



Effective Dose and Persistence of Rhodamine-B in Wild Pig Vibrissae

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ABSTRACT As a result of substantial ecological and economic damage attributed to wild pigs (*Sus scrofa*), there is international interest in using pharmaceutical baits to control populations. To assess the efficacy and specificity of baiting programs, chemical biomarkers can be used to evaluate uptake of pharmaceutical baits. Rhodamine B (RB) is known to be an effective biomarker in wild pigs. However, significant data gaps exist regarding the minimum effective dosage and persistence of RB in wild pigs. We used a controlled double-blind study experiment conducted in spring of 2014 on the Savannah River Site, Aiken, South Carolina, USA, wherein we administered a one-time dose of RB at 3 treatment levels (5 mg/kg, 15 mg/kg, or 30 mg/kg) to 15 captive pigs, with 5 pigs/treatment group to investigate persistence of RB. Facial vibrissae were collected pre-RB ingestion as a control and every 2 weeks post-RB ingestion for 12 weeks. We examined samples for RB presence and used a generalized linear mixed model (GLMM) to determine the influence of treatment dose on persistence of RB. Additionally, we measured distance moved by the RB mark away from the vibrissae root and used a GLMM to assess movement rates of RB bands along growing vibrissae. We found consistently greater persistence of RB in the 15- and 30-mg/kg treatments across the sampling period. A significant, positive movement trend in RB bands was observed within the 15 mg/kg and 30 mg/kg groups. Based on our results, a 15 mg/kg dosage can be considered a minimum effective dose for wild pigs and will reliably produce a detectable RB mark up to and likely beyond 12 weeks after ingestion. © 2017 This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS baiting, biomarker, invasive species, Rhodamine-B, Savannah River Site, *Sus scrofa*, wild pigs.

Wild pigs (*Sus scrofa*) have become one of the most widespread invasive large mammals globally, and currently occur on all continents except Antarctica. In North America, wild pigs were introduced in localized areas during the 16th century by European settlers as a food resource, but populations have since expanded and are now found in more than two-thirds of the continental United States (West et al. 2009). Wild pigs cause >US\$1.5 billion in damage and that cost is expected to rise as populations continue to expand in the United States (Pimentel et al. 2008). Although some localized control efforts have proven successful (McCann

and Garcelon 2008, Parkes et al. 2010), eradication of wild pig populations at a landscape scale is a considerable challenge because of continued human-instigated translocations—introductions of wild pigs to create hunting opportunities, pigs' ability to reproduce prolifically, and generalist life-history characteristics that allow pigs to persist in numerous habitat types and climates (Keiter and Beasley 2017).

Several methods are currently employed to mitigate the spread of wild pigs, including but not limited to lethal trapping and snaring, aerial removal with firearms, and baited shooting. Large-scale baiting programs distributing either lethal toxicants or contraceptives have been used in some areas of Australia and New Zealand to control wild pigs and other invasive species (Saunders et al. 1990, Cowled et al. 2006). Indeed, researchers have previously determined that

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lethal toxicant baits are both cheaper and more effective for eliminating wild pig populations than other control methods under certain circumstances, such as on islands (Coblentz and Baber 1987). Although baiting programs alone generally are not 100% effective because of variable bait acceptance among individuals (Hone 1983), baiting programs are an effective tool when used in conjunction with other control methods and can be an important component of integrated pest management programs (Mitchell 1998, Fleming et al. 2000, Twigg et al. 2005). As a result of the potential for use of toxicants to control wild pig populations, there is growing international interest in developing effective pharmaceutical baits for use in the control of wild pigs that simultaneously pose little threat to nontarget species (Cowled et al. 2008, Bengsen et al. 2011, Massei et al. 2011). Therefore, prior to and throughout large-scale implementation of any toxicant baiting program, research is needed to elucidate efficacy and acceptance of baits by target species and insure minimal effects to nontarget species.

Chemical biomarkers are increasingly being used in wildlife research and have become an important tool to quantify the effectiveness of baiting programs and bait delivery systems. For example, biomarkers are routinely used to evaluate the proportion of target populations ingesting baits, demographic patterns of bait consumption, and assess potential risks of exposure by nontarget species (Savarie et al. 1992, Beasley et al. 2015a). Biomarkers are nontoxic substances that, upon ingestion, are metabolized and produce a “mark,” sometimes a “band” fluorescent in nature, on the hair, facial vibrissae (i.e., whiskers), teeth, tissue, or bone of the individual (Fry and Dunbar 2007). Several biomarkers allow for verification of bait ingestion using noninvasive sampling, facilitating their use in both lethal and nonlethal management programs. However, for a chemical biomarker to be an effective verification tool of bait ingestion in both target and nontarget species, it must persist within the organism for a substantial time period after ingestion and should not require lethal dispatch of nontarget species to assess marker presence.

Rhodamine-B (RB; Sigma-Aldrich, St. Louis, MO, USA) is a biomarker that is metabolized in wild pigs and has been used in previous studies of bait uptake in raccoons (*Procyon lotor*), coyotes (*Canis latrans*), and several other terrestrial mammalian species, such as ungulates and domestic livestock, that may be of management interest in areas where wild pigs exist (Farry et al. 1998a, b; Fisher 1999; Fisher et al. 1999; Beasley et al. 2015a). Ingestion of RB results in the formation of a fluorescent band in the hairs of the individual that can be collected via nonlethal sampling methods. However, the ability to detect RB across mammalian species is known to differ, and in some species factors such as age, sex, or body mass can influence metabolism and thus detectability of RB (Fisher 1999). Notable knowledge gaps exist regarding the minimum effective RB dose for wild pigs or the duration of time that bands may persist in an individual.

Previous studies have shown that RB administered at a dose of $\geq 0.5\%$ mass RB/mass of individual results in a visible, external mark on the pelage or skin of wild pigs for

up to 5 days (Clarke 1992). Additionally, Beasley et al. (2015b) found that a dose of 0.03% mass RB/mass of individual produced a fluorescent band in hair for up to 14 days. Baruzzi et al. (2017) reported that a dose of 0.0013% mass RB/body mass of individual was effective for marking adult female wild pigs. However, RB persistence for ≥ 14 days has not been evaluated in wild pig populations, a component essential to accurately determine bait acceptance in wild pigs during long-term baiting programs where individuals of differing sex and age classes may be sampled to assess consumption over a several-week or -month period post-RB bait deployment. Although RB is considered safe for animal ingestion in doses below 1% of body mass, there are still potential risks to individual survival, growth, and reproduction, an important consideration for nontarget species of conservation concern when developing bait dose concentrations (Fisher 1999). Previous research has determined 30 mg/kg to be an effective dose of RB for wildlife species such as opossums (*Didelphis virginiana*), raccoons, and coyotes (Farry et al. 1998a, b; Smyser et al. 2010). However, because wild pigs can vary greatly in mass (<5 kg for juv to >200 kg in ad males), and thus volume of food consumption, determining a minimum dosage that will be effective on large pigs without being concentrated to the point of harmful toxicity for smaller individuals or nontargets is critical. Although the minimum effective dosage in wild pigs is currently unknown, dosages as low as 3.9 mg/kg have been shown to produce a fluorescent band in vibrissae in small mammal species, such as house mouse (*Mus musculus*; Weekaroon et al. 2013). Our objective was to determine the minimum effective dose for detection of RB in wild pigs, as well as the persistence of RB over 12-week period postconsumption to develop practical guidelines for the use of RB in research on bait uptake by invasive wild pigs. Based on work by Beasley et al. (2015), we predicted that a minimum dosage for large wild pigs (25–150 kg) would be <30 mg/kg. We also predicted the position of the RB mark on a vibrissae would move away from the root of the vibrissae over time, but at a slow enough rate such that marks would be visible for the entire 12-week duration of the study.

STUDY AREA

We conducted this study on the U.S. Department of Energy’s Savannah River Site, a 78,000-ha National Environmental Research Park in the Upper Coastal Plain physiographic region of western South Carolina, USA. The Savannah River Site landscape was dominated by managed upland loblolly (*Pinus taeda*) and longleaf pine (*P. palustris*) forest with interspersed bottomland hardwood forests in riparian areas. Wild pigs were abundant on the Savannah River Site and known to cause extensive ecological and economic damage, including dozens of vehicle collisions annually (Mayer and Brisbin 1991, Beasley et al. 2013). The population was actively controlled because of vehicle collision risks and extensive ecological damage caused by pigs on the Savannah River Site; 1,605 pigs were culled during federal fiscal year 2014 (2.1 pigs/km²).

METHODS

Sample Collection

We trapped 15 wild pigs throughout the Savannah River Site during spring 2014 using box or corral traps baited with shelled corn, and randomly assigned 5 pigs to each of 3 RB dosage-treatment levels (5, 15, and 30 mg/kg). We immobilized captured pigs using a combination of Telazol (4.4 mg/kg) and Xylazine (2.2 mg/kg) or Ketamine (10 mg/kg) and Xylazine (0.5 mg/kg), which we administered using a blow gun or jab stick (Kreeger and Arnemo 2012). Once immobilized, we collected 2 facial vibrissae from each individual, including dark and light samples of each if available, to serve as control samples prior to RB administration. We recorded mass, body length, chest girth, and age class of each individual. We differentiated individuals into 3 different age classes, juvenile, subadult, and adult, where delineations were based on mass of the individual. We defined juveniles as individuals <13.6 kg, subadults as individuals 13.6–45.3 kg, and adults as individuals >45.3 kg. Subsequent to immobilization, we orally administered RB (Sigma–Aldrich) via syringe at either 5 mg/kg, 15 mg/kg, or 30 mg/kg dosage. Although administration of the biomarker in this manner is not consistent with field administration of baits, it was used to ensure the individual ingested the entire RB dose. We then transferred anesthetized pigs via truck to a captive facility on Savannah River Site, where they were held for 12 weeks. We monitored body temperature of pigs rectally throughout the immobilization and transportation process to ensure the safety and well-being of captured animals.

The captive care facility consisted of covered 2.5 × 3-m or 2.5 × 6-m pens constructed of high-quality chain-link fencing with a concrete floor (Beasley et al. 2015*b*). We placed pigs in individual pens except for juveniles that were trapped together, which we housed in the same pen (2.5 × 6 m). We provided each individual 0.5–1.8 kg of corn or hog feed daily (depending on the mass of each animal) and constant access to fresh water. We cooled pigs with a misting of water 2–3 times daily when ambient temperatures exceeded 32°C. Throughout the 12-week sampling period, we visually monitored body condition, ectoparasite presence, parasite presence in feces, and food consumption of individuals. Additionally, some individuals required treatment for minor internal parasite infections or minor external injuries. Baytril (Bayer Pharmaceuticals, Leverkusen, Germany), Penicillin (Durvet Inc., Blue Springs, MT, USA), Phenylbut (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), and dewormer (Sykes Vet International PTY LTD, Dandenong, Victoria, Australia) were administered to individuals as needed. We catalogued all dates and dosages for each medication administered and included each as a random effect in analyses. We resampled each individual at 2-week intervals by anesthetizing them as described above and pulling 4 facial vibrissae. To ensure collection of vibrissae that were growing at the time of RB administration and not newly emerging hairs, we initially collected vibrissae at the top of the snout and rotated sample

collection clockwise around the snout on subsequent sampling occasions. We stored samples in envelopes in a dark, cool environment until analysis. We immobilized pigs and euthanized them via gunshot to the head at the end of the 12-week holding period. All animal handling practices and euthanasia were carried out in accordance with University of Georgia Animal Care and Use guidelines under protocol A2013 04-27-Y1-A0.

Sample Analysis

To determine the presence or absence of RB, we mounted all samples (including controls) on microscope slides with Fluoromount Aqueous Mounting Medium (Sigma–Aldrich) and then examined them using an Olympus BX 61 fluorescent microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) with a tetramethylrhodamine isothiocyanate filter set (narrow-band excitation filter and a red-shifted emission filter) under 4× and 10× magnification. We defined sampling units (hereafter referred to as “samples”) as all of the vibrissae collected from an individual at a sampling occasion. Thus each sample included multiple vibrissae from one individual collected at a specific sampling occasion. We considered a sample “RB-present” when we detected a RB band on ≥1 hair. Two personnel trained to identify RB in wild pig vibrissae scored each sample as RB-present or RB-absent. The analysis followed a double-blind format, with all identifying information about the samples obscured from scorers and samples mixed randomly prior to scoring. Any samples with disagreement between scorers were scored blindly by a third party, and the majority score was used in subsequent analysis.

Once all samples were scored, we used a binomially distributed generalized linear mixed-effects model (GLMM) to assess the effect of several covariates including the fixed effect of treatment dosage (5, 15, or 30 mg/kg) and the random effects of sex, individual (accounting for the repeated measurements of an individual over time), mass (in kg), and administration of medication such as dewormer or antibiotics (a binary “presence” or “absence” of medication in the individual’s capture history at the time of each sampling). We conducted all modeling in Program R 3.2.5 using the package “lme4” (Bates et al. 2015). Use of GLMM allowed us to estimate the magnitude of the effect size of each level of the covariates of interest and thus quantify the extent to which each covariate influenced the probability of RB persistence.

In addition to scoring presence or absence of RB in samples, personnel also measured (in millimeters) the distance of all detected RB bands from the base of the root of vibrissae to quantify the distance traveled by the band through time. This measurement allowed us to quantify how, if at all, an individual’s natural metabolic hair growth over time may influence the persistence and position of the RB band on the vibrissae at different dosage levels. To determine the rate at which RB bands may move along vibrissae, we pooled those samples from the treatment groups deemed effective (see results) to create a single data set of RB-positive samples for analysis. We then analyzed this subset using

GLMM to determine whether a trend in RB movement over time existed, as well as to predict the average rate of movement for the RB band within individuals over time. Time period, the only variable of interest, was included as a fixed effect; while sex, mass, and individual were all included in the analysis as residual random effects.

RESULTS

Fourteen of the 15 individuals sampled in this study tested negative for RB in their control samples collected prior to oral administration of RB. The single individual that scored positive for RB in the control sample was removed from further analysis to prevent bias in postexposure RB presence assessments. The origin of markings resembling RB in vibrissae from this individual are unknown, but possibly resulted from abnormal levels of autofluorescence (naturally occurring fluorescence in the vibrissae) or exposure to RB or a similar biomarker through other sources on our study site. Overall, 393 vibrissae samples were collected and RB was present in 72% of samples collected throughout the entirety of the study. Persistence of RB varied among treatment groups, with lower proportions of RB-marked vibrissae in the 5 mg/kg group than either the 15 mg/kg or 30 mg/kg treatment groups ($P < 0.001$ and $P = 0.004$, respectively; Fig. 1). Dosages of 15 mg/kg and 30 mg/kg were both highly effective in marking individuals over a 12-week period, with 89% of all vibrissae samples from the 15 mg/kg group and 84% of all vibrissae samples from the 30 mg/kg group exhibiting RB presence. Comparatively, only 53% of vibrissae from the 5 mg/kg group exhibited RB presence. At the individual level, RB was found to persist in the 30 mg/kg treatment group for a minimum of 8 weeks in all individuals, and throughout the entire 12-week period in 4 out of 5 individuals. Additionally, RB persisted throughout the 12-week study period for all individuals within the 15 mg/kg group. In contrast, only 1 of 5 individuals in the 5 mg/kg group exhibited RB markings at 12 weeks, and our

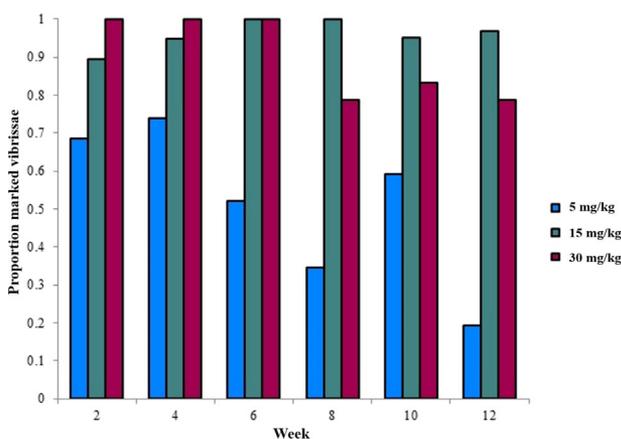


Figure 1. Proportion of wild pig facial vibrissae with Rhodamine-B (RB) bands from each treatment dosage group (5 mg/kg, 15 mg/kg, and 30 mg/kg) for 4 vibrissae/individual sampled biweekly across a 12-week sampling period following RB challenge of 15 individuals captured in spring of 2014 on the Savannah River Site, Aiken, South Carolina, USA, and housed in a captive facility for the duration of the experiment.

ability to detect RB across sampling occasions was not consistent for 4 of 5 individuals within this treatment group (Table 1).

The mixed-effects model revealed that the 30 mg/kg and 15 mg/kg treatment groups had greater persistence of RB ($\beta = 2.92$, $P = 0.004$ and $\beta = 4.36$, $P < 0.001$, respectively) when compared with the 5 mg/kg treatment group ($\beta = 0.129$, $P = 0.796$) over the 12-week sampling period. The random effects of individual ($\sigma^2 = 3.648e-07$), mass ($\sigma^2 = 1.80$), medicine administration ($\sigma^2 = 1.57$), and sex ($\sigma^2 = 5.393e-09$) were not found to explain a substantial amount of model variation.

Only the 15 mg/kg and 30 mg/kg treatment groups consistently produced RB bands in vibrissae; therefore, only samples from these treatment groups were included in the distance analysis. The positioning of RB bands moved away from the root of the vibrissae over time as a result of whisker growth ($\beta = 0.85$, $SE = 0.26$, $P = 0.001$; Fig. 2) by an estimated 0.85 mm/2 weeks. Neither animal sex nor mass explained a significant amount of variation in RB movement in our model. Further, almost 73% (185 of 255) of vibrissae samples with RB presence from the 15 and 30 mg/kg treatment groups exhibited movement of ≥ 5 mm along the vibrissae shaft.

DISCUSSION

Overall, RB persisted in the facial vibrissae of wild pigs at doses ≥ 15 mg/kg throughout the 12-week sampling period, making it a useful tool for management and research programs assessing pharmaceutical bait uptake and efficacy. Based on our results, a dosage of 15 mg/kg may be considered a minimum effective dose for RB in wild pigs, although some individuals in the 5 mg/kg treatment group did display a detectable, but inconsistent, RB mark. Our sample sizes of 5 individuals for each treatment group, while relatively small, was sufficient to ensure that each treatment group was representative of all age classes and sexes. Further, we found that factors such as sex, mass, and individual did not influence persistence or detectability of RB. Additionally, although both 15 mg/kg and 30 mg/kg groups showed significant positive RB movement trends over time indicative of vibrissae growth, movement of RB bands was slow and, given the high persistence of marked vibrissae over 12 weeks, detection of RB presence is likely feasible over a several-month period.

Our findings are consistent with previous research that has found RB to effectively mark actively growing hairs of several mammalian species over an extended time period, including coyotes (25 weeks), European badger (*Meles meles*, up to 22 weeks; Johns and Pan 1981, Cagnacci et al. 2006), stoats (*Mustela ermine*, up to 19 weeks; Spurr 2002), house mice (12 weeks; Jacob et al. 2002), and mountain beaver (*Aplodontia rufa*, 28 weeks; Lindsey 1983). Cagnacci et al. (2006) found that RB detection in European badger hair and whiskers was dependent on the molting cycle of the species. Specifically, accurate RB detection depended on timing the marking event so that hair or whisker follicles are in a “growth phase” (i.e., during molting cycle) rather than a “resting phase” when

Table 1. Detection of Rhodamine B (RB) in wild pig vibrissae collected every 2 weeks over a 12-week sampling period (2 vibrissae collected prior to RB challenge and 4 thereafter biweekly) among 3 RB treatment dosages (5 mg/kg, 15 mg/kg, and 30 mg/kg) by age and sex class for 15 individuals captured in spring of 2014 on the Savannah River Site, Aiken, South Carolina, USA, and housed in a captive facility for the duration of the experiment. Rhodamine B persistence is indicated as the number of vibrissae with RB presence/number of vibrissae scored.

Pig ID	Sex	Mass (kg)	Age class	Dosage (mg/kg)	Sampling occasion (week)						
					0	2	4	6	8	10	12
78	M	43.1	Subad	5	0/2	4/4	4/4	0/4	0/4	0/4	1/4
794	M	45.3	Ad	5	0/2	0/4	0/4	4/4	4/4	4/4	0/4
4691	F	3.17	Juv	5	0/2	4/4	4/4	0/4	0/4	0/4	0/4
39	F	52.2	Ad	5	0/2	2/4	3/4	2/4	0/4	4/4	0/4
800	F	10.9	Subad	5	0/2	4/4	4/4	4/4	4/4	4/4	0/4
801	F	8.24	Subad	15	0/2	4/4	4/4	4/4	4/4	3/4	4/4
785	F	59.9	Ad	15	0/2	3/4	2/4	4/4	4/4	4/4	3/4
789	M	43.1	Subad	15	0/2	3/4	4/4	4/4	4/4	4/4	4/4
786	F	43.1	Subad	15	0/2	4/4	4/4	4/4	4/4	4/4	4/4
788	M	41.7	Subad	15	0/2	4/4	4/4	4/4	4/4	4/4	4/4
4688	F	2.72	Juv	30	0/2	4/4	4/4	4/4	4/4	0/4	2/4
790	F	38.6	Subad	30	0/2	4/4	4/4	4/4	4/4	4/4	4/4
799	M	11.3	Subad	30	0/2	4/4	4/4	4/4	4/4	4/4	4/4
796	M	28.3	Subad	30	0/2	4/4	4/4	4/4	3/4	4/4	2/4
798	M	10.0	Subad	30	1/2	4/4	4/4	4/4	4/4	4/4	4/4

the animal is not molting. All individuals in our study were observed to actively shed hair during the sampling period; however, shedding did not appear to affect our ability to detect RB in facial vibrissae at dosages ≥ 15 mg/kg. It is important to acknowledge that seasonal shedding of hairs or whiskers may influence the ability to detect RB over time and across seasons.

Previous research in black rats (*Rattus rattus*) found the minimum effective dose to detect RB in vibrissae to be 3.9 mg/kg (Weekaroon et al. 2013), and a recent study found that the effective dose to mark adult female wild pigs was 1.3 mg/kg (Baruzzi et al. 2017). However, our findings suggest the minimum effective dose for wild pigs is greater, likely between 5 mg/kg and 15 mg/kg. This is not unexpected because the ability to detect RB across species is known to differ (Fisher 1999). One factor that may have hindered our ability to consistently detect RB was the relatively low number of facial vibrissae (4) collected at each sampling occasion. Although sampling 4 whiskers was sufficient to detect RB in individuals in the higher dosage

groups, it is possible that RB persisted in individuals at low dosages scored as RB-negative but we failed to detect it. This potential for false negatives in our data set may be due to shedding of marked hairs or the limitations of collecting vibrissae over time. Limitations can include having a finite number of vibrissae from which to sample, difficulty determining whether the vibrissae sampled were present at the time of ingestion (i.e., not new growth after RB was metabolized out of individual), and uncertainty that vibrissae have not been broken off or damaged since time of RB ingestion in such a way that would impede accurate RB detection. However, given the high efficacy of the 15 mg/kg and 30 mg/kg dosages at individuals of varying age, sex, and mass, we recommend that field studies should target a minimum dose of 15 mg/kg across all individual wild pigs.

Understanding minimum effective dose and persistence of RB in wild pigs will allow for assessment of the efficacy of large-scale pharmaceutical baiting programs prior to deploying chemicals or toxicants into an ecosystem. To be effective for both small and large individuals, doses of RB in baits should not to exceed 1% body mass to maximize bait acceptance due to palatability while minimizing the potential for negative health effects (Fisher 1999). Our findings indicate that 0.015% mass RB/body mass of individual is effective for wild pigs, regardless of the individual's mass; thus, bait delivery methods should be designed in such a way as to effectively mark wild pigs while not approaching the 1% threshold, even for individuals with small body mass (i.e., juveniles). Although there is a small risk of negative health effects resulting from RB ingestion, toxic doses of orally administered RB in small mammals have been reported at $\geq 50\%$ body mass, making the likelihood of an individual pig ingesting toxic amounts of RB extremely unlikely (Rochat et al. 1979, Smart 1984). Hypothetically, these delivery systems could either focus on developing various mediums to contain the bait that pose little to no threat of approaching the threshold of safety, or focus on designing an effective delivery system for bait that exclude small individuals and

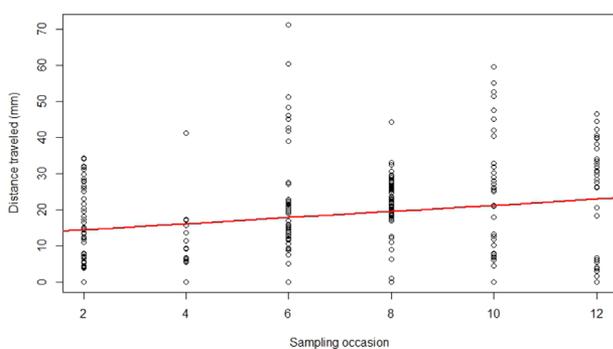


Figure 2. Distance in millimeters traveled by Rhodamine-B band over a 12-week biweekly sampling period from the root of facial vibrissae of 10 wild pigs challenged with 15 mg/kg and 30 mg/kg of Rhodamine-B. All individuals were captured in spring of 2014 on the Savannah River Site, Aiken, South Carolina, USA, and housed in a captive facility for the duration of the experiment.

nontarget species. Although our research presents an advancement in assessing the efficacy and potential role of RB in developing an effective toxicant baiting program, we acknowledge that further research to assess baits for effectiveness, both in bait acceptance and effective mediums to hold RB in a field setting, is needed.

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