United States Department of the Interior

FISH AND WILDLIFE SERVICE

Ecological Services
420 South Garfield Avenue, Suite 400
Pierre, South Dakota 57501-5408

August 22, 2011

MEMORANDUM

SUBJECT: Review and Management Recommendations for study titled "Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone bait"

FROM: Matt Schwarz, Environmental Contaminants Specialist, South Dakota Ecological Services Field Office, and Joy Gober, Fish and Wildlife Biologist, Natural Resource Program Center, Colorado

THRU: Scott Larson, Field Office Supervisor, South Dakota Ecological Services Field Office

TO: Kevin Johnson, Regional EC Coordinator, Region 6 Office

Attached is the final report and management recommendations for the Off-Refuge Environmental Contaminants Investigation titled "Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone." The main component of the investigation included a laboratory study performed by the U.S. Department of Agriculture's National Wildlife Research Center (Center) in Fort Collins, Colorado. The South Dakota Ecological Services Field Office has reviewed the study report submitted by the Center (Witmer, 2011) and has prepared comments and management recommendations in light of the study findings.

Witmer (2011) provides valuable information on prairie dog chlorophacinone excretion, residue concentrations, and observed effects from toxicity; however, the limited prairie dog exposure to chlorophacinone bait in the current study underestimates what would occur during a field application. For reasons specified in our enclosed review, the amount of bait (53 grams each) and time of exposure (1-2 days) for the lab study are much lower than what would be expected for a field application.

Concentrations of chlorophacinone in prairie dog tissues from the current study exceeded those previously reported for common voles (Vidal et al., 2009) but were lower than those previously reported for prairie dogs carcasses retrieved 11 days after a field application (Primus, 2007). Study results also indicate that chlorophacinone is metabolized over time so that death may occur after the parent compound is nearly gone. Therefore, "trace amounts" of chlorophacinone, as

reported previously in raptor diagnostic necropsy examination reports, should be considered as acceptable evidence that rodenticide exposure may have attributed to untimely death. Furthermore the behavioral incapacitation and lethargy effects of chlorophacinone may result in raptors being more susceptible to being shot or hit by vehicles or succumbing to injuries that would typically be considered non-life threatening (e.g., territorial infighting or capture of prey).

The primary weakness of the current study is that it underestimates the amount of bait available, and the length of time bait is available, compared to field applications. Although three prairie dogs were offered *ad libitum* Rozol for two days (T2 group), valid conclusions cannot inferred from the T2 group due to small sample size, the limited 2 days of *ad libitum* availability and variability in the amount of bait consumed.

Further field evaluations are needed to evaluate concentrations of chlorophacinone in prairie dogs exposed during actual field applications to supplement less robust assessments that are currently available. However, results of the current study combined with knowledge from other studies and field observations indicate that chlorophacinone residues in prairie dogs are elevated enough to cause concern for secondary poisoning of non-target avian and mammalian species. Therefore, we recommend that future assessments focus more on the effects of chlorophacinone toxicity to non-targets that consume poisoned prairie dogs. More studies are needed to determine how sub-lethal exposure may result in decreased non-target reproduction and survival.

References

See enclosure

U.S. FISH AND WILDLIFE SERVICE
DIVISION OF ENVIRONMENTAL QUALITY

REGION 6

RETENTION TIME OF CHLOROPHACINONE IN THE TISSUES OF BLACK-TAILED PRAIRIE DOGS EXPOSED TO CHLOROPHACINONE BAIT

Final Report
Region 6
DEC ID : 200960003
FFS: **6F56**
Congressional District: SD00

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Reviewer's Comments

The current study (Witmer, 2011) was funded by the U.S. Fish and Wildlife Service through the Environmental Contaminants Program as an Environmental Contaminants Investigation. The objectives of this investigation were:

1) To determine chlorophacinone tissue residues in prairie dogs at incremental time periods post-exposure from a limited feeding.

2) To determine potential exposure of predators from consuming prairie dogs killed by chlorophacinone at incremental time periods post-exposure.

The current study provides data on Objective 1: however, the limited exposure of bait to prairie dogs (i.e., short feeding duration and limited amount of bait) is not reflective of actual prairie dog consumption rates when chlorophacinone bait is used in the field as a rodenticide. Therefore, while inferences can be made towards Objective 2, they are limited because the current study was not designed to replicate how prairie dogs forage on poisoned bait in the field. We recognize that to examine the issue of secondary poisoning of predators and scavengers from consumption of poisoned prairie dogs, it was necessary to start with a controlled exposure experiment that could provide levels of chlorophacinone in various prairie dog tissues at specific time intervals post exposure. However in doing so, study Objective 2 was not fully satisfied because the limited feeding duration in the current study underestimates exposure that may occur from repeated small daily doses consumed during an actual field application of chlorophacinone to control prairie dogs. Prairie dogs that feed on chlorophacinone bait over several days following a field application would likely have higher tissue residues than those in the current study. Secondary exposure risk to prairie dog predators would also be expected to occur for a longer duration and at higher uptake levels than the current study results suggest.

Chlorophacinone bait availability is expected to be greater after a field application to control prairie dogs than in the current study. In the current study, prairie dogs in group T1 were provided bait for two consecutive days (53 grams total) and excess bait was removed. Prairie dogs were then either euthanized at predetermined time intervals or maintained on a clean diet for up to 25 days. For field applications, the Rozol label recommends a dose of 53 grams per active burrow and there are on average 3.9 active burrow-entrances per black-tailed prairie dog (Biggins et al., 1993). Inactive burrows may also be mistakenly baited resulting in further bait availability. Furthermore, there is no effort to remove excess bait or provide clean food during field applications and thus prairie dogs may continue to consume bait after they have accumulated a lethal dose. As

noted on page 35 of the report, it has been observed that “animals continue to feed on the baits for several days, then become lethargic and eventually stop feeding." Lee and Hygnstrom (2007) reported finding prairie dogs carcasses 10 - 25 days after field applications.

Observations during the current study also indicate that prairie dogs were exposed to less bait than what would typically occur during a field application. For example, only 3 of 36 poisoned prairie dogs died and only 9 showed signs of being poisoned. The current study also reported that only 56 percent of T1 animals showed evidence of hemorrhaging. Although first generation indandiones (i.e., chlorophacinone and diphacinone) can cause mortality without showing hemorrhaging in mammals, the low number of dead and morbid prairie dogs in the current study indicates a lower dose was received than reported elsewhere for field applications. For example, Lee and Hygnstrom (2007) reported prairie dog population reductions between 85 – 96 percent following field applications of chlorophacinone.

Previous reports (Lee and Hygnstrom, 2007; Primus, 2007) that include chlorophacinone residues in prairie dog tissues after a field application also suggest that prairie dogs in the current study consumed less bait than what would likely occur under a field application. The current study reported a mean concentration of chlorophacinone in liver of 0.82 ± 0.70 micrograms per gram (µg/g) in prairie dogs euthanized 11 days after bait (0.005% chlorophacinone) was first presented. In comparison, eight prairie dog carcasses collected 10 to 25 days after a field application of similar bait had a mean concentration of chlorophacinone in liver of 2.19 ± 1.80 µg/g (Lee and Hygnstrom, 2007; Primus, 2007). Eight additional prairie dog carcasses collected from other non-experimental field sites (dates of application unknown) reported a mean concentration of chlorophacinone in liver of 5.86 ± 1.88 (Primus, 2007). Residues in live rodents are expected to be greater than in their carcasses, especially for relatively short-lived, chronic rodenticides such as diphacinone and chlorophacinone as the rodent may continue to consume bait above a lethal dose and close to the onset of morbidity. The longer the lag time (between exposure and death), the more time is available for the target rodent to continue consuming bait. Therefore, chlorophacinone residues in live prairie dogs could be substantially higher than those in carcasses, but further study is needed to predict residue levels in live prairie dogs that have undergone multiple feedings of poisoned bait.

Repeated small daily doses of anticoagulants are also known to result in higher toxicity than a single acute dose (Godfrey et al., 1981; Jackson and Ashton, 1992). For example, the median lethal dose (LD50) from a single exposure of chlorophacinone to Norway rats (*Rattus norvegicus*) is 20.5 milligrams per kilograms (mg/kg) whereas a 5-day daily dose LD50 is twenty times lower at 0.95 mg/kg (Jackson and Ashton, 1992). We expect that

the difference in toxicity between a single acute dose and repeated daily doses would also apply to non-target species that feed on prairie dogs. Additionally, the extended period of 1 to 4 weeks between application of poisoned bait and observed deaths of prairie dogs (Vyas, 2010, Lee and Hygnstrom, 2007) extends the time during which prairie dogs are available to non-target predatory animals.

Results from the current study indicate that tissue concentrations of chlorophacinone were highly variable among individuals necropsied on the same day (not evident in Figures 3 and 4 but note standard deviations in Table 1). Silberhorn and others (2003) also reported large variations in residues in individual ground squirrel carcasses even for those squirrels that died on the same day. All the prairie dogs in the current study were exposed at the same time so the variability may be due to individual differences in the amount of bait consumed, size, and metabolism and excretion of chlorophacinone. Due to variability in individual residue loads, it is recommended that future assessments include at least 10 individuals collected on the same day post application.

Toxicity to chlorophacinone among individuals also appeared to be highly variable and not necessarily related to the amount of bait consumed. Observational data on prairie dog response after consuming the maximum amount of bait (53 grams) ranged from appearing normal to severe incapacitation and death. Animal health logs indicate that the three prairie dogs found dead had appeared normal during prior observation checks and only one showed signs of external bleeding. Variability in the susceptibility of target species to chlorophacinone toxicity may also apply to non-target species and should be further evaluated.

Although chlorophacinone residues in the current study likely underestimate exposure from field applications, dead and euthanized prairie dogs still had residue levels that were high enough to warrant concern for secondary exposure to non-target birds or mammals. Non-target species that feed on prairie dogs would be exposed to chlorophacinone in whole-body and liver tissues. In the current study, prairie dogs had concentrations of chlorophacinone in whole-body and liver that ranged from 0.053 - 1.78 µg/g and 0.061 - 8.407 µg/g, respectively. Liver chlorophacinone concentrations exceeded those previously reported for common voles (0.082 - 3.800 µg/g) but secondary exposure risk to vole predators was not evaluated (Vidal et al., 2009). Whole-body concentrations in prairie dogs from the current study were also greater than those estimated in laboratory rats (0.18 – 0.81 µg/g) that resulted in the death of 11 of 20 domestic ferrets when fed upon for five consecutive days (Ahmed et al., 1996 as cited by Erickson and Urban, 2004).

Observations from the current study indicate that chlorophacinone is metabolized over time so that death can occur after the parent compound is nearly gone. Prairie dogs that lived the longest but eventually were euthanized based on condition had liver chlorophacinone levels similar to "trace amounts" reported for wildlife mortality incident investigations. For example, two prairie dogs (KQ-18 and KQ-26) ate 36 and 53 grams of bait during the first two days, respectively. They were then euthanized due to their condition on days 22 and 26 and had liver concentrations of chlorophacinone of 0.265 and 0.187 µg/g, respectively. These concentrations are similar to those previously reported as "trace" amounts of chlorophacinone (e.g., 0.25 µg/g) in wild raptors opportunistically found. For example, a bald eagle found near a chlorophacinone poisoned prairie dog town in Nebraska had a liver concentration of 0.3 µg/g chlorophacinone and forensic necropsy results indicated that the eagle died from chlorophacinone ingestion (USFWS, 2007). Chlorophacinone was also detected at 0.18 µg/g in a red-tailed hawk from New York State (Stone et al., 2003). Other raptors, for which chlorophacinone exposure may have contributed to death, include a ferruginous hawk and great-horned owl, both collected from Kansas with “trace amounts” of 0.25 µg/g chlorophacinone (USFWS, 2009).

The current study indicates that lethargy can persist in poisoned prairie dogs for several days before they die or need to be euthanized based on morbidity. Despite only a single exposure to rozol bait, many of the prairie dogs suffered from delayed incapacitation. For example, prairie dog KQ26 was lethargic for 16 days starting 10 days after exposure and was euthanized on Day 26 due to poor condition. Incapacitation in these animals occurred despite receiving a clean maintenance diet post exposure to rozol. Prolonged lethargy would likely result in increased susceptibility to predation and these same prairie dogs that are more easily captured by predators may present the highest risk of secondary exposure if they continue to eat chlorophacinone after receiving a lethal dose and thus accumulate higher tissue residues.

The current study included a T2 group of three prairie dogs that were provided chlorophacinone bait *ad libitum* for two days. Valid conclusions cannot be made from this T2 group. The small sample size of this group (n = 3) and high variability in both the amount of bait consumed per individual (i.e., range of 7.0 - 54.6) and tissue concentrations (see Table 2B) preclude statistical analysis. The prairie dog that consumed only 7.0 g of bait had the lowest tissue concentrations of chlorophacinone in the T2 group and ingested much less bait than any other prairie dog in either treatment group (the next lowest was 27.5 g of bait consumed), leading to the question of whether some other factor was affecting this test animal. Furthermore, the time period of two days for *ad libitum* exposure is less than what would be expected in a field application.

We do not agree with the current study conclusions that "the highest risk of secondary exposure to chlorophacinone residues by non-target animals consuming prairie dogs exposed to the bait would occur within a few days after bait application and would drop quickly thereafter." As specified above, the current study is not representative of prairie dog exposure to chlorophacinone from a typical field application and prairie dogs that continue to consume bait after they have accumulated a lethal dose may have the highest chlorophacinone tissue residues. This would result in risk of secondary exposure to non-target animals over a more extended time period.

The current study also suggests that "because birds are less susceptible to chlorophacinone poisoning than mammals, secondary risks are probably higher for predatory or scavenging mammals (coyotes) than for predatory birds" and based this conclusion on a review by Primus and others (2001). The risk assessment by Primus and others (2001) did not evaluate sub-lethal effects leading to indirect mortality, which is our greatest concern regarding avian consumption of chlorophacinone poisoned prairie dogs. Sub-lethal effects have been documented in raptors exposed to anticoagulants and can occur despite low tissue residue concentrations. For example, American kestrels (*Falco sparverius*) administered diphacinone and with liver residues just above the diphacinone method detection limits of 0.263 and 0.280 µg/g diphacinone had histological evidence of hemorrhage in lung and liver (Rattner et al., 2011a). Golden eagles (*Aquila chrysaetos*) fed muscle from diphacinone-treated sheep exhibited extreme weakness, hemorrhages, and ataxia (Savarie et al., 1979). These studies indicate that raptors are susceptible to indandione's multiple modes of action which include both the blocking of prothrombin formation and the uncoupling of oxidative phosphorylation (Van Den Berg and Nauta, 1975). Ample evidence exists to indicate that avian predators and scavengers are susceptible to secondary toxicity risks and additional study is needed to further evaluate the issue.

Management Recommendations

More data that are representative of field conditions are needed to adequately evaluate Objectives 1 and 2. We recommend a more robust assessment of chlorophacinone residues in prairie dogs that mimics operational application exposures of chlorophacinone bait. The assessment is needed to determine residues in prairie dogs that receive repeated small doses of chlorophacinone and should include at least 10 individuals that are euthanized as soon as they exhibit signs of lethargy or morbidity.

Studies indicate that avian lethality tests required to register first generation indandione rodenticides can result in toxicity values that ultimately underestimate risk and that new test requirements are needed. Standardized tests for avian lethality that are required by the U.S. Environmental Protection Agency (USEPA) to support pesticide registration include the single-dose acute oral toxicity test and the five-day sub-acute dietary toxicity test that are used to derive an LD50 and median lethal concentration (LC50), respectively (USEPA, 2007). First generation indandione rodenticides have a mode of action that results in cumulative effects over several days of feeding, thus the required single-dose acute oral toxicity test tends to result in large LD50s values that ultimately underestimate risk (Ashton et al., 1986; Jackson and Ashton, 1992). The standardized five-day sub-acute dietary toxicity test includes multiple exposures over several days but has little value as a quantitative descriptor of lethal toxicity and is more of a measure of vulnerability to a contaminated diet, with results that can be highly dependent on a species willingness to eat the bait and ability to cope with reduced nutriment (Hill, 1993; Mineau et al., 1994; Hoffman, 2003). Studies that do not follow required methodologies for registration but provide supplemental information, such as the previously mentioned five-day sub-acute oral toxicity tests (Godfrey et al., 1981; Jackson and Ashton, 1992), indicate that a repeated low dose oral sub-acute toxicity test for anticoagulant rodenticides can result in a more toxic LD50 than a single-dose acute oral test. Likewise, a dietary toxicity test that measured the diphacinone-treated diet consumed daily by Eastern screech-owls (*Megascops asio*) found that repeated low-dosage exposure over seven days increased diphacinone toxicity by more than an order of magnitude compared to an acute oral toxicity test (Rattner et al., 2011b; N. Vyas pers comm.). These studies indicate a need to change current required avian oral and dietary lethality tests for first generation indandione rodenticides to include multiple day low-dose exposures that measures individual daily dosage. Factors associated with extrapolating laboratory derived risk quotients to the field can further underestimate risk (Matz et al, 1998; Vyas et al., 2006), and this may be especially true when considering the sub-lethal effects from first generation indandione rodenticides. Thus, methods for the lethality tests should also be expanded to include observational periods for sub-lethal effects and protocols that include gross pathology and histopathological examination of tissues to evaluate internal hemorrhaging. The USEPA has the responsibility and authority under the Federal Insecticide, Fungicide, and Rodenticide Act to determine the potential of a pesticide to cause adverse effects and require further testing when needed (USEPA, 2007). We recommend that USEPA develop new standardized testing requirements for first generation indandione rodenticides and require additional long term field studies to allow for a more adequate determination of whether continued registration approval is warranted for use of first generation indandione rodenticides to control prairie dogs.

Active surveillance is needed to further examine the extent of non-target mortalities from the use of anticoagulant rodenticides to control prairie dogs. Lee and Hygnstrom (2007) included searchers for non-target carcasses on and immediately around baited plots while performing field assessments designed to assess the efficacy of chlorophacinone and did not report any indications that avian non-targets were adversely affected from feeding on poisoned prairie dogs. However, recovery of poisoned raptors from baited prairie dog downs is expected to be highly unlikely given the chronic nature of chlorophacinone that allows wide ranging birds to move away from the site of application.

Additional assessments of secondary risks to avian species from exposure to chlorophacinone are needed and should consider interspecific differences in exposure and susceptibility. Although there is a paucity of sub-lethal threshold effects data following repeated exposure for birds of prey to chlorophacinone; a few studies of diphacinone toxicity to raptors (Savarie et al., 1979; Mendenhall and Pank, 1980; Rattner et al., 2011a) indicate that they may be especially sensitive to anticoagulants. Acute diphacinone toxicity tests indicate that American kestrels are over 20 times more sensitive than Northern bobwhite (*Colinus virginianus*), and over 30 times more sensitive than mallards (*Anas platyrhynchos*), two test species required by USEPA for pesticide registration (Rattner et al., 2010 and 2011a). Furthermore, golden eagles appear to be even more sensitive to diphacinone than kestrels (Savarie et al., 1979; Rattner et al., 2011a). Mendenhall and Pank (1980) observed differences in diphacinone toxicity between great-horned owls and barn owls and suggested that explanations for such a discrepancy may include interspecific differences in susceptibility or differences in prey species that result in dissimilar exposure. These studies indicate that future assessments on the effects of chlorophacinone on avian species that consume prairie dogs should include multiple species. Ferruginous hawks may be especially susceptible to anticoagulant use on prairie dogs as they are a primary predator of prairie dogs and have been frequently reported near prairie dog towns poisoned with anticoagulants. In 2010, Audubon of Kansas reported finding the remains of 17 dead hawks in 2009 following anticoagulant use to control prairie dogs in the area. Unfortunately, these carcasses were not recovered for necropsy or chemical analysis.

Based on sub-lethal effects to non-target species as reported from laboratory studies as well as reported mortalities and concerns based on opportunistic recoveries (Littrell, 1990; Ruder et al., 2008), there is clearly a need for field studies that evaluate anticoagulant exposure and effects to the many species that may consume poisoned prairie dogs. Littrell (1990) ranked exposure to diphacinone/chlorophacinone second only to strychnine as the most hazardous vertebrate pesticide to non-targets based on his 10 years of experience in reviewing vertebrate pesticides. Ruder and others (2008)

reported three mortality events involving several species, including wild turkeys (*Meleagris gallopavo*), a raccoon (*Procyon lotor*), and an American badger (*Taxidea taxus*) after a chlorophacinone application to control black-tailed prairie dogs in Kansas. The authors concluded that their opportunistic findings of non-target mortalities likely underestimate actual non-target losses and warrant further investigation. This conclusion seems justified as a four year survey of possible anticoagulant poisonings of wildlife in France that was based on a wildlife disease surveillance network yielded 59 confirmed diagnoses for bromadiolone and 41 for chlorophacinone (Berny et al., 1997 as cited by Stone et al., 1999). A similar surveillance network is needed to evaluate non-targets after anticoagulant use to control prairie dogs in the United States, especially given all of the avian predators that key in on and consume prairie dogs including golden eagles, northern goshawks, northern harriers, peregrine falcons, prairie falcons, Cooper's hawks, ferruginous hawks and red-tailed hawks. A few laboratory studies indicate that some of these species survive after being fed anticoagulant poisoned rodents, at least until time of necropsy (Savarie et al., 1979; Mendenhall and Pank, 1980; Radvanyi et al., 1988). However, the sub-lethal effects described in these studies (e.g., fatigue, wing-dropping, and lung, heart and liver hematomas) are likely to result in decreased survival or reproduction and need to be evaluated under field conditions.

Chlorophacinone is slow acting and non-target species are often highly mobile, thus tracking individual birds via radio or satellite telemetry is needed to evaluate secondary toxicity to avian predators that consume chlorophacinone poisoned prairie dogs. Avian carcasses are also quickly scavenged in the wild (Vyas, 1999) and tracking may aid in recovering intact carcasses for necropsy and residue analyses. Tracking studies also have the added benefit of allowing for evaluation in the field where animals also are exposed to other stressors and can also help assess potential sub-lethal effects that can include decreased survival and reproduction.

Conclusions

The current study provided some information on chlorophacinone residue concentrations over time and observed effects from toxicity; however, the limited exposure of chlorophacinone in the current study underestimates what would occur during a field application. Chlorophacinone loss from metabolism and excretion indicates that prairie dogs that continue to consume bait over several days will likely have higher chlorophacinone residues than prairie dogs that are found dead or euthanized after several days of being too sick to eat. Field observations and results from previous studies indicate that field applications would likely result in higher prairie dog residue burdens

than indicated in the current study and risk of secondary exposure to prairie dogs predators could last for weeks after application. Predators that consume prairie dogs would also have an increased risk to secondary toxicity from repeated small doses above what is inferred from a single reference dose.

Study results indicate that injury from exposure to chlorophacinone may not be related to concentrations of chlorophacinone in whole-body or liver tissues as measured at the time of death. Chlorophacinone is ingested, results in internal and sometimes external hemorrhaging and is then metabolized and excreted. Internal bleeding in non-targets from repeated exposure to chlorophacinone likely results in sub-lethal effects that contribute to increased mortality without resulting in high concentrations in tissue. Thus even low concentrations of chlorophacinone detected in poisoned carcasses may be indicative of cause of death, either directly or indirectly, and should be considered with other biological evidence in determining whether harmful exposure to anticoagulants occurred.

This study was an acute dietary toxicity test that provided some useful information, as previously noted. However, chlorophacinone lethality increases with multiple low-dose feedings and prairie dogs exposed to chlorophacinone bait under field conditions can live for several weeks before death occurs. Therefore, further study is needed to adequately evaluate Objectives 1 and 2. We recommend a more robust assessment of chlorophacinone residues in prairie dogs that are exposed to repeated low doses of chlorophacinone and are immediately euthanized after they exhibit signs of morbidity. Previous studies indicate that raptors are likely more sensitive to the first generation indandione rodenticides than traditional avian test species and further evaluation of threshold effects from repeated daily doses are needed. Lastly, we recommend that future field assessments incorporate tracking techniques to evaluate decreased survival and reproduction for multiple avian species.

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VOLUME

STUDY TITLE

Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone bait

DATA REQUIREMENT(S):

None

AUTHORS

Gary Witmer, Ph.D., Study Director

STUDY COMPLETION DATE

March 23, 2011

PERFORMING LABORATORY

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LABORATORY PROJECT ID:

QA-1682

CITATION

Witmer, Gary. 2011. Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone bait. Final Report: QA-1682. USDA/APHIS/WS National Wildlife Research Center, Fort Collins, CO. 59 pp.

FINAL REPORT Study ID: QA-1682

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d) 1(A), (B), or (C).

Submitter: U. S. Fish and Wildlife Service

Agent: _____

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FINAL REPORT Study ID: QA-1682

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study QA-1682, entitled, Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone bait, was performed in accordance with the Good Laboratory Practice Standards (GLPS) as outlined in 40 CFR Part 160, August 19, 1989 with the following exception:

1. HOBO data loggers used to monitor environmental conditions during product storage may not meet all GLP criteria but were utilized under written, authorized SOPS.

Sponsor: _____ 4/4/11

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FINAL REPORT Study ID: QA-1682

QUALITY ASSURANCE STATEMENT

This study (QA-1682) was inspected by NWRC Quality Assurance on the dates listed below. QA Inspection Reports were submitted to the Study Director and Test Facility Management as follows:

Phase	Inspection Date	Date to Study Director	Date to Test Facility Management
Protocol Inspection	10/22/10	10/22/10	10/22/10
Study Conduct - Animal weights/sexing	1/25/10	3/12/10	3/12/10
Study Conduct – Test material application	1/28/10	3/12/10	3/12/10
Study Conduct – Animal sacrifice and necropsy	2/3/10	3/12/10	3/12/10
Study Conduct – Sample preparation	3/3/10	3/12/10	3/12/10
Study Conduct –Sample extraction/analysis	3/31/10	4/14/10	4/14/10
Study Conduct – test material analysis	7/21-22/10	8/12/10	8/12/10
Draft Final Report/ Raw Data Review	2/17-3/22/11	3/18/11	3/18/11
Final Report	3/23/11	3/23/11	3/23/11

The Final Report was found to reflect the raw data.

Catherine M. Bens
Quality Assurance Manager

3/23/11
Date

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EXECUTIVE SUMMARY

Rozol prairie dog bait (0.005% chlorophacinone) was fed to male and female adult/subadult black-tailed prairie dogs (*Cynomys ludovicianus*) over a 2-day period. The residue levels of chlorophacinone in prairie dogs were determined over a 27 day period. Most prairie dogs (n=36; T1 group) were allowed to eat up to 53 g (EPA label application rate) while a group of 3 prairie dogs (T2 group) were allowed to eat bait *ad libitum* for a 2-day period. All remaining animals (n = 11) served as the control group. The T1 group consumed an average of 48.5 g (SD = 7.2 g) of bait, while the T2 group consumed an average of 33.0 g (SD = 24.1 g) of bait. Highest residue levels were found on Day 3 after the bait was first offered: an average of 5.499 µg/g (SD = 2.034 µg/g) in livers and 1.281 µg/g (SD = 0.369 µg/g) in whole bodies. Levels quickly declined after Day 3 and a half life of about 5-6 days in livers and whole bodies was interpolated from the graphed results. No chlorophacinone was found in the control animals (all values below the Method Limit of Detection). Residue levels were not significantly different in males and females nor in animals that died versus those euthanized in the same time period.

No control animals died during the course of the study. The 3 T2 animals were euthanized 3 days after bait was offered and no conclusive signs of anticoagulant poisoning (hemorrhaging) were observed from this limited time of exposure. Of the 36 T1 animals, 3 died of anticoagulant poisoning and 9 were considered to be moribund or had bleeding injuries and were euthanized. The remaining 24 animals in the T1 group appeared healthy when they were euthanized as per the schedule. The first clinical symptoms of anticoagulant poisoning (lethargy) were observed on Day 5, while main symptoms (external bleeding or blood in feces) began to be observed in Day 8 after the bait was first offered. Twenty of the 36 (56%) T1 animals showed evidence of hemorrhaging (external and/or internal) when necropsied.

INTRODUCTION

Black-tailed prairie dogs (*Cynomys ludovicianus*) are one of five species of prairie dogs found in North America. Black-tailed prairie dog population sizes are sometimes controlled because of the conflicts that arise with humans (e.g., property damage, consumption of range forage meant for livestock, threat of plague to humans and companion animals) and citizen attitudes about prairie dogs and their management vary widely (Zinn and Andelt 1999). Management of prairie dogs in the past has included poisoning, fumigants, barriers, and relocation (Franklin and Garrett 1989, Robinette et al. 1995, Andelt and Hopper 1998). Anticoagulants are commonly used to control rodent populations, but have not been registered for use with prairie dogs until recent years (Witmer and Fagerstone 2003). Fisher and Timm (1987) demonstrated in a cage trial that chlorophacinone was an effective rodenticide for prairie dogs, but also demonstrated the potential for secondary hazards to carnivores (using domestic ferrets) from consumption of poisoned prairie dogs. Lee et al. (2005) demonstrated the field efficacy of a chlorophacinone bait when placed in prairie dog burrows. Unlike zinc phosphide, the traditional toxicant for prairie dogs, anticoagulants persist in tissue (Eason et al. 2010). Symptoms of chlorophacinone exposure typically take several days after ingestion to manifest, and it may take 7-20 days for mortality to occur after a single gavage dose (Yoder 2007). Chlorophacinone (Rozol®) was approved under a Special Local Need or 24(c) registration for use on prairie dogs in several states. Because prairie dog colonies are utilized by various mammalian and avian predators, the

US Fish and Wildlife Service was concerned about the potential poisoning of these animals. The concern seems well-founded because, for example, Fournier-Chambrillon et al. (2004) and Albert et al. (2010), found chlorophacinone residues in the livers of mustelids and owls, respectively. To allow that assessment, managers need information on the levels of chlorophacinone levels that can occur in prairie dog tissues after feeding on rodenticide baits. This study is designed to provide the requested data set of the sponsor, USFWS. It will also be submitted to the US EPA to assist in making registration decisions on this anticoagulant rodenticide.

STUDY OBJECTIVE

The specific objective of this study was to determine the chlorophacinone residue levels in prairie dog livers and whole bodies at various time intervals after the animals have consumed chlorophacinone rodenticide baits. We hypothesized that residue levels would peak at some point and then decline over time.

MATERIALS

Test Material

Name: Rozol for Prairie Dogs
EPA Reg. No.: 7173-286
CAS number: CAS #3691-35-8 (chlorophacinone)
Lot/Batch No.: 28709A
Source: LiphaTech, Inc., Milwaukee WI
Description: Coated grain rodenticide food bait
Purity: 0.005% active ingredient
Active Ingredient: chlorophacinone
Stability of Compound
Under Test Conditions: Listed as stable on MSDS

Storage Conditions of
Test Chemicals: Test material was maintained in a plastic, sealed, black container (original container) in a fume hood at room temperature (maintained at about 21^{o}C).

Test Organism

Species: Black-tailed prairie dog (*Cynomys ludovicianus)*

Age at study initiation: all > 9 months (all adults or subadults)

Weight at study initiation: Ave. = 813.2 g (range: 590-1,060 g)

Source: Wild capture at Buckley Air Force Base, Aurora CO

METHODS

The protocol for this study was prepared according to NWRC standards and procedures and approved on 11/23/2009 (note: study initiation is considered as the date of Study Director signature, 11/20/2009). It was assigned NWRC Study Number QA-1682 (Appendix I). Details of the methods of the approved protocol are presented in Appendix I; amendments and deviations to the protocol are provided in Appendix II.

Test Conditions

Quarantine Period: Approximately 2 weeks.

Conditions: Animals were dusted with an insecticide (Drione) powder while in their capture cages in the field. When brought to NWRC, animals were held individually in raccoon-sized cage traps (25 cm wide, 81 cm deep, 30 cm height) in an outdoor building under ambient conditions for the approximately 2-week quarantine period. They were then brought into a climate controlled animal room where conditions were maintained at about 5.6 ^{o}C, a relative humidity of 25-30%, and a 12 hrs on:12 hrs off light cycle. Room conditions were monitored by daily checking the room condition panel in the antechamber room and recording the temperature. Additionally, a HOBO data logger in the actual animal room was maintained and checked periodically to assure that the room's settings were actually occurring as programmed. Animals were allowed 3 days to acclimate indoors in their new cages before rodenticide bait was added.

Feeding: Animals were fed a maintenance diet of grass hay, a slice of apple, and a slice of carrot each day.

Health: A health log was maintained for each animal and each animal was checked twice daily (morning and afternoon) beginning with the afternoon check on 1/27/10. The Study Director and Attending Veterinarian were consulted when any abnormalities were observed and animals were euthanized if deemed appropriate.

Pen size and construction materials: Stainless steel rabbit rack cages (48 cm wide, 61 cm deep, 41 cm height) were used to house the animals during the study with one animal per cage.

Test duration: 27 days from the day the Rozol Prairie Dog Bait was first offered (January 28, 2010 = Day 1).

Test Material Application: All maintenance food was removed from treatment animal cages in late afternoon the day (1/27/10) before bait was offered. At 8 am, 1/28/10 (henceforth called Day 1), rodenticide bait was offered to each animal in a ceramic bowl for 2 days with no alternative food available. At 8 am on 1/30/10, all remaining rodenticide bait was removed. The animals were then put back on the maintenance diet. Bait was weighed before being offered and when removed so that the amount consumed could be determined. Animals were randomly assigned to one of 3 groups: T1 (received 53 g of bait for a 2-day period, T2 (received *ad limitum* bait for a 2-day period), and a control group maintained on the maintenance diet throughout the study.

Chemical analysis: The Study Director received a certificate of analysis of the Rozol bait at the time of receipt from the manusfacturer. Additionally, the Analytical Chemistry Unit of NWRC analyzed the bait using NWRC Analytical Method 163A. Liver residue levels were determined with NWRC Analytical Method 143A; whole body residue levels were determined with NWRC Analytical Method 142A.

Observations

Parameters recorded: initial and final body weight, bait consumption, animal condition (twice daily), mortality, necropsy results (external/internal hemorrhaging). Animals were observed twice daily. Any animal appearing to be moribund (substantial lethargy, unresponsive to probing, and/or substantial bleeding) was euthanized for purposes of humaneness after consultation with the Study Director and/or Attending Veterinarian. Otherwise, animals were euthanized according to a predetermined schedule. A group of 4 randomly selected animals was euthanized according to a predetermined schedule; that is, on days 3, 5, 7, 9, 11, 14, 18, 27 with the bait presentation day being Day 1. Animals were euthanized by anesthetizing with isoflourane gas and then exposing to carbon dioxide. Animals were then necropsied and prepared for residue analysis with signs of external and internal hemorrhaging noted. Samples taken and frozen for chemical analysis were livers, whole bodies (less pelt, head, paws, tail), and rodenticide bait samples (freezer temperature maintained at about -10.6 ^{o}C). Animals found dead in their cages were processed in the same way.

Statistical Analysis

Statistical tests: Software program "Statistix 9" (Analytical Software, Tallahassee FL) was used to perform ANOVA and t tests to determine the significance of differences in the variables food consumption, body weight, and residue levels. A P value of ≤ 0.05 was considered to indicate a significant difference. Non-linear regression was performed on the average residue levels to generate the decay curves, associated regression coefficients, and regression equations.

Randomization method: The random numbers table from the book, Tables for Statisticians by Arkin and Colton (1963) was used to assign animals to treatment groups and to select animals for euthanasia.

RESULTS

The chlorophacinone concentration as determined by the NWRC Analytical Chemistry Unit was 0.005% (Appendix IV). The concentration of active ingredient (chlorophacinone) in the Rozol prairie dog bait used in this study was also determined by the manufacturer (LiphaTech, Inc.) to be 44.86 mg/kg or 0.0045% (Appendix IV).

The levels of chlorophacinone residues in black-tailed prairie dogs were determined over a 27 day period (Tables 1 and 2). Rozol prairie dog bait (0.005% chlorophacinone) was fed to male and female adult/subadult prairie dogs over a 2-day period with no other food present during

those 2 days (Day 1 and 2). Most prairie dogs (n=36; T1 group) were allowed to eat up to 53.0 g (EPA label application rate) while a group of 3 prairie dogs (T2 group) were allowed to eat bait *ad libitum* for the 2-day period. An additional 11 prairie dogs served as a control group. Table 2 provides the data set for all animals. The T1 group consumed an average of 48.5 g (SD = 7.3 g) of bait, while the T2 group consumed an average of 33.0 g (SD = 24.1 g) of bait (Table 2). The difference in food consumption between the two groups was not significant (t = 0.82, P = 0.4565). Table 1 provides a summary of the residue levels data set for T1 animals with values averaged by days after bait first presented. Highest residue levels in T1 animals were found on Day 3 after the bait was offered: an average of 5.499 µg/g (SD = 2.034 µg/g) in livers and 1.281 µg/g (SD = 0.369 µg/g) in whole bodies. [Note: µg/g = ppm.] Residue levels declined significantly over time in livers (F = 20.88, P = 0.0000) and in whole bodies (F = 25.67, P = 0.0000). Levels quickly declined after Day 3 (Tables 1 and 2; Figure 1) and the levels on Day 7 averaged 1.069 µg/g (SD = 0.409 µg/g) in livers and 0.251 µg/g (SD = 0.124µg/g) in whole bodies. These levels are significantly lower than the levels on Day 3 in livers (t = 4.27, P = 0.0053) and in whole bodies (t = 5.34, P = 0.0018). Non-linear regression fit a curvilinear line very well to the decline in liver residues (pseudo R^2 = 0.82) and to whole body residues (pseudo R^2 = 0.94; Figure 1). A half life of about 5-6 days in livers and whole bodies can be interpolated from the graphed results (Figure 1, Figure 2). The rate of decline in residue levels slowed after Day 7, suggesting a biphasic degradation curve (Figure 2) which is common of other anticoagulants such as diphacinone (J. Eisemann, pers. comm.). Levels of residues were not significantly different in the livers (t = 1.34, P = 0.2371) of T1 Day 3 animals versus T2 animals. Levels of residues were significantly higher (t = 3.13, P = 0.0259) in whole bodies of T1 Day 3 animals versus T2 animals, but were not significantly different (t = 1.22, P = 0.2756) between T1 Day 5 animals and T2 animals (Table 2, Figures 3 and 4). No chlorophacinone was found in the control animals (all values below the Method Limit of Detection).

We compared the residue levels between T1 males and females that had been euthanized on Day 3 and Day 5 (4 males; 4 females). The highest residue levels occurred in animals euthanized on those two days. There were no significant differences in residue levels in the livers (t = 0.07, P = 0.9448) of males (mean = 4.4150 µg/g, SD = 2.8166) versus females (mean = 4.5200 µg/g, SD = 0.7386) or in whole bodies (t = 0.13, P = 0.8996) of males (mean = 0.9675 µg/g, SD = 0.6091) versus females (mean = 1.0100 µg/g, SD = 0.2149). We also compared residue levels in animals that were found dead (n = 3) versus levels in animals that were euthanized in that same time period (n = 11). There were no significant differences in residue levels in the livers (t = -1.23, P = 0.2408) of animals that were found dead (mean = 0.4300 µg/g, SD = 0.3579) versus those euthanized (mean = 0.8391 µg/g, SD = 0.5341) or in whole bodies (t = -0.77, P = 0.4564) of animals found dead (mean = 0.1700 µg/g, SD = 0.1908) versus those euthanized (mean = 0.2690 µg/g, SD = 0.1957).

No control animals died during the course of the study. The 3 T2 animals were euthanized 3 days after bait was offered and no conclusive signs of anticoagulant poisoning (hemorrhaging) were observed from this limited time of exposure. Of the 36 T1 animals, 3 died of anticoagulant poisoning and 9 were considered to be moribund and were euthanized for purposes of humaneness. The average days to death (or moribund state resulting in euthanasia) was 15.3 days (n = 12, range = 9-26, SD = 5.5). This is similar to the days to death reported by Yoder (2007) in her LD50 determination study: most deaths in 9-14 days with a smaller peak in deaths

in 17-20 days. The remaining 24 animals in the T1 group appeared healthy when they were euthanized as per the schedule. The first clinical symptoms of anticoagulant poisoning were observed on Day 5 (lethargy) and especially on Day 8 (external bleeding or blood in feces) after the bait was first offered. Twenty of the 36 (56%) T1 animals showed evidence of hemorrhaging (external and/or internal) when necropsied.

All animals, including those of the control group, lost a significant amount of weight over the course of the study (for control animals: $t = -6.48$, $P = 0.0001$; for T1 animals: $t = -10.16$, $P = 0.0000$). This may be attributed to the fact that a relatively low nutrition maintenance diet was provided (grass hay, apple, carrot) which was done to avoid confounding the anticoagulant effects by providing a diet relatively high in vitamin K (the antidote to anticoagulant poisoning). This would have occurred if the standard rodent chow pellets were provided to study animals. An additional factor that may have played a role in weight loss was that the study was conducted in winter (albeit indoors) when the wild-caught animals would normally be less active, would have only low nutrition foods available, and would be losing weight.

CONCLUSIONS AND DISCUSSION

Chlorophacinone levels quickly peaked in prairie dogs after being fed Rozol prairie dog bait. Highest levels were obtained from animals euthanized on the third day after being offered the bait. Levels quickly declined thereafter and were significantly lower by Day 7. Chlorophacinone residues in our liver samples (maximum average on Day 3 of 5.499 µg/g) were higher than the 2008 data reported by the Colorado Division of Wildlife (L. Baeten, unpubl. data; received from Francie Pusateri) for prairie dogs recovered dead after a field application of Rozol prairie dog bait (average = 1.34 µg/g, SD = 1.21) perhaps because of the relatively rapid metabolism and excretion of chlorophacinone residues after consumption of the bait and/or a late collection date of carcasses after death in the field study (see review by Primus et al. 2001). Primus et al (2001) reported varying levels of residues, depending on the rodent species. Vidal et al. (2009) reported somewhat lower levels of chlorophacinone residues (0.082-3.800 µg/g) in the livers of voles (*Microtus arvalis*) than our maximum average levels in prairie dogs. In their risk assessment, they suggested that the risks to avian scavengers are minimal to negligible while there may be higher risks to some mammalian scavengers.

Our results also demonstrated that prairie dogs allowed to feed *ad libitum* on the bait did not consume more bait nor did they have higher residue levels than those offered only 53 g of bait. The overall study results suggest that the highest risk of secondary exposure to chlorophacinone residues by non-target animals consuming prairie dogs exposed to the bait would occur within a few days after bait application and would drop quickly thereafter. Additionally, it has been suggested that because birds are less susceptible to chlorophacinone poisoning than mammals the secondary risks are probably higher for predatory or scavenging mammals (coyotes) than for predatory birds (barn owls, American kestrels; see review by Primus et al. 2001).

ARCHIVE

All raw data, documentation, records, protocols, specimens, correspondence and other

documents relating to interpretation and evaluation of data, and final reports generated as a result of this study are retained in the archives of the National Wildlife Research Center at Fort Collins, Colorado.

KEY PERSONNEL

Key personnel involved in the study include the following staff of the NWRC:

Name	Title	Duties related to study
Gary Witmer	Supervisory Research Wildlife Biologist	Study Director, major participant in all aspects of study
Nathan Snow	Biological Science Technician	Major participant in all aspects of study
Rachael Piergross	Biological Science Technician	Major participant in all aspects of study
David Goldade	Supervisory Chemist	Residue analysis
Doreen Griffin	QA-QC Specialist	Sample log-in, archiving
Christopher Campton	Biol. Sci. Lab Technician	Tissue preparation
Dustin Keller	CO State Univ. Work-Study Student	Tissue preparation

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Table 1. Average liver and whole body chlorophacinone residue levels of T1 sacrificed prairie dogs by days after bait first presented. T1 prairie dogs were presented with 53 g of Rozol for Prairie Dogs on Day 1 (January 28, 2010).

T1 Groups Sacrificed (No. Animals in Group[a])	Days After Bait First Presented	Ave. Liver Residues, µg/g (S.D.)	Ave. Whole Body Residues, µg/g (S.D.)
1 (4)	3	5.499 (2.034)	1.281 (0.369)
2 (4)	5	3.431 (1.223)	0.691 (0.225)
3 (4)	7	1.069 (0.409)	0.251 (0.124)
4 (4)	9	1.101 (0.310)	0.435 (0.070)
5 (4)	11	0.821 (0.698)	0.224 (0.191)
6 (6)	14	0.470 (0.389)	0.106 (0.130)
7 (5)	18	0.216 (0.137)	0.053 (0.000)
8 (5)	27	0.217 (0.146)	0.072 (0.028)

[a] Groups with more than 4 animals resulted from animals dying or having to be euthanized for humaneness purposes within a few days of a scheduled euthanasia day.

Table 2. Data set for all animals by animal number, sex, weights, bait consumption, fate and date, and residue levels and treatment group.

(A) Animals offered 53 g of Rozol for Prairie Dogs (T1 group) on January 28, 2010.

Prairie Dog No.	Sex (F/M)	Assigned Treatment	Initial Weight (g)	End Weight (g)	Difference in Weights (g)	Bait Offered (g)	Bait Remaining (g)	Bait Consumed (g)	Euthanized or died	Date of fate	Liver Residue (µg/g)	Whole Body Residue (µg/g)
KQ-02	Male	T_1	710.0	665.0	-45.0	53.0	0.0	53.0	Euthanized	1/30/2010	3.660	1.085
KQ-04	Female	T_1	925.0	910.0	-15.0	53.0	0.0	53.0	Euthanized	1/30/2010	4.905	0.935
KQ-28	Female	T_1	655.0	605.0	-50.0	53.0	7.5	45.5	Euthanized	1/30/2010	5.025	1.325
KQ-41	Male	T_1	810.0	745.0	-65.0	53.0	0.2	52.8	Euthanized	1/30/2010	8.407	1.78
KQ-17	Male	T_1	935.0	895.0	-40.0	53.0	0.0	53.0	Euthanized	2/1/2010	3.803	0.518
KQ-27	Female	T_1	675.0	610.0	-65.0	53.0	0.4	52.6	Euthanized	2/1/2010	4.710	0.89
KQ-32	Female	T_1	630.0	565.0	-65.0	53.0	7.1	45.9	Euthanized	2/1/2010	3.425	0.881
KQ-48	Male	T_1	740.0	700.0	-40.0	53.0	0.9	52.1	Euthanized	2/1/2010	1.785	0.477
KQ-15	Female	T_1	895.0	835.0	-60.0	53.0	21.0	32.0	Euthanized	2/3/2010	0.794	0.096
KQ-29	Female	T_1	715.0	665.0	-50.0	53.0	25.5	27.5	Euthanized	2/3/2010	1.675	0.309
KQ-34	Male	T_1	915.0	825.0	-90.0	53.0	0.9	52.1	Euthanized	2/3/2010	0.945	0.218
KQ-37	Male	T_1	730.0	635.0	-95.0	53.0	0.6	52.4	Euthanized	2/3/2010	0.864	0.382
KQ-20	Male	T_1	915.0	815.0	-100.0	53.0	0.1	52.9	Euthanized due to condition	2/5/2010	1.537	0.377
KQ-21	Female	T_1	840.0	755.0	-85.0	53.0	15.3	37.7	Euthanized	2/5/2010	0.937	0.532
KQ-40	Female	T_1	825.0	770.0	-55.0	53.0	0.4	52.6	Euthanized	2/5/2010	1.096	0.44
KQ-50	Male	T_1	1045.0	960.0	-85.0	53.0	0.8	52.2	Died	2/5/2010	0.834	0.393
KQ-08	Female	T_1	670.0	500.0	-170.0	53.0	18.6	34.4	Euthanized	2/7/2010	0.877	0.330
KQ-13	Male	T_1	800.0	705.0	-95.0	53.0	0.0	53.0	Euthanized	2/7/2010	0.502	0.053
KQ-24	Male	T_1	1060.0	900.0	-160.0	53.0	0.0	53.0	Died	2/7/2010	0.141	0.073
KQ-35	Female	T_1	805.0	660.0	-145.0	53.0	14.3	38.7	Euthanized due to condition	2/7/2010	1.765	0.439
KQ-12	Male	T_1	765.0	700.0	-65.0	53.0	0.0	53.0	Died	2/9/2010	0.321	0.053
KQ-01	Female	T_1	590.0	530.0	-60.0	53.0	0.1	52.9	Euthanized due to condition	2/10/2010	0.090	0.053
KQ-30	Male	T_1	870.0	760.0	-110.0	53.0	0.1	52.9	Euthanized	2/10/2010	1.190	0.053
KQ-33	Female	T_1	675.0	540.0	-135.0	53.0	0.1	52.9	Euthanized due to condition	2/10/2010	0.576	0.372
KQ-45	Male	T_1	820.0	745.0	-75.0	53.0	1.8	51.2	Euthanized	2/10/2010	0.235	0.053
KQ-19	Male	T_1	775.0	635.0	-140.0	53.0	0.0	53.0	Euthanized due to condition	2/11/2010	0.413	0.053
KQ-03	Male	T_1	765.0	720.0	-45.0	53.0	0.1	52.9	Euthanized	2/14/2010	0.127	0.053
KQ-39	Female	T_1	855.0	840.0	-15.0	53.0	4.0	49.0	Euthanized	2/14/2010	0.145	0.053

KQ-42	Male	T_1	895.0	860.0	-35.0	53.0	0.2	52.8	Euthanized	2/14/2010	0.131	0.053
KQ-44	Female	T_1	895.0	805.0	-90.0	53.0	11.4	41.6	Euthanized	2/14/2010	0.229	0.053
KQ-49	Female	T_1	810.0	730.0	-80.0	53.0	13.0	40.0	Euthanized due to condition	2/15/2010	0.451	0.053
KQ-46	Male	T_1	985.0	695.0	-290.0	53.0	0.8	52.2	Euthanized due to condition	2/17/2010	0.442	0.116
KQ-06	Male	T_1	760.0	660.0	-100.0	53.0	0.0	53.0	Euthanized	2/18/2010	0.132	0.053
KQ-18	Female	T_1	855.0	660.0	-195.0	53.0	16.6	36.4	Euthanized due to condition	2/18/2010	0.265	0.053
KQ-26	Female	T_1	875.0	505.0	-370.0	53.0	0.0	53.0	Euthanized due to condition	2/22/2010	0.187	0.084
KQ-47	Female	T_1	790.0	730.0	-60.0	53.0	0.2	52.8	Euthanized	2/23/2010	0.061	0.053
		Average	813.2	717.6	-95.6	53.0	4.5	48.5			1.463	0.355
		SD	111.3	117.5	71.9	0.00	7.3	7.3				

(B) Animals offered *ad libitum* Rozol for Prairie Dogs (T2 group): bait was presented on January 28, 2010.

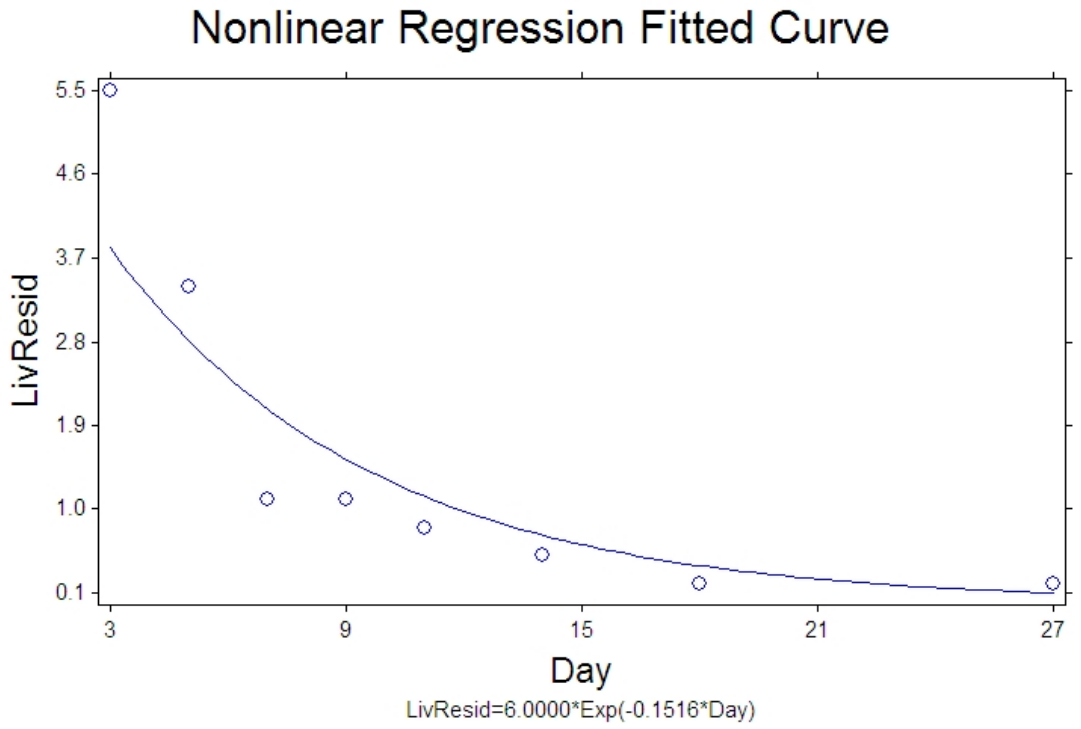
Prairie Dog No.	Sex (F/M)	Assigned Treatment	Initial Weight (g)	End Weight (g)	Difference in Weights (g)	Bait Offered (g)	Bait Remaining (g)	Bait Consumed (g)	Euthanized or died	Date of fate	Liver Residue (µg/g)	Whole Body Residue (µg/g)
KQ-07	Male	T_2	880.0	810.0	-70.0	150.0	143.0	7.0	Euthanized	1/31/2010	0.146	0.053
KQ-25	Female	T_2	925.0	875.0	-50.0	150.0	95.4	54.6	Euthanized	1/31/2010	3.02	0.648
KQ-31	Female	T_2	765.0	730.0	-35.0	150.0	112.5	37.5	Euthanized	1/31/2010	5.923	0.609
		Average	856.7	805.0	-51.7	150.0	117.0	33.0			3.030	0.437
		SD	82.5	72.6	17.6	0.0	24.1	24.1			2.889	0.333

(C) Animals in control (C) group (fed only maintenance diet). All residue levels below the Minimum Limit of Detection.

Prairie Dog No.	Sex (F/M)	Assigned Treatment	Initial Weight (g)	End Weight (g)	Difference in Weights (g)	Bait Offered (g)	Bait Remaining (g)	Bait Consumed (g)	Euthanized or died	Date of fate	Liver Residue (µg/g)	Whole Body Residue (µg/g)
KQ-05	Male	C	795.0	775.0	-20.0	0.0	0.0	0.0	Euthanized	1/30/2010	<0.061	<0.053
KQ-43	Female	C	950.0	915.0	-35.0	0.0	0.0	0.0	Euthanized	1/30/2010	<0.061	<0.053
KQ-10	Male	C	900.0	760.0	-140.0	0.0	0.0	0.0	Euthanized	2/7/2010	<0.061	<0.053

KQ-11	Female	C	805.0	725.0	-80.0	0.0	0.0	0.0	Euthanized	2/7/2010	<0.061	<0.053
KQ-14	Female	C	825.0	765.0	-60.0	0.0	0.0	0.0	Euthanized	2/18/2010	<0.061	<0.053
KQ-16	Male	C	860.0	715.0	-145.0	0.0	0.0	0.0	Euthanized	2/18/2010	<0.061	<0.053
KQ-09	Male	C	965.0	850.0	-115.0	0.0	0.0	0.0	Euthanized	2/23/2010	<0.061	<0.053
KQ-22	Male	C	645.0	580.0	-65.0	0.0	0.0	0.0	Euthanized	2/23/2010	<0.061	<0.053
KQ-23	Female	C	840.0	690.0	-150.0	0.0	0.0	0.0	Euthanized	2/23/2010	<0.061	<0.053
KQ-36	Female	C	885.0	760.0	-125.0	0.0	0.0	0.0	Euthanized	2/23/2010	<0.061	<0.053
KQ-38	Female	C	745.0	685.0	-60.0	0.0	0.0	0.0	Euthanized	2/23/2010	<0.061	<0.053
		Average	837.7	747.3	-90.5	0.0	0.0	0.0				
		SD	91.8	87.6	46.3	0.00	0.00	0.00				

Figure 1. Non-linear regression of average chlorophacinone residue levels in prairie dog livers (top) and whole bodies (bottom) by date of euthanasia or death. Animals were offered Rozol for Prairie Dogs on January 28, 2010 (Day 1).



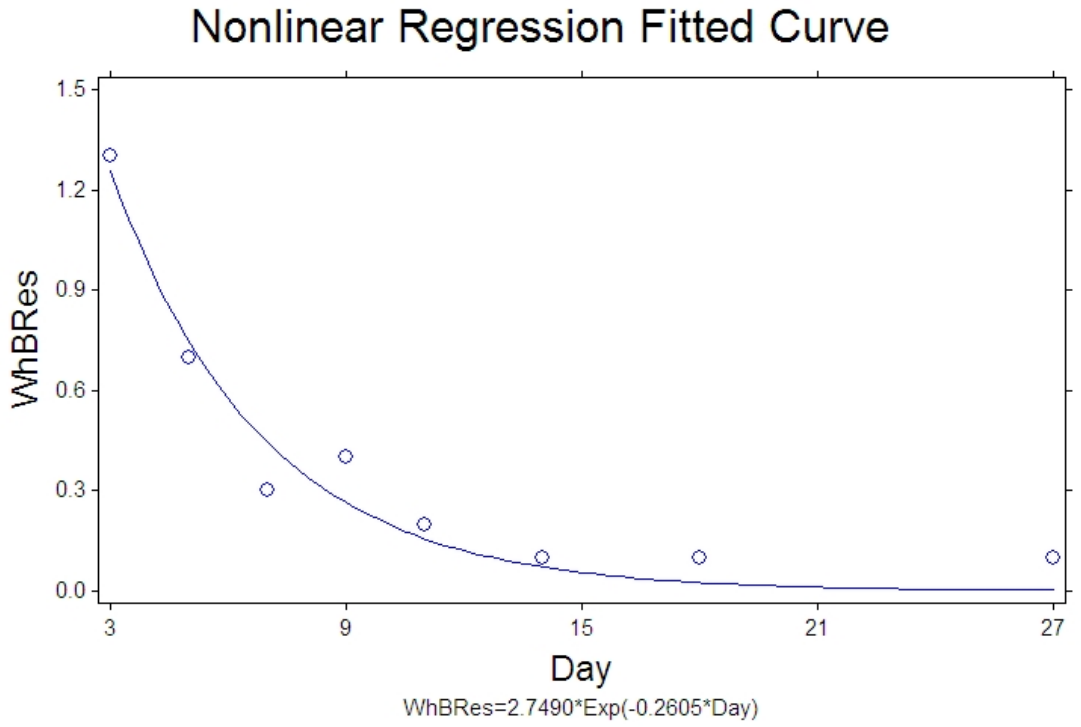


Figure 2. Average chlorophacinone residue levels in prairie dog livers (upper line) and whole bodies (lower line) by date of euthanasia or death. Animals were offered Rozol for Prairie Dogs on January 28, 2010 (Day 1).

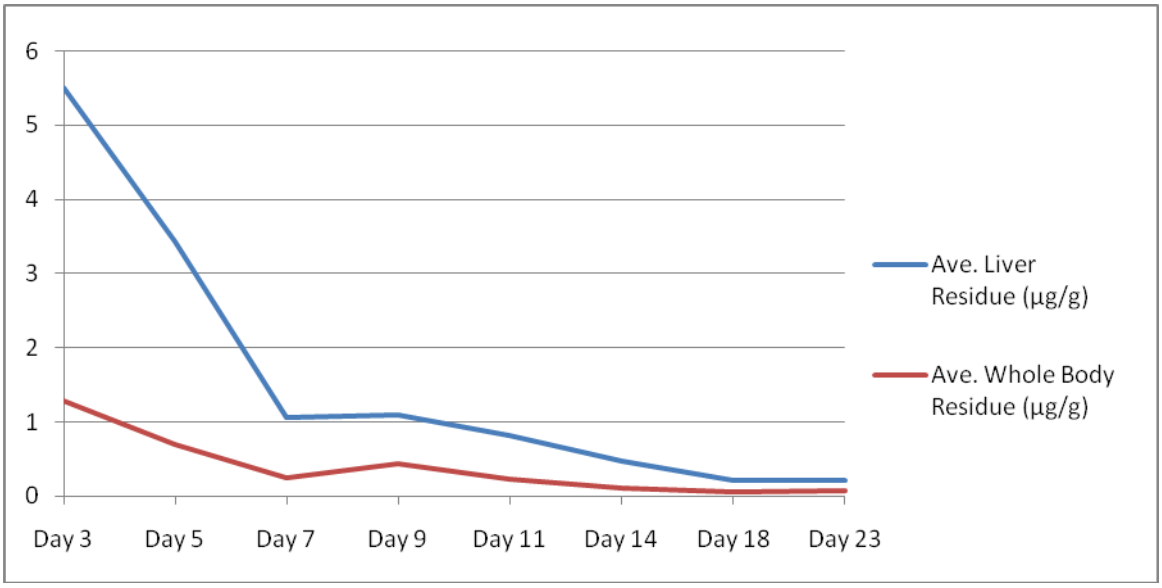


Figure 3. Average chlorophacinone residue levels in livers of control animals, over time in animals presented with 53 g of bait, and in animals allowed to feed *ad libitum* for 2 days. Animals were offered Rozol for Prairie Dogs on January 28, 2010 (Day 1). All control animal values were below the Method Limit of Detection (MLOD). For liver samples the MLOD = 0.061 µg/g.

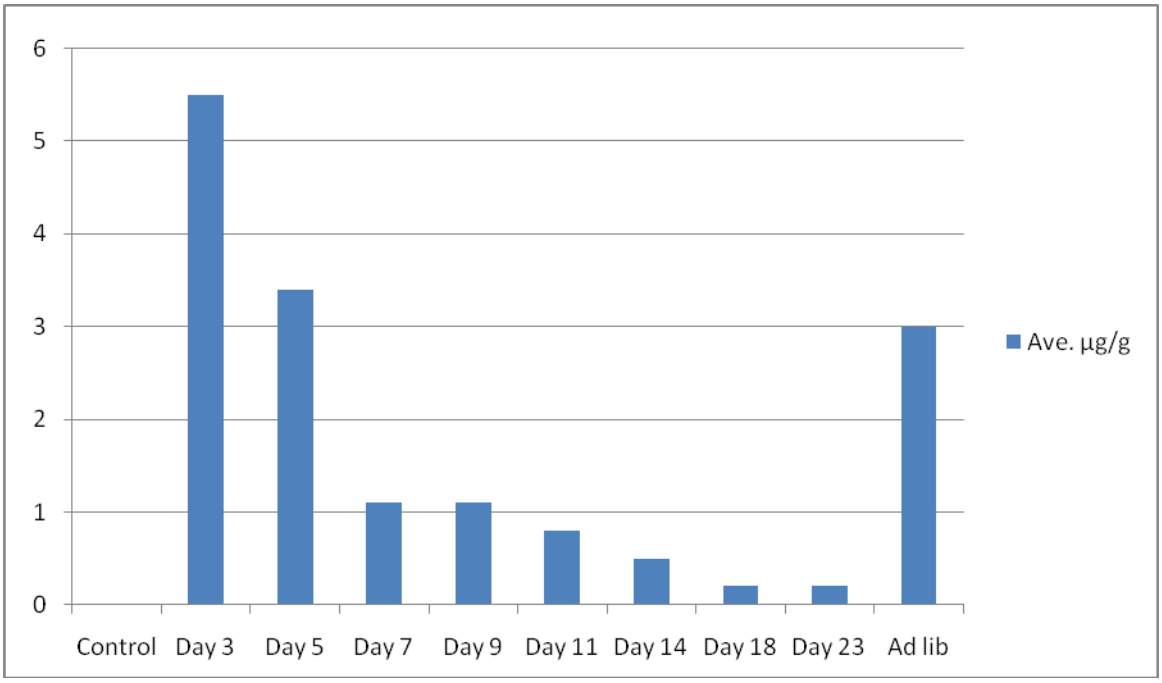
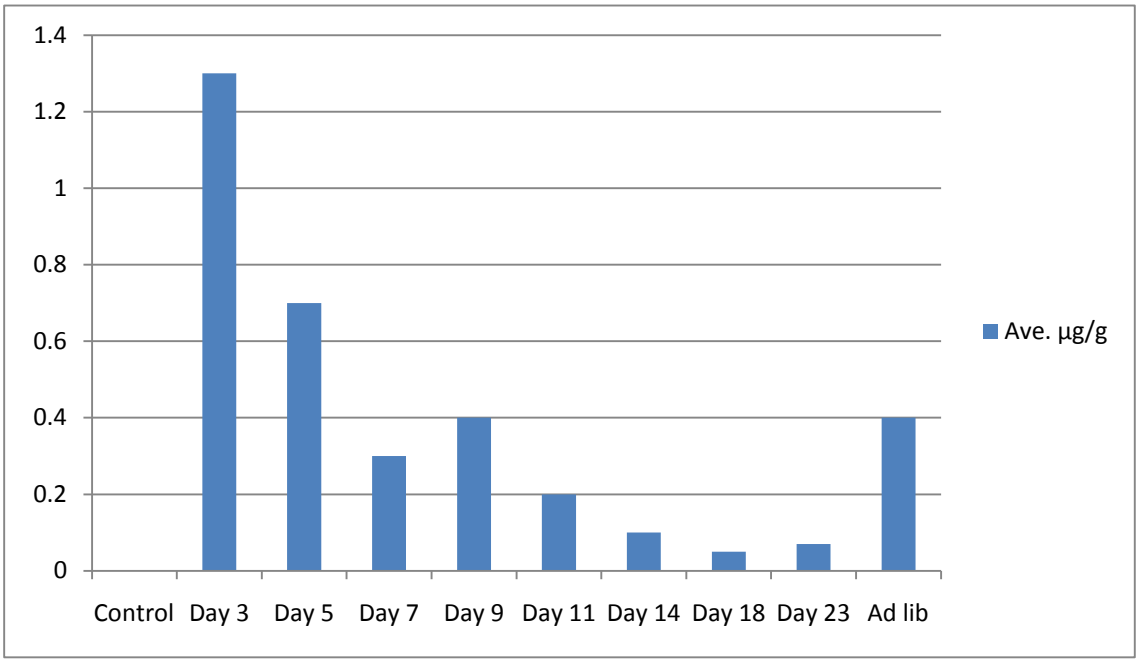


Figure 4. Average chlorophacinone residue levels in whole bodies of control animals, over time in animals presented with 53 g of bait, and in animals allowed to feed *ad libitum* for 2 days. Animals were offered Rozol for Prairie Dogs on January 28, 2010 (Day 1). All control animal values were below the Method Limit of Detection (MLOD). For whole body samples the MLOD = 0.053 µg/g.



APPENDICES

Appendix I - Study Protocol

Appendix II - Protocol Amendments/Deviations

Appendix III - ACP Analytical Services Report (Liver and Whole Body Residue Levels)

Appendix IV – NWRC Bait Analysis and Certificate Provided by the Manufacturer

Appendix I - Study Protocol

QA- 1682

National Wildlife Research Center
Wildlife Services
Animal and Plant Health Inspection Service
United States Department of Agriculture

Study Protocol

1. **Title:**
   Retention time of chlorophacinone in the tissues of black-tailed prairie dogs exposed to chlorophacinone bait

2. **Study Director:**
   Gary Witmer, Ph.D.

3. **Sponsor:**
   Name: USFWS
   Address: 420 S. Garfield Ave., Suite 400
   Pierre, SD 57501

4. **Testing Facility:**
   Name: USDA/APHIS/WS National Wildlife Research Center
   Address: 4101 LaPorte Ave.
   Fort Collins, CO 80521

5. **Background and Justification:**
   Black-tailed prairie dogs (*Cynomus ludovicianus*) are one of five species of prairie dogs found in North America. Their habitat covers the Great Plains from northern Mexico to southern Canada. Although they currently occupy less than 2% of their original range (Miller et al. 2000), they are frequently the subject of controversy. Ranchers typically dislike them because of the perception that wildlife can break a leg by stepping into a burrow entrance, although this rarely actually occurs (Hoogland 1995). In addition, ranchers believe prairie dogs compete with their livestock for forage. Estimates of dietary overlap with cattle range from 64-90% (Hygnstrom and Virchow 1994), although the magnitude of the effect on livestock is controversial (Fagerstone 1982). Prairie dogs may also carry fleas infected with sylvatic plague, leading to a potential health hazard for humans and pets that come in contact with an infected animal (Barnes 1982, Menkens and Anderson 1991, Cully 1997).

   Management of prairie dogs in the past has included poisoning, fumigants, barriers, and relocation (Franklin and Garrett 1989, Robinette et al. 1995, Andelt and Hopper 1998). A survey of Fort Collins residents in 1993 showed residents that experienced no prairie dog related damage supported relocation over lethal control. Residents experiencing conflicts with prairie dogs were more likely to support lethal control measures (Zinn and Andelt 1999). Barriers and relocation tend to be expensive, can be ineffective, and are dependent on available sites.

   Anticoagulants are commonly used to control rodent populations. With the emergence of warfarin-resistant rodent strains, so-called "superwarfarins" were developed. Among the new first generation anticoagulants was chlorophacinone, an indandione derivative (Timm

1994). Chlorophacinone works by inhibiting the vitamin K(1)-2,3 epoxide reductase enzyme responsible for recycling of vitamin K to its active form (Silverman 1980, Hadler and Buckle 1992, Watt et al. 2005). Active vitamin K is a cofactor used in the carboxylation of the glutamic acid residues on clotting factors II, VII, IX, and X. A reduction in the synthesis of these clotting factors leads to hemorrhage, and ultimately death from hypovolemic shock (Watt et al. 2005). In addition, chlorophacinone causes damage to capillary walls (Timm 1994). In rodents, it may also result in neurologic and cardiopulmonary damage that leads to morbidity before hemorrhage begins (International Programme on Chemical Safety).

Unlike zinc phosphide, the traditional toxicant for prairie dogs, anticoagulants persist in tissue. Symptoms of chlorophacinone exposure typically take several days after ingestion to manifest, and it may take 7-20 days for mortality to occur after a single gavage dose (Yoder, unpublished data). Chlorophacinone (Rozol®) was recently approved under a Special Local Need or 24(c) registration for use on prairie dogs in several states. Because prairie dog colonies are utilized by various mammalian and avian predators, the US Fish and Wildlife Service is concerned about the potential poisoning of these animals. Chlorophacinone-related mortality was documented in a badger in Kansas and a bald eagle in Nebraska (Peter Gober, USFWS, pers. commun.). Rozol® is currently being used to control prairie dogs at a black-footed ferret recovery site in Kansas despite its documented toxicity to ferrets. More information is needed to accurately assess the secondary risks associated with chlorophacinone use (e.g., Fisher and Timm 1987). To allow that assessment, managers need information on the levels of chlorophacinone levels that can occur in prairie dog tissues after feeding on rodenticide baits. This study is designed to provide the requested data set of the sponsor, USFWS, and for purposes of submission to the US EPA as a GLP data set to assist in making registration decisions on this anticoagulant rodenticide. The study is designed as an Acute Oral Toxicity study, and hence, follows the published guidelines of the EPA (2002).

6. Objective/Hypotheses:

To determine the chlorophacinone residue levels in prairie dog livers and whole bodies at various time intervals after the animals have consumed chlorophacinone rodenticide baits. We hypothesize that residue levels will peak at some point and then decline over time.

7. NWRC Approved Project Title:

Development of methods to control rodent populations and damage with an emphasis on invasive house mice and native voles

8. Regulatory Compliance/Guidelines:

	None, non-regulated study
X	CFR Title 40, Part 160: Good Laboratory Practice Standards (FIFRA);
	CFR Title 21, Part 58: GLP Standards for Nonclinical Laboratory Studies, (FFDCA)
	Other:

U.S. EPA. 1996. Ecological Effects Test Guidelines: Wild Mammal Acute Toxicity. OPPTS 850.2400. OPP Pesticide Assessment Subdivsion G: Product Performance. Section 96-12: Rodentcides on Farm and Rangeland.

9. Study Classification Information

X	Animals -- please complete and attach **Animal Use Appendix**
	Plants -- no additional appendix required
	Microbiological/Biohazardous Materials -- please complete and attach **Microbiological/Biohazardous Materials Use Appendix**
X	Chemical Analysis -- please complete and attach **Analytical Chemistry Appendix**
	Literature review only -- no additional appendix required
	Statistical or economic analysis only -- no additional appendix required
X	Use of a test, control, references substance, bait or device -- complete and attach **Test, Control and Reference Materials / Device Formulation and Use Appendix**

10. Methods/Procedures:

Prairie dogs will be obtained from the USFWS or Colorado counties or municipalities that are already conducting trap and euthanasia programs for nuisance animals. Only females ≥ 600 g and males ≥ 700 g will be used for the study. Because only adults will be used for the study, prairie dogs will be weighed in the field (SOP FP 029.00) and aged as either a juvenile or an adult based on body weight (SOP FP 026.00). No juveniles or lactating females will be used. The treatment group will consist of 18 males and 18 females. Another 9 animals will serve as control animals.

Prior to transport, prairie dogs will be dusted for fleas with a pyrethrin-based flea powder or another suitable parasiticide approved by the Attending Veterinarian and Study Director. Prairie dogs will be transported to the National Wildlife Research Center either in individual Tomahawk traps or a dog kennel (approximately 3' x 2' x 3'). Transport time is not expected to exceed several hours. Upon arrival, prairie dogs will be quarantined in an Outdoor Rodent Building for 14 days as long as the weather permits; otherwise they will be quarantined inside an animal room of the ARB or ISRB (SOP AC/CO 016.00). All prairie dogs will be dusted again for fleas at the end of the quarantine period.

Prairie dogs will be individually housed indoors in individually-numbered 2' x 1.5' x 1' cages that contain a length of PVC pipe to serve as a hide. Because rodent block and alfalfa contain small quantities of vitamin K1 (phylloquinone), animals will be maintained on grass hay, apples, and carrots throughout the test (Haroon and Hauschka 1983, Arjo and Nolte 2004). Grass hay should more closely mimic the levels of vitamin K1 prairie dogs are likely to be exposed to in the wild.

All animals will be weighed the day prior to treatment. Food will be removed from all cages the evening prior to treatment. On the morning of treatment, clean tray liners will be placed under each cage. Each prairie dog will be given ¼ cup Rozol® bait (approximately 53 g) as the sole source of food for the day per the Rozol® label. Each food ration will be weighed prior to feeding. Food consumption will be monitored periodically throughout the day. Any prairie dog that has completely consumed the bait will be given maintenance diet. After 2 days, if bait remains in the cage, it will be collected and weighed to determine bait consumption and dose. Prairie dogs will be maintained on maintenance diet for the remainder of the study. Control animals will not receive the rodenticide bait, but will be maintained on the maintenance diet during the entire study. The chlorophacinone

concentration in the Rozol® bait used for the study will be confirmed by the bait manufacturer prior to the start of the study.

The Organization for Economic Cooperation and Development (OECD 2000) recommends that observations be made daily on animals after dosing, however, the prairie dogs in this study will be monitored twice a day for health and mortality (dead vs. alive) throughout the study and a health log will be maintained for each animal. Animals will be observed at 7-9:00 am and again at 4-6 pm each day. Animals will not be disturbed during the 12-hr dark portion of the light-dark light cycle so as to not disturb resting animals; this is also important so as to not influence the onset of distress in animals which could lead to the onset of clinical symptoms requiring intervention and euthanasia. Humane practices recommended by the EPA (2002) for acute oral toxicity studies will be followed: "moribund animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed." The EPA recommends use of the guidelines published by the OECD (2000) which states: "a humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, sever distress, suffering, or impending death." Signs of severe pain and distress and of a moribund condition to be used as criteria for humane killing of study animals listed by OECD (2000) include abnormal vocalization, persistent difficult labored breathing, prolonged impaired ambulation preventing the animal from reaching or water, persistent convulsions, and significant blood loss. If these signs are observed, the Study Director, Attending Veterinarian, or their appropriately-trained designees will decide if the animal should be euthanized.

One treatment group of 4 animals (generally 2 females and 2 males) will be sacrificed on days 1, 3, 5, 7, 9, 12, 16, 20, and 25, post-chlorophacinone dosing. Animals will be randomly selected, using the SAS statistical program or Excel software, from all remaining, treated animals one day before a sacrifice day. Animals in each treatment group will be euthanized on the scheduled date with CO_2 (SOP AC/CO 008.00). But because some animals may be found dead on that day, we will count up to a maximum of 2 dead animals as part of the group of 4 animals to be sacrificed that day. Hence, if 2 animals are found dead on day X, only 2 of the animals selected and scheduled for euthanasia on day X will be sacrificed. Those 2 animals will be randomly selected from the 4 that had been previously selected for euthanasia that day. However, no matter how many animals are found dead on any given day, all will be processed for tissues and residue analyses. Additionally, 2 control animals will be randomly selected and euthanized at days 1, 9, and 20 after the start of the study (date of dosing of treatment animals). An additional treatment group of 3 animals will be allowed to feed on the rodenticide bait *ad libitum* for 2 days. These 3 animals will then be euthanized 2 days later. Euthanized prairie dogs will be weighed, skinned and frozen in labeled, resealable plastic bags until analysis by Analytical Chemistry personnel. Both the liver (Method 143 A) and the whole body (Method 142 A) will be analyzed for chlorophacinone residues. The liver and body will be homogenized separately for each prairie dog, and the chlorophacinone extracted. The extract will be analyzed by HPLC for chlorophacinone concentration. Any prairie dogs found dead during the study will be processed as above and the day of death will be recorded. Any animals surviving after 25 days will be euthanized with CO_2 (SOP AC/CO 008.00).

11. Experimental Design and Statistical Analyses:

For the purpose of data analysis, we will determine residue levels in 5 ways: 1) by including only animals that are sacrificed on an assigned day, 2) by including only animals that die on their own, 3) including all animals (both of the previous groups), 4) compare residue levels in animals that are sacrificed versus those that die on their own, and 5) compare residue levels between males and females. The mean and standard deviation of residue levels will be determined at each testing time period. A residue decay curve will be generated using regression analysis. Residue data will be analyzed using logistic regression (PROC PROBIT) and the slope of the residue-response line will be calculated. Residue levels will be compared between the various data sets (1-5) with ANOVA and t-tests.

12. Description of Environmental Conditions and Monitoring Requirements:
All prairie dogs will be maintained on a 12L:12D light schedule, 60-70° F, and ambient humidity conditions.

13. List number and title of Standard Operating Procedures (SOPs):

AC/CO 008.00	Euthanasia With CO_2
AC/CO 016.00	Animal Quarantine Procedures at Fort Collins
AD 004.01	Archiving Studies
AD 007.01	Final Reports
AD 008.01	Personnel Qualification Records
AD 010.01	Standard Format for Data Submissions to EPA
AD 011.02	Data Recording and Error Correction
AD 012.02	Test, Control, and Reference Substance Chain of Custody
HS 004.00	Personal Protective Equipment
FP 023.00	Live-trapping Prairie Dogs
FP 026.00	Sexing and Aging of Black-tailed Prairie Dogs
FP 029.00	Use of a Spring Scale for Body Mass Measurements

14. List of Records to be Maintained:
Analytical chemistry results
Animal accession data (animal/cage number and sex)
Animal health observation log
Body weights
Rodenticide bait consumption during trial
Mortality
Record of accidental deaths or injuries
Statistical analysis results

15. Permits/Certifications:
Trapping of prairie dogs will be conducted under an existing prairie dog collecting permit of the USFWS or a Colorado county or municipality.

16. Endangered Species Act Compliance:
Is there a possibility that the study, as proposed, will or may affect threatened or endangered (T&E) species?
Yes:____ No:__X__, this study will have no effect on any T&E species.

17. Historical Resources:

Does the study involve any major ground disturbance or otherwise have the potential to adversely affect historic resources?

Yes:_____ No: X

18. National Environmental Policy Act Compliance:
Does this study qualify for categorical exclusion[1] from further NEPA analysis?

Yes: X No: _____ Unsure: _____

19. Employee and Public Safety:
All personnel handling prairie dogs will be required to wear thick gloves to help prevent injury from bites. Prairie dogs will be dusted with a pyrethrin-based flea powder upon arrival at the National Wildlife Research Center prior to quarantine and again at the end of quarantine. All personnel handling prairie dogs will be made aware of the risks of animal bites, and will be provided with appropriate protective equipment. Employees will also be made aware of the symptoms and risk of plague transmission. Employees may, at their discretion, employ additional protective measures as they deem necessary (SOP HS 004.00). Personnel handling chlorophacinone will wear latex or nitrile gloves.

20. Schedule:
Proposed Experiment Start Date: November 10, 2009

Proposed Experiment Termination Date: June 30, 2010

Proposed Study Completion/Archive Date: September 30, 2010

21. Staffing:

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[1] Categorical exclusion is based on consideration of all environmental issues relevant to this study, including consideration of cumulative impacts on wild animals and other environmental parameters, such as removal caused by the study combined with other reasonably foreseeable removals by other causes (e.g., sport harvest, wildlife damage management actions, and any other known causes of mortality) pursuant to APHIS NEPA Implementing Procedures at 7 CFR Part 372.5(c)(2)(i) which categorically exclude:

"Research and development activities . . . that are carried out in laboratories, facilities, or other areas designed to eliminate the potential for harmful environmental effects--internal or external--and to provide for lawful waste disposal.

or at 7 CFR Part 372.5(c)(1)(i) which categorically exclude:

ARoutine measures, such as . . . surveys, sampling that does not cause physical alteration of the environment, testing . . . removals . . . (This) may include the (lawful) use . . . of chemicals, pesticides, or other potentially hazardous or harmful substances, materials, and target-specific devices or remedies, provided that such use . . . : (A) . . . is localized or contained in areas where humans are not likely to be exposed, and is limited in terms of quantity . ... B) . . . will not cause contaminants to enter water bodies . . . (C) . . . does not adversely affect any federally protected species or critical habitat; and (D) . . . does not cause bioaccumulation.@

Title	FTE FY-10
Wildlife Biologist	0.15
Technician	0.15
Chemist	0.15

22. Principal Investigators, Cooperators and Consultants:

David Goldade, Chemist
USDA/APHIS/WS NWRC
4101 Laporte Avenue
Fort Collins, Colorado 80521

23. Related protocols:

N/A

24. Cost Estimate for Each Fiscal Year:

	FY-10
A. Salaries and Benefits	$ 35,035.00
B. Analytical Chemistry	$ 35,000.00
C. Animal Care	$ 7,310.00
D. Supplies	$ 750.00
E. Travel	$ 250.00
F. Communication/copying	$ 500.00
G. Indirect Costs (16.15%)	$ 12,733.00
TOTAL	$ 91,578.00

25. Staff qualifications:

All study participants have documentation on file, which verifies their training and qualifications for the work they will perform in this study, including SOP training logs. All SOPs and study specific training logs will be completed and documented in study or personnel records prior to participation in that aspect of the study. Study participants include Gary Witmer, Nate Snow, Rachael Piergross, David Goldade, and Christi Yoder.

26. Archiving:

All raw data, documentation, records, protocols, specimens, correspondence and other documents relating to interpretation and evaluation of data, and final reports generated as a result of this study will be retained in the archives of the National Wildlife Research Center at Fort Collins, Colorado.

27. Protocol Amendments:

Any changes in this protocol will be documented on the Study Protocol Amendment Form, reviewed by appropriate personnel (e.g., IACUC, IBC, ACP, QA, etc.), and signed and dated by the Study Director, Research Program Manager and Sponsor. Amendments will be distributed to all study participants as appropriate.

28. References:

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29 Appendices:
Animal Use Appendix
Analytical Chemistry Appendix
Test, Control and Reference Materials/Device Use Appendix

QA- 1682

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Signature Page:

Study Director Gary W. Palmer Date 11/20/09

Position (check one):

☐ Biologist/Chemist/Technician
Supervisor signature required:
_____ Date_____

☐ Research Scientist

X Project Leader

☐ Visiting Scientist NWRC Representative/Contact: _____

Concur: NWRC Acting Research Program Manager John D. Eisemann Date 11/20/09

QAU Review and Processing: 11/20/09 L. Greiner

Approved: NWRC Director Mark E. Tobin Date 11/23/09
acting

QA- 1682

Animal Use Appendix

A. Animal description:

1) Species: black-tailed prairie dog (*Cynomys ludovicianus*)
2) Strain and substrain (if applicable): N/A
3) Number and Sex: 50 (25 females, 25 males)
4) Body weight range: 800-1400 g
5) Age: ≥ 1 year

B. Rationale for involving animals, for appropriateness of species, and for numbers:

1) Rationale for involving animals: There is no *in vitro* model for determining the residues of chlorphacinone in dosed black-tailed prairie dogs.
2) Rationale for appropriateness: Because black-tailed prairie dogs are the target of chlorophacinone rodenticide treatment, it is appropriate to utilize them. This study is needed to for evaluation of the non-target hazard posed by the continued EPA registration of chlorophacinone for prairie dogs.
3) Rationale for numbers (include calculations as appropriate): The numbers of prairie dogs in each treatment group are based on recommended EPA guidelines (OPPTS 850.2400).

C. Source:

Prairie dogs will be trapped by the USFWS or at a Colorado county or municipality. These will be nuisance animals planned to be removed for development or other reason.

D. Method of identification of animals:

Prairie dogs will be individually identified by placement in individually-numbered cages.

E. Trapping/Collecting:

Prairie dogs will be trapped using single or double door Tomahawk live traps according to the procedures outlined in SOP FP 023.00. Briefly, traps will be baited with rolled oats coated with molasses and wired open for several days prior to the actual trapping period. During the actual trapping period (estimated ten days), traps will be closed during the night. Trapping will be conducting under an prairie dog collecting permit of the USFWS, the Study Director or a Colorado county or municipality.

F. Transport:

Prairie dogs will be transported to the National Wildlife Research Center either in individual Tomahawk traps or a dog kennel (approximately 3' x 2' x 3'). Animals will not be trapped or transported if daily temperatures are expected to be below 40 degrees F or in excess of 80 degrees F. Animals will only be trapped during the day. If animals are trapped in Fort Collins or Boulder, Colorado areas, transportation is not expected to take more than an hour. If animals are trapped in South Dakota, transportation may require 6-7 hours. In either case, each animal will be given a half

apple to provide a source of moisture during the trip. Individual traps will be covered with burlap to help keep animals calm during transport. Animals will be transported in a pick-up truck with a canopy.

G. Handling/restraint:
Prairie dogs will be manually restrained by personnel wearing thick leather gloves.

H. Quarantine:
Prairie dogs will be quarantined in the Outdoor Animal Research Facilities for 14 days as long as the weather permits; otherwise they will be quarantined inside the ARB or the ISRB (SOP AC/CO 016.00).

I. Housing/maintenance:
Prairie dogs will be individually housed indoors in 2' x 1.5' x 1' cages that contain a length of PVC pipe to serve as a hide. Because rodent block and alfalfa contain significant quantities of vitamin K1 (phylloquinone), an antidote for chlorophacinone, animals will be maintained on grass hay, apples, and carrots throughout the study (Haroon and Hauschka 1983, Arjo and Nolte 2004). Grass hay should more closely mimic the levels of vitamin K1 that prairie dogs are likely to be exposed to in the wild.

J. Disposition of animals:
After chemical analyses are conducted, animal remains will be incinerated at NWRC. (No SOP will be developed due to the simple nature of the procedure.) Any animals surviving after 25 days will be euthanized with CO_2 (SOP AC/CO 008.00).

K. Duplication of prior studies:
There are no existing decay curves and residue levels over time for chlorophacinone in black-tailed prairie dogs.

L. Pain or distress:

Consultation with Attending Veterinarian:

Name of Attending Veterinarian: Gordon Gathright
Date of Consultation: September 1, 2009

Is this study expected to cause more than momentary or slight pain or distress?

Yes: X No: _____

It is not known for sure whether consumption of anticoagulants in oral grain baits produces significant pain or stress in rodents, although it has been commonly assumed that they do not by rodent control professionals: "The rate of blood clotting gradually decreases and blood loss leads to an apparently painless death." (Timm 1994). It has been the experience of the study director and colleague John Baroch (pers. comm.) both of whom had conducted numerous anticoagulant efficacy studies with numerous species of rodents that consumption of a lethal dose of an anticoagulant rodenticide bait does not result in overt signs of more than momentary

or slight pain or distress, perhaps because of the slow-acting nature of low-concentration anticoagulants. Animals continue to feed on the baits for several days, then become lethargic and eventually stop feeding. Death usually occurs a short time (1-2 days) later. Rowsell (1979 as cited in Corrigan 2001) studied nervous system responses, including the EEG, of rodents poisoned with anticoagulants. He reported that the EEG remained normal until a terminal condition was achieved at which time the EEG was depressed then flat. He found that clinical evidence of pain or distress was absent. The UK's Department for Environment, Food and Rural Affairs (1997) produced an assessment of humaneness of vertebrate control agents. They cite a review of the toxicity of chlorophacinone that states the clinical observations of poisoned rats, pigs, and dogs included lethargy with breathlessness, increased heart rate, and weak pulse. Those findings were considered not necessarily to be indicative of pain or discomfort. On the other hand, in another study at NWRC, a single, large liquid dose of chlorophacinone by oral gavage was placed in the stomachs of test animals to determine the LD50. In this case, some animals appeared to suffer severe pain. Hence, we have checked the box that animals in this residue study may experience more than momentary or slight pain or distress. Animals will be observed twice daily (at 7-9:00 am and again at 4-6 pm each day) after dosing for signs of pain or distress and observations will be recorded in the daily health log for each animal. Animals will not be disturbed during the 12-hr dark portion of the light-dark light cycle so as to not disturb resting animals; this is also important so as to not influence the onset of distress in animals which could lead to the onset of clinical symptoms requiring intervention and euthanasia. Humane practices recommended by the EPA (2002) for acute oral toxicity studies will be followed: "moribund animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed." The EPA recommends use of the guidelines published by the OECD (2000) which states: "a humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, sever distress, suffering, or impending death." Signs of severe pain and distress and of a moribund condition to be used as criteria for humane killing of study animals listed by OECD (2000) include abnormal vocalization, persistent difficult labored breathing, prolonged impaired ambulation preventing the animal from reaching or water, persistent convulsions, and significant blood loss. If these signs are observed, the Study Director, Attending Veterinarian, or their appropriately-trained designees will decide if the animal should be euthanized.

1) Alternative procedures:
   There are no alternatives for determining the residue levels of chlorophacinone in black-tailed prairie dog tissues.

2) Sedatives, analgesics, or anesthetics:
   a) No sedatives, analgesics, or anesthetics will be used because their use might affect normal metabolism and activity of dosed animals, possibly compromising the final data set.

   b) A Column E justification will be provided if it is determined that chlorophacinone treatment results in pain or distress to the animal.

3) Surgery: N/A

M. Euthanasia:

On each scheduled day for euthanasia, they will be euthanized with CO_2 (SOP AC/CO 008.00). Any animals surviving after 25 days will be euthanized with CO_2 (SOP AC/CO 008.00).

N. IACUC approval:

Date of IACUC Approval Letter: 11/18/09

Analytical Chemistry Appendix

A. Number of samples to be analyzed (by type): 45 samples of prairie dog liver and 45 whole bodies will be analyzed.

B. Storage conditions (temperature, container type, light/dark, duration):
Samples will be frozen at -2 to -4° C until the chemical analysis for chlorophacinone residues is performed.

C. Method title and number:
Method 142 A – Determination of chlorophacinone residues in whole body prairie dog
Method 143 A – Determination of chlorophacinone residues in prairie dog livers

D. ACP Leader consultation: Thomas Primus/David Goldade **Date:** July and Sept, 2009

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Test, Control and Reference Material/Devices Formulation and Use Appendix

A. Describe the test material:

1) Rozol Prairie Dog rodenticide bait: chlorophacinone (CAS #.3691-35-8; 2-(2-(4-chlorophenyl)-phenylacetyl)-1H-indene-1,3(2H)-dione
   a) concentration: 0.005% active ingredient
   b) source: LiphaTech, Inc., Milwaukee, WI
   c) batch number: Will be recorded upon receipt

B. Describe any control or reference materials/devices:

N/A

C. Carriers, mixtures and material preparation:

The rodenticide bait will be obtained from a commercial supplier.

D. Route of administration:

Chlorphacinone bait will be administered as per the EPA label. The bait (53 g) will be provided for free-feeding by each test animal after light fasting.

E. Dosage:

Each test animal will receive 53 g of the chlorophacinone bait and will be allowed to consume the entire amount.

F. Test, control, and reference substance accountability:

Chlorophacinone bait will be tracked according to SOP AD 012.02 (Test, Control, and Reference Substance Chain of Custody). Eventually all remaining bait will be disposed of as hazardous waste by appropriate means.

G. Material verification:

The manufacturer of the bait used in the study will provide verification of the % active ingredient in the bait used in the study. NWRC's Analytical Chemistry Unit does not have a validated method for chlorophacinone concentration in a pelleted bait.

ACP Consultation: Thomas Primus/David Goldade **Date:** July and Sept. 2009

Appendix II - Protocol Amendments/Deviations

National Wildlife Research Center **QA-** 1682
AMENDMENT TO STUDY PROTOCOL

Study Director Gary Witmer **Amendment No.** 1 **Page** 1 **of** 1

Changes in dates:

[X]	No date changes		
[ ]	Experiment Start Date:	(current)_____	(revised) _____
[ ]	Experiment Termination/Completion Date:	(current)_____	(revised) _____
[ ]	Study Completion/Archive Date:	(current)_____	(revised) _____

Additional protocol section/subsection/appendix to be changed:

Methods Section

Description of revisions:

Weighing and sexing of prairie dogs timing and place changed. This activity was ~~preformed~~ performed at once the prairie dogs were brought in from quarantine (Bldg. 11) to the ISRB SNE 163 for transfer to the rack cages. This activity was performed a few days before the animals were put under test not the day before the test began. Use of only adult animals for the study changed---because of trapping timing, all animals captured were essentially adults and would be breeding a month and a half later.

Justification/reason(s) for changes and impact on study:

Sexing the prairie dogs in their capture cages in the field proved difficult and required having the prairie dog in hand to do accurately. I decided to do this once we were transferring the animals from their quarantine cages to their rack cages in which they would remain for the duration of the study. This occurred a few days before the study was initiated (i.e., rodenticide bait added to cages). Animals were accurately sexed while in hand, and then weighed by placing each animal in a tared, small cardboard box as part of this transfer process. This was a more accurate method of weighing than weighing the animal in its quarantine cage and then weighing the cage after removing the animal. Transferring the animals and performing these procedures a few days before the test began allowed them to acclimate to their new rack cages and animal room in the ISRB. Also, it was expected that animals would be captured early in the fall when there would be a sizable difference between adult and juvenile animals. Animals were not captured until early January when all were adult size. This combined with the need to capture all animals for the study in a brief window of mild weather when animals are active and can be captured, required us to take the first 50 animals captured. Only one female was slightly (590 g) below the lowest acceptable weight (600 g) for females used in the study. None of these changes are considered a significant change in conduction of the study or to the approved study protocol.

Gary Witmer 1-29-10
Study Director **Date**

Research Program Manager 1-29-10 **Date**

QAU Received: 2/1/10 **QAU Processed:** 2-1-10

AD 003.03 - Attachment 2

National Wildlife Research Center **QA-** 1682

PROTOCOL AMENDMENT / CHANGE / REVISION

Study Director Gary Witmer **Amendment No.** 2 **Page** 1 **of** 1

Changes in schedule:

☐	No schedule changes		
☐	Experiment Start Date:	(current)_____	(revised) _____
☐	Experiment Termination Date:	(current)_____	(revised) _____
☒	Study Completion/Archive Date:	(current) 9-30-10	(revised) 1-15-11

Protocol section/subsection/appendix to be changed:

N/A

Description of revisions: *(Please provide the level of detail normally required in the protocol)*

Change of Study Completion/Archive date from: Sept. 30, 2010 to: January 15, 2011

Justification/reason(s) for changes and impact on study: *(If dates are changed, please provide a description of current status of study and remaining study plan/schedule.)*

Because of some technical difficulties, the analytical chemistry report on the residue levels in tissues was not received until early October (i.e., after the study completion/archive data had passed). Because the residue levels are the central component of the study, the data analysis and writing of the final report could not begin until well into October. Hence, it will be a few months before the final report can be written, reviewed, and the study archived.

Study Director: Gary Witmer Date 10-19-10

NWRC Project Leader: Same Date _____

QAU received: 10/21/10 L Greiner QAU reviewed: 10/21/10

NWRC IACUC / IBC (as needed): _____ Date 10/22/10

NWRC Assistant Director: Mark E. Tobin Date 10/22/10

Note: Sponsor approval is needed for all non-NWRC sponsored research

AD 003.03 - Attachment 2

National Wildlife Research Center QA- 1682

PROTOCOL AMENDMENT / CHANGE / REVISION

Study Director Gary Witmer **Amendment No.** 3 **Page** 1 **of** 1

Changes in schedule:

[X]	No schedule changes		
[ ]	Experiment Start Date:	(current)_____	(revised) _____
[ ]	Experiment Termination Date:	(current)_____	(revised) _____
[ ]	Study Completion/Archive Date:	(current)_____	(revised) _____

Protocol section/subsection/appendix to be changed:

11. Experimental Design and Statistical Analyses

Description of revisions:

The protocol states that logistic regression (PROC PROBIT) would be used to plot the residue decay curve and to determine the slope of the residue-response curve. Instead, I used non-linear regression to plot the residue decay curve.

Justification/reason(s) for changes and impact on study:

The data set indicated a non-linear decay in residue levels, so a non-linear regression analysis was used to generate the decay curve and associated regression coefficient and regression equation.

Study Director: Gary Witmer Date 3/21/11

NWRC Project Leader: [signature] Date 3/21/11

QAU received: [signature] 3/22/11 QAU reviewed: [signature] 3/21/11

NWRC IACUC / IBC (as needed): NA Date NA

NWRC Assistant Director: Mark E. Tobin Date 3/23/11

Appendix II, cont. Amendments to, and deviations from, the approved study protocol.
The deviations to the approved protocol (as identified in the quality assurance inspection reports and described in the two amendments attached to this appendix and in the analytical chemistry report (Appendix III) were:

1. Animals were not weighed and sexed in the field; instead, they were weighed and sexed when brought into the animal research building after quarantine. This allowed us to determine the weight and sex more accurately and closer to the start of the study.
2. A few (5 females and 1 male) of the 50 prairie dogs used in the study were below the minimum weight cut-off levels of 600 g for females and 700 g for males. However, because all animals were captured in January, all were considered to be adults or subadults approaching adult size.
3. A random numbers table was used instead of a statistical software program to assign animals to treatment group. Memo-to-File on this change was put in the study records.
4. The study completion date and date of archiving was extended because of a delay experienced in getting the final analytical chemistry report on residue levels.
5. The analytical chemistry method used in the study was slightly modified (as detailed in the report in Appendix III) when some difficulties were encountered in achieving consistent residue levels from tissue samples.

Appendix III - ACP Analytical Services Report (Liver and Whole Body Residue Levels)

Wildlife Services NWRC National Wildlife Research Center Analytical Services Report	United States Department of Agriculture Animal Plant Health Inspection Service Wildlife Services National Wildlife Research Center Invasive Species and Technology Development Research Program Analytical Chemistry Project	Invoice #: 10-008 Date: 9/28/10 Page: 1 of 15

To: Gary Witmer
Research Wildlife Biologist, NWRC

Subject: Analysis of Chlorophacinone in Prairie Dog Whole Body and Liver (QA-1682)

Method: 142A and 143A

Analysis Date: 03/15/2010 – 08/12/2010

AC Notebook Reference: AC 106, pp. 110-140, 147-153, 156-157, 159-160
AC 150, pp. 1-9

QC Notebook Reference: QC 29, pp. 186-189, 193-196, 199, 202-203, 207
QC 30, pp. 7, 9-10, 18-19, 22, 33, 41-43, 56, 61, 66-67, 75-77

Analyst: David A. Goldade, Dustin Keller and Laura Hulslander

---

Additional Comments:

- Samples were analyzed in duplicate, with a third replicate added when the initial two extractions did not closely agree (± 25%). Additional replicates were performed on some samples as needed to address poor method performance due to SPE column overload or other SPE performance issues. In all cases, the first data from a valid analysis is reported with the extraneous observations omitted.
- Observed concentrations are corrected for recovery of the surrogate compound (Diphacinone).
- Quality Control Standard match failed for the SPE investigative quality control experiment (analysis date 7/12/2010). These data were used for investigative purposes only and are not reported.

The following modifications were made to the method:

1. Phenomenex Strata solid phase extraction (SPE) columns were used in place of the Isolute NH_2 SPE columns as follows:
   a. Runs between 3/15/2010 and 6/21/2010 used Phenomenex Strata NH2 SPE columns.
   b. Runs between 6/21/2010 and 8/12/2010 used Phenomenex Strata X-AW SPE columns.
2. Sample weights were decreased from 1 gram to 0.5 gram for the following analysis dates:6/15, 6/16, 6/21, 7/14, 8/3, 8/5, 8/12. Sample weights were decreased due to overloading of the SPE columns resulting in high recovery of the Quality Control samples due to excessive matrix peaks.
3. A diode array detector was used to produce spectra for all positive samples to confirm the presence of chlorophacinone.
4. A Phenomenex Gemini C-18; 3 µm; 150 x 3.0 mm column was used.
5. The mobile phase was changed from Aqueous Ion-Pairing Reagent on channel A to a 1:1 mixture of Aqueous Ion-Pairing Reagent: Methanolic Ion-Pairing Reagent.
6. The column temperature was increased to 50°C.
7. The run time was shortened to 24 minutes for standards and 30 minutes for samples.
8. The mobile phase gradient was changed as follows:

Time	%A	%B
0	95	5
8	95	5
19	60	40
21	50	50
23	20	80

Analyst	10/1/10 Date	QC Specialist	10/1/10 Date	Reviewer	10.1.10 Date

Results:

Chlorophacinone in Prairie Dog Whole Body

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100201-03 A	Whole Body Prairie Dog KQ-02 M 1/30/10 C	4/7/2010	1.08§
S100201-03 B		4/7/2010	1.09§
S100201-04 A	Whole Body Prairie Dog KQ-04 1/30/10 C	4/7/2010	0.939§
S100201-04 B		4/7/2010	0.931§
S100201-05 F†	Whole Body Prairie Dog KQ-05 1/30/10 C	8/17/2010	< MLOD‡ [a]
S100201-05 G		8/17/2010	< MLOD‡ [a]
S100201-06 A	Whole Body Prairie Dog KQ-28 F 1/30/10 C	4/7/2010	1.33§
S100201-06 B		4/7/2010	1.32§
S100201-07 A	Whole Body Prairie Dog KQ-41 M 1/30/10 C	4/7/2010	1.75§
S100201-07 B		4/7/2010	1.81§
S100201-08 F†	Whole Body Prairie Dog KQ-43 1/30/10 C	8/17/2010	< MLOD‡ [a]
S100201-08 G		8/17/2010	< MLOD‡ [a]
S100201-15 F†	Whole Body Prairie Dog KQ-07 M 1/31/10 C	8/17/2010	< MLOD‡ [a]
S100201-15 G		8/17/2010	< MLOD‡ [a]
S100201-16 A	Whole Body Prairie Dog KQ-31 F 1/31/10 C	4/7/2010	0.619§
S100201-16 B		4/7/2010	0.599§
S100201-17 A	Whole Body Prairie Dog KQ-25 F 1/31/10 C	4/8/2010	0.680
S100201-17 B		4/8/2010	0.616
S100204-01 E†	Whole Body Prairie Dog KQ-15 F 2/3/10 C	8/17/2010	0.0950‡ [a]
S100204-01 F		8/17/2010	0.0961‡ [a]
S100204-02 A	Whole Body Prairie Dog KQ-29 F 2/3/10 C	4/8/2010	0.338
S100204-02 B		4/8/2010	0.280

Results (continued):

Chlorophacinone in Prairie Dog Whole Body

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100204-03 A	Whole Body Prairie Dog KQ-34 M 2/3/10 C	4/8/2010	0.224
S100204-03 B		4/8/2010	0.212
S100204-04 A	Whole Body Prairie Dog KQ-37 M 2/3/10 C	4/8/2010	0.398
S100204-04 B		4/8/2010	0.366
S100204-09 A	Whole Body Prairie Dog KQ-17 M 2/1/10 C	4/8/2010	< MLOD
S100204-09 B		4/8/2010	0.631
S100204-09 C		4/14/2010	0.869§
S100204-10 A	Whole Body Prairie Dog KQ-27 F 2/1/10 C	4/8/2010	0.949
S100204-10 B		4/8/2010	0.831
S100204-11 A	Whole Body Prairie Dog KQ-32 F 2/1/10 C	4/8/2010	0.826
S100204-11 B		4/8/2010	0.936
S100204-12 A	Whole Body Prairie Dog KQ-48 M 2/1/10 C	4/9/2010	0.478§
S100204-12 B		4/9/2010	0.475§
S100208-01 A	Whole Body Prairie Dog KQ 20 M 2/5/10 C	4/9/2010	0.392§
S100208-01 B		4/9/2010	0.361§
S100208-02 A	Whole Body Prairie Dog KQ 21 F/5/10 C	4/9/2010	0.583§
S100208-02 B		4/9/2010	0.480§
S100208-03 A	Whole Body Prairie Dog KQ 40 F 2/5/10 C	4/9/2010	0.465§
S100208-03 B		4/9/2010	0.415§
S100208-04 A	Whole Body Prairie Dog KQ 50 M 2/5/10 C	4/9/2010	0.502§
S100208-04 B		4/9/2010	0.366§
S100208-04 C		4/14/2010	0.312§

Results (continued):

Chlorophacinone in Prairie Dog Whole Body

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100208-09 A	Whole Body Prairie Dog KQ 08 F 2/7/10 C	4/9/2010	0.355§
S100208-09 B		4/9/2010	0.304§
S100208-10 E†	Whole Body Prairie Dog KQ 10 M 2/7/10 C	8/17/2010	< MLOD‡ [a]
S100208-10 F		8/17/2010	< MLOD‡ [a]
S100208-11 F†	Whole Body Prairie Dog KQ 11 F 2/7/10 C	8/17/2010	< MLOD‡ [a]
S100208-11 G		8/17/2010	< MLOD‡ [a]
S100208-12 E†	Whole Body Prairie Dog KQ 13 M 2/7/10 C	8/17/2010	< MLOD‡ [a]
S100208-12 F		8/17/2010	< MLOD‡ [a]
S100208-13 E†	Whole Body Prairie Dog KQ 24 M 2/7/10 C	8/17/2010	0.0928‡ [a]
S100208-13 F		8/17/2010	< MLOD‡ [a]
S100208-14 A	Whole Body Prairie Dog KQ 35 F 2/7/10 C	4/10/2010	0.439§
S100208-14 B		4/10/2010	0.439§
S100210-01 E†	Whole Body Prairie Dog KQ 12 M 2/9/10 C	8/17/2010	< MLOD‡ [a]
S100210-01 F		8/17/2010	< MLOD‡ [a]
S100211-01 F†	Whole Body Prairie Dog KQ 01 F 2/10/10 C	8/17/2010	< MLOD‡ [a]
S100211-01 G		8/17/2010	< MLOD‡ [a]
S100211-02 F†	Whole Body Prairie Dog KQ 30 M 2/10/10 C	8/5/2010	< MLOD‡
S100211-02 G		8/5/2010	< MLOD‡
S100211-03 A	Whole Body Prairie Dog KQ 33 F 2/10/10 C	4/10/2010	0.423§
S100211-03 B		4/10/2010	0.321§
S100211-04 E†	Whole Body Prairie Dog KQ 45 M 2/10/10 C	8/5/2010	< MLOD‡
S100211-04 F		8/5/2010	< MLOD‡

Results (continued):

Chlorophacinone in Prairie Dog Whole Body

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100212-01 E†	Whole Body Prairie Dog KQ 19 M 2/11/10 C	8/5/2010	< MLOD‡
S100212-01 F		8/5/2010	< MLOD‡
S100216-01 E†	Whole Body Prairie Dog KQ 03 M 2/14/10 C	8/5/2010	< MLOD‡
S100216-01 F		8/5/2010	< MLOD‡
S100216-02 E†	Whole Body Prairie Dog KQ 39 F 2/14/10 C	8/5/2010	< MLOD‡
S100216-02 F		8/5/2010	< MLOD‡
S100216-03 E†	Whole Body Prairie Dog KQ 42 M 2/14/10 C	8/5/2010	< MLOD‡
S100216-03 F		8/5/2010	< MLOD‡
S100216-04 E†	Whole Body Prairie Dog KQ 44 F 2/14/10 C	8/5/2010	< MLOD‡
S100216-04 F		8/5/2010	< MLOD‡
S100216-09 E†	Whole Body Prairie Dog KQ 49 F 2/15/10 C	8/5/2010	< MLOD‡
S100216-09 F		8/5/2010	< MLOD‡
S100218-01 E†	Whole Body Prairie Dog KQ 46 M 2/17/10 C	8/5/2010	0.116‡
S100218-01 F		8/5/2010	0.116‡
S100219-01 E†	Whole Body Prairie Dog KQ 06 M 2/18/10 C	8/5/2010	< MLOD‡
S100219-01 F		8/5/2010	< MLOD‡
S100219-03 C†	Whole Body Prairie Dog KQ 16 M 2/18/10 C	8/12/2010	< MLOD‡
S100219-03 D		8/12/2010	< MLOD‡
S100219-04 C†	Whole Body Prairie Dog KQ 18 F 2/18/10 C	8/12/2010	< MLOD‡
S100219-04 D		8/12/2010	< MLOD‡
S100219-02 C†	Whole Body Prairie Dog KQ 14 F 2/18/10 C	8/12/2010	< MLOD‡
S100219-02 D		8/12/2010	< MLOD‡
S100223-01 C†	Whole Body Prairie Dog KQ 26 F 2/22/10 C	8/12/2010	< MLOD‡
S100223-01 D		8/12/2010	0.115‡

Results (continued):

Chlorophacinone in Prairie Dog Whole Body

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100224-01 C†	Whole Body Prairie Dog KQ 09 M 2/23/10 C	8/12/2010	< MLOD‡
S100224-01 D		8/12/2010	< MLOD‡
S100224-02 C†	Whole Body Prairie Dog KQ 22 M 2/23/10 C	8/12/2010	< MLOD‡
S100224-02 D		8/12/2010	< MLOD‡
S100224-03 C†	Whole Body Prairie Dog KQ 23 F 2/23/10 C	8/12/2010	< MLOD‡
S100224-03 D		8/12/2010	< MLOD‡
S100224-04 C†	Whole Body Prairie Dog KQ 36 F 2/23/10 C	8/12/2010	< MLOD‡
S100224-04 D		8/12/2010	< MLOD‡
S100224-05 C†	Whole Body Prairie Dog KQ 38 F 2/23/10 C	8/12/2010	< MLOD‡
S100224-05 D		8/12/2010	< MLOD‡
S100224-06 C†	Whole Body Prairie Dog KQ 47 F 2/23/10 C	8/12/2010	< MLOD‡
S100224-06 D		8/12/2010	< MLOD‡

MLOD = Method Limit of Detection – 0.053 µg/g
† = Quality control recoveries from prior runs were determined to be out of control.
‡ = Sample size reduced to approximately 0.5g. Sample matrix was adversely affecting recovery of surrogate; therefore the sample size was reduced and surrogate recoveries returned to acceptable levels.
§ = Quality control recoveries at the 0.2 µg/g level for this analysis date fell outside of control limits. Values above 0.3 µg/g were accepted.
[a] = High level QC samples were fortified using incorrect stock solution. Values <MLOD were accepted.

Results:

Chlorophacinone in Prairie Dog Liver

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100201-09 A	Prairie Dog Liver KQ-02 M 1/30/10 L	4/15/2010	3.80
S100201-09 B		4/15/2010	3.52
S100201-10 A	Prairie Dog Liver KQ-04 F 1/30/10 L	4/15/2010	5.06
S100201-10 B		4/15/2010	4.75
S100201-11 A	Prairie Dog Liver KQ-05 1/30/10 L	4/15/2010	< MLOD
S100201-11 B		4/15/2010	< MLOD
S100201-12 A	Prairie Dog Liver KQ-28 F 1/30/10 L	4/15/2010	5.14
S100201-12 B		4/15/2010	4.91
S100201-13 A	Prairie Dog Liver KQ-41 M 1/30/10 L	4/15/2010	8.48
S100201-13 B		4/15/2010	5.64
S100201-13 E†		6/25/2010	11.1‡§
S100201-14 A	Prairie Dog Liver KQ-43 1/30/10 L	4/15/2010	< MLOD
S100201-14 B		4/15/2010	< MLOD
S100201-18 A	Prairie Dog Liver KQ-07 M 1/31/10 L	4/15/2010	0.144
S100201-18 B		4/15/2010	0.147
S100201-19 A	Prairie Dog Liver KQ-25 F 1/31/10 L	4/15/2010	2.89
S100201-19 B		4/15/2010	3.15
S100201-20 A	Prairie Dog Liver KQ-31 F 1/31/10 L	4/16/2010	3.61
S100201-20 B		4/16/2010	5.11
S100201-20 E†		6/25/2010	9.05‡§
S100204-05 A	Prairie Dog Liver KQ-15 F 2/3/10 L	4/16/2010	0.813
S100204-05 B		4/16/2010	0.774
S100204-06 A	Prairie Dog Liver KQ-29 F 2/3/10 L	4/16/2010	1.80
S100204-06 B		4/16/2010	1.55

Results (continued):

Chlorophacinone in Prairie Dog Liver

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100204-07 A	Prairie Dog Liver KQ-34 M 2/3/10 L	4/16/2010	0.921
S100204-07 B		4/16/2010	0.969
S100204-08 A	Prairie Dog Liver KQ-37 M 2/3/10 L	4/16/2010	0.934
S100204-08 B		4/16/2010	0.794
S100204-13 A	Prairie Dog Liver KQ-17 M 2/1/10 L	4/16/2010	3.76
S100204-13 B		4/16/2010	2.71
S100204-13 E†		6/25/2010	4.94‡§
S100204-14 A	Prairie Dog Liver KQ-27 F 2/1/10 L	4/16/2010	5.09
S100204-14 B		4/16/2010	3.41
S100204-14 E†		6/25/2010	5.63‡§
S100204-15 A	Prairie Dog Liver KQ-32 F 2/1/10 L	4/16/2010	3.43
S100204-15 B		4/16/2010	3.42
S100204-16 A	Prairie Dog LiverKQ-48 M 2/1/10 L	4/17/2010	1.75
S100204-16 B		4/17/2010	1.82
S100208-05 A	Prairie Dog Liver KQ 20 M 2/5/10 L	4/17/2010	1.02
S100208-05 B		4/17/2010	1.57
S100208-05 E†		6/25/2010	2.02‡§
S100208-06 A	Prairie Dog Liver KQ 21 F/5/10 L	4/17/2010	0.928
S100208-06 B		4/17/2010	0.946
S100208-07 A	Prairie Dog Liver KQ 40 F 2/5/10 L	4/17/2010	0.843
S100208-07 B		4/17/2010	0.536
S100208-07 E†		6/25/2010	1.91‡§
S100208-08 A	Prairie Dog Liver KQ 50 M 2/5/10 L	4/17/2010	0.789
S100208-08 B		4/17/2010	0.878

Results (continued):

Chlorophacinone in Prairie Dog Liver

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100208-15 A	Prairie Dog Liver KQ 08 F 2/7/10 L	4/17/2010	0.891
S100208-15 B		4/17/2010	0.862
S100208-16 A	Prairie Dog Liver KQ 10 M 2/7/10 L	4/17/2010	< MLOD
S100208-16 B		4/17/2010	< MLOD
S100208-17 A	Prairie Dog Liver KQ 11 F 2/7/10 L	4/17/2010	< MLOD
S100208-17 B		4/17/2010	< MLOD
S100208-18 E†	Prairie Dog Liver KQ 13 M 2/7/10 L	6/16/2010	0.493‡
S100208-18 F		6/16/2010	0.511‡
S100208-19 E†	Prairie Dog Liver KQ 24 M 2/7/10 L	6/16/2010	0.171‡
S100208-19 F		6/16/2010	0.108‡
S100208-20 E†	Prairie Dog Liver KQ 35 F 2/7/10 L	6/16/2010	1.89‡
S100208-20 F		6/16/2010	1.64‡
S100210-02 E†	Prairie Dog Liver KQ 12 M 2/9/10 L	6/16/2010	0.248‡
S100210-02 F		6/16/2010	0.393‡
S100211-05 E†	Prairie Dog Liver KQ 01 F 2/10/10 L	6/16/2010	0.118‡
S100211-05 F		6/16/2010	< MLOD‡
S100211-06 E†	Prairie Dog Liver KQ 30 M 2/10/10 L	6/16/2010	1.27‡
S100211-06 F		6/16/2010	1.11‡
S100211-07 E†	Prairie Dog Liver KQ 33 F 2/10/10 L	6/16/2010	0.560‡
S100211-07 F		6/16/2010	0.587‡
S100211-08 E†	Prairie Dog Liver KQ 45 M 2/10/10 L	6/16/2010	0.219‡
S100211-08 F		6/16/2010	0.251‡

Results (continued):

Chlorophacinone in Prairie Dog Liver

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100212-02 A	Prairie Dog Liver KQ 19 M 2/11/10 L	4/19/2010	0.413
S100212-02 B		4/19/2010	0.413
S100216-05 A	Prairie Dog Liver KQ 03 M 2/14/10 L	4/19/2010	0.136
S100216-05 B		4/19/2010	0.118
S100216-06 A	Prairie Dog Liver KQ 39 F 2/14/10 L	4/19/2010	0.147
S100216-06 B		4/19/2010	0.142
S100216-07 A	Prairie Dog Liver KQ 42 M 2/14/10 L	4/19/2010	0.136
S100216-07 B		4/19/2010	0.125
S100216-08 A	Prairie Dog Liver KQ 44 F 2/14/10 L	4/19/2010	0.301
S100216-08 B		4/19/2010	< MLOD
S100216-08 F†		7/19/2010	0.324‡
S100216-10 A	Prairie Dog Liver KQ 49 F 2/15/10 L	4/19/2010	0.455
S100216-10 B		4/19/2010	0.446
S100218-02 A	Prairie Dog Liver KQ 46 M 2/17/10 L	4/19/2010	0.445
S100218-02 B		4/19/2010	0.438
S100219-05 A	Prairie Dog Liver KQ 06 M 2/18/10 L	4/19/2010	0.130
S100219-05 B		4/19/2010	0.134
S100219-06 G†	Prairie Dog Liver KQ 14 F 2/18/10 L	7/19/2010	< MLOD‡
S100219-06 H		7/19/2010	< MLOD‡
S100219-07 G†	Prairie Dog Liver KQ 16 M 2/18/10 L	7/19/2010	< MLOD‡
S100219-07 H		7/19/2010	< MLOD‡
S100219-08 G†	Prairie Dog Liver KQ 18 F 2/18/10 L	7/19/2010	0.277‡
S100219-08 H		7/19/2010	0.253‡

Results (continued):

Chlorophacinone in Prairie Dog Liver

Lab Sample ID #	Sample Description	Analysis Date	Chlorophacinone Concentration (µg/g)
S100223-02 G†	Prairie Dog Liver KQ 26 F 2/22/10 L	7/19/2010	0.199‡
S100223-02 H		7/19/2010	0.175‡
S100224-07 G†	Prairie Dog Liver KQ 09 M 2/23/10 L	7/19/2010	< MLOD‡
S100224-07 H		7/19/2010	< MLOD‡
S100224-08 G†	Prairie Dog Liver KQ 22 M 2/23/10 L	7/19/2010	< MLOD‡
S100224-08 H		7/19/2010	< MLOD‡
S100224-09 G†	Prairie Dog Liver KQ 23 F 2/23/10 L	7/19/2010	< MLOD‡
S100224-09 H		7/19/2010	< MLOD‡
S100224-10 G†	Prairie Dog Liver KQ 36 F 2/23/10 L	7/19/2010	< MLOD‡
S100224-10 H		7/19/2010	< MLOD‡
S100224-11 G†	Prairie Dog Liver KQ 38 F 2/23/10 L	7/19/2010	< MLOD‡
S100224-11 H		7/19/2010	< MLOD‡
S100224-12 G†	Prairie Dog Liver KQ 47 F 2/23/10 L	7/19/2010	< MLOD‡
S100224-12 H		7/19/2010	< MLOD‡

MLOD = Method Limit of Detection – 0.061 µg/g
† = Quality control recoveries from prior runs were determined to be out of control.
‡ = Sample size reduced to approximately 0.5g. Sample matrix was adversely affecting recovery of surrogate; therefore the sample size was reduced and surrogate recoveries returned to acceptable levels.
§ = Quality control recoveries at the 0.4 µg/g level for this analysis date fell outside of control limits. Values above 0.6 µg/g were accepted.

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Quality Control Results:

Chlorophacinone in Prairie Dog Whole Body

Sample ID	Analysis Date	Target Content (µg/g) Chlorophacinone	Surrogate Corrected Recovery
QC-1	4/7/2010	Control	------
QC-2		Control	------
QC-3		0.208	95.3%§
QC-4		0.206	131%§
QC-5		2.04	94.6%
QC-6		1.96	85.4%
QC-7	4/8/2010	Control	------
QC-8		Control	------
QC-9		0.196	91.8%
QC-10		0.206	79.5%
QC-11		1.94	104%
QC-12		2.04	114%
QC-13	4/9/2010	Control	------
QC-14		Control	------
QC-15		0.196	212%§
QC-16		0.200	193%§
QC-17		2.06	112%
QC-18		1.98	101%
QC-19	4/10/2010	Control	------
QC-20		Control	------
QC-21		0.200	159%§
QC-22		0.200	50.7%§
QC-23		1.98	96.7%
QC-24		2.08	91.8%
QC-25	4/12/2010	Control	------
QC-26		Control	------
QC-27		0.206	210%§
QC-28		0.210	215%§
QC-29		1.91	112%
QC-30		2.08	143%
QC-31	4/12/2010	Control	------
QC-32		Control	------
QC-33		0.193	183%§
QC-34		0.200	138%§
QC-35		2.04	112%
QC-36		2.06	110%
QC-37	4/14/2010	Control	------
QC-38		Control	------
QC-39		0.204	200%§
QC-40		0.198	239%§
QC-41		1.94	105%
QC-42		2.06	109%

Invoice #: 10-008	Date: 9/28/10	Page: 14 of 15

Quality Control Results (continued):

Chlorophacinone in Prairie Dog Liver

Sample ID	Analysis Date	Target Content (µg/g) Chlorophacinone	Surrogate Corrected Recovery
QC-49	4/16/2010	Control	------
QC-50		Control	------
QC-51		0.416	79.4%
QC-52		0.416	79.9%
QC-53		4.16	79.9%
QC-54		4.08	81.8%
QC-55	4/17/2010	Control	------
QC-56		Control	------
QC-57		0.382	97.6%
QC-58		0.382	73.0%
QC-59		4.12	88.5%
QC-60		4.12	69.0%
QC-61	4/19/2010	Control	------
QC-62		Control	------
QC-63		0.408	50.3%
QC-64		0.400	52.3%
QC-65		4.20	64.6%
QC-66		4.12	55.0%
QC-67	4/19/2010	Control	------
QC-68		Control	------
QC-69		0.412	95.3%
QC-70		0.420	96.9%
QC-71		4.12	95.9%
QC-72		4.16	96.3%
QC-73	4/26/2010	Control	------
QC-74		Control	------
QC-75		0.420	52.2%
QC-76		0.412	49.1%
QC-77		4.04	55.6%
QC-78		4.12	57.8%
QC-79	5/3/2010	Control	------
QC-80		Control	------
QC-81		0.412	24.5%
QC-82		0.389	22.5%
QC-83		4.08	65.2%
QC-84		4.20	41.1%
QC-85	5/10/2010	Control	------
QC-86		Control	------
QC-87		0.382	61.8%[§]
QC-88		0.404	61.4%[§]
QC-89		3.89	86.7%
QC-90		4.08	88.4%

Quality Control Results (continued):

Chlorophacinone in Prairie Dog Liver

Sample ID	Analysis Date	Target Content (µg/g) Chlorophacinone	Surrogate Corrected Recovery
QC-91	5/13/2010	Control	------
QC-92		Control	------
QC-93		0.420	27.0%
QC-94		0.404	26.4%
QC-95		4.16	51.9%
QC-96		4.08	40.8%
QC-97	5/20/2010	Control	------
QC-98		Control	------
QC-99		0.400	45.3%
QC-100		0.412	44.5%
QC-101		4.33	**
QC-102		4.00	**
QC-103	6/16/2010	Control	------‡
QC-104		Control	------‡
QC-105		0.392	92.2%‡
QC-106		0.378	100%‡
QC-107		3.97	95.8%‡
QC-108		3.80	85.2%‡
QC-109	6/18/2010	Control	------‡
QC-110		Control	------‡
QC-111		0.390	79.2%‡§
QC-112		0.385	52.6%‡§
QC-113		3.91	84.2%‡
QC-114		3.93	81.3%‡
QC-115	6/25/2010	Control	------‡
QC-116		Control	------‡
QC-117		0.380	166%‡§
QC-118		0.387	259%‡§
QC-119		3.94	109%‡
QC-120		3.87	94.3%‡
QC-121	7/19/2010	Control	------‡
QC-122		Control	------‡
QC-123		0.397	77.8%‡
QC-124		0.390	73.5%‡
QC-125		4.04	91.5%‡
QC-126		3.98	89.6%‡

[a] = Samples were fortified using incorrect stock solution. Results not used.
** = Samples was not analyzed.
‡ = Sample size reduced to approximately 0.5g. Sample matrix was adversely affecting recovery of surrogate; therefore the sample size was reduced and surrogate recoveries returned to acceptable levels.
§ = Quality control recoveries at the 0.4 µg/g level for this analysis date fell outside of control limits. Therefore, data from this analysis date below 0.6 µg/g were not reported. Values above 0.6 µg/g were accepted.

Appendix IV – NWRC Bait Analysis and Certificate Provided by the Manufacturer

Wildlife Services NWRC National Wildlife Research Center Analytical Services Report	United States Department of Agriculture Animal Plant Health Inspection Service Wildlife Services National Wildlife Research Center Invasive Species and Technology Development Research Program Analytical Chemistry Project	Invoice #: 10-009 Date: 7/23/2010 Page: 1 of 2

To: Dr. Gary Witmer
Research Wildlife Biologist
NWRC

Subject: Chlorophacinone Rozol Bait

Method: 163A

Analysis Date: 7/22/2010

AC Notebook Reference: AC 130: pages 52-57

QC Notebook Reference: QC 30: pages 69 and 72

Analyst: Doreen Griffin

Sample Description:

Three Rozol Grain Bait samples were submitted. Sample descriptions and results are provided on page 2 of this report.

Additional Comments:

Three replicate weighings of each submitted sample were assayed according to the procedures outlined in the method.

Analyst	Date	QC Specialist	Date	Reviewer	Date
[signature]	7/28/10	[signature]	7.26.10	[signature]	7.27.10

Invoice #: 10-009	Date: 7/23/2010	Page: 2 of 2

Results:

Chlorophacinone Rozol Bait Assay

Lab Sample ID #	Observed % Chlorophacinone (w/w)	
S100205-01A	0.00512	$Mean_3$ = 0.00511
S100205-01B	0.00510	sd = 0.000010%
S100205-01C	0.00511	cv = 0.20%
S100205-02A	0.00512	$Mean_3$ = 0.00509%
S100205-02B	0.00507	sd = 0.000029%
S100205-02C	0.00507	cv = 0.57%
S100205-03A	0.00499	$Mean_3$ = 0.00505%
S100205-03B	0.00511	sd = 0.000060%
S100205-03C	0.00504	cv = 1.2%

QC Results

Lab Sample ID #	Observed % Chlorophacinone	Target % Chlorophacinone	% Recovery
QC-1	No Response Detected	Control	NA
QC-2	0.00511	0.00503	102
QC-3	0.00511	0.00499	102
QC-4	0.00490	0.00497	98.6

LIPHATECH

3600 WEST ELM STREET
MILWAUKEE, WI 53209
Tel: 414/351 1476 800/351 1476
Fax: 414/247 8166

CERTIFICATE OF ANALYSIS

PRODUCT NAME:	Rozol Prairie Dog Bait		
LOT NUMBER:	28709A	**TECHNICAL REFERENCE:**	635101
MANUFACTURING DATE:	10/14/2009	**DATE OF ANALYSIS:**	10/14/2009

ASSAY	SPECIFICATION		RESULTS
	Lower Limit	Upper Limit	
Chlorophacinone Assay	40 mg/kg	60 mg/kg	44.86 mg/kg

DATE OF ISSUE:	10/26/2009	**CONCLUSIONS:**	Pass

Shane G. Nimmer
Quality Control Chemist

10-26-09
Date

Quality Control Chemist

LiphaTech Home Page: http//www.liphatech.com
E-mail: rodentcontrol@liphatech.com