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Development of Dietary-Based Toxicity Reference Values to Assess the Risk of Chlorophacinone to Non-Target Raptorial Birds

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ABSTRACT: Regulatory changes in the use of some second-generation anticoagulant rodenticides in parts of North America may result in expanded use of first-generation anticoagulant rodenticides (FGARs). Recent toxicological studies with captive raptors have demonstrated that these species are considerably more sensitive to the FGAR diphacinone than traditional avian wildlife test species (mallard, bobwhite). We have now examined the toxicity of the FGAR chlorophacinone (CPN) to American kestrels fed rat tissue mechanically amended with CPN, or rat tissue containing biologically-incorporated CPN, for 7 days. Nominal CPN concentrations in these diets were 0.15, 0.75, and 1.5 µg/g food wet weight, and actual CPN concentration in diets were analytically verified as being close to target values. Food intake was consistent among groups, body weight fluctuated by less than 6%, exposure and adverse effects were generally dose-dependent, and there were no dramatic differences in toxicity between mechanically-amended and biologically-incorporated CPN diets. Using benchmark dose statistical methods, toxicity reference values at which clotting times were prolonged in 50% of the kestrels was estimated to be about 80 µg CPN consumed/kg body weight-day for prothrombin time and 40 µg CPN/kg body weight-day for Russell's viper venom time. Based upon carcass CPN residues reported in rodents from field baiting studies, empirical measures of food consumption in kestrels, and dietary-based toxicity reference values derived from the 7-day exposure scenario, some free-ranging raptors consuming CPN-exposed prey might exhibit coagulopathy and hemorrhage. These sublethal responses associated with exposure to environmentally realistic concentrations of CPN could compromise survival of exposed birds.

KEY WORDS: anticoagulant rodenticides, birds, chlorophacinone, clotting time, hazard, non-target effects, risk assessment, rodenticides, secondary poisoning

INTRODUCTION

Anticoagulant rodenticides are used worldwide for the control of vertebrate pests in urban and suburban settings, in agriculture, and for the eradication of invasive species as part of ecological restoration projects. Despite widespread use and benefit, there is growing concern about the risk of anticoagulant rodenticides to children, companion and domestic animals, and non-target wildlife. In much of North America, recent restrictions placed on the use of the more hazardous second-generation anticoagulant rodenticides (SGARs) (Health Canada Pest Management Regulatory Agency 2012, US EPA 2012) are likely to result in expanded use of first-generation anticoagulant rodenticides (FGARs). These first-generation compounds are considered to be less hazardous to non-target wildlife than SGARs (Erikson and Urban 2004, US EPA 2011a), although recent diphacinone studies with captive raptors indicate that they may be more hazardous than previously recognized (Rattner et al. 2011, Rattner et al. 2012a, Rattner et al. 2012b, Rattner et al. 2014).

Chlorophacinone (CPN) is an extensively-used FGAR that has been detected in tissues of wild birds in various monitoring studies, albeit at a low frequency (Berny et al. 1997, Stone et al. 2003, Albert et al. 2010, Hughes et al. 2013, Vyas et al. 2013). There have been several reported secondary exposure incidents in which the death of raptors was attributed to CPN exposure (Berny et al. 1997, CA EPA 2012, US EPA 2012). Studies of CPN in bobwhite quail (*Colinus virginianus*) and mallard (*Anas platyrhynchos*) submitted to the US EPA in support of the registration suggest that this FGAR falls in the category of being highly toxic to birds (US EPA 2011a). However, controlled studies using various exposure scenarios in several species of raptors documented sublethal responses including hemorrhage and coagulopathy, but not mortality of test subjects (Mendenhall and Pank 1980, Radvanyi et al. 1988, Riedel et al. 1988, Askham and Poché 1992). Few of these controlled-exposure studies determined actual CPN consumption of the birds, so there are no quantitative data on exposure. Herein we describe a subset of exposure and effect data from a study conducted in American kestrels (*Falco sparverius*) that examined the toxicity of graded concentrations of CPN that had been either mechanically amended or biologically incorporated into rat tissue diets. These data were used to estimate the dietary exposure threshold associated with impaired blood clotting (toxicity reference value) to assist in comparative risk analyses of various anticoagulant rodenticides to predatory birds.

MATERIALS AND METHODS
Animals

Adult 2- and 3-year-old captive male American kestrels, propagated from the colony at Patuxent, were moved from flight pens to small outdoor cages (1.2 × 0.8 × 0.6 m) with a shade roof, perches, food tray, and water

bowl. Individually-housed kestrels (n = 40) were acclimated for 2 weeks (March 2013) during which time they were fed dead mice or chicks. A 0.9-ml jugular venipuncture sample was collected into 0.1 ml of 3.2% sodium citrate from each bird to determine baseline clotting time, and then birds were shifted to Classic Bird of Prey diet (Nebraska Brand, North Platte, NE) for 1 week.

Young adult (220-225 g) SAS Sprague Dawley rats (*Rattus norvegicus*; n = 63 males and 63 females), obtained from Charles River Laboratories (Wilmington, MA) were used to prepare test diets for the kestrels. Rats housed in groups (segregated by sex) were provided standard rodent chow and water *ad libitum* for a 1-week acclimation period.

Preparation of Diets

Two control diets were used in this study. These consisted of 1) Classic Bird of Prey diet (hereafter NBP) formed into 25 ± 0.1-g wet weight (ww) meat balls, and 2) ground tissue from untreated laboratory rats (euthanized with carbon dioxide; fur, paws, gastrointestinal tract, and skull removed), amended with 15 ml vegetable oil/kg rat tissue (hereafter RT), formed into 18 ± 0.1-g ww meat balls. The two groups were used to determine if there was any difference in feeding Bird of Prey diet (two 25-g meatballs/day) or ground rat tissue (two 18-g meatballs/day) on various measurement endpoints.

Three diets mechanically-amended with CPN (nominal concentrations of 0.15, 0.75, and 1.5 µg CPN/g ww) were prepared from RT. Neat CPN was dissolved in acetone and then added to vegetable oil (1:20). Varying quantities of this solution were mixed into RT in a fixed volume of vegetable oil (15 ml/kg for constant caloric value). These mechanically-amended CPN diets were formed into 18 ± 0.1-g ww meat balls. In addition, three diets containing biologically-incorporated CPN were prepared from rats that had been fed Rozol® bait (0.005% CPN) for 3 days and then euthanized. Ground tissue from CPN-fed rats (fur, paws, gastrointestinal tract, and skull removed) were mixed with untreated RT to yield nominal concentrations of 0.15, 0.75, and 1.5 µg CPN/g ww. Each of these diets was amended with 15 ml vegetable oil/kg tissue and formed into 18 ± 0.1-g ww meat balls.

The two control diets, three mechanically-amended CPN diets, and three biologically-incorporated CPN diets were supplemented with Vionate® (Gimborn, OH). During the course of diet preparation, subsamples were chemically analyzed to verify CPN concentrations. The purity of the CPN was determined using the same reversed phase ion-paired high-performance liquid chromatography (RP-HPLC) with UV detection at 285 nm previously described (Rattner et al. 2011, Rattner 2012a) with diphacinone as an internal standard. The purity of chlorophacinone was found to be 99.6% (% ww). Mechanically-amended and biologically-incorporated CPN diets were homogenized, solvent extracted, cleaned up using solid phase extraction cartridges, and CPN quantified by RP-HPLC (Rattner et al. 2012a). Recovery of CPN from spiked ground rat tissue (mean ± SD, n = 6) was $82.9 \pm 12.4\%$ at 0.2 µg CPN/g ww and

89.4 ± 4.9 % at 2.0 µg CPN/g ww. Chlorophacinone in diets used in the toxicity trial were analytically verified and averaged 93.9% of target concentrations.

Chlorophacinone Toxicity Trial in American Kestrels

One control group (n = 5 kestrels) was fed NBP meatballs throughout the course of the study. The other control group (n = 5) and the six treatment groups that were to receive mechanically-amended or biologically-incorporated CPN diets (n = 5 per group) were fed untreated RT meatballs for 3 days prior to the study. At the initiation of the toxicity trial (Day 0), one control group received NBP meatballs (two 25-g meatballs/day), and the other control group received RT meatballs (two 18-g meatballs/day) for 7 days. The six CPN treatment groups then received either mechanically-amended diets (nominal concentrations of 0.15, 0.75, and 1.5 µg CPN/g ww) or biologically-incorporated diets (nominal concentrations of 0.15, 0.75, and 1.5 µg CPN/g ww) in the form of two 18-g meatballs per day for a 7-day exposure period. All birds were fed daily between 1200 and 1300 hours, and uneaten food scraps were carefully removed from each kraft paper-lined pen the following day between 1000 and 1200 hours. The scraps for each kestrel were pooled, weighed, stored, dried, and converted back to ww as previously described (Rattner et al. 2012a). The difference between the meatballs provided and scraps collected on a ww basis was defined as total food intake over the 7-day exposure period, and thus total CPN exposure.

Kestrels were visually observed in their individual pens 3 times daily during the toxicity trial, and were weighed and physically examined on Days 0, 3, and 5 of the trial. On Day 7, each bird was examined, weighed, bled (0.9-ml jugular venipuncture blood draw into 0.1 ml 3.2% sodium citrate), sacrificed using carbon dioxide, and necropsied. Each blood sample was centrifuged (2,000 g for 5 min), citrated plasma harvested, and various volumes were pipetted into cryotubes that were stored at -80ºC for subsequent clotting time assays.

Clotting Time Assays

Prothrombin time (PT) and Russell's viper venom time (RVVT) of citrated kestrel plasma samples were used to evaluate CPN 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 a plasma sample and is insensitive to deficiency of vitamin K-dependent clotting factors. Fibrinogen deficiency resulting from improper sample collection can prolong clotting time, and in rodenticide toxicity studies it is important to verify that its concentration is adequate to promote clot formation. The conduct, performance, and use of these assays in raptors have been previously described (Rattner et al. 2011, Rattner et al. 2012a, Rattner et al. 2014).

Statistical Methods

All measurement endpoints (body weight change, PT, RVVT and TCT, and CPN consumption) were tested for homogeneity of variances and normality and evaluated by one-way analysis of variance followed by Tukey's HSD

test. Body weight change (Days 3, 5, and 7) relative to initial Day 0 weight (weight change/100 g body weight) was evaluated over time using a repeated measures analysis of variance.

The ingested CPN concentration at which clotting time was prolonged in kestrels was calculated to derive estimated dietary-based toxicity reference values. Response parameters included estimated CPN consumption /kg body weight-day (average per treatment group) and clotting time (dichotomous: number of kestrels in each treatment group with clotting time exceeding mean baseline clotting time by 2 standard deviations). Using the dose-response curve, a benchmark dose at the 50% effect level (BMD50) and a benchmark dose low (BMDL50; lower bound of the 95% CI of BMD50) was calculated using gamma, multi-stage, Weibull, quantal-linear, logistic, log-logistic, probit, and log-probit models (Filipsson et al. 2003, US EPA 2011b). Akaike's Information Criterion and P-values were used to select the most appropriate model for these data.

RESULTS AND DISCUSSION

All kestrels survived the 7-day feeding trial. Measurement endpoints did not differ between control kestrels receiving untreated NBP diet and control kestrels fed untreated RT, so these birds were combined into a single control group (n = 10). Slight differences in body weight were detected among groups, but these changes were modest (weight fluctuation was <6 g/100 g body weight) and did not seem biologically meaningful. Food consumption did not differ among groups; daily consumption ranged from 225.6 to 273.6 g ww/kg body weight-day. In general, exposure and adverse effects were dose-dependent, and there were no dramatic differences in toxicity between the mechanically-amended and biologically-incorporated CPN diets. Overt signs of toxicity (external bruising) and gross evidence of hemorrhage at necropsy were apparent in half of the kestrels receiving a diet of 1.5 µg CPN/g food.

Before the toxicity trial, baseline PT values ranged from 7.65-17.65 seconds (mean ± SD, 12.02 ± 2.03 s) and RVVT values ranged from 13.3-23.3 seconds (mean ± SD, 18.13 ± 2.42 s). Prothrombin time and RVVT of kestrels fed 0.75 and 1.5 µg CPN/g food for 7 days were significantly prolonged ($P < 0.05$) compared to the 0.15 µg CPN/g and controls groups. This response did not differ between mechanically-amended or biologically-incorporated diets.

Prolonged clotting time was defined as the average baseline PT value + 2 standard deviations and the average baseline RVVT value + 2 standard deviations. Estimates of the ingested quantity of CPN associated with prolonged PT and RVVT (dietary-base toxicity reference values) are presented in Figures 1 and 2. The mean quantity of CPN consumed by the 6 treatment groups ranged from 30.7 to 354.1 µg/kg body weight-day. The dose-response curve for PT was very steep such that PT was not affected at the low CPN dietary dose, but all kestrels exceeded baseline values at both the intermediate and high CPN doses. The log-logistic model provided the best fit for this data set, yielding an estimate that 50% of the kestrels would exhibit prolonged PT at a daily dietary dose of 79.2 µg CPN consumed/kg body weight-day (toxicity reference value) for a 7-day period (lower bound of the 95% confidence interval: 39.6 µg CPN consumed/kg body weight-day). Likewise, the dose-response curve for RVVT was also steep, but RVVT values of several kestrels in the 0.15 µg CPN/g groups exceeded baseline clotting time values. The logistic model provided the best fit for this data set (shape of curve better defined than for PT), yielding an estimate that 50% of the kestrels would exhibit prolonged RVVT at a daily dietary dose of 39.1 µg CPN consumed/kg body weight-day (toxicity reference value) for a 7-day period (lower bound of the 95% confidence interval: 32.6 µg CPN consumed/kg body weight-day).

The American kestrel is a test species often used as a toxicological model for raptors (Bardo and Bird 2009), and the toxicity reference values estimated in the present study can be used to better evaluate the hazard of CPN to predatory birds. Several studies have determined CPN in carcass and tissues of target mammals following application to fields used for grazing livestock. Following use of bait stations containing 0.05% CPN, carcasses of Belding's ground squirrels (*Spermophilus beldingi*), pocket gophers (*Thomomys bottae*), and voles (*Microtus* spp.) had mean CPN residues of 0.131, 0.357, and 1.58 µg CPN/g ww, respectively (Primus et al. 2001). In another study involving spot baiting and hand baiting around burrows with 0.01% CPN bait, carcass residues of Belding's ground squirrels averaged 0.159 µg CPN/g ww (Ramey et al. 2007). More recently, Rozol[®] (0.005% CPN) use in pastures resulted in liver residues ranging from 0.44 to 7.56 µg CPN/g ww in black-tailed prairie dogs (*Cynomys ludovicianus*) and thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) found dead or moribund on Days 5 through 29 post-application (Vyas et al. 2012). Notably, CPN in RT fed to kestrels in the present study (0.15 to 1.5 µg CPN/g ww) falls in the range of values found in target species in these prairie ecosystem field studies.

In the present study, kestrels ingested on average 246 g of RT per kilogram body weight each day. Thus, at the intermediate dietary concentration (i.e., 0.75 µg CPN/ g ww food), a kestrel would consume about 185 µg CPN/kg body weight-day. This daily exposure is 2.3 times greater than the toxicity reference value that would result in prolonged PT in 50% of birds exposed for a 7-day period (i.e., 79.2 µg CPN consumed/kg body weight-day) and 4.7 times greater than the toxicity reference value associated with prolonged RVVT in 50% of birds exposed for a 7-day period (i.e., 39.1 µg CPN consumed /kg body weight-day). Although prey availability, daily consumption, and carcass CPN residues are likely to vary considerably, these data suggest that coagulopathy could certainly occur in raptors feeding on CPN-exposed prey for an extended period (e.g., 7 days) following bait application in large-scale agricultural settings and island eradication projects. Using an adverse outcome pathway (Rattner et al. 2013), coagulopathy has been linked to
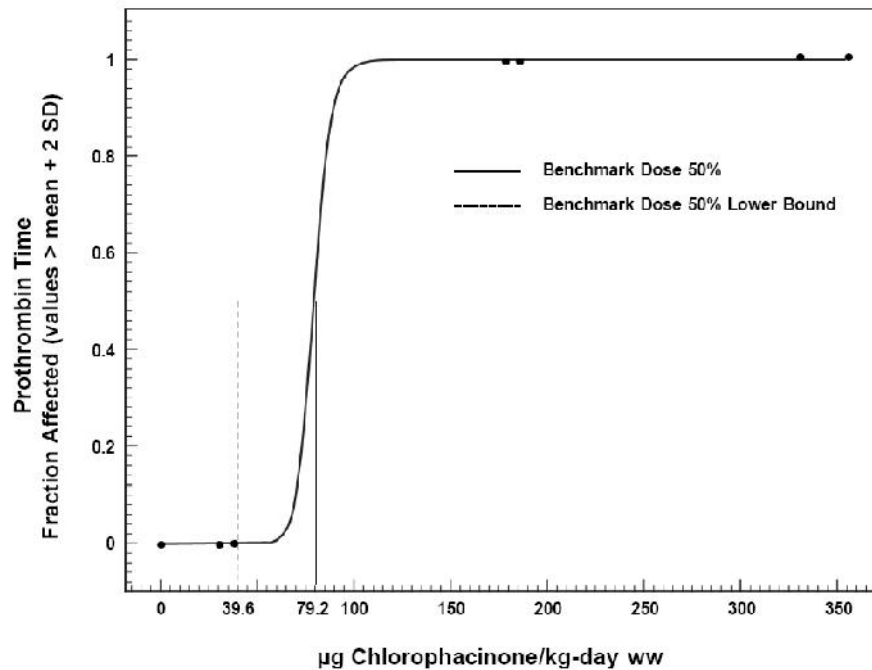
Figure 1. Dose-response relation of CPN consumed and PT in American kestrels. Using the benchmark dose method, a dietary-based toxicity reference value at which PT in 50% of the test population exceeded the baseline mean by 2 standard deviations occurred at 79.2 µg CPN consumed/kg body weight-day (lower bound of the 95% confidence interval: 39.6 µg CPN consumed/kg body weight-day).
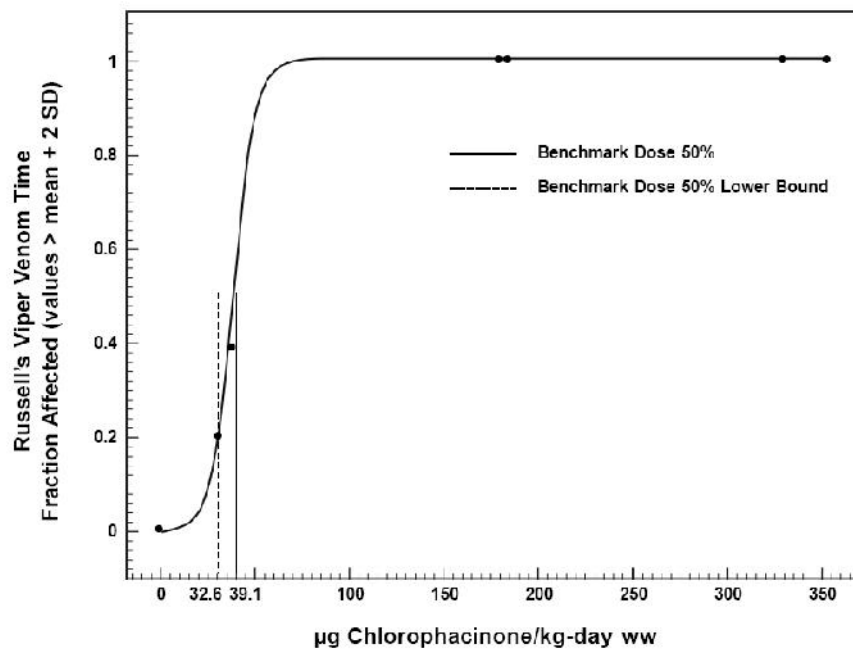

Figure 2. Dose-response relation of CPN consumed and RVVT in American kestrels. Using the benchmark dose method, a dietary-based toxicity reference value at which RVVT in 50% of the test population exceeded the baseline mean by 2 standard deviations occurred at 39.1 µg CPN consumed/kg body weight-day (lower bound of the 95% confidence interval: 32.6 µg CPN consumed/kg body weight-day).

hemorrhage at the organ system level, and anemia and even mortality at the organismal level. It has often been suggested that sublethal toxicological effects are one of many stressors affecting survival. Nonetheless, effects of FGARs on non-target predatory birds and mammals at the population level have not been established.

CONCLUSIONS

Our findings demonstrate that dietary exposure to environmentally realistic levels of CPN can evoke gross evidence of hemorrhage and coagulopathy in American kestrels, a model raptorial species. Based upon registration data, peer-reviewed published literature, and risk assessments, CPN appears to be somewhat less hazardous than SGARs. However, because SGARs have been used in areas more often frequented by people, detection of mortality incidents is more likely. Furthermore, most FGAR toxicity studies have used diets supplemented with vitamin K, which could differentially affect FGAR and SGAR toxicity. In addition, many FGAR risk assessments have inappropriately used acute toxicity data rather than multi-day exposure data to evaluate and compare the risk (Vyas and Rattner 2012). Accordingly, the hazard of CPN, and perhaps other FGARs, to non-target predators may be greater than previously perceived. In order to translate findings in captive kestrels to free-ranging raptors, natural resource managers must consider relevant exposure scenarios (duration of access and availability of contaminated prey) in order to predict potential adverse effects and to weigh the costs and benefits of anticoagulant rodenticide use in pest control and eradication programs.

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