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# CHLOROPHACINONE RESIDUES IN MAMMALIAN PREY AT A BLACK-TAILED PRAIRIE DOG COLONY

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**Abstract**—Black-tailed prairie dogs (BTPDs), *Cynomys ludovicianus*, are an important prey for raptors; therefore, the use of the rodenticide Rozol (0.005% chlorophacinone active ingredient) to control BTPDs raises concern for secondary poisonings resulting from the consumption of contaminated prey by raptors. In the present study, the authors observed Rozol exposure and adverse effects to mammalian prey on 11 of 12 search days of the study. Mammalian hepatic chlorophacinone residues ranged from 0.44 to 7.56 μg/g. Poisoned prey availability was greater than previously reported. Environ. Toxicol. Chem. 2012;31:2513–2516. © 2012 SETAC

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## INTRODUCTION

Black-tailed prairie dogs (BTPDs), *Cynomys ludovicianus*, are colonial, diurnal, fossorial rodents and are considered a keystone species because they influence the prairie ecosystem's structure, function, and composition [1]. Many in the agricultural community regard BTPDs as a major pest [1] and, in 2009, the U.S. Environmental Protection Agency (U.S. EPA) registered the rodenticide Rozol (0.005% active ingredient chlorophacinone; 2-[(p-chlorophenyl)phenylacetyl]1,3-indandione) for BTPD control in Colorado, Kansas, Montana, Nebraska, New Mexico, North Dakota, Oklahoma, South Dakota, Texas, and Wyoming (U.S. EPA registration number 7173-286) [2] (http://www.regulations.gov/#!searchResults;rpp=25;po=0;s=EPA-hq-opp-2009-0684-0164). Chlorophacinone is a first-generation anticoagulant rodenticide, and its mode of action involves inhibition of vitamin K epoxide reductase, resulting in the disruption of blood-clotting activity, subsequent hemorrhaging, and death [3]. The Rozol prairie dog bait formulation consists of green-colored chlorophacinone-treated winter wheat.

Black-tailed prairie dog colonies are a valuable concentrated food source for raptors [4,5], and Rozol-poisoned BTPDs create opportunities for secondary poisoning of raptors through the consumption of contaminated prey. Although little is known about the hazards of Rozol to nontarget wildlife at BTPD colonies, the few serendipitous wildlife carcass recoveries confirm the risks to avian predators and scavengers (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0003; http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0140). The objective of the present study was to measure chlorophacinone residues in poisoned mammalian prey following operational Rozol applications at a BTPD colony to determine the potential exposures that raptors can receive via consumption of contaminated prey.

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## MATERIALS AND METHODS

Rozol's effects on target and nontarget mammals were documented at a BTPD colony (~17.3 ha) located on a private pasture approximately 1.3 km south of Vernon, Colorado, USA. The colony was poisoned with Rozol by the Yuma County Pest Control District as part of their ongoing BTPD control program. Rozol was applied according to the label by certified pesticide applicators of the Yuma County Pest Control District: approximately 216 kg of Rozol was applied, treating 4,080 BTPD burrows. Observations on Rozol's effects on mammals were conducted on 12 d over a 29-d postapplication period in March and April, 2010 (Fig. 1). No field work was conducted on days 3, 6 to 11, or 14 to 22 postapplication. East–west transects (~3 m apart) were walked through the colony on 10 of the 12 d, and on the remaining 2 d (days 23 and 29 postapplication), transects were not followed but the area was still perused on foot for evidence of effects. Each search lasted for 2 to 4 h and was conducted in late afternoon as recommended on the Rozol label. Dead and moribund mammals were collected at the treated colony for chlorophacinone residue analysis. Reference BTPDs were collected by shooting the BTPDs in the head (to preserve the livers) at an untreated colony from the Cheyenne River Sioux Reservation, South Dakota, USA. All samples were stored in a freezer before shipping them to Patuxent Wildlife Research Center.

Livers of animals collected from the field were submitted to the U.S. Department of Agriculture, Beltsville Agricultural Research Center for chlorophacinone residue analyses. Analysis methods followed those described by Albert et al. [6], with the following modifications. The 0.5 g of sample was ground with approximately 5.0 g sodium sulfate (anhydrous $Na_2SO_4$, granular powder; Mallinckrodt Baker) using a mortar and pestle, and the ground mixture was transferred to a screw-top plastic 50-ml graduated tube and extracted three times with 5.0 ml acetonitrile (all solvents used in these analyses were of high purity and pesticide grade; Burdick & Jackson Honeywell International). The 5.0-ml extracts were hand shaken for 1.5 min and then mechanically shaken for 15 min. They were then centrifuged at room temperature at 1,000 rpm for 15 min, and the three supernatants from each sample were combined

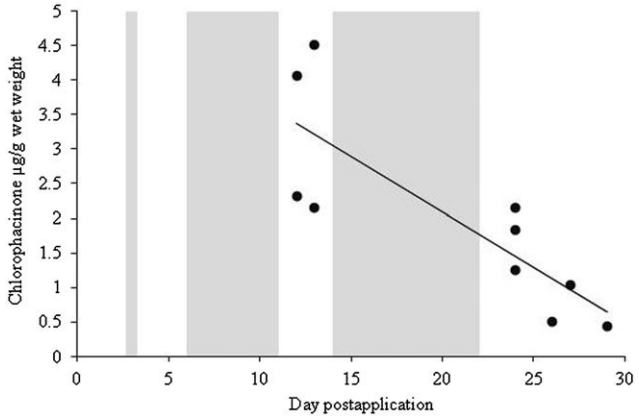


Fig. 1. Chlorophacinone residues (μg/g wet wt) in black-tailed prairie dog (*Cynomys ludovicianus*) livers from animals collected at a colony treated with Rozol. Gray bars indicate days on which carcass searches were not conducted. Chlorophacinone residues in mammalian livers decreased over time, $r(8) = -0.82$, $p < 0.01$.

and transferred to a plastic 15-ml screw-top graduated tube. The total supernatant was then concentrated to 5.0 ml under nitrogen in a water bath maintained at approximately 60°C. A 1.0-ml aliquot (corresponding to 0.1 g liver) was then added to a Sep-Pak Plus tC18 cartridge (Waters Corporation), which was conditioned with 5.0 ml MeOH, 5.0 ml deionized, carbon-free water (DI water) purified in a Nano-Pure water purification system (Barnstead International), and 10 ml acetonitrile. The cartridge was not allowed to go dry between any of the conditioning steps and the introduction of the sample. This Sep-Pak Plus tC18 cartridge conditioning regimen was used by Albert et al. [6] but was not mentioned in the publication (F. Maisonneuve, Environment Canada, Ottawa, Ontario, Canada, personal communication). After the 1.0-ml sample had been introduced to the cartridge, the tube containing the sample was rinsed with 9.0 ml acetonitrile, and this rinsate was then added to the cartridge. The resultant 10-ml eluate was then concentrated, as described above, to 2.0 ml, and a 10.0-μl volume was analyzed by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). The running solvents for these analyses were 10 mM ammonium acetate (solvent A) and acetonitrile (solvent D), and the gradient that we used went from 70:30 A:D initially to 30:70 A:D over 5 min (normal gradient), then 5 to 12 min (normal gradient) from 70:30 A:D to 10:90 A:D, after which the instrument was returned to the initial conditions 70:30 A:D (12–13 min) in 1 min, where it was held for the next 7 min (e.g., 13–20 min) until it was ready again for the next sample. The solvent flow throughout was 250 μl/min, and the column temperature was maintained at 35°C. We used an X-bridge C-18, 5 μm, $2.2 \times 150$ mm column (Waters Corporation). The elution times were approximately 6.7 min for chlorophacinone. The analyses were conducted with a model 2695 LC (Waters Corporation) and a triple quadrupole mass spectrometer, Micromass model Quattro Ultima (Waters Corporation) with an electrospray ionization source operating in negative ionization mode for quantitation. Positive ionization was used to confirm identity. Parent-to-daughter transition for negative identification and quantitation was mass 373.4 transition to 201.3 m/z with a cone voltage setting of 96 V and collision voltage of 22 eV. Acquisitions were performed in multiple reaction monitoring mode, and peak integration and quantitation using external standard methods were performed in MassLynx 4.0 software (Micromass). The lowest chlorophacinone standard (Sigma-Aldrich) used was 0.005 μg/ml. Two additional concentrations (0.001 and 0.05 μg/ml) were used to establish linearity, and $r^2$ generally exceeded 0.990. The limit of detection was 0.03 μg/g. Recoveries from spiked tissue samples averaged 106%. Microsoft Excel was used to develop a correlation coefficient for BTPD chlorophacinone residues over time.

## RESULTS

Signs of Rozol exposure and adverse effects on mammals were observed on 11 of the 12 search days and included the following: two dead, intact thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*); eight dead, intact BTPDs; seven scavenged BTPDs; eight moribund BTPDs; and green BTPD droppings. Several BTPD remains (i.e., feet, hide, and head) were also found but were not collected. All carcasses were found above ground, and all intact BTPD carcasses were within 3 m of burrow entrances. The first observed mortality, a thirteen-lined ground squirrel, was found on day 5 postapplication, and the first BTPD poisonings were observed on day 12 postapplication. Most of the moribund BTPDs were close to their burrow entrances and appeared listless. Signs of BTPD poisoning were observed up to the last day of the study; three moribund BTPDs were observed on day 29 postapplication. One of the three moribund BTPDs was captured in a net but died shortly afterward. Green BTPD droppings (an indication that BTPDs had been exposed to Rozol) were seen around the burrow entrances beginning at day 2 postapplication. The number of BTPDs observed above ground declined over the study period, and no thirteen-lined ground squirrels were found in the study colony by day 29 postapplication. The tails and hind legs of several of the dead and moribund BTPDs were blood stained, as confirmed by a phenolphthalein presumptive blood test (Doje's Forensic Supplies).

Chlorophacinone residues were detected in all liver samples collected from the study colony (BTPDs $n = 10$ and thirteen-lined ground squirrels $n = 2$). Chlorophacinone residues in BTPD livers decreased over time, $r(8) = -0.82$, $p < 0.01$ (Fig. 1). The greatest residue concentrations were detected in two thirteen-lined ground squirrels, both collected early in the sampling period (7.04 μg/g and 7.56 μg/g on days 5 and 13 postapplication, respectively). Chlorophacinone residues in reference livers collected from an untreated BTPD colony were below the detection limit (0.03 μg/g). All chlorophacinone values are presented based on the sample wet weight.

## DISCUSSION

The staggered onset of adverse effects and their persistence for at least 29 d after Rozol application are in agreement with the mode of action and the time course of effects for first-generation anticoagulant rodenticides. First-generation anticoagulant rodenticides generally require multiple feedings over several days to achieve a threshold concentration in tissue and cause adverse effects [7] (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2006-0955-0764). Sublethal adverse effects (e.g., prolonged clotting time) can occur within 48 h of exposure, but mortality may occur one week or more postexposure [8–11]. In our study, the first mortality (thirteen-lined ground squirrel) was found on day 5 postapplication, and the first BTPD mortality was documented on day 12. However, on day 12 postapplication, based on the presence of previously scavenged BTPD carcasses, the onset of mortalities appears to have started earlier, but it was not recorded because

no field work was conducted from days 6 to 11 postapplication (Fig. 1). Only two other field studies have addressed Rozol's effects and residues in wildlife at BTPD colonies. Lee and Hygnstrom (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2009-0684-0013) conducted carcass searches every other day for 16 to 25 d post-Rozol applications in 10 treated BTPD colonies (~58 ha). They recovered nine BTPD carcasses and one cottontail rabbit (*Sylvilagus* sp.) carcass and observed five impaired BTPDs. All carcasses were found above ground, and adverse effects (dead and moribund BTPDs) were observed from day 10 to day 25 postapplication; however, the authors hypothesized that the majority of BTPD mortalities occurred underground. Forgacs (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0140) monitored a BTPD colony for 21 d post application but, because of bad weather that limited observations, failed to find effects.

We measured hepatic residues because the greatest chlorophacinone concentrations are generally found in the liver (http://www.epa.gov/oppsrrd1/REDs/2100red.pdf; http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0028) and because our observations have shown that raptors preferentially feed on certain BTPD organs, especially the liver. Chlorophacinone residues in BTPD livers ($n = 10$) and in thirteen-lined ground squirrels ($n = 2$) from the study colony ranged from 0.44 to 7.56 μg/g (Fig. 1). Lee and Hygnstrom reported mammalian hepatic residues from BTPD colonies ranging from 0.45 to 8.31 μg/g (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2009-0684-0014). In a laboratory study, Witmer (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0028) provided a diet containing 53 g Rozol for 2 d to captive BTPDs that either died from Rozol or were euthanized over a 27-d period post-Rozol introduction. Liver chlorophacinone residues ranged from 0.061 to 8.4 μg/g, and the highest chlorophacinone concentrations were reported for BTPDs euthanized 3 d after introduction of Rozol (mean ± SE, $5.5 \pm 1.0$ μg/g, $n = 4$) but then declined rapidly. In the present study, several factors might have contributed to the decline of hepatic chlorophacinone residues over time (Fig. 1), including the short (<7 d) hepatic half-life of chlorophacinone in prairie dogs in conjunction with chlorophacinone's chronic time course of adverse effects (i.e., poisoned animals may stop feeding, and it may take several days before they die from secondary causes such as starvation and extreme weather), small amounts of multiple feedings, and a reduced availability of Rozol bait in the latter part of the study period [12] (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2006-0955-0764; http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0028). Although the chlorophacinone residues in the livers from the study colony may seem small, they are of biological significance because first-generation anticoagulant rodenticides are designed for low-level multiple feedings over time, so a single high exposure is not necessary for adverse effects [7,12,13].

All adverse effects reported in our study were observed in animals above ground; therefore, the affected animals provided an opportunity for secondary poisoning to avian predators and scavengers (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0003; http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0140). Typically, BTPDs sound alarm calls and run to their burrows when threatened; however, the moribund BTPDs were weak and slow to react and as a consequence were likely more vulnerable to predation. The fact that one of the moribund BTPDs was caught using a 1.8-m-long landing net shows that poisoned BTPDs are easy prey [14–16].

Lee and Hygnstrom (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2009-0684-0013) found 15 affected mammals from 10 BTPD colonies (~58 ha) that were searched for 16 to 25 d postapplications, and Forgacs (http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0909-0140) found no evidence of poisoning above ground. By contrast, we document 25 adversely affected mammals from a single BTPD colony (17.3 ha). Our results show a much greater availability of poisoned animals for raptors. It is also of note that, although hepatic residue levels continued to decline over the study period, BTPDs were still dying with these low residue levels 29 d after a single Rozol application. At this time, no avian toxicity data generated from testing methodologies that mimic real-world exposures are available for chlorophacinone. Appropriate chlorophacinone avian toxicity data are needed to complement our chlorophacinone residues from the field for conducting meaningful risk assessments for raptors.

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