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ABSTRACT

The worldwide presence of vertebrate pests such as rodents has created a need for non-lethal control methods that can be applied to integrated pest management plans. Chemical repellents are often a useful wildlife management tool as they can be directly applied to a commodity or structure to prevent infringement and damage. We assessed the efficacy of an anthraquinone (AQ)-based repellent in a structural barrier model against Richardson's ground squirrels (*Urocitellus richardsonii* (Sabine)) (RGS) and house mice (*Mus musculus* L.). The AQ-based repellent was applied to pieces of burlap which were secured over each end of a small section of PVC pipe. Unadulterated enrichment food was then offered within the enclosed PVC pipe to motivate interactions with repellent-treated and untreated burlap barriers. Defeat of the barrier was defined as a physical breach by means of chewing the burlap or burlap/repellent barrier such that the test animal was able to gain entry to the hide and the enrichment food. RGS defeated 55% (± 7.9) of untreated barriers, 25% (± 6.8) of barriers treated with 50% dilution AQ-based repellent, and 27.5% (± 5.6) of barriers treated with 0% dilution AQ-based repellent. House mice defeated 100% (± 0.0) of untreated barriers, 20.5% (± 6.4) of barriers treated with 50% dilution AQ-based repellent, and 45.5% (± 7.8) of barriers treated with 0% dilution AQ-based repellent. Relative to untreated barriers, AQ treatments reduced defeat of the barrier by 50–55% for RGS and 55–80% for house mice. RGS showed a marked decrease in consumption of enrichment food after exposure to AQ. The 0% dilution of AQ-treated structural barrier had more individuals of both RGS and house mice chew through the structural barrier than the 50% dilution despite the increased concentration of AQ. We hypothesized that the additional water in the 50% dilution may have allowed for greater absorption of the repellent throughout the burlap fibers, thus enabling greater interaction with the AQ-treated barriers. Our results indicate that AQ-based repellents show promise as structural barriers for RGS and house mice.

1. Introduction

Public concerns regarding lethal control of wildlife have led to an increased interest in non-lethal control methods that target individuals or local groups of animals (Gibson, 1988; Fall and Jackson, 2002; Baker et al., 2007). Non-lethal control methods include those that alter individual behavior (e.g. learned food aversions, repellents, and diversionary feeding; (Fall and Jackson, 2002). Chemical repellents are often a useful wildlife management tool as they can be directly applied to a commodity or structure to prevent infringement and damage. The use of non-lethal wildlife repellents and context-specific motivations has the potential to protect agricultural crops, commodities and valued structures from rodent damage.

Rodents are considered major agricultural pests worldwide, spreading disease and reducing human food availability (Stenseth et al., 2003). Ground squirrel species make up approximately 4% of rodent species worldwide. The extensive range of *Urocitellus spp*. in the U.S. and Canada coinciding with crop and pasture lands has led to Richardson's ground squirrels (*Urocitellus richardsonii* (Sabine)) (RGS)[1] being a prominent rodent pest in North America (Alsager and Yaremko, 1972; Johnson-Nistler et al., 2005; Proulx and Mackenzie, 2009). Marsh (Marsh, 1998) documented $12-16 million dollars in damage due to crop loss and $8-12 million dollars in physical damage from ground squirrels to materials such as structures, levees, and earthen dams.

House mice (*Mus musculus* L.) are the most widespread invasive mammal in the world (Witmer and Jojola, 2006). Commonly found in close association with humans, house mice have established populations in North and South America, sub-Saharan Africa, Australia, and

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[1] Richardson's ground squirrel.

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many oceanic islands (Macholan, 1999). In addition to causing direct damage to crops (Nolte and Barnett, 2000; Tobin and Fall, 2004), house mice are implicated in the contamination of stored grains as well as pig and poultry production facilities (Henzler and Opitz, 1992; Adhikari et al., 2002; Meerburg and Kijlstra, 2007). Contamination of commercial animal facilities and the storage of bulk grains are particularly problematic due to the large availability of food, water, and shelter. Limitations to the use of traps and rodenticides in close proximity to other animals (i.e. livestock, zoo animals) make effective rodent population control in concentrated animal feeding operations and other animal facilities challenging (Corrigan, 2001).

Non-lethal methods for rodent control can include physical or structural barriers and non-lethal chemical repellents or combinations of these two types of control methods. Structural barriers can take many forms from wrapping tree trunks with barriers such as paper, cloth, or yucca leaves (Allan, 1942) to the use of electric fences to exclude rodents from larger areas (Shumake et al., 1979; McKillop and Sibly, 1988; Smith and Meyer, 2015). In some instances, chemical repellents are applied directly on a commodity or structure (e.g. tree trunk or building) to prevent rodent or woodpecker damage. Barriers for rodent control have grown in importance as food commodities have come to be packaged and stored for longer periods of time and in larger warehouses and feed bins. Food or feed storage can be penetrated by rodents causing contamination issues, spillage, and disease spread (Smith and Meyer, 2015).

Methods to deter rodents have evolved through time, from employing traps and rodenticides to constructing structural barriers and administering chemical repellents. Beginning in the late 1940's through the 1960's, the U.S. Army supported barrier penetration bioassays to evaluate chemical repellents for rodents and the protection of food packaging, electric cable coatings, and other materials (Bowles et al., 1974). The barrier penetration assay consisted of a paperboard, corrugated cardboard or burlap barrier impregnated with repellents that had been pre-screened for rodent repellent activity. The procedure for the barrier penetration assays included controlling the motivation of the test subjects and training the test subjects to better ensure each test subject would perform consistently (Stolurow, 1948). Previous testing achieved motivation control by limiting the availability of maintenance food through food rationing and fasting or by maintaining test subjects at 70% of their normal body weight to ensure proper motivation (Bellack and DeWitt, 1950; Bendig and Stolurow, 1952; Welch, 1954; Glahn and LaVoie, 1983). In addition, test subjects were subjected to intensive training programs to eliminate animals unwilling to perform the required task (Welch, 1954; Glahn and LaVoie, 1983). These studies evaluated the success of rodent repellents based upon the time required by test subjects to penetrate treated barriers (Bendig and Stolurow, 1952).

There are few registered repellents for non-lethal control of rodents and those that are available have limited effectiveness (Gurney et al., 1996; Agnello et al., 2014). For example, Ropel® (a.i. 0.065% denatonium saccharide; 0.035% thymol) failed to protect surface drip irrigation tubing from rodent damage on peanut farms (Sorensen et al., 2006). Denatonium benzoate and capsaicin also failed to protect cables from pocket gophers (*Thomomys talpoides*) unless encased in electrical shrink tubing (Shumake et al., 1999). Repellents incorporated into fibrous and elastomer barriers and then installed on wood, plant, or other surfaces have had limited success against burrowing damage caused by rodents (e.g. moles, voles, gophers) (Hoffmann et al., 2003; Agnello et al., 2014). Although not registered for use with rodents, an anthraquinone (AQ)[2]-based repellent has shown some promise as a repellent for voles (Hansen et al., 2015; Werner et al., 2016), and moderate success with ground squirrels and mice in laboratory testing (Werner et al., 2016). These authors have indicated that application strategies for rodent management of stored products and farm structures be evaluated (Hansen et al., 2016; Werner et al., 2016). Therefore, we conducted a barrier penetration assay modified from previous literature to comparatively investigate the behavioral response of RGS and house mice to burlap treated with an AQ-based repellent barrier (Arkion® Life Sciences, New Castle, DE, USA) under controlled conditions. We used unadulterated enrichment food as motivation for test subjects to penetrate the barrier. As a means to keep the number of test animals low we did not evaluate test animals for chewing behavior prior to study initiation or condition test animals with decreased maintenance diet or to penetrate burlap. This testing paradigm represents a wild animal's encounter with a barrier and potential prevention measure (e.g. chemical repellent) and demonstrates natural behavioral responses to treated and untreated barriers.

2. Experimental methods

2.1. Capture and care

We captured 30 RGS within alfalfa fields in Montana during June 2016 using 76 × 18 × 18 cm live traps (Tomahawk Live Traps, Hazelhurst, WI, USA). Adult RGS (i.e. body weight ≥250 g) were weighed, dusted for fleas (Drione; Bayer, Leverkusen, Germany), and transported in 41 × 19 × 20 cm cages that we equipped with 20 cm sections of PVC pipe to serve as hides. Upon arrival at the headquarters of U.S. Department of Agriculture's National Wildlife Research Center (NWRC), RGS were individually housed indoors in 41 × 19 × 20 cm cages with a 20 cm hide, maintenance diet, and water *ad libitum*. The maintenance diet for RGS consisted of rodent blocks (LabDiet® Land O'Lakes, St. Louis, MO, USA), apple slices, and alfalfa hay. We set the light conditions to 12 h of light and 12 h of dark. We quarantined and monitored the health of the RGS for two weeks before the study was initiated. This study was approved by the NWRC Institutional Animal Care and Use Committee (QA-2243A1).

We captured 32 house mice at dairies and feedlots in Northern Colorado during August 2016 using 7.5 × 9 × 23 cm live traps (Sherman Live Traps, Tallahassee, FL). Weaned house mice (i.e. body weight ≥7 g) were dusted for fleas (Drione) and transported in cage traps. Upon arrival at the NWRC, house mice were individually housed indoors in 18 × 29 × 13 cm cages with a 10 cm hide, maintenance diet, and water *ad libitum*. The maintenance diet for house mice consisted of rodent blocks (LabDiet® Land O'Lakes, St. Louis, MO, USA) and an apple slice. We set the light conditions to 12 h of light and 12 h of dark. We quarantined and monitored the health of the house mice for two weeks before the study was initiated. This study was approved by the NWRC Institutional Animal Care and Use Committee (QA-2679).

2.2. Behavioral assay

2.2.1. Acclimation

We acclimated RGS and house mice within individual test cages (62 × 50 × 42-cm for RGS and 41 × 19 × 20 cm for house mice) for five days (Wed-Sun; Week 1). The maintenance diet was presented within each cage at 0800, daily throughout the experiment (acclimation, pre-test, and test).

2.2.2. Enrichment food

Ground squirrels and other rodents are known to damage watermelon in the wild and have been fed watermelon as a supplement to maintenance diet in captivity (Dyche, 1889; Hollister, 1916; Hall, 1981; Marsh, 1998; Pasztor et al., 2001). Off-test we found that all of the RGS readily consumed pieces of watermelon in addition to maintenance diet, therefore watermelon was selected as the RGS enrichment food during testing. Unadulterated watermelon sections without the rind were offered to RGS each day during the pre-test and test.

House mice are commonly trapped using peanut butter and it has

[2] anthraquinone.

been used as a lure food in captive trials (Brown et al., 2004; Witmer and Burke, 2008; Witmer, 2014). Other foods preferred by mice include nut meats and foods high in fat, protein, or sugar, over grain and seed (Timm, 1994). Enrichment blocks consisting of peanut butter combined with toasted oat cereal, sunflower seed, and honey were readily consumed by off-test house mice in addition to maintenance diet, therefore this combination was selected as the house mouse enrichment food during testing. Unadulterated enrichment blocks were offered to house mice each day during the pre-test and test.

2.2.3. Pre-test

We presented enrichment food within a black PVC hide (17.7 cm or 3.8 cm diameter; open on both ends) in each cage at 0800, daily for four days (Mon-Thur; Week 2). We visually determined consumption of the enrichment food after 24 h of exposure (i.e. Tuesday-Friday of Week 2) as "0" no consumption, "0.5" half consumed and "1" fully consumed. Based upon the visual, pre-test consumption determinations of the enrichment food we assigned each RGS to one of three test groups (n = 10–11 subjects per group) such that each group had similar numbers of high and low consumers. We then randomly assigned test treatments among groups.

2.2.4. Test

We presented enrichment food within a black PVC hide (17.7 cm or 3.8 cm diameter) in each cage at 0800, daily for four days (Mon-Thur; Week 3). Watermelon offered to RGS had weight values that varied between a minimum of 28.9 g to a maximum of 109.3 g over the course of the test. Mouse enrichment block weight values varied between a minimum of 0.62 g to a maximum of 4.38 g over the course of the test. The barrier component that covered each end of the PVC hide was a piece of burlap secured with a zip tie (Fig. 1). Groups 1–3 received a PVC hide with burlap structural barriers treated as follows, Group 1: no repellent (untreated control); Group 2: a 50% dilution (1:1 AQ:water) of an AQ-based repellent (Arkion® Life Sciences, New Castle, DE, USA, a.i. 50% AQ); and Group 3: a 0% dilution of the AQ-based repellent. We treated both sides of the burlap coverings using a CO_2-pressurized sprayer to ensure even and complete coating. Enrichment-food consumption (g) and defeat of the structural barrier were measured after 24 h of exposure on Tuesday-Friday of Week 3. Defeat of the structural barrier was defined as a physical breach by means of chewing the burlap or burlap/repellent barrier such that the test animal was able to gain entry to the hide and enrichment food.

2.3. Statistical analysis

We calculated the percentage of animals (RGS or house mice) that defeated (as defined in the methods) each structural barrier treatment by day throughout the experiment. We used descriptive statistics ($\bar{x}$ ± binomial SE of sample proportion) to describe the percent of animals that defeated each structural barrier by treatment throughout the experiment (Fig. 2 and 3) and analyzed using multiple comparisons with a Bonferroni type adjustment (Pearson chi-square test SAS v.9.2) to determine if the treatment medians differed (Tables 1–2). Bonferroni type adjustments are used when conducting multiple analyses on the same dependent variable. The adjusted significance level was calculated using the formula: $\alpha_{altered} = (\alpha/k)$ where α is 0.05 and k is the number of pairwise comparisons.

Pre-test consumption of enrichment food was summarized using descriptive statistics. The initial weight of enrichment offered to test animals varied. Therefore to account for these differences we calculated the percent change in the consumption of enrichment food during the test as ([(test enrichment food offered (g) − test enrichment food taken back (g))/(test enrichment food offered (g))*100]. We analyzed the percent change in consumption of test enrichment food using multiple pair wise nonparametric comparisons (Kruskal-Wallis tests; SAS v.9.4) and adjusted the significance level used for the decision criteria using a Bonferroni type adjustment to determine if the treatment medians differed (Tables 1–2).

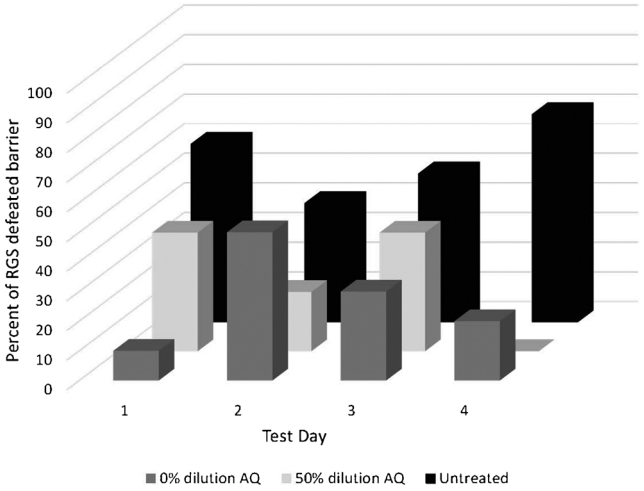

Fig. 2. Percent of Richardson's ground squirrels (*Urocitellus richardsonii* (Sabine)) that defeated the barrier component by test day and treatment group during testing.

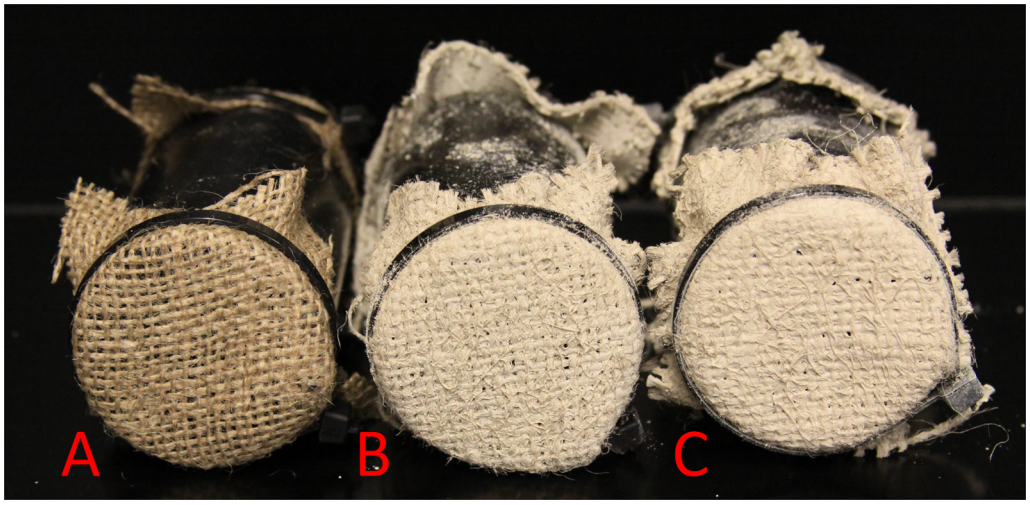

Fig. 1. Photo of PVC hide with barrier components as used during week 3 of experiments evaluating anthraquinone-treated structural barriers with Richardson's ground squirrels (*Urocitellus richardsonii* (Sabine)) and house mice (*Mus musculus* L.). A = untreated burlap B = burlap treated with 50% dilution of anthraquinone; C = burlap treated with 0% dilution of anthraquinone.

Table 1
Average percent consumption of test enrichment-food and percent barrier defeat by Richardson's Ground Squirrels during structural barrier testing. Means with different letters are significantly different; Bonferroni adjusted Kruskal Wallis test and Pearson chi-square test, $\alpha = 0.0167$.

Treatment group	Avg. percent test enrichment-food consumption (%)		Percent barrier defeat (%)	
Untreated Control	90.3	A	55.0	A
50% Dilution	43.3	B	25.0	B
0% Dilution	46.8	B	27.5	B

Table 2
Average percent consumption of test enrichment-food and percent barrier defeat by house mice during structural barrier testing. Means with different letters are significantly different; Bonferroni adjusted Kruskal Wallis test and Pearson chi-square test, $\alpha = 0.0167$.

Treatment group	Avg. percent test enrichment-food consumption (%)		Percent barrier defeat (%)	
Untreated Control	92.8	A	100.0	A
50% Dilution	82.6	A	20.5	B
0% Dilution	76.4	A	45.5	C

3. Results

3.1. RGS results

3.1.1. Pre-test

RGS consumption of the enrichment food was the same between 24 of 30 test subjects as they consumed all of the enrichment food each day of the pre-test. Of the six RGS that did not consume all of the enrichment food, only one animal consumed none of the enrichment food and only on days 3–4 of the pre-test. The remaining five RGS consumed at least half of the enrichment food each day of the pre-test.

3.1.2. Test

RGS defeat of the burlap barrier varied among treatment groups and test days (Fig. 2). Across the four test days, RGS in Group 1 (untreated control) chewed through the untreated burlap an average of 55.0% (± 7.9). Three RGS in the untreated group did not chew through the barrier during the four-day test. An average of 25.0% (± 6.8) and 27.5% (± 5.6) of RGS in Group 2 (50% dilution of an AQ-based repellent) and Group 3 (0% dilution of an AQ-based repellent) chewed through the treated barriers, respectively. Four RGS in each of Groups 2 and 3 did not chew through the barriers on any of the four test days. We detected a difference in the percent defeat of barriers between RGS in Group 1 (untreated control) versus that in Groups 2 and 3. Compared to the control group, the AQ treatment reduced RGS defeat of the barrier by 50–55%. We also detected a difference in the percentage of enrichment food consumed between RGS in Group 1 (untreated control) versus that in Groups 2 and 3 (Table 1). RGS in Groups 2 and 3 ate 48–52% less of the enrichment food than RGS in the untreated control group. RGS in the 50% dilution group showed a decreasing trend in consumption of enrichment food from 67.9% consumed on test day 1 to 0.0% consumed on day 4. RGS in the 0% dilution group showed decreased consumption from day 1 to day 2, but consumption among days 2, 3 and 4 remained approximately the same.

3.2. House mouse results

3.2.1. Pre-test

House mouse consumption of the enrichment food during the pre-test was the same between 30 of 32 animals as they consumed all of the enrichment food each day. Pre-test consumption of enrichment food was consistently a "1" or fully consumed with only two animals consuming "0.5" or half on one day.

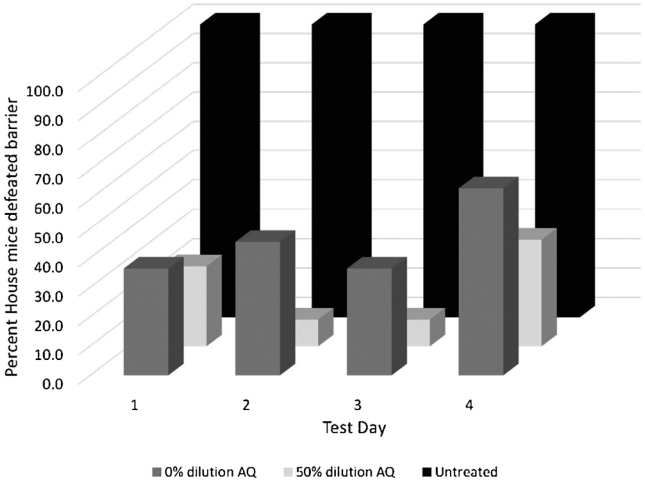

Fig. 3. Percent of house mice (*Mus musculus* L.) that defeated the barrier component by test day and treatment group during testing.

3.2.2. Test

House mice defeat of the burlap barrier varied among treatment groups and test days (Fig. 3). Across the four test days an average of 100% (± 0.0) of the house mice in Group 1 (untreated control) chewed through the untreated burlap structural barrier. An average of 20.5% (± 6.4) and 45.5% (± 7.8) of house mice in Group 2 (50% dilution of an AQ-based repellent) and Group 3 (0% dilution of an AQ-based repellent), respectively chewed through the treated burlap structural barrier (Fig. 3). Seven house mice in Group 2 and four house mice in Group 3 did not chew through the burlap during the four-day test. We detected a difference in the percent barrier defeat between house mice in Group 1 (untreated control), Group 2, and Group 3 (Table 2). Compared to the control group, the AQ treatment reduced house mouse defeat of the barrier by 55–80%. We did not detect a difference in the percentage of enrichment food consumed among house mice in Groups 1–3 (Table 2).

4. Discussion

Previous studies conducted with rats exposed many test subjects to weeks of intensive barrier defeat training to ensure equal performance among test subjects and even removed animals from testing for failure to perform (Tigner and Besser, 1962). Previous examples of barrier testing also routinely utilized some form of starvation or food limitation to motivate test subjects to defeat the barrier to access either a maintenance diet or an enrichment food (e.g. peanuts) (Bendig and Stolurow, 1952; Welch, 1954; Weeks, 1959). We selected species-specific enrichment foods to motivate our unconditioned, wild-captured test subjects to interact with the experimental structural barriers.

Both Richardson's ground squirrels (Werner et al., 2016) and house mice (Werner, SJ unpublished data) have shown moderate repellency to AQ treated whole oats. Despite the selection of burlap for our barrier, a material that rodents readily learn to penetrate (Bowles et al., 1974), there were three RGS that did not chew through the untreated burlap structural barrier and eight RGS that did not chew through the AQ treated burlap structural barrier (four from each treated group). One RGS from each of the treatment groups that did not chew through the AQ treated burlap showed a decreased interest in watermelon consumption during the pre-test. The three RGS in the untreated group that did not chew through the untreated burlap structural barrier ate all of their watermelon each pre-test day. These results lead to some unclear inferences as to whether the AQ treatment caused the eight RGS to not chew through the burlap or if these individuals showed a lesser

propensity for chewing behavior. Captive testing conducted with gray squirrels (*Sciurus carolinensis* Gmelin) found that most gnawing was done by the youngest and most active squirrels (Morris, 1953). In addition to treatment, the age of RGS may have played a role in the amount of gnawing each animal was inclined to, regardless of interest in enrichment food. Indeed, even in test subjects trained to defeat barriers Bendig and Stolurow (Bendig and Stolurow, 1952) observed variation among test days and trials.

Interestingly, the 0% dilution (Group 3) of AQ treated barriers had more individuals of both RGS and house mice chew through the structural barrier than the 50% dilution (Group 2) despite the increased concentration of AQ in Group 3. This effect was especially pronounced in the house mice testing where individual mice defeated the 50% burlap barrier only 9 times out of 44 tries. Previous testing with house mice dissolved candidate repellents in a solvent then soaked burlap bags in the solution to ensure uniform distribution of the repellent treatment (Tigner and Besser, 1962). Barrier testing conducted with paperboard, instead of burlap, had to be coated on both sides to ensure a rat's tongue would come in contact with the repellent (Weeks, 1959). The AQ-based repellent used in this study had a thick, paint-like consistency and we hypothesize that the additional water in the 50% dilution may have allowed for greater absorption of the repellent throughout the burlap fibers whereas more AQ in the 0% dilution may have remained on the burlap surface. Thus, RGS or mice may have ingested less AQ in the 0% dilution treatments causing less repellency than the 50% dilution treatments.

Anthraquinone is an UV-absorbent, postingestive repellent (Werner et al., 2012). When California voles (*Mircrotus californicus* Peale) and red-winged blackbirds (*Agelaius phoeniceus* L.) are conditioned with AQ-treated food, both voles and blackbirds subsequently avoid UV-treated food relative to unconditioned animals (Werner et al., 2012; Werner et al., 2016). House mice transmit 50% of incident illumination at 313–337 nm while Richardson's ground squirrels transmit 50% of incident illumination at 462 nm (Douglas and Jeffery, 2014). Thus, house mice are more sensitive to UV and potentially more visually sensitive to the AQ repellent than RGS. There were 11 house mice that never chewed through the AQ treated barrier, none of which showed a decreased interest in enrichment block consumption during the pretest, while all house mice in the untreated group defeated the burlap structural barrier. These results may be attributed to a pre-ingestive visual cue associated with the AQ treatments in house mice.

Previous studies have shown large variation in repellency of AQ among various species of mammals (Werner et al., 2011; Werner et al., 2016). Even within species differences in repellency have been shown between male and female common voles (Hansen et al., 2016). We observed differences in consumption of enrichment food after defeat of an AQ-treated barrier as compared to an untreated barrier in RGS. RGS decreased consumption of enrichment food after defeat of an AQ-treated barrier on days 2–4 compared to day 1, while RGS consumption of enrichment food after defeat of an untreated barrier remained the same on days 2–4 compared to day 1. This suggests a decrease in RGS appetite due to an interaction with the AQ treatment. This trend was more pronounced in the 50% dilution group than in the 0% dilution group, supporting the hypothesis that the 50% dilution group consumed more AQ due to the absorption of the AQ into the burlap barrier.

5. Conclusion

Relative to untreated barriers, AQ treatments reduced the defeat of structural barriers by 50–55% in RGS and 55–80% in house mice. We therefore recommend additional efficacy testing of AQ-treated barriers for these and other rodents under field conditions. In addition to the decreased defeat of treated barriers, AQ treatments also decreased consumption of the enrichment food by RGS. Because AQ is a post-ingestive repellent (Werner and Provenza, 2011), we conclude that RGS consumed AQ through interacting with treated barriers and then subsequently avoided untreated enrichment food associated with barriers. Additionally, we conclude that uniform absorption of the repellent throughout or across the barrier is integral to success of the repellent. Structural barriers including AQ-based repellents should therefore be designed to ensure consumption of the active ingredient as rodents interact with the treated surface. We recommend further testing of combined food treatments and structural treatments of AQ-based repellents. Such testing should include context-specific motivations associated with food reinforcement versus structural reinforcement (e.g. rodent harborage, barriers to food resources). These species-specific efficacy data can be used for the development of non-lethal wildlife repellents and the protection of agricultural crops, commodities and valued structures from rodent damage.

Conflict of interest

None.

Acknowledgements

Our barrier experiments were conducted with Avipel® Shield repellent (Arkion® Life Sciences, New Castle, DE, USA). Corporate collaborations do not imply endorsement by the United States Department of Agriculture. We are grateful for the daily care of all test subjects provided by the NWRC Animal Care Staff throughout the quarantine and holding periods of each experiment.

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