



# Evaluation of Rhodamine B as a Biomarker for Assessing Bait Acceptance in Wild Pigs

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**ABSTRACT** Worldwide, there is growing interest in the use of pharmaceutical baits to control populations of wild pigs (*Sus scrofa*). In this study we evaluated the utility of Rhodamine B (RB), a chemical marker commonly used in wildlife research and management, as a potential biomarker for quantifying bait uptake in wild pigs. Thirty wild pigs were live-trapped, transported to a captive facility on the Department of Energy's Savannah River Site located in South Carolina, USA, during autumn 2013, and administered RB orally at a dosage of 30 mg/kg. Eight vibrissae and guard hairs were collected pre- and post-RB exposure (7 or 14 days) and evaluated for the presence of RB using fluorescence microscopy. No evidence of RB marking was observed in any samples collected pre-RB administration. In contrast, we observed fluorescent marking post-RB exposure that was indicative of the presence of RB for all individuals, with 98% of vibrissae and 100% of guard hairs exhibiting RB marks. The uniform detection of RB among individuals and consistent manifestation of marks in both guard hair and vibrissae, samples that easily can be collected and stored by untrained field personnel from live or deceased pigs, suggests that RB is an effective biomarker for use in large-scale management programs to control wild pigs. In particular, our results, combined with previous studies evaluating uptake of RB in other species, suggest that RB can be used to develop baiting programs to deliver pharmaceuticals to free-ranging wild pigs, as well as evaluate the potential impacts of pig baits on non-target species. © 2014 The Wildlife Society.

**KEY WORDS** bait, boar, feral swine, hair, pharmaceutical, Rhodamine B, *Sus scrofa*, toxicant, vaccination, wild pig.

Wild pigs (*Sus scrofa*) are one of the most widespread species of terrestrial large mammal, currently found on all continents except Antarctica and many oceanic islands as a result of accidental and intentional introductions over the past few centuries (Barrios-Garcia and Ballari 2012). Particularly in regions where they are non-native, wild pigs are a substantial source of human-wildlife conflict, causing extensive ecological and economic damages, including destruction of agricultural and forestry crops, vehicle collisions, and reduced abundance of plant and animal species (Engeman et al. 2004, Seward et al. 2004, Fordham et al. 2007, Beasley et al. 2013). Wild pigs also serve as potential reservoirs for infectious diseases of importance to human and livestock health, including foreign animal diseases (e.g., foot and mouth); thus, reducing or eliminating wild pigs from landscapes where they are invasive often is a high priority for management agencies (Witmer et al. 2003, Barrios-Garcia and Ballari 2012).

In the United States, in particular, populations of wild pigs have been expanding in size and distribution at an

increasing rate, despite extensive efforts to control populations through a combination of trapping, hunting, and aerial shooting programs (Sweeney et al. 2003, West et al. 2009). Confounding these efforts, wild pigs can exhibit a density-dependent response to increased mortality through a combination of larger litter sizes and increased reproductive rates of young pigs (Gamelon et al. 2011, Servanty et al. 2011, but see Ditchkoff et al. 2012). Thus, more effective methods or approaches for controlling populations of wild pigs are needed to successfully manage populations. Dissemination of baits to deliver pharmaceuticals (e.g., vaccines, fertility control agents, and toxicants) is now a widely used tool in the management and conservation of wildlife species and there is growing interest in the use of pharmaceuticals to manage populations of wild pigs (Kaden and Lange 2001, Brauer et al. 2006, Campbell et al. 2006). Indeed, baiting programs have been established to deliver toxicants to wild pigs throughout portions of Australia (Twigg et al. 2005, Cowled et al. 2006) and there are increasing efforts globally to develop pig-specific toxicant delivery mechanisms (Ballesteros et al. 2009, Massei et al. 2010) and evaluate uptake rates of various bait types by both wild pigs and non-target species (Campbell and Long 2009, Massei et al. 2010, Ballesteros et al. 2011).

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Chemical markers have long been used to quantify consumption of pharmaceutical baits and forage in free-ranging wildlife populations. An ideal marker is one that can easily be incorporated into baits or foodstuffs without altering palatability, can be detected in tissues easily collected from live or culled animals, can be stored under a variety of field conditions by personnel with varying levels of expertise, and can be detected subsequent to consumption both cheaply and with a high degree of certainty. Of the available chemical markers for wildlife, both tetracycline and iophenoxic acid have been evaluated for use as potential markers in wild pigs (Campbell et al. 2006, Massei et al. 2009, Ballesteros et al. 2011, Reidy et al. 2011). However, these markers have drawbacks which may alter their utility or use in management programs. Specifically, presence of iophenoxic acid is determined through liquid chromatography analysis of serum samples. Although blood can be collected from pigs, there are both expertise and logistic issues that limit the feasibility of blood-based markers, particularly in large-scale management scenarios. For example, collection of blood requires extensive training (particularly from live pigs), post-collection processing (i.e., centrifugation) and storage, and must be collected shortly after euthanasia to avoid clotting. Similarly, evaluation of tetracycline marking is typically assessed in tooth or bone samples collected from harvested individuals and may not be practical in many situations. Thus additional markers appropriate for large-scale management programs should be evaluated for use in pigs.

Rhodamine B (RB) is a fluorescent dye that has been widely used in ecological and behavioral studies of a variety of wildlife taxa, including carnivores, small mammals, ungulates, and birds (reviewed in Fisher 1999). As a highly soluble compound that acts as a systemic marker of keratinized tissue, RB is particularly useful for studies assessing bait uptake because it easily can be integrated into most bait materials and can be detected non-invasively through collection of vibrissae, guard hair, or other keratinized tissues. These samples can be collected easily by untrained personnel from either living or dead animals without the difficulties mentioned previously for serum-based markers. One previous study evaluated the use of RB in wild pigs by screening serum, hoof, and jaw samples but did not characterize uptake in vibrissae or guard hair samples (Fleming et al. 2000). Collection of hooves and jaws may not be practical in many management scenarios and RB is only present in blood for a few days after ingestion (Fisher 1999). The objective of this study, therefore, was to evaluate the potential utility of RB as a biomarker for use in quantification of bait uptake in wild pigs by quantifying presence in both guard hair and vibrissae of male and female wild pigs of various age classes at 1 and 2 weeks post-exposure.

## METHODS

### Sample Collection

We conducted this study on the Department of Energy's Savannah River Site (SRS) located in South Carolina, USA. Wild pigs are highly invasive throughout the SRS

and cause considerable ecological and economical damage, including collisions with vehicles (Beasley et al. 2013). We trapped wild pigs throughout the SRS during autumn 2013 using box or corral traps baited with corn. 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), administered using a blow gun (Kreeger and Arnemo 2012). Once immobilized, we collected a minimum of eight facial vibrissae and eight guard hairs along the spine, including dark and light samples of each if available, from each individual to serve as control samples prior to RB administration. We also collected age, morphometric data including weight, body length, and girth, and tissue samples. While under anesthesia we administered RB (Sigma-Aldrich, St. Louis, MO) orally at a dose of 30 mg/kg to each individual (Smyser et al. 2010). We then transferred pigs via truck to a captive facility (see below) while immobilized (South Carolina Department of Natural Resources permit HR14-06). We monitored temperature of pigs during the immobilization and transportation process to ensure the safety and well-being of captured animals.

We placed captured pigs in individual pens at a captive facility and fed them 1.5–2.0 lbs (680–907 g) of corn twice daily with constant access to fresh water. Pens were 2.5 × 3 m or 2.5 × 6 m in size and consisted of high-quality chain-link construction materials with a concrete floor. In some instances, adult and juvenile pigs or two juvenile pigs were housed in the same pen (2.5 × 6 m) if they were trapped together. Once in the captive care facility, we held individuals for either 7 or 14 days. At the end of the holding period, we immobilized animals using the same method described above and collected post-RB exposure facial vibrissae and guard hairs (min. of eight each, matching pre-exposure sample collection). We then euthanized all pigs while under anesthesia via gunshot to the head. 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

Subsequent to collection we stored vibrissae and guard hair samples in paper envelopes at room temperature and out of direct exposure to light from the time we removed them from the field until we prepared them for microscopic analysis. Both guard-hair and vibrissae samples underwent identical preparation generally following methods outlined in Fisher et al. (1999) and Weerakoon et al. (2013). For each individual, we prepared eight guard hairs and vibrissae for both control (pre-RB exposure) and treatment (post-RB exposure) samples. We prepared samples by first removing dirt and debris using isopropyl alcohol wipes. We then placed hairs in distilled water baths for 3 min; we used separate water baths for each sample to avoid cross-contamination. We then air dried samples at room temperature before mounting them on slides using Fluoromount (Sigma-Aldrich) and a cover slip.

We initially used a subset of control and treatment samples for training purposes as a reference to differentiate between RB presence and any potential natural fluorescence. We

subsequently mixed and randomly scored control and experimental samples blindly. To determine presence of RB, a single observer evaluated each slide using an Olympus BX 61 fluorescent microscope with a tetramethylrhodamine isothiocyanate filter set (narrow-band excitation filter and a red-shifted emission filter) under 4× and 10× magnification. Guard hairs and vibrissae were classified by the observer as ‘marked’ when we detected a band on >1 hair (Smyser et al. 2010). We also recorded the proportion of hair samples (vibrissae and guard hair separately) for which we observed RB marking for each individual. A random subset of samples was screened by a second observer to ensure consistency and accuracy in sample screening.

From our treatment samples, we estimated the proportion of pigs correctly identified as positive for RB ( $\hat{\pi}$ ) as the number of individuals for which  $\geq 1$  positive sample was identified, divided by the total number of individuals evaluated. Furthermore, to determine the power of 8 vibrissae or guard-hair samples to determine whether an individual was positive or negative for RB consumption, we estimated the probability of mark detection ( $\hat{d}$ ) following Smyser et al. (2010). To estimate  $\hat{d}$ , we divided the total number of vibrissae and guard-hair samples manifesting an RB mark by the total number of each sample collected during post-RB sampling. We then expressed the probability of failing to detect an RB-marked individual, given that that individual had consumed a RB bait, as

$$\prod_{j=1}^j (1 - \hat{d}),$$

where  $j$  represents the number of guard hairs or vibrissae sampled from an individual.

## RESULTS

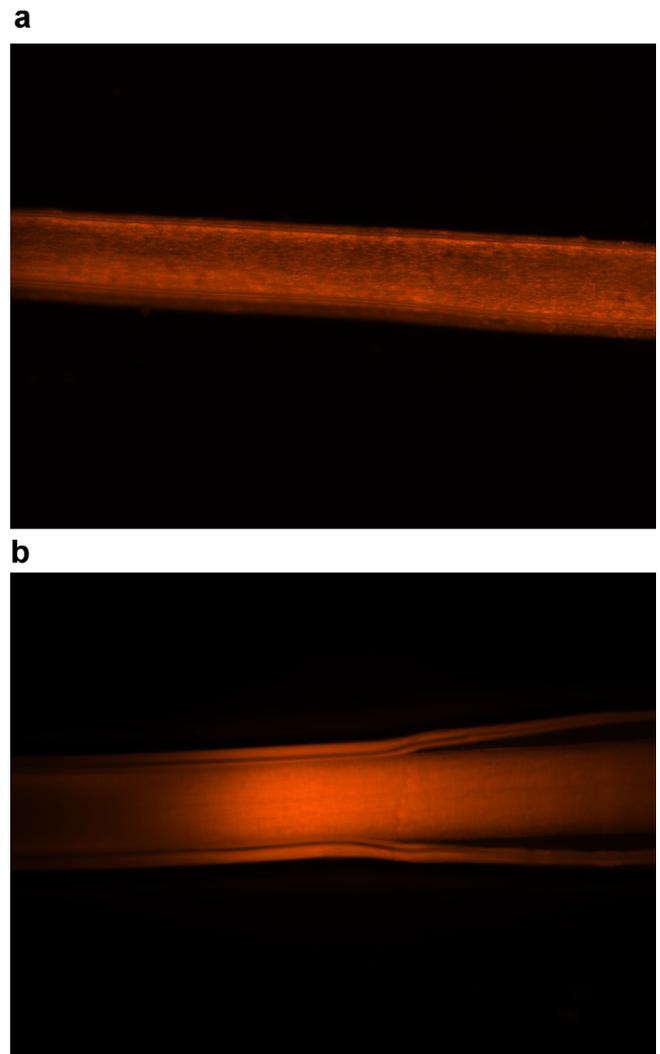
Thirty wild pigs (15 M, 15 F) were trapped and transported to captive pens to evaluate uptake of Rhodamine B in this study. These individuals ranged in size from 16 kg to 87 kg and included a range of age classes (juv–ad). Eleven pigs (5 M, 6 F) were euthanized 7 days post-RB administration and the remaining 19 (10 M, 9 F) were held for 14 days.

Under fluorescence microscopy, we failed to detect RB in any control sample of the 240 vibrissae and 240 guard-hair samples screened. From the 240 samples of each hair type collected post-RB administration, we observed RB marking in all individuals ( $\hat{\pi} = 1.0$ ) for both sample types and in 98% of screened vibrissae and 100% of guard-hair samples. The minimum number of vibrissae manifesting an RB mark was 6 (of 8) and all guard-hair samples screened exhibited evidence of RB marking. Accordingly,  $\hat{d}$  was estimated as 0.975 among post-RB vibrissae samples and 1.0 among guard-hair samples. These results suggest that a sample of 8 vibrissae or guard hairs provided exceptional statistical power to quantify RB uptake with a probability of false detection of essentially zero. Although both guard-hair and vibrissae samples consistently exhibited marking indicative of RB presence, the manifestation of RB staining differed between sample types. In vibrissae, RB manifested as a distinct bright band, whereas in guard-hair RB was visible as more uniform, lower

intensity marking across a longer section of the sample (Fig. 1), although more discrete banding was observed for some samples. Despite the lack of clear bands, guard-hair samples from RB-marked individuals could clearly be distinguished from control samples because no marking consistent with RB was observed in any control samples. No differences were observed between males and females or between 1- and 2-week marking periods in the number of individuals for which we observed evidence of RB marking.

## DISCUSSION

Efficacy of RB as a bait marker has been demonstrated for a wide array of taxa (reviewed in Fisher 1999), although few studies have evaluated RB for use in ungulates (Fleming et al. 2000, Webb et al. 2000). Our results suggest that RB can be used as an efficient biomarker to elucidate the proportion of wild pigs consuming baits that contain toxicants, vaccines, or



**Figure 1.** Guard hair (a) and vibrissae (b) of wild pigs (*Sus scrofa*) held captive on the Department of Energy’s Savannah River Site located in South Carolina, USA, during autumn 2013. Hairs show fluorescent markings following ingestion of Rhodamine B. Examples of control vibrissae are not shown because all slides were evaluated against a black background and controls are not easily visible due to the lack of fluorescence.

other pharmaceuticals because markings consistent with RB exposure were observed in 100% of individuals after RB ingestion, regardless of age, weight, or gender. These findings were consistent among individuals sampled 7 and 14 days post-exposure, indicating that uptake rates can be assessed shortly after the dissemination of baits.

Worldwide, there is growing interest in the use of baits to deliver vaccines and toxicants to wild pigs, particularly in landscapes where traditional control methods have been ineffective at reducing or eliminating populations (Fleming et al. 2000, Brauer et al. 2006, Campbell et al. 2006). In some regions these programs have been successful at controlling localized populations of pigs (e.g., Twigg et al. 2005), although questions remain regarding appropriate designs for baiting operations and the potential effects to non-target species. Given that RB has been shown to efficiently mark numerous non-target species likely to encounter baits (e.g., coyotes [*Canis latrans*; Fisher 1999], raccoons [*Procyon lotor*] and Virginia opossums [*Didelphis virginiana*; Smyser et al. 2010]), our results suggest that RB baits could be a cost-effective means of assessing bait-distribution methods and potential risks to non-target species prior to implementation of baiting programs for wild pigs.

As observed for other species, evidence of RB marking can be distinguished through sampling of either guard hair or vibrissae in pigs with a high degree of certainty. However, assessments of RB uptake in guard hair should be limited to fluorescence microscopy because guard hairs evaluated using ambient-light or ultraviolet-lamp detection methods may greatly underestimate the proportion of marked individuals (Weerakoon et al. 2013). Our results support these findings because evidence of RB marking was generally less intense in guard hairs, suggesting sampling of vibrissae may be better suited for quantifying RB uptake in pigs. This may be particularly important for individuals with white or light-colored hair due to low levels of natural autofluorescence in these hair colors that may be indistinguishable from low-intensity marks. Rhodamine B also is only available in the bloodstream for a few days and thus may be more efficiently integrated into vibrissae, which exhibit continuous growth and contain more vascularized follicles than guard hairs, which undergo active growth during discrete times of the year (Fisher 1999).

Although evidence of RB marking was observed for all pigs used in this study, individuals were dosed at 30 mg/kg and thus the minimum dosage needed to observe evidence of RB marking is unknown. High concentrations of RB also could potentially alter bait palatability, although the influence of RB concentration on bait acceptance has not been evaluated for pigs. Weerakoon et al. (2013) reported RB can be detected in rodents at doses as low as 3.9 mg/kg when using fluorescence microscopy, suggesting baits incorporating standardized doses of RB based on average body sizes should be sufficient to discern uptake for even the largest individuals. Wild pigs also are likely to consume multiple baits at bait stations, further increasing the utility of RB as a systemic biomarker in pig management. Although the number of RB baits consumed by an individual in a single day

cannot be quantitatively assessed using fluorescence microscopy (Massei et al. 2009), consumption of RB baits on multiple occasions may be distinguishable, particularly if multiple days occur between exposures (Fisher 1999). However, for many species a single bait often can contain sufficient toxicant or vaccine to treat an individual, and thus the ability to discern the actual number of baits generally is unnecessary for most species targeted with vaccine or toxicant baits.

Other biomarkers (e.g., iophenoxic acid, tetracycline – Campbell et al. 2006, Massei et al. 2009, Ballesteros et al. 2011, Reidy et al. 2011) also have shown promise for assessing bait uptake in mammals, including wild pigs, and may be suitable for long-term or quantitative assessments of bait uptake (Massei et al. 2009). However, these markers may have limited utility in large-scale management operations for wild pigs because they require collection of blood or bone samples. Although implementation of baiting programs for wild pigs often involve lethal sampling or collection of samples from deceased individuals, collection of blood or bone may not be practical in all circumstances and requires specialized training, storage, and processing of samples. In contrast, guard hair or vibrissae can easily be collected by untrained personnel, require minimal storage or collection equipment, and can be analyzed for presence of RB at a minimal cost. Despite these advantages, the ability to detect RB is limited by the persistence of hair and vibrissae, which are shed periodically. Previous studies have documented persistence of RB markings ranging from 7 (house mouse [*Mus musculus*]) to 28 (e.g., mountain beaver [*Aplodontia rufa*]) weeks for a variety of taxa (Lindsey 1983, Jacob et al. 2002, Spurr 2002). Similarly, Robertson et al. (2013) estimated an average retention period for vibrissae in Eurasian badgers (*Meles meles*) of 104 days. These studies, combined with our data, suggest that assessment of bait uptake in pigs can conservatively be performed between 1 and 12 weeks post-RB treatment. Thus, RB alone, or in combination with other markers, could be used to develop comprehensive bait-distribution strategies for the dissemination of pharmaceuticals to wild pigs.

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