FINAL REPORT

for

Vertebrate Pest Control Research Advisory Committee

STUDY TITLE:

An assessment of secondary toxicity risk for 0.005% diphacinone treated grain via three application strategies for California ground squirrels.

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EXECUTIVE SUMMARY

Anticoagulant rodenticides are one of the primary tools used to mitigate rodent damage in agricultural systems, but they have received increased scrutiny over the last several decades given concerns about secondary exposure in non-target wildlife. Various strategies could potentially reduce secondary exposure. One approach is to use rodenticide application strategies that minimize risk. To better understand this approach, we tested residual concentrations of a first-generation anticoagulant (diphacinone) in radiotransmittered California ground squirrel (*Otospermophilus beecheyi*) liver samples following spot treatments, broadcast applications, and bait stations in rangelands in central California during summer and autumn 2018–2019. Specific details for our sampling methodology and findings include:

1. We established 4 study plots in rangelands in San Joaquin and Stanislaus Counties in summer and autumn 2018 and 2019. The 4 plots each season were randomly assigned into one of 3 treatment categories (spot treatment, broadcast application, or bait station) or served as a control.
2. We trapped and radiotransmittered ground squirrels in each plot to allow us to track mortality rates for each application strategy. We transmittered 7 ground squirrels in each plot during 2018. In 2019, we transmittered 8 ground squirrels in each treatment plot and 4 in the control plots. Ground squirrels were tracked daily to determine individual mortalities.
3. For bait station plots, we used 64 inverted "T"-shaped bait stations that were spread out in an 8 × 8 grid. Spot treatments involved the spreading of bait by hand around the entrance of active burrow systems. For broadcast applications, we used a calibrated seed spreader to distribute bait around burrow entrances. We used the California Department of Food and Agriculture's Rodent Bait Diphacinone Treated Grain for all applications. We used the 0.005% concentration for all applications except for broadcast applications during autumn 2018 and summer and autumn 2019. We switched to the 0.01% concentration bait for these broadcast applications given poor efficacy experienced in summer 2018. We recorded the amount of bait applied for each application.
4. We tracked ground squirrels daily and documented mortalities. We noted if the ground squirrel died aboveground or within their burrow system, we documented the number of days from original diphacinone application until death, and we collected the animal for liver sampling. All transmittered ground squirrels that survived until the end of the study were trapped to collect livers for sampling as well. All liver samples were tested for residual diphacinone levels to allow for comparisons across the different application strategies.
5. We documented substantially greater amounts of bait applied via bait station applications, followed by spot treatments, then broadcast applications. Some bait removed from bait stations was likely cached underground; potential non-target risks from caching are unknown.
6. We did not document any difference in residual diphacinone concentrations across the 3 application strategies. However, the primary concentration of diphacinone bait used for broadcast applications was twice that applied in bait stations and spot treatments (0.01% vs. 0.005%, respectively). Further testing is needed to determine if

a broadcast application of 0.005% diphacinone bait could reduce residual concentrations in target rodents.

7. We did not note any difference in time from bait application to death, nor did we note any impact of seasonality on any of the factors we tested.
8. The vast majority of mortalities occurred belowground (82–91%), which helped to reduce secondary exposure. Applicators could further reduce secondary exposure by conducting daily searches to remove carcasses from the landscape.

Collectively, results from this study can be used to better identify risk associated with first-generation anticoagulant applications for control of field rodents in agriculture, which may allow for decreased non-target exposure when incorporating anticoagulants into integrated pest management programs.

As part of this study, we also determined the applicability of using visual counts to document efficacy associated with ground squirrel management practices. Specific details for our sampling methodology and findings for this portion of the study include:

1. We conducted visual counts at each plot previously described. Visual counts were conducted in morning and afternoon periods across 3 consecutive days. We used the highest count for each plot in analyses. Efficacy was defined as the ratio between the number of ground squirrels counted pretreatment vs. the number counted posttreatment.
2. We used radiotransmittered ground squirrels in plots previously described to provide a corroborative estimate of efficacy, with efficacy defined as the number of mortalities divided by the number of uncensored individuals. We compared paired efficacy values derived from visual counts and radiotransmittered individuals to determine if any difference between the 2 was apparent.
3. We tracked location data for each transmittered ground squirrel, and we compared the proportion of individual ground squirrel locations found within treatment areas to survival or mortality for each ground squirrel to determine the relationship between rodenticide exposure and mortality.
4. We did not observe a difference in efficacy associated with the 2 monitoring strategies, indicating that visual counts are an effective strategy for this rodent species. However, we did generally document greater efficacy associated with tracking of transmittered individuals, suggesting that estimates derived from visual counts may be conservative.
5. The somewhat greater efficacy observed with transmittered individuals may be due to rapid immigration from adjacent areas. Furthermore, we observed low efficacy in 2 treatment plots, likely due to low usage of those plots by ground squirrels. Increasing the size of buffer zones would increase the usage of treatment areas by the target population, and would also help to minimize concerns of reinvasion by adjacent ground squirrel populations, which could bias efficacy values low.
6. We suggest a minimum of a 61-m buffer surrounding census plots. Increasing to 66 m or more would further benefit efficacy assessments, but an increased size of the buffer zone must be balanced with greater costs and regulatory constraints.

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FOREWORD

This report is divided into two chapters based on different stated goals. They have been written in such a way to allow for publication as two separate manuscripts. Please note that Table 1 in Chapter 1 and Table 1 in Chapter 2 have slightly different values. This is because of the different stated goals of each chapter. In Chapter 1, we were interested in the most accurate assessment of efficacy, but in Chapter 2, we were interested in comparing monitoring tools that required the inclusion of data that were censored in Chapter 1. For assessments of efficacy, please refer to the data provided in Chapter 1 as it is considered the most accurate representation of the effectiveness of each rodenticide application strategy.

Chapter 1: Impact of application strategy on residual concentrations of diphacinone

INTRODUCTION

Rodents cause extensive damage in many agricultural settings worldwide. One of the primary tools used to mitigate this damage has been anticoagulant rodenticides given the efficacy and cost effectiveness of this approach (Witmer and Eisemann 2007, Baldwin et al. 2014, Capizzi et al. 2014, Jacob and Buckle 2018). Perhaps the primary drawback of anticoagulant rodenticides is the potential for secondary poisoning of predators and scavengers. Substantial effort has been undertaken over the last several decades to address secondary toxicity risks associated with anticoagulant rodenticides (see van den Brink et al. 2018 for a substantive review).

One proposed strategy for reducing secondary toxicity is to use application strategies that lower exposure risk to scavengers and predators (Buckle and Prescott 2018). Several researchers have postulated that the method by which rodenticide baits are applied could substantially affect residual levels of anticoagulants in rodents (Dubock 1982, Record and Marsh 1988, Whisson and Salmon 2009). Commonly used strategies for rodenticide application in agricultural fields include spot treatments, broadcast applications, and bait stations (Tobin and Richmond 1993, Marsh 1994a,b, Salmon 2007, Jacob and Buckle 2018). Spot treatments involve the spreading of rodenticide baits over a label-specified area around a burrow entrance or rodent trail. This strategy is generally used over small areas given the time-consuming nature of this approach. Broadcast applications are used for treating larger areas. Broadcast applications involve the use of a spreader that is calibrated for distribution of bait over areas frequented by target species. Both spot treatment and broadcast applications take advantage of the natural foraging patterns of target rodent species (Marsh 1968, Matschke et al. 1983). However, these approaches do not limit access to the bait, which is a substantial concern in areas frequented by domesticated animals, humans, and some non-target wildlife species. In such areas, bait stations are preferred. Many bait station designs exist, but the general premise is to eliminate non-target access to the rodenticide by animals that are larger than the opening of the bait station.

Many rodenticide labels now require the use of bait stations for rodenticide application given the potential reduction in primary non-target exposure. That said, bait stations could potentially increase secondary exposure by allowing repeated feedings at the station, as rodents do not reduce bait consumption until several days after initiating feeding (e.g., Whisson and Salmon 2002). This repeated feeding could ultimately allow for higher residual concentrations within the target rodent (Hindmarch and Elliott 2018). Conversely, broadcast applications have been postulated to have the lowest risk for secondary exposure given a sparse distribution of bait over target areas. Spot treatments are believed to be intermediate; bait availability is lower than that for bait stations, but greater than that for broadcast applications given that target levels of bait from spot treatments are established to allow for removal of multiple rodents per burrow system (Record and Marsh 1988, Salmon et al. 1997). However, this assertion has not been rigorously tested. Knowing how differing rodenticide application strategies contribute to secondary toxicity risk would assist in the development of a rodenticide application program that could reduce this risk.

An alternative strategy for reducing secondary exposure is to limit the amount of time between rodenticide consumption and mortality. Anticoagulant rodenticides require an extended timeframe for mortality to occur, often 4–13 days or more (Clark 1978, Hindmarch and Elliott 2018). As previously stated, animals can repeatedly feed on the bait during this timeframe, potentially leading to higher residual concentrations of rodenticides within the body (Hindmarch and Elliott 2018). Additionally, the longer an intoxicated rodent is alive and active on the landscape, the greater the opportunity for it to be predated upon (Buckle and Prescott 2018). This is of note given that several studies have suggested variable timeframes from bait application until death for target rodents depending on the application strategy used (e.g., Baroch 1996, Whisson and Salmon 2009). A better understanding of how rodenticide application strategies influence time to death is needed to better guide applicators as to how to lower secondary toxicity risk to non-target predators and scavenger.

Ultimately, the best way to reduce secondary exposure is to eliminate intoxicated rodents from the food web (Record and Marsh 1988). This could be accomplished by eliminating the use of the anticoagulant. However, anticoagulant rodenticides continue to be a key part of many rodent management programs given their high efficacy and cost effectiveness, ease of use, reduced exposure risk when proper mitigation actions are taken, and a lack of effective and practical alternative management tools in many settings (Baldwin et al. 2014, Jacob and Buckle 2018); their use is likely to continue into at least the near future. Fortunately, many intoxicated rodents die within burrow systems, where they are unavailable to many predators and scavengers. However, the proportion that die belowground is largely unknown. In a minimal study with California ground squirrels (*Otospermophilus beecheyi*), Whisson and Salmon (2009) determined that 3 of 8 (38%) radiotransmittered individuals died aboveground, while Saucy et al. (2001) reported similar results for water voles (*Arvicola terrestris*; 38% died aboveground). Knowing the proportion of the target species that die aboveground and remain available to scavengers is a key step toward devising management programs that reduce this secondary-exposure risk.

Discussions about the secondary-toxicity effects of anticoagulants have been increasing in many parts of North America over the last decade (e.g., Serieys et al. 2015, Gabriel et al. 2018, Quinn 2019), leading to numerous legislative attempts to limit or eliminate their use in many settings (Quinn et al. 2019). That said, anticoagulant rodenticides are still considered an important tool for minimizing rodent damage in both agricultural and urban areas (Baldwin et al. 2014, Quinn et al. 2019). Ground squirrels (Sciuridae) provide an excellent example of this importance. Ground squirrels are broadly distributed throughout much of North America, and anticoagulant rodenticides are extensively used to mitigate damage caused by many of these ground squirrel species (Askham 1994, Marsh 1994a, Baldwin et al. 2014). In California, the California ground squirrel is widely considered one of the two most damaging rodent species in agriculture (Marsh 1998, Baldwin et al. 2014). Diphacinone is the most commonly used rodenticide for field rodents in California, and is available for use via spot treatments, broadcast applications, and bait stations depending on the particular product used (Timm et al. 2004). However, application strategy may influence non-target exposure risk, and little is known about the availability of ground squirrel carcasses to predators and scavengers following anticoagulant baiting programs. Therefore, we established the following objectives to better elucidate potential risks associated with these factors. Specifically, we tested for: 1) differences in amounts of diphacinone-treated oat groats applied via spot treatments, broadcast applications, and bait stations, 2) differences in time from consumption to death for each application strategy, 3) differences in residual concentrations of diphacinone for each application strategy, and 4) the proportion of ground squirrels that died belowground. Collectively, this information should allow for the development of management actions that can minimize non-target exposure associated with anticoagulant baiting programs.

STUDY AREA

This study was centered on rangelands located on the western side of San Joaquin and Stanislaus Counties, California, USA. These rangelands were seasonally grazed by cattle, with grazing typically occurring from October through March. The soils in the area consisted of Carbona clay loam and Zacharias gravely clay loam with a small portion of Stomar clay loam up to an 8% slope. Annual precipitation in the area averages from 25.4–30.5 cm, with the majority of precipitation occurring from October through March. Average temperatures range from a low of 4ºC in January to a high of 35ºC in July. Species composition was primarily annual grasses (non-native) and annual forbs such as *Hordeum murinum, Bromus madritensis, Bromus diandrus, Bromus hordeaceus, Avena fatua, Medicago polymorpha*, and *Erodium* spp. Forage production on Carbona clay soils can range from 2668–2825 kg/ha and 2522 kg/ha for the other soils in the area (Web Soil Survey 2020). Local forage production conducted on the ranch close to our study sites averaged forage production of 1636 kg/ha with a range of 479 to 2697 kg/ha (Becchetti et al. 2016, T. Becchetti, unpublished data).

MATERIALS AND METHODS

Plot establishment

During summer 2018, we established four 186 × 186 m plots (3.4 ha) in areas that had abundant ground squirrel numbers to allow for collaring with radiotransmitters. Plots were generally located a minimum of 192 m ($\bar{x}$ minimum distance = 418 m) from any other plot to minimize the likelihood of a ground squirrel moving between plots, although the control and broadcast plots were separated by only 87 m during summer 2018. We recorded only one ground squirrel that was ever located within any treatment plot other than where they were captured. This individual was originally located in a bait station plot and showed up in a broadcast plot. However, the location within the broadcast plot did not occur until 7 days after the final broadcast application. Because bait rarely lasts 7 days in broadcast plots (Dochtermann 2005), it seemed unlikely that this ground squirrel ingested any appreciable amount of bait from the broadcast plot. As such, we considered all plots independent within each sampling season. Each plot was randomly assigned a treatment (spot, broadcast, or bait station) or served as the control. This process was repeated at new locations during summer 2019 and autumn 2018–2019.

Capture and collaring

To radiotransmitter ground squirrels, we established a trapping grid of 20–25 traps within the 0.4-ha core area of each plot. We selected this size to allow a buffer of 61 m on all sides to reduce the likelihood of ground squirrels moving off the treatment areas. This buffer distance is similar to other studies that have tested efficacy of rodenticides for California ground squirrel management (e.g., Baroch 1996, Salmon et al. 2007). We used Tomahawk wire cage traps (combination of 13 × 13 × 46 cm and 15 × 15 × 61 cm traps; Tomahawk Live Trap, Hazelhurst, WI) to capture ground squirrels. Traps were initially prebaited with plain oat groats for 1–2 days, and then were activated and again baited with oat groats to allow captures. Trapping occurred from early morning until 11:00 to reduce heat exposure concerns to ground squirrels. Traps were checked approximately every hour. Upon capture, ground squirrels were moved within the traps to a shaded area for processing. We initially dusted ground squirrels with a 0.25% permethrin dust (Hi-Yield Garden, Pet & Livestock Dust, Voluntary Purchasing Groups, Inc., Bonham, TX) to reduce potential concerns with ectoparasites. We handled ground squirrels via a cloth handling cone outlined by Koprowski (2002). They were weighed, sexed, and fitted with a VHF transmitter via a cable tie around the neck (Model M1535, weight = 14 g; Advanced Telemetry Systems, Isanti, MN). The transmitters were retrofitted with a mortality signal that would trigger after 12 hours of inactivity. No ground squirrels were collared that weighed <266 g to ensure that the transmitter did not constitute more than 5% of the ground squirrels body weight. Upon completion, we placed each ground squirrel back into the trap and released them at the site of capture. During summer and autumn 2018, we captured and collared 7 ground squirrels in each treatment and control plot. We altered this strategy slightly in 2019 to collar 8 ground squirrels in each treatment plot and only 4 ground squirrels in control plots given the lack of mortalities that occurred in the control plots.

Radiotracking

We always allowed several days ($\bar{x}$ = 8.6 days, SE = 0.2) between collaring and the start of bait application to allow the ground squirrels a period of adjustment to wearing the collars. During this timeframe, we generally obtained locations of ground squirrels daily, though occasionally other activities kept us from collecting locations. That said, we always identified ground squirrel locations daily following completion of initial rodenticide application until the termination of the trial. Locations were determined by walking up to ground squirrel locations. We documented if a ground squirrel was visually observed. Likewise, we documented any mortalities that occurred, and any removed collars that were detected aboveground. In some situations, we could not easily find a ground squirrel location. If we could not find a location, we drove around for a minimum of 500 m beyond the buffer zone to continue searching for locations. If we could not detect a location, it was noted as missing for that day. Otherwise, we recorded all locations using a hand-held GPS unit.

Bait application

For bait station plots, we used inverted "T" shaped PVC pipe bait stations that were commonly used for California ground squirrel control (Whisson and Salmon 2009). The stations were made of 10 cm pipe with end caps cut in half and glued to the end of each bait station to keep bait from spilling onto the ground. The stations were 1.2 m in length and 0.9 m in height with an endcap on top to close off bait access. We attached stations to metal T-posts that were staked into the ground. Following Baroch (1996), we spaced bait stations 23 m apart following an 8 × 8 grid pattern that covered the entire treatment area. On day 0 for each bait application trial, we placed 0.9 kg of Rodent Bait Diphacinone Treated Grain (0.005%; California Department of Food and Agriculture, Sacramento, CA) into each bait station. We then checked bait stations approximately every three days to maintain a bait supply within each station. If a station required refilling, we documented the amount of bait that was added. Bait station trials were conducted until bait was no longer being removed from the stations. Upon completion of the trial, we collected and weighed all bait from the bait stations. We subtracted this amount from the total amount applied to determine the total amount removed by ground squirrels in each plot.

We also used the Rodent Bait Diphacinone Treated Grain (0.005%) for spot treatments. Before treatment, we identified all burrow openings within the 3.4 ha treatment area that appeared to house ground squirrels by looking for fresh footprints, scrapings, fecal pellets, and clear openings (i.e., devoid of detritus and spider webs, not overgrown with vegetation, etc.). We then treated each of these active burrow openings on day 0 of the trial with approximately 37 g of grain spread evenly over a 3.7–4.6 m^2 area. However, if an individual treatment area overlapped multiple burrow openings, bait was applied only once over these openings to minimize rodenticide availability. This process was repeated 4 days later to ensure that ground squirrels had access to the bait over the period required to maximize efficacy (Whisson and Salmon 2002). We recorded the total amount of bait applied for comparison to other application strategies.

For broadcast applications, we initially used the Rodent Bait Diphacinone Treated Grain (0.005%) to allow for a more direct comparison in residual diphacinone concentrations within ground squirrels across the different application strategies. However, we did not observe any mortalities following application during summer 2018. As such, for the remaining three trial periods, we defaulted back to the label-specified rate of 0.01% diphacinone for broadcast applications (Rodent Bait Diphacinone Treated Grain [0.01%]; California Department of Food and Agriculture, Sacramento, CA). For broadcast applications, we first calibrated a seed spreader (Solo 421-S, Newport News, VA) to discharge the bait at the label-specified rate of approximately 11.4 kg ha^{-1}. We then flagged out transects that intersected active burrow systems throughout the treatment area to allow for efficient application. We applied bait along these transects on day 0 and again four days later to attain target exposure levels required for effective population reduction (Whisson and Salmon 2002). We compared the amount of bait applied across all three treatment types and two seasons using a two-factor ANOVA. If the model was significant, we used Fisher's least significant difference *post hoc* test to determine which application strategies or seasons differed (Zar 1999).

<u>Fate of ground squirrels</u>

We expected several outcomes of radiotransmittered ground squirrels including dropped collars, lost signals, unknown fates (collars that were recovered far from previous locations suggesting scavenging or for which we were unable to find a collar or ground squirrel when digging), squirrels that moved out of treatment areas before rodenticide applications occurred, unknown causes of mortality, rodenticide mortality, and survival. As such, we placed each radiotransmittered ground squirrel into one of these categories at the completion of each trial period, but for the purposes of this study, we censored all ground squirrels that were not included in the survival or rodenticide mortality fates. We determined mortality rates by dividing the number of radiotransmittered ground squirrels that died from diphacinone exposure by the number of uncensored individuals remaining at the end of the trial period.

For mortalities, if the ground squirrel carcass was aboveground, we dusted the ground squirrel with 0.25% permethrin dust, we noted the location, and collected the animal. If the ground squirrel was belowground, we first pinpointed the location and then began digging. Soils were extremely hard and compact, requiring the use of a jackhammer to retrieve the ground squirrels. Depths of ground squirrels varied, but generally ranged from 0.5–1.2 m below ground. Once found, the ground squirrel was dusted with 0.25% permethrin dust, the condition of the carcass was noted, and the animal was stored in a freezer bag. We prioritized digging up ground squirrels the day the mortality signal was first heard, but initial staff limitations sometimes precluded us from digging up the ground squirrel until the next day. Upon liver collection, we noted that waiting an extra day was often too lengthy to recover a viable liver, so adjustments were made to ensure carcass collection the day a mortality signal was first noted. This substantially improved our ability to retrieve viable livers. We also searched daily for any additional carcasses located aboveground, and during digging activities, we collected any dead non-transmittered ground squirrels within burrow systems. All collected ground squirrels were transported back to the laboratory where they were frozen for future laboratory assessment. We used Fisher's exact test (Zar 1999) to test for seasonal differences in the proportion of ground squirrels that died belowground.

At the end of each trial period, we again used Tomahawk wire cage traps to recapture any surviving radiotransmittered ground squirrels using the same protocols outlined above (although we did not prebait during this process). When we captured a radiotransmittered ground squirrel, we dusted it with 0.25% Permethrin and then euthanized it via a carbon dioxide euthanasia chamber. All euthanized ground squirrels were collected and frozen for future liver extraction. Any non-transmittered ground squirrels that were captured were immediately released. All aspects of this project were approved by the University of California, Davis, Institutional Animal Care and Use Committee (Protocol no. 20025).

<u>Time to death</u>

We estimated time from bait application to death by noting the day of initial application as day 0. Occasionally, a ground squirrel was retrieved aboveground without the collar emitting a mortality signal. Because the timeframe to initiate a mortality signal was 12 hours, we considered those ground squirrels to have died the day when they recovered. For most ground squirrels that we collected that had emitted a mortality signal, we considered them to have died the day prior to initial detection given that all signal detections were completed before 12:00 each day. However, in some situations, the state of decay of the ground squirrel made it obvious that they had been dead for a longer period of time. We believe that surviving ground squirrels bumped or pulled on dead ground squirrels occasionally, keeping mortality switches from activating. In these situations, we made the assumption that the second day that the location did not change was the date of mortality, and we compared that estimated date of mortality to the condition of the carcass. For example, if initial instars of maggots were present, we considered the ground squirrel to have died 2–3 days prior. These two factors were corroborative in their estimation of when the ground squirrel likely died for all but one ground squirrel. For that ground squirrel, the carcass had completely decomposed, and as such, it was eliminated from further analysis. We used a two-factor ANOVA to test for potential differences in time from bait application to death and season. If the model was significant, we used Fisher's least significant difference *post hoc* test to determine which application strategies or seasons differed (Zar 1999).

Diphacinone residue analysis

We removed whole livers in the lab and shipped them to the Texas A&M Veterinary Medical Diagnostic Laboratory in College Station, TX, for testing. We analyzed liver tissues for the presence of diphacinone using the quick, easy, cheap, effective, rugged, and safe method (QuEChERS; Anastassiades et al. 2003). QuEChERS cleanup tubes were prepared for each sample and control. We added the following to a 15 mL disposable centrifuge tube: 250 mg of C18 sorbent, 500 mg of basic alumina, 250 mg of Florisil, 175 mg of $MgSO_4$, and 50 mg of PSA. We then weighed liver samples (1.0 ± 0.2g) and transferred the samples to 15 mL centrifuge tubes. We added 20 µL of an internal standard solution to each tube, followed by 3.0 mL of Acetonitrile and 0.2 g NaCl. Samples were then homogenized using an OMNI bead ruptor mill. We added 1.0 mL of Acetonitrile to each tube, subsequently mixing tubes by vortexing. We transferred the supernatant to a clean QuEChERS tube, which was vortexed and then centrifuged at ~2,000 rpm for ~5 min. We transferred the supernatant to a clean 13 × 100 mm culture tube and evaporated just to dryness at 40 ± 5° C. The dried extracts were reconstituted with 100 µL of Acetonitrile, vortexed and transferred to 1.5 mL microtubes. We then centrifuged the microtubes to separate the flocculent material, and sample extracts were analyzed for the diphacinone compound using an Agilent 6400 series LC-MS triple quadrupole mass spectrometer. Positive identification and quantitation were based on retention time, spectral matching, and transition ions compared to a concurrently analyzed certified reference standard.

We compared residual concentrations of diphacinone using a one-factor ANOVA across five categories: spot treatment mortalities, broadcast application mortalities, bait station mortalities, survivors from rodenticide application plots, and control-plot survivors. If the model was significant, we used Fisher's least significant difference *post hoc* test to determine which application strategies differed (Zar 1999). We also tested for differences in residual diphacinone concentrations between 0.005% and 0.01% broadcast applications to determine if initial concentration of the rodenticide affected residual levels in ground squirrels (Student's *t*-test; Zar 1999). Significance for all tests was set at $\alpha = 0.05$.

RESULTS

The amount of bait applied varied substantially across application strategies ($F_{2,8} = 290.5$, $P < 0.001$), but did not vary across seasons ($F_{1,8} = 0.6$, $P = 0.476$). The greatest amount of bait was applied via bait stations ($\bar{x} = 64.1$ kg, SE = 3.2), followed by spot treatments ($\bar{x} = 11.1$ kg, SE = 0.4) and broadcast applications ($\bar{x} = 3.5$ kg, SE = 0.1).

We observed 46 ground squirrel mortality events from treatment plots, with 20, 19, and 7 occurring in bait station, spot treatment and broadcast application plots, respectively (Table 1). This resulted in 100% mortality for bait station and spot treatment applications, but only 39% mortality was observed in the broadcast application plots. Of the mortalities in the broadcast plot, 100% occurred during autumn (Trials 2 and 4). We documented no mortalities in the control plot indicating that observed mortalities were due to diphacinone bait application (Table 1). We also censored a large number of ground squirrels ($n = 33$), effectively lowering the number of ground squirrels used in further analyses. Reasons for censoring included: dropped collar = 13, lost signal = 9, unknown fate = 6, unknown cause of mortality = 3, and moved completely out of application site before application occurred = 2.

We did not observe an impact of application strategy ($F_{2,41} = 0.8$, $P = 0.462$) or season ($F_{1,41} = 1.5$, $P = 0.224$) on mean time from bait application to death (collective $\bar{x} = 9.1$ days, SE = 0.5, range = 4–19; Table 2). We did not detect a seasonal impact on the number of ground squirrels that died belowground (Fisher's exact $P = 0.336$), with 91% of documented mortalities occurring belowground (Table 3). We did observe five instances of ground squirrels where we suspected predation or scavenging. If we considered those as aboveground mortalities that occurred from diphacinone exposure, then the adjusted belowground mortality rate drops to 82%. We still did not observe a seasonal effect following this scenario (Fisher's exact $P = 0.714$).

For residual diphacinone concentrations, we did observe an overall model difference ($F_{4,60} = 19.2$, $P < 0.001$, $R^2 = 0.561$), although this difference was driven by lower values observed in control plots and for ground squirrels that did not succumb to the toxicant (Fig. 1). We observed no difference in residual diphacinone concentrations across any of the three application strategies (Fig. 1), with an average residual concentration of 1,399 ppb (SE = 129) for mortalities across all three application strategies. All ground squirrels that survived the diphacinone applications occurred in the broadcast plots ($n = 10$). We did not detect a difference in residual concentrations of ground squirrels surviving broadcast applications of 0.005% ($\bar{x} = 85$ ppb) and 0.01% ($\bar{x} = 130$ ppb) diphacinone-treated grain ($t_8 = 0.9$, $P = 0.393$). Collectively, residual concentrations for surviving individuals averaged 112 ppb (SE = 24).

DISCUSSION

Although bait stations are often considered a safer option for limiting primary non-target exposure to rodenticides, Whisson and Salmon (2009) speculated that bait stations might increase secondary exposure risk given an abundance of bait available for consumption by the target species ultimately allowing for the build-up of higher residual concentrations of toxicants in those animals. Similar to Baroch (1996), we did not observe this pattern with California ground squirrels in our study system. As such, there does not appear to be an increased risk of higher residual concentrations of anticoagulants in ground squirrels following application via bait stations. That said, ground squirrels did remove a substantial amount of grain, far exceeding the amount that was distributed by other application strategies. It is likely that much of that grain was stored underground for potential consumption later (Marsh 1994b, Whisson and Salmon 2009). The fate and risk of this stored grain remains unanswered. Baroch (1996) determined that diphacinone on 0.005% treated oats exposed aboveground degraded by 72% over a 9-day period, but only by 7% in bait stations. In a study on water voles (*Arvicola terrestris*), Sage et al. (2007) found that half-lives of bromadiolone baits in a simulated underground cache ranged from 24.6 to 42.7 days depending on the season assessed. However, their study used wheat grains as a carrier. Rolled oat groats likely degrade more rapidly in the environment given the lack of a hard external coating, so degradation of diphacinone oat groats may occur more rapidly; at this point, it is unknown. At a minimum, caching behavior should be considered in locations where there is a substantial concern of primary exposure from these food stores (Whisson 1999, Whisson and Salmon 2009).

Table 1. The proportion (Mortality) and associated efficacy values of California ground squirrels that died from diphacinone intoxication following application of a diphacinone-coated oat bait (0.005% concentration unless otherwise noted) applied via bait station, spot treatment, and broadcast applications across four trial periods in rangelands located in central California during summer and autumn 2018–2019. Results from control plots are provided for comparative purposes. Censored individuals were removed for a variety of reasons including a dropped collar, transmitter failure, unknown fate or causes of mortality, and ground squirrel movement out of the study area. Composite (Comp) data are provide for comparative purposes.

	Control			Bait station			Spot treatment			Broadcast		
	Censored	Mortality	Efficacy	Censored	Mortality	Efficacy	Censored	Mortality	Efficacy	Censored	Mortality	Efficacy
Trial 1	0	0/7	0%	2	5/5	100%	2	5/5	100%	3	0/4	0%
Trial 2	0	0/7	0%	1	6/6	100%	5	2/2	100%	4	3/3	100%[a]
Trial 3	0	0/4	0%	2	6/6	100%	2	6/6	100%	1	0/7	0%[a]
Trial 4	0	0/4	0%	5	3/3	100%	2	6/6	100%	4	4/4	100%[a]
Comp	0	0/22	0%	10	20/20	100%	11	19/19	100%	12	7/18	39%

[a] these broadcast treatments were applied using 0.01% diphacinone-treated oats.

Table 2. Mean, standard error, and range of the number of days from application of diphacinone-treated grain until death for California ground squirrels during summer and autumn in central California rangelands, 2018-2019. Bait was applied via bait stations, spot treatments, and broadcast applications.

	Bait station			Spot treatment			Broadcast application		
	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range
Summer	8.5	1.1	5-16	8.9	0.6	6-12			
Autumn	10.0	1.8	4-19	10.3	1.5	4-17	8.1	0.7	5-11
Combined	9.2	1.0	4-19	9.4	0.7	4-17			

Table 3. Number of radiotransmittered California ground squirrel carcasses that were located belowground, aboveground, and the proportion located belowground at rangeland locations in central California during summer and autumn, 2018–2019. We have also included information on ground squirrels that were potentially scavenged to represent the minimum proportion (Adjusted proportion) that may have died belowground. Composite (Comp) data are provide for descriptive purposes.

	Belowground	Aboveground	Proportion belowground	Potentially scavenged	Adjusted proportion
Summer	19	3	0.86	0	0.86
Autumn	23	1	0.96	5	0.79
Comp	42	4	0.91	5	0.82

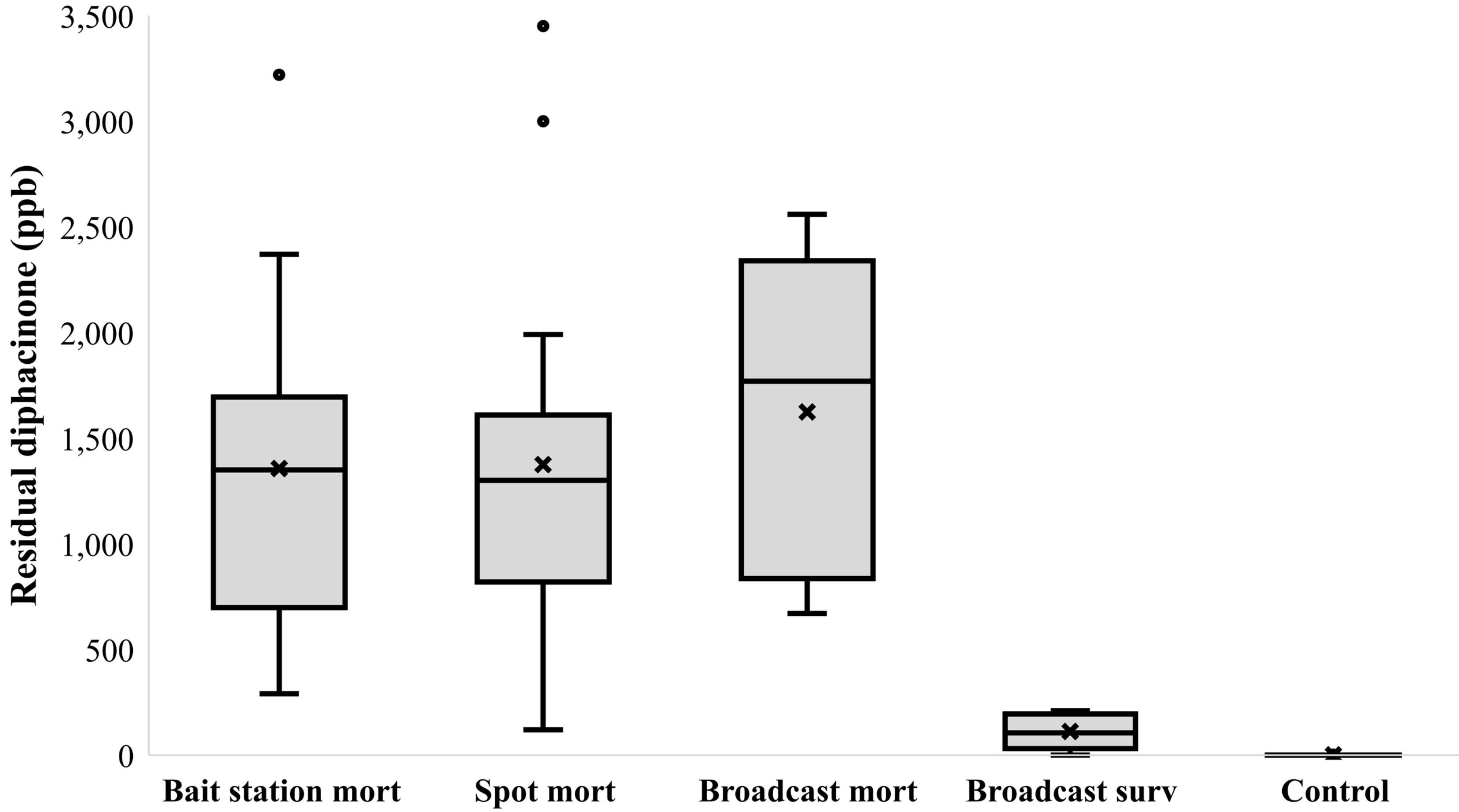

Figure 1. Box and whisker plot showing the residual concentrations of diphacinone in California ground squirrel livers following applications of grain bait via spot treatment, broadcast, and bait station application strategies in rangelands in central California. Most bait application strategies resulted in 100% mortality (mort), although some ground squirrels in the broadcast plots survived (surv). As such, residual concentrations from broadcast application mortalities and survivors were analyzed separately. Differences in mean values are denoted by different letters ($P \leq 0.05$).

It is interesting to note that although less bait was applied via broadcast applications, the residual concentrations were as high as that observed in ground squirrels from spot treatments and bait station applications. This may have been driven at least in part by the higher concentration of diphacinone used in 3 of 4 broadcast applications, as other studies have noted a similar response in rodents when using higher-concentrations of anticoagulants (e.g., Kaukeinen 1982, Ward 2003, Silberhorn et al. 2006). However, the relationship between diphacinone concentration in treated grain and residual concentration in ground squirrel carcasses is not entirely clear. For example, Baroch (1996) noted no difference in residual concentrations of diphacinone from ground squirrel carcasses exposed to 0.005% and 0.01% diphacinone-treated grain. Likewise, we did not observe a significant difference in residual concentrations of diphacinone from ground squirrels that survived 0.005% and 0.01% applications, although we did note a general trend toward lower residual concentrations when using the lower bait concentration (0.01% $\bar{x}$ = 130 ppb, 0.005% $\bar{x}$ = 85 ppb). That said, it makes intuitive sense that lower concentrations of diphacinone should result in lower residual concentrations in target animals. Our initial goal was to use 0.005% diphacinone-treated grain for broadcast applications, but given a lack of mortality following our first application, we defaulted back to the label rate of 0.01% diphacinone-treated grain. In retrospect, this may not have been necessary, as the primary reason for the low efficacy observed in Trials 1 and 3 was likely due to low usage of the treatment areas by ground squirrels in these plots. For example, in the broadcast plots for Trials 1 and 3, ground squirrels were located within the treatment areas only 54% of the time (R. Baldwin, unpublished data). Conversely, ground squirrels were located within broadcast treatment areas 88% of the time for Trials 2 and 4 where 100% mortality occurred. Obviously, if ground squirrels do not have access to the bait, then a rodenticide application will be ineffective. It is also possible that the difference in efficacy could have been due to a seasonal effect, but this seems unlikely given that bait station and spot treatment applications worked equally well between summer and autumn periods. This all is of note, as previous research has indicated that 0.005% diphacinone applications work as well as 0.01% concentrations in rangeland settings (Baroch 1996, Salmon et al. 2007). Given the potential of lower residual concentrations and high levels of efficacy previously reported with 0.005% diphacinone broadcast applications, a switch to a lower-concentration product for broadcast applications in rangelands may be worthwhile. Further investigation would be needed to determine the efficacy of broadcasting a lower concentration diphacinone bait in cropland settings where more preferred foods might lessen bait uptake, potentially reducing efficacy of lower concentration baits (Whisson and Salmon 2009).

Although we did not observe a difference across treatment types in residual diphacinone concentrations for ground squirrels dying from intoxication, we did notice a dramatically lower concentration in ground squirrels that survived. This suggests a substantially lower risk of exposure for non-target predators should they predate on a ground squirrel that was sublethally exposed. This risk would be further mitigated by the short half-life of diphacinone in ground squirrels (65 hours; Ward 2003), indicating that long-term risk from sublethally-exposed ground squirrels is substantially lower than that observed from scavenged ground squirrels or from predation on ground squirrels that had consumed a lethal dose but had not yet died.

Time from initial consumption to death can be a concern with anticoagulant rodenticides given the extended timeframe needed for them to work (Record and Marsh 1988, Buckle and Prescott 2018). We did not note any difference in average time to death across any of the application strategies we tested, suggesting little impact of application type on this potential secondary exposure variable. Our investigation looked at the time from bait application to death, thereby accounting for the timeframe that it took for ground squirrels to first find and consume the toxicant, as well as how long it took for mortality to occur following ingestion. Therefore, we expected longer timeframes than those experienced in more controlled investigations, but results were consistent across studies (9–10 days; Clark 1978, Whisson and Salmon 2002). That said, previous investigations have suggested or observed increased time from application to ground squirrel population reduction across varying rodenticide application strategies, with bait stations generally taking longer given potential neophobic responses to bait stations, as well as territoriality of dominant males limiting conspecific access to bait stations (Baroch 1996, Whisson and Salmon 2009). Less availability of bait may have been a limiting factor in some previous studies, as Whisson and Salmon (2009) used much wider spacing of bait stations (39–92 m). This wider spacing likely led to exclusion of bait stations by dominant individuals, and perhaps took longer for ground squirrels to find and encounter bait stations. The tradeoff between cost of additional bait stations compared to quicker population reduction may be worthy of additional investigation. Material cost will certainly be much higher with shorter spacing, but quicker time from application to death will reduce damage and will result in substantially less time required to perform daily carcass searches that are frequently required by most rodenticide labels.

Likely the best way to reduce anticoagulant exposure of predators and scavengers is to remove intoxicated individuals from the food chain. Most predators and scavengers of ground squirrels primarily hunt aboveground (e.g., raptors, coyotes, and bobcats). The vast majority of documented ground squirrel mortalities occurred within burrow systems (91%), effectively removing them from the food chain. We did note an additional five incidents where ground squirrels may have been scavenged or predated on. If we assume that all these ground squirrels did or would succumb to diphacinone intoxication, this still resulted in >82% of the ground squirrels functionally unavailable to most predators and scavengers. The true proportion may have been somewhere in between. Regardless, the ultimate goal is to minimize the availability of carcasses to scavengers. One way to further reduce this risk is to perform daily carcass searches to remove any dead ground squirrels from the food chain (Montaz et al. 2014, Buckle and Prescott 2018). Our daily searches resulted in the detection of only three non-transmittered ground squirrels that died aboveground. When combined with the four radiotransmittered ground squirrel carcasses located aboveground, we documented only 0.17 carcasses/ha. Because of additional field activities and constraints on our time, we were only able to search for carcasses during mornings. Given that ground squirrels are diurnal while many predators and scavengers are nocturnal, carcass searches conducted shortly before nightfall might be most beneficial at removing these individuals from the landscape; the likelihood of ground squirrels dying aboveground at night seems low, but the potential advantage of conducting carcass searches in late afternoon has not yet been tested.

CONCLUSIONS

Many factors must be considered when determining how to manage rodent pests. How rodenticides are applied is an important consideration, as application strategies can influence the cost and practicality of each application strategy. For example, broadcast applications are generally considered the easiest and most economical strategy for ground squirrel management in large open areas (Kaukeinen 1982, Kowalski et al. 2006). Although we did not discern any difference in residual concentrations of diphacinone in ground squirrels following any of the tested application strategies, we were unable to adequately test residual concentrations following a broadcast application of a lower concentration 0.005% diphacinone bait. Previous research suggests that such an application should be efficacious (Salmon et al. 2007), and may lower residual concentrations, although collectively, this has not been rigorously examined. If effective, such a strategy would likely make broadcast applications the preferred rodenticide application approach for ground squirrel management in rangelands. That said, at the time of this study, 0.01% diphacinone-treated grain products were registered for broadcast applications in California. Our study reflects the current application protocols for California ground squirrels in the state, and we observed no difference in residual concentrations across all three application strategies. Likewise, we did not identify any difference in time to death for any application strategy. As such, spot treatments, broadcast applications, and bait stations appear to have equivalent secondary-exposure risks based on the diphacinone concentrations included in this study. This risk is further mitigated by the fact that surviving ground squirrels had an order of magnitude lower residual concentration of diphacinone 2–3 weeks post-application, and the vast majority of ground squirrels died belowground, further reducing risk of secondary exposure. What's more, ground squirrels rapidly decayed belowground. If not recovered within 48 hours, they were too deteriorated to use for residual diphacinone analysis and were covered in maggots within 72 hours. This essentially eliminated fossorial scavenging unless it occurred within a few days post-mortality. Collectively, the low proportion of ground squirrels exposed aboveground, combined with daily carcass searches, should substantially reduce secondary exposure risk. That said, rodenticide application should be only one part of an integrated pest management program for rodent management (Baldwin et al. 2014, Hindmarch et al. 2018, Witmer 2018). Relying on anticoagulant rodenticides only when needed is the best strategy for minimizing secondary exposure risk.

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Chapter 2: Utility of visual counts for monitoring changes in ground squirrel numbers

INTRODUCTION

California ground squirrels (*Otospermophilus* spp.) cause extensive damage in many agricultural commodities including rangelands (Marsh 1998, Fleming et al. 2013, Baldwin et al. 2014). Many tools are used to manage ground squirrel populations including habitat modification, rodenticides, burrow fumigants, trapping, and shooting (Salmon and Schmidt 1984, Marsh 1994, Baldwin et al. 2014). Development of new tools requires methods to assess the efficacy of those tools. Changes in animal numbers is one of the primary methods for detecting the effectiveness of management tools. This can be done in a variety of ways including official assessments of population size, as well as the use of indices that reflect population size (Stroud 1981). Indices are often the preferred tool for efficacy assessments given that they can be quicker and easier to employ, and they have less onerous assumptions to be met. That said, indices must be sensitive to changes in population size to be effective (see Engeman 2005 for detailed discussion on indices). A common indexing approach for ground squirrel species is visual counts. Fagerstone (1984) provided an early assessment on the utility of visual counts for tracking population size of Richardson's ground squirrels (*Urocitellus richardsonii*), and found this approach to be effective. Visual counts have subsequently been used extensively to assess efficacy of a variety of ground squirrel species (e.g., Whisson et al. 1999, Salmon et al. 2007, Nelson et al. 2012, Baldwin et al. 2017), although it has not been officially verified for other species. Such an assessment would lend credence to this approach for other ground squirrel species.

One of the potential problems with visual counts is that some of the target population may move in or out of the study area between the pre and posttreatment visual counts. This is generally not a problem with management tools that reduce populations within a couple of days (e.g., burrow fumigants and acute toxicants such as zinc phosphide). However, first-generation anticoagulant rodenticides such as diphacinone and chlorophacinone require a more extended timeframe to reduce populations (Marsh 1994). This timeframe can vary depending on the application strategy used, as bait stations sometimes take longer to reduce populations than do broadcast applications or spot treatments given some individuals' neophobic response to bait stations (Whisson and Salmon 2009). Regardless, it generally takes 2 weeks and sometimes longer to reduce a ground squirrel population with anticoagulant rodenticides; during that timeframe, adjacent ground squirrels may reinvade treatment areas (Alsager 1972, Fagerstone et al. 1981), thereby confounding results from visual counts. Increasing the size of buffer zones around censusing plots where visual counts occur can minimize the risk of adjacent ground squirrels repopulating treatment areas, but the necessary width of this buffer zone is unknown (Stroud 1982). Creation of buffer zones sufficiently sized to minimize ground squirrel reinvasion of censusing plots would increase the utility of visual counts as a monitoring approach.

Radiotransmittered rodents are also used to track efficacy of various management approaches. This approach monitors survival of radiotransmittered individuals and determines efficacy based on the ratio of mortalities vs. uncensored individuals at the end of the study period (Fagerstone et al. 1981). Using radiotransmittered individuals is often considered a more sensitive approach for assessing efficacy given direct knowledge of mortality vs. survival ratios of a subset of the population (Fagerstone et al. 1981). However, this approach is more costly and invasive given the need to capture and transmitter individuals, so it is not used as widely as other less-invasive approaches. That said, radiotransmittered individuals can also provide movement data, which can be useful in establishing protocols for management practices, as well as better defining the size and spacing of treatment areas for efficacy assessments. Therefore, comparing efficacy values estimated from visual counts to those derived from radiotransmittered individuals should provide a good test of the applicability of visual counts for monitoring changes in population size, and should provide information on plot size needed to accurately reflect efficacy of management tools of interest. Specifically, our goals for this project were to: 1.) compare efficacy values derived from visual counts and radiotransmittered individuals from a diphacinone bait application to determine the validity of visual counts for California ground squirrels, and 2.) determine the appropriate plot size for efficacy studies of California ground squirrels. This information would greatly assist researchers, regulatory agencies, and land managers on how to monitor this common agricultural pest.

STUDY AREA

We conducted this study in seasonally grazed rangelands in west-central California in Stanislaus and San Joaquin Counties. Grazing occurred from October to March, which coincided with the timeframe when most precipitation fell in this region ($\bar{x}$ = 25.4–30.5 cm annually). Annual temperatures for the area range from 4–35ºC. Soils were similar throughout and consisted of Zacharias gravely clay loam and Carbona clay loam. A small portion of the study area was comprised of Stomar clay loam that exhibited up to an 8% slope. Plant composition was primarily non-native annual grasses and forbs including *Hordeum murinum, Bromus madritensis, Bromus diandrus, Bromus hordeaceus, Avena fatua, Medicago polymorpha*, and *Erodium* spp. Forage production on our study sites ranged from 479 kg/ha to 2,697 kg/ha, with a mean of 1,636 kg/ha (Becchetti et al. 2016; T. A. Becchetti, University of California, unpublished data).

MATERIALS AND METHODS

Plot establishment

We established four 64 × 64 m censusing plots (0.4 ha) in summer 2018 in areas that had abundant ground squirrel numbers. Similar to past studies (e.g., Baldwin et al. 2017), we surrounded these interior censusing plots by a 61-m buffer on all sides (3.4 ha). These combined census plots and buffer zones served as our treatment plots for rodenticide bait application. This process was repeated in autumn 2018, and in summer and autumn 2019. Plots were generally located a minimum of 192 m from one another ($\bar{x}$ minimum distance = 418 m) to minimize the likelihood that any ground squirrels would move from one plot to another, although in summer 2018, the control and broadcast application plots were separated by only 87 m. These distances appeared to be sufficient to maintain independence as only once did we ever record a location of a radiotransmittered ground squirrel in a treatment plot other than where it was captured. Each season, we randomly assigned the four plots into one of three bait application strategies or as a control plot.

Capture, collaring, and radiotracking

To track ground squirrel movements and mortality, we trapped ground squirrels to allow us to collar individuals with a VHF transmitter attached around the neck via a cable tie (Model M1535, weight = 14 g; Advanced Telemetry Systems, Isanti, MN, USA). For trapping, we used 20–25 Tomahawk cage traps (combination of 13 × 13 × 46 cm and 15 × 15 × 61 cm traps; Tomahawk Live Trap, Hazelhurst, WI, USA) distributed throughout each censusing plot. We focused collaring efforts on the censusing plots to reduce the likelihood that a ground squirrel would move off the treatment area ($\bar{x}$ diameter of home range = 20–34 m; Boellstorff and Owings 1995). We initially tied traps open and prebaited traps with oat groats for 1–2 days, and then activated the traps for capture. We operated traps from early morning until 11:00 to reduce potential problems with heat exposure. Traps were checked every hour. Upon capture, trapped ground squirrels were moved to a shaded location for processing, and we dusted all captured ground squirrels with a 0.25% permethrin dust (Hi-Yield Garden, Pet & Livestock Dust, Voluntary Purchasing Groups, Inc., Bonham, TX, USA) to remove ectoparasites. We sexed and weighed captured ground squirrels to ensure that the transmitter did not constitute more than 5% of their body weight. We used a cloth handling cone as described by Koprowski (2002) to allow us to collar captured ground squirrels, and we retrofitted all transmitters with a mortality signal that would trigger after 12 hours of inactivity. Captured ground squirrels were then taken back to the site of capture and released. We radiotransmittered 7 ground squirrels in all 4 plots during both summer and autumn 2018. In summer and autumn 2019, we collared 8 individuals in each treatment plot and 4 in the control plots to increase treatment sample sizes for a separate study (see previous chapter). This kept the total number of collared ground squirrels consistent across all sampling periods (n = 28). We did not initiate bait application until several days after the end of collaring activities ($\bar{x}$ = 8.6 days, SE = 0.2) to allow the ground squirrels time to adjust to wearing the collar.

Upon release, we generally obtained ground squirrel locations daily, although occasionally other activities kept us from tracking locations before bait application occurred. That said, we always tracked ground squirrels daily following bait application. To identify locations, we walked to where the ground squirrel was located and documented if the ground squirrel was visually observed. If a mortality was observed aboveground, we noted this and removed the ground squirrel and collar from the site. Occasionally, we could not find a ground squirrel during normal telemetry scans. If a ground squirrel was not located, we used a vehicle to attempt to locate ground squirrels within a 500-m perimeter around the treatment plot. If we still could not find it, we recorded it as missing for that day. We recorded all locations with a hand-held Global Positioning System (GPS) unit, and we plotted all locations in ArcMap 10.7 (Environmental Systems Research Institute, Redlands, CA, USA) to allow for a comparison of each ground squirrel's location data to their respective treatment plot. We used a one-factor analysis of variance to determine if the proportion of ground squirrel locations found within a treatment plot varied across the different bait application strategies. If the model was significant (α = 0.05), we used Fisher's least significant difference *post hoc* test to determine which application strategies differed (Zar 1999). We also used a Student's t-test (α = 0.05; Zar 1999) to test for potential differences in the proportion of ground squirrel locations observed within treatment plots for ground squirrels that survived versus those that succumbed to diphacinone exposure, as access to bait could influence the efficacy of the application strategy.

Visual counts

We conducted visual counts of ground squirrels upon completion of collaring activities. Our protocol followed the general approach originally outlined by Fagerstone (1984) and subsequently modified for use in numerous ground squirrel studies (e.g., Salmon et al. 2000, 2007, Baldwin et al. 2017). This approach was comprised of 5 counts separated by 5-minute intervals, with all counts occurring from a fixed location outside the core area. Counts were conducted once in the morning (7:10–11:07) and once in the evening (16:00–18:48) to coincide with periods of high ground squirrel activity (Fitch 1948). Counts occurred across 3 consecutive days for a total of 30 counts per treatment or control plot. We used the maximum number of ground squirrels counted in each plot in subsequent analyses. These counts occurred before bait application and at the end of the bait application period (between 14 and 19 days post-application depending on the year and season) to allow for comparison of numbers before and after treatment. We determined efficacy of the 3 different bait application strategies for each season using:

$$\text{Efficacy (\%)} = [(\text{pretreatment} - \text{posttreatment}) / \text{pretreatment}] \times 100$$

where pretreatment and posttreatment equal the maximum number of ground squirrels observed before and after treatment. Natural changes in population size can influence visual counts as well. Therefore, we applied a correction factor for all bait application approaches in a given season if we observed a >30% change in maximum ground squirrel counts from the pretreatment to the posttreatment survey period in the control plot. The correction factor for this study was calculated following O'Connell and Clark (1992):

$$\text{Posttreat expected GS bait} = (\text{pretreat GS bait} \times \text{posttreat GS control}) / \text{pretreat GS control}$$

$$\text{Percent adjusted efficacy} = [1 - (\text{posttreat GS bait} / \text{posttreat expected GS bait})] \times 100$$

where posttreat = posttreatment survey, pretreat = pretreatment survey, GS = maximum number of ground squirrels counted, and bait = bait application strategy. Following U.S. EPA standards, we considered population reductions of ≥ 70% efficacious (Schneider 1982).

Bait application

We initiated bait application the day following the completion of pretreatment ground squirrel counts for each trial period. For spot treatments, we identified all active burrow entrances within the treatment area, and we applied 37 g of Rodent Bait Diphacinone Treated Grain (0.005%; California Department of Food and Agriculture, Sacramento, CA, USA) in a 3.7–4.6 m^2 area around the entrance. We identified active burrow entrances by the presence of new footprints, fresh fecal pellets, scrapings, or clear openings (i.e., were devoid of leaf litter, spider webs, and were not overgrown with vegetation). We noted the initial date of bait application as day 0. Following the label specification, we again applied bait in the same manner on day 4 to ensure adequate exposure to diphacinone (Whisson and Salmon 2002).

For bait stations, we used inverted T-shaped bait stations that were constructed of 10-cm polyvinyl chloride pipe. These stations were 1.2 m in length and 0.9 m in height. We cut end caps in half and glued them on to both horizontal ends of the bait station to keep ground squirrels from kicking bait out onto the ground. We placed an endcap on the vertical arm of the station to eliminate access to bait from the top. We attached all bait stations to metal T-posts that were staked into the ground. We spaced all bait stations in an 8 × 8 grid structure with all stations 23 m apart (Baroch 1996); the bait stations covered the entire treatment plot. We applied 0.9 kg of Rodent Bait Diphacinone Treated Grain (0.005%) to each bait station on day 0. Bait stations were checked at least every 3 days to ensure that they maintained a constant bait supply. If we determined that additional bait was needed, we documented the amount that was added. We continued to add bait to the bait stations until bait was no longer removed by ground squirrels.

We applied bait via a broadcast approach through the use of a seed spreader (Solo 421-S, Newport News, VA, USA). The seed spreader was calibrated to discharge bait at a rate of 11.4 kg/ha. To allow for efficient application of bait, we flagged out transects that intersected active burrow systems. We applied bait along these transects on day 0 and day 4 to ensure access to bait over the timeframe required to maximize efficacy of diphacinone (Whisson and Salmon 2002). We initially used the Rodent Bait Diphacinone Treated Grain (0.005%) to allow us to most directly compare results across the 3 different application strategies. However, we observed no mortalities following the initial trial period for broadcast applications in summer 2018. At the time of this study, the label-specified concentration of diphacinone for broadcast applications was 0.01%. Therefore, we defaulted back to this label-specified rate (Rodent Bait Diphacinone Treated Grain [0.01%]; California Department of Food and Agriculture, Sacramento, CA, USA) for the remaining 3 trial periods.

Fate of ground squirrels

We anticipated a variety of outcomes for radiotransmittered ground squirrels including lost signals, dropped collars, mortality from diphacinone exposure, unknown causes of mortality, and survivors. As such, we defined the specific fate of each ground squirrel, but for the purposes of this study, we placed all ground squirrels into 3 categories: 1.) mortality from diphacinone exposure, 2.) survival, and 3.) censored individuals (all ground squirrels that did not fit into the first 2 categories). If we observed a dead ground squirrel aboveground, we dusted it with a 0.25% permethrin dust, recorded the location with a hand-held GPS unit, and collected the animal. For belowground mortalities, we dug the ground squirrel up to document mortality, dusted it with a 0.25% permethrin dust, recorded the location, and collected the ground squirrel. At the completion of all activities associated with this project, we used Tomahawk live traps to capture remaining radiotransmittered ground squirrels following the same protocols listed previously (except we did not prebait for recaptures). Recaptured radiotransmittered ground squirrels were euthanized via a carbon dioxide euthanasia chamber to allow us to collect livers for rodenticide residue testing. All collected livers were tested for residual diphacinone concentrations to verify mortality from diphacinone exposure (see Baldwin et al. 2020 for additional details on this process and subsequent results). We determined efficacy for each bait application strategy by dividing the number of radiotransmittered ground squirrels that died from diphacinone exposure by the number of uncensored ground squirrels for that particular treatment plot. We used a paired *t*-test to compare efficacy between visual counts and telemetry results to determine if counts were representative of what we observed via telemetry estimates (Zar 1999). We also used logistic regression to model the relationship between efficacy (binary response included survival or mortality for each individual) and the proportion of locations found within bait application areas (Hosmer and Lemeshow 2000). The model was validated using the area under curve (AUC) approach, with AUC scores <0.7 = uninformative, 0.7–0.9 = good, and >0.9 = very good (Swets 1988). All aspects of this project were approved by the University of California, Davis' Institutional Animal Care and Use Committee (protocol no. 20025).

RESULTS

We censored a large number of radiotransmittered ground squirrels for a variety of reasons including dropped collar = 13, lost signal = 9, unknown fate = 6, and unknown cause of mortality = 3. This left 81 for inclusion in efficacy assessments (Table 1). We observed 100% efficacy from spot treatments across all trial periods (Table 1). For bait stations, we observed 75–100% efficacy collectively. The one survivor was a ground squirrel that was located within the treatment area only once out of 33 locations. We did not observe a single mortality during summer trials in broadcast application plots. In contrast, we observed 75–100% efficacy in broadcast plots during autumn, with the sole survivor located only 3 times within the treatment area out of 25 total locations during the trial period. We observed no mortality events in control plots during any trial period (Table 1). We did note a difference in the proportion of time spent within treatment areas across different diphacinone application strategies ($F_{3,77} = 4.3$, $P = 0.007$, $r^2 = 0.14$), with the number of locations within broadcast plots ($\bar{x} = 68\%$,) different than that observed in spot treatment ($\bar{x} = 93\%$,) and bait station plots ($\bar{x} = 89\%$, $P \leq 0.007$). We did not observe a difference across any other application strategies ($P \geq 0.052$). Furthermore, the low efficacy we observed for broadcast plots during summer may have been driven by low usage of treatment areas, as the amount of time spent in broadcast plots was lower for ground squirrel survivors ($\bar{x} = 54\%$, SE = 7) than for mortalities ($\bar{x} = 87\%$, SE = 10; $t_{16} = -2.4$, $P = 0.029$)

Based on visual counts, we observed a substantial reduction in numbers of ground squirrels within the control plot during the first trial period, so we adjusted efficacy for all treatment types within that trial period accordingly (Table 2). We did not observe substantive changes in ground squirrel numbers in control plots during any other trial period, so we did not adjust efficacy values for those periods. All efficacy values exceeded the 70% threshold for bait station and spot treatment plots during trial periods 2–4, except for spot treatments during trial period 4, where efficacy was close to the desired threshold (67%; Table 2). We observed adjusted efficacy values under the 70% threshold during trial period 1 for both bait station and spot treatment plots. However, efficacy values were well above this threshold if using the unadjusted rates (Table 2). We observed low efficacy for broadcast plots during trial periods 1 and 3, and high efficacy during trial periods 2 and 4 (Table 2). We did not observe a difference in efficacy values between visual counts ($\bar{x} = 68\%$, SE = 9) and radiotransmittered estimates ($\bar{x} = 79\%$, SE = 11; $t_{11} = -2.0$, $P = 0.073$), although in general, efficacy from radiotransmittered individuals was higher.

We observed a strong relationship between the number of ground squirrel locations within bait application areas and efficacy ($\chi^2 = 12.1$, $P = <0.001$; $\beta = 0.071$, SE = 0.020). The accuracy of this model was very good (AUC = 0.92), and indicated that efficacy was higher when a greater proportion of locations were found within treatment areas. Expected efficacy met the 70% U.S. EPA threshold when the percentage of ground squirrel locations within the treatment area surpassed 73% (Fig. 1). Trial periods 1 ($\bar{x} = 63\%$) and 3 ($\bar{x} = 53\%$) for broadcast plots were substantially below this proportion suggesting that this played a role in their low observed efficacy.

Table 1. The proportion of California ground squirrels that died (Mortality) following application of diphacinone-treated oats (0.005% unless otherwise noted) following 3 application strategies, as well as a concomitant control plot, in rangelands in central California during summer and autumn 2018–2019. Efficacy was defined as the ratio between the number of mortalities divided by the number of uncensored individuals. We censored individuals for a variety of reasons including a dropped collar, transmitter failure, and unknown fate or causes of mortality. Combined (Comb) values are provided across treatment types for comparative purposes.

	Bait station			Spot treatment			Broadcast			Control		
	Censored	Mortality	Eff (%)	Censored	Mortality	Eff (%)	Censored	Mortality	Eff (%)	Censored	Mortality	Eff (%)
Trial 1	2	5/5	100	2	5/5	100	3	0/4	0	0	0/7	0
Trial 2	1	6/6	100	5	2/2	100	3	3/4	75[a]	0	0/7	0
Trial 3	2	6/6	100	2	6/6	100	1	0/7	0[a]	0	0/4	0
Trial 4	4	3/4	75	2	6/6	100	4	4/4	100[a]	0	0/4	0
Comb	9	20/21	95	11	19/19	100	11	7/19	37	0	0/22	0

[a] these broadcast treatments were applied using 0.01% diphacinone-treated oats.

Table 2. The number of California ground squirrels observed before (Pre) and after (Post) application of diphacinone-treated grain (0.005% unless otherwise noted), as well as the associated unadjusted efficacy (Eff) for 4 trial periods across 3 different bait application strategies and control plots in rangelands in central California during summer and autumn 2018–2019. Adjusted efficacy (A eff) is provided for trial 1 given a substantial reduction in ground squirrels in the control plot during that period.

	Trial 1				Trial 2			Trial 3			Trial 4		
	Pre	Post	Eff (%)	A eff (%)	Pre	Post	Eff (%)	Pre	Post	Eff (%)	Pre	Post	Eff (%)
BS	18	3	83	60	15	0	100	11	2	82	12	0	100
Spot	15	2	87	68	11	2	82	11	1	91	9	3	67
Broad	17	6	65	14	7	2	71[a]	11	11	0[a]	11	2	82[a]
Control	17	7	59		17	15	12	10	11	-10	7	6	14

[a] these broadcast treatments were applied using 0.01% diphacinone-treated oats.

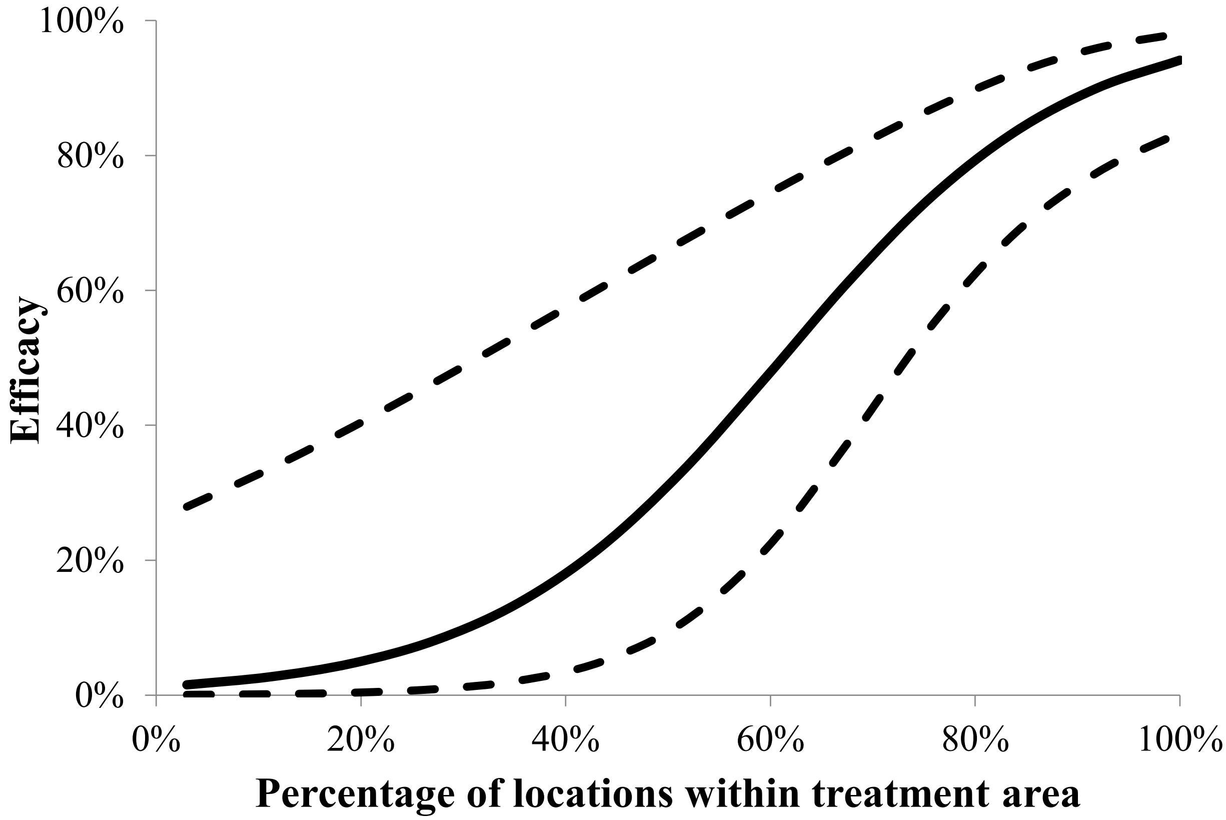

Figure 1. Relationship between the percentage of California ground squirrel locations found within treatment areas and efficacy (derived from radiotransmittered individuals) associated with consumption of diphacinone-treated grain. 95% confidence intervals are represented by dashed lines.

DISCUSSION

We did not observe a difference in efficacy values derived from visual counts and radiotransmittered individuals, corroborating the findings of Fagerstone (1984) with Richardson's ground squirrels. Visual counts are widely used to assess efficacy of various management strategies for ground squirrels (e.g., Whisson et al. 1999, Salmon et al. 2007, Nelson et al. 2012, Baldwin et al. 2017), as the approach is far less costly, time-consuming, and invasive than using radiotransmittered individuals. This is particularly important for the registration of new pesticides (e.g., rodenticides, burrow fumigants, repellents, and chemosterilants), as multiple indexing tools are usually required by the U.S. EPA for their registration (Schneider 1982). Even if radiotransmittered individuals are used, an additional strategy such as ground squirrel counts will be needed to register these products. Our findings indicate that visual counts can be effectively used to monitor ground squirrel populations moving forward.

Although ground squirrel counts effectively tracked changes in ground squirrel numbers, the use of radiotransmittered individuals appeared to be somewhat more effective. For example, we regularly observed higher efficacy values with radiotelemetry data than we observed with ground squirrel counts ($\bar{x}$ = 79% vs. $\bar{x}$ = 68%, respectively). This difference may be driven by reinvasion of adjacent ground squirrel populations into treated areas, as ground squirrels will often quickly reinvade depopulated sites (Stroud 1982, Salmon et al. 1987). We attempted to minimize this effect by conducting counts soon after bait application. We could not cut this time down much more given the extended length of time required for first-generation anticoagulants such as diphacinone to lead to mortality (often 4–13 days or more; Clark 1978, Hindmarch and Elliott 2018). Such reinvasion would be most impactful on broadcast and spot treatments given that bait stations continued to supply bait throughout the duration of the project, and in fact, we observed greater efficacy associated with bait stations (bait station $\bar{x}$ = 86%, spot treatment $\bar{x}$ = 77%, broadcast $\bar{x}$ = 42%). In short, ground squirrel counts do generally reflect true efficacy of management tools, but may provide somewhat conservative estimates when compared to results from radiotelemetry.

Although limited reinvasion by adjacent ground squirrel populations may marginally lower efficacy estimates, the biggest concern with ground squirrel counts likely stems from the potential for ground squirrels to move out of application plots during the trial period. Such movements were most notable in broadcast plots as the number of locations within treatment areas was much lower for broadcast plots (68%) than for spot treatments (93%) or bait stations (89%). This reduced use of diphacinone-treated areas seemed to influence efficacy of the rodenticide bait, as ground squirrels that succumbed to diphacinone spent substantially more time in treatment areas than survivors (87% vs. 54%, respectively). Interestingly, many ground squirrels also vacated the control plot during the summer 2018 trial period as well, with an average of only 50% of locations found within the treatment area during this timeframe. Given this substantial reduction in the control plot, we adjusted our estimates of efficacy in all 3 treatment plots accordingly. However, these adjusted values may be overly conservative for the bait station and spot treatment plots given that radiotelemetry data indicated that ground squirrels spent the majority of their time within the treatment areas (83% and 94%, respectively), reinforcing the idea that combining radiotelemetry data with another indexing tool will likely provide an improved assessment of efficacy. When such location data are unavailable, researchers and practitioners will likely need to rely on the use of adjusted efficacy values to counteract the potential for natural reductions in animal numbers at treatment sites (e.g., mortality associated with a disease outbreak or estivation in the local population).

One method to minimize the impact that ground squirrel movement patterns have on efficacy assessments would be to increase the size of the buffer zone surrounding plots. However, plot size is often constrained by a number of factors. For example, treatment plots must be separated by some minimum distance to keep plots independent. If multiple management approaches are to be tested, then fields of sufficient size with substantial ground squirrel numbers will be needed to incorporate all treatment replicates. This becomes increasingly challenging as the size of treatment plots increase. Not only does it become more challenging to find appropriate field sites as plot sizes increase, but it also becomes more costly and logistically challenging to treat large areas. It also bears noting that for pesticide testing, U.S. EPA generally limits the area where an unregistered pesticide can be tested to 4.05 ha (U.S. EPA 2020). As such, it is important to keep treatment areas as small as possible. For our study, we determined that we would meet the U.S. EPA threshold of 70% efficacy if 73% of ground squirrel locations occurred within the bait application area. We surpassed this 73% level for all spot treatment and bait station plots, but were substantially below it for broadcast plots during summer 2018 and 2019. Increasing the size of buffers from 61 m to 66 m would have allowed us to surpass this 73% threshold for the broadcast plot in summer 2018, and would have only increased the treatment area from 3.4 ha to 3.8 ha. However, in summer 2019, we would have had to increase the buffer zone to 96 m to surpass the 73% threshold, which would have come close to doubling the treatment area (3.4 ha to 6.5 ha). The treatment area for the broadcast plot in summer 2019 was unique in that it was located close to a farm with large alfalfa haystacks (minimum distance of 75 m from the closest edge of the buffer zone). Unexpectedly, the ground squirrels spent considerable time in and around these haystacks, substantially affecting the presence of ground squirrels within the buffer zone. If we exclude this outlier plot, then an addition of 5 m to the edge of each buffer zone should increase the utility of our study design while minimizing additional costs and logistical concerns. At present, we recommend a minimum of a 61-m buffer zone for similar ground squirrel efficacy studies, and increasing the treatment area to 66 m may yield more robust results.

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