). Diphacinone prolonged prothrombin time ($P < 0.036$) and RVVT ($P < 0.032$) in blood samples collected 48 hrs after administration of the final capsule of the divided dose, but after 7 days clotting times returned to control values (Figures 5 and 6). Fibrinogen concentration was detectable in all samples (range: 45 to 147 mg/dL) and did not differ ($P > 0.15$) among the 4 groups.

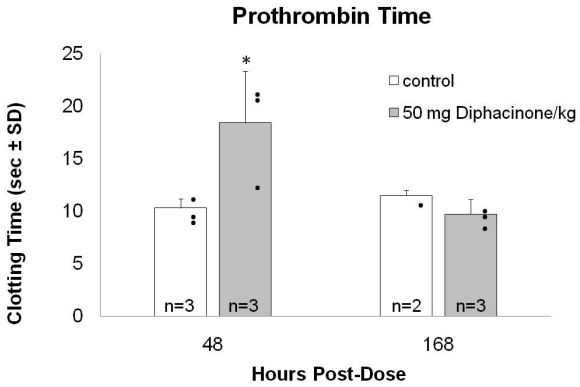

Figure 5. Prothrombin time of kestrels administered 4 capsules containing diphacinone (50 mg/kg over 24 hrs in divided doses) or capsules without rodenticide (control) at 48 and 168 hrs post-dose.
* = significantly different (P < 0.05) than concurrent control.

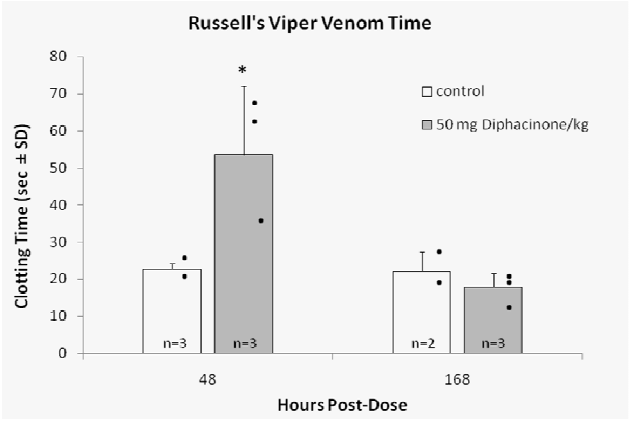

Figure 6. Russell's Viper venom time of kestrels administered 4 capsules containing diphacinone (50 mg/kg over 24 hrs in divided doses) or capsules without rodenticide (control) at 48 and 168 hrs post-dose.
* = significantly different (P < 0.05) than concurrent control.

DISCUSSION

Diphacinone may be categorized as only slightly toxic (LD_{50} 2,014 mg/kg) to northern bobwhite using traditional categories that classify harm (Loomis 1978). A reliable diphacinone median lethal dose for bobwhite could not be estimated in a previous study (Campbell et al. 1991) as dosages were separated by a factor of 5, but inspection of the data suggest that the theoretical value fell between 400 and 2,000 mg/kg (US EPA 1998). Our estimated LD_{50} in quail is of the same order of magnitude as reported for mallards (*Anas platyhrynchos*; 3,158 mg/kg) (Erikson and Urban 2004). Based upon data from avian species commonly used in pesticide registration tests (viz., northern bobwhite, mallards), diphacinone appears to be less toxic to captive birds than to laboratory rats and domesticated mammals (range of estimated LD_{50}: 0.8 to 15 mg/kg), and to wild mammals (0.2 to 340 mg/kg), and its risk to wild birds would seemingly be minimal (reviewed in Erikson and Urban 2004, Eisemann and Swift 2006). However, results of the diphacinone acute toxicity test in American kestrels (LD_{50} 97 mg/kg) indicate that they are over 20 times more sensitive than bobwhite, and over 30 times more sensitive than mallards. Furthermore, a small dosing trial in which diphacinone-poisoned mice (*Peromyscus maniculatus*) were fed to great-horned owls (*Buto virginianus*) and a saw-whet owl (*Aegolius acadicus*) also suggests that they are more sensitive than bobwhite (Mendenhall and Pank 1980). Notably, diphacinone has been linked to secondary poisoning in raptors (Stone et al. 1999, 2003), and in general, raptors are more sensitive to pesticides than other groups of birds (Wiemeyer and Sparling 1991, Vyas et al. 1998, Mineau et al. 1999). These findings indicate that extrapolation of diphacinone toxicity data from quail and mallards to other avian Orders (e.g., Falconiformes, Stringiformes) may be dubious, and protection of raptors may require substantial safety factors.

Some kestrels that survived dosing trials exhibited behavioral changes (e.g., 1 dosed at 266.7 mg/kg, and 1 of 3 dosed at 79.0 mg/kg) and prolonged clotting time (2 of 3 dosed at 50 mg/kg). Similarly, golden eagles (*Aquila chrysaetos*) fed muscle from sheep dosed with diphacinone (30 mg/kg) were weakened and debilitated and had prolonged prothrombin time (Savarie et al. 1979). In both of these studies animals recovered, but such behavioral and physiological deficits could affect survival of free-ranging birds.

Several coagulation assays were adapted that yielded short and precise clotting times with EDTA-treated plasma from quail and citrated plasma from kestrels. Using a thromboplastin extract from chick hatchlings, prothrombin time of untreated quail (mean ± standard deviation: 15.2 ± 1.5 sec, Figure 3) and kestrels (10.6 ± 0.8, Figure 5) was in the range of values reported for many species of domesticated and wild birds (Martin et al. 1994, reviewed in Powers 2000, Thomson et al. 2002, Morrisey et al. 2003, Rattner et al. 2009, Webster 2009). Using RVV that activates Factor X in the common pathway, clotting time of plasma from untreated quail (39.5 ± 8.9 sec, Figure 4) and kestrels (22.6 ± 2.8, Figure 6) was slightly greater than reported in other avian species (~9 to 21 sec; Tahira et al. 1977, Timms 1977), although precision for duplicate RVVT determinations and standard deviation of control samples with >45 mg fibrinogen/dL, seems acceptable.

Anticoagulant rodenticides inhibit vitamin K-dependent post-translational processing of clotting Factors II (prothrombin), VII, IX, and X (reviewed in Powers 2000), but do not affect the synthesis of fibrinogen. Fibrinogen deficiency resulting from improper sample collection and from pathophysiologic conditions (e.g., hepatic synthetic failure, disseminated intravascular coagulation) can prolong *in vitro* clotting time. Avian studies examining anticoagulant rodenticide toxicity have failed to determine if sample fibrinogen content supports *in vitro* clot formation. A concentration threshold that supports clotting has yet to be established for birds, and in the interim, we used 60 mg/dL in quail and 45 mg/dL in kestrels. A conservative diagnostic approach for anticoagulant rodenticide studies that evaluate vitamin K-dependent coagulopathies would entail a combination of assays, namely prothrombin time and/or RVVT, plus determination of fibrinogen (TCT) to rule out any nonspecific influence on clotting time.

In the present study, several quail plasma samples did not contain detectable quantities of fibrinogen, failed to clot in both the *in vitro* one-stage prothrombin time and RVVT assays, and thus were excluded from our evaluation of diphacinone on hemostasis in quail. Clotting time of samples with detectable fibrinogen was not affected at 6 and 12 hrs after administration of diphacinone, but was prolonged at 24 and 48 hrs post-dose. In kestrels, effects on clotting time were apparent at 48 hrs after administration of the final divided dose of diphacinone. This time course roughly corresponds with, and may precede, the onset of overt toxicity and lethality at greater dosage levels used in the acute toxicity studies. The lag time between dosing and development of coagulopathy reflects the half-life clearance of functional coagulation factors and the increasing circulation of descarboxy, dysfunctional factors. Prothrombin time is used as an early indicator of anticoagulant rodenticide ingestion in domestic mammals (Mount and Feldman 1983), and is routinely measured to monitor coumadin anticoagulant therapy in humans (Spinler et al. 2005). Prolonged prothrombin times have been reported within days of 1) dietary exposure to the anticoagulant rodenticides diphacinone (golden eagles, Savarie et al. 1979; American crow, *Corvus brachyrhynchos*, Massey et al. 1997), warfarin (chickens, Veltmann et al. 1981) and brodificoum (Japanese quail, *Coturnix coturnix*, Webster 2009), and 2) repeated gavage with pindone (wedge-tailed eagles, *Aquila audax*, bronzewing pigeons, *Phaps chalcoptera*, Port Lincoln parrots, *Barnardius zonarius*, black ducks, *Anas supercillosa*, Australian magpies, *Gymnorhina tibicen*, Martin et al. 1994).

In conclusion, diphacinone was found to be considerably more toxic to American kestrels than to northern bobwhite. A group of clotting assays were developed and applied for use in quail and kestrels that are sensitive, precise, linked to the pathogenesis of toxicity (and ultimately mortality), and together have applicability as biomarkers in both laboratory studies and field monitoring. These findings and assay methods will assist

in rodenticide hazard and risk assessments of secondary poisoning in non-target avian species.

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