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Evaluation of rhodamine B as a biomarker for raccoons

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***Abstract*:** The USDA/APHIS/Wildlife Services (WS) oral rabies vaccination program uses tetracycline, a broad-spectrum antibiotic and relatively reliable biomarker, to quantify vaccine-bait uptake by raccoons (*Procyon lotor*). However, obtaining samples (e.g., bone or teeth) to assess tetracycline uptake is highly invasive, and sample preparation can be expensive. By contrast, rhodamine B, a commercially available dye, is absorbed systemically in growing tissues, including hair and whiskers, and can be observed under ultraviolet (UV) light as fluorescent orange bands. Our goal was to evaluate whether rhodamine B can be used as a biomarker to monitor bait uptake by raccoons. We began by orally administering a solution containing 100 mg, 150 mg, or 200 mg of rhodamine B to captive raccoons. We monitored whisker and hair samples for fluorescence using a hand-held UV lamp and a fluorescent microscope for 13-weeks post-treatment. All raccoons that were administered rhodamine B exhibited fluorescence in their whisker and hair samples during the course of the study. Our ability to detect fluorescing whiskers varied based on the method of detection and time interval, but not with rhodamine B dosage level. We detected rhodamine B in 81% of marked individuals using the fluorescent microscope and 58% of marked subjects using the UV lamp. We were able to detect rhodamine B when doses as low as 1 mg/kg were given. Raccoons did not exhibit a taste aversion to baits containing ≤3% rhodamine B. We believe that rhodamine B can be a useful biomarker for raccoons and has potential as an application to monitor the uptake of oral rabies vaccine.

Key words: bait marker, biomarker, human–wildlife conflicts, oral rabies program, *Procyon lotor*, rabies, raccoon, rhodamine B

BIOLOGICAL MARKERS (i.e., biomarkers) cause alterations in baseline cellular, biochemical, or molecular characteristics that can be used to identify when an event or physiological process of interest has occurred in an individual. In wildlife disease management, biomarkers have been used to provide evidence of exposure to vaccine baits. The purpose of distributing vaccine baits is to lower the proportion of susceptible (i.e., nonvaccinated) animals in a population so that the probability of an infected animal encountering and infecting a susceptible animal becomes too low to maintain the chain of infection. Generally, vaccination coverage of 70% is considered sufficient to break disease transmission cycles (Hethcote 1978). A biomarker-based baiting strategy, such as that used by the U.S. Department of Agriculture, Wildlife Services' (WS) national oral rabies vaccination program, should include a post-hoc assessment of bait uptake, followed by additional bait deployment if vaccination coverage is determined to be <70%. For such a strategy to be successful, timely information on bait uptake is essential.

Wildlife Services has incorporated tetracycline as a biomarker in bait blocks used to distribute the RABORAL V-RG® (Merial, Ltd., Duluth, Ga.) oral rabies vaccine to raccoons since 1990. Tetracycline chelates with calcium ions in teeth and bones and produces a fluorescent mark under ultra-violet (UV) light (Savarie et al. 1992). Although tetracycline has proven to be a reliable biomarker for bait consumption, several concerns exist regarding its practicality (Fry and Dunbar 2007). First, recovering samples from animals is laborious and requires either euthanasia to retrieve a bone sample or anesthesia to extract a tooth. Second, sample preparation and analysis are intensive processes requiring a highly-trained individual, diamond-blade saw, and a fluorescent microscope (Johnston et al. 1999). There also is controversy related to introducing tetracycline into the environment. Wide-scale use of antibiotics has been linked to the

development of antimicrobial resistance and the perpetuation of antibiotic-resistant genes in bacteria (Levy 1998). Given those concerns, a better biomarker is desirable.

Rhodamine B, a pink dye, has been used as coloration of lipstick by the cosmetic industry, in microscopy, and as a water-tracing agent (Smart 1984). Rhodamine B is used extensively as a biomarker in Australia (Fisher et al. 1999), Africa (Knobel et al. 2002), and Europe (Southey et al. 2002), and has been tested on a number of wildlife species native to the United States, including coyotes (*Canis latrans*; Johns and Pan 1981), mountain beavers (*Aplodontia rufa*; Lindsey 1983), and jackrabbits (*Lepus* spp.; Evans and Griffith 1973). Rhodamine B, like tetracycline, is deposited in annuli of teeth (Ellenton and Johnston 1975) and can be used to monitor serial exposure to baits, and offers several practical advantages over tetracycline. Rhodamine B stains the buccal cavity and is subsequently absorbed through diffusion in growing keratinous tissues, such as nails, hair, and whiskers (Clark 1953). Unlike tetracycline, which requires tooth extraction, rhodamine B requires a simple noninvasive procedure—extraction of whiskers and hair—to detect its presence. Moveover, whisker and hair samples can easily be examined under UV light for the presence of a fluorescent orange band, confirming exposure to rhodamine B. Finally, multiple exposures to rhodamine B, and, thus, exposure to multiple baits over time, can be observed as discrete fluorescent bands in the hair or whisker as long as they are growing when rhodamine B is ingested.

We evaluated the utility of rhodamine B as a biomarker to indicate bait uptake by raccoons (*Procyon lotor*). Our objectives were to (1) develop methods for sampling and evaluating exposure to rhodamine B, (2) determine the duration over which rhodamine B is detectable in raccoons, and (3) identify a minimum dosage that will mark raccoons.

Methods

We used Tomahawk™ box traps (Tomahawk Live Trap Co., Tomahawk, Wis.) to capture raccoons in Larimer County, Colorado, during the spring of 2006 and the summer of 2007. We transported captured individuals to the Outdoor Animal Research Facility at the National Wildlife Research Center (NWRC), in Fort Collins, Colorado. Raccoons were held in accordance with Institutional Animal Care and Use Committee guidelines under USDA-APHIS-QA-1410, QA-1483, and QA-1557. We gave animals a health screening, administered anti-helmintic medication, and housed them in 3- × 3- × 2.5-m pens that included den boxes and enrichment structures. We used raccoons captured in the spring of 2006 (hereafter, 2006 cohort) to identify sampling and evaluation methodology and persistence of rhodamine B. We used a second cohort of raccoons captured in summer of 2007 (hereafter, 2007 cohort) to identify a minimum dosage and assess potential taste aversion to rhodamine B.

To evaluate rhodamine B exposure and extent of detectability evidence, we randomly assigned 20 raccoons (14 females, 6 males) from the 2006 cohort to 1 of 3 treatment groups (n = 6 individuals per group) or a control group (n = 2 individuals). We dissolved 23.3 mg of rhodamine B in 1 ml of distilled water to create an aqueous solution suitable for oral dosage and evaluated which of 3 dosage levels (100 mg, 150 mg, and 200 mg; see Table 1 for corresponding dosages) most effectively marked raccoons. We lightly sedated all raccoons using a mixture of ketamine hydrochloride (10 mg/kg) and xylazine hydrochloride (2 mg/kg), and used a 10-cc needleless syringe to administer oral doses of the rhodamine B solution. Dosage by weight ranged from 9.2 to 32.2 mg/kg, with an average of 18.5 mg/kg (Table 1). We orally dosed individuals assigned to the control group with 6.4 ml of water. We chose to orally dose raccoons, rather than allowing them to ingest baits containing rhodamine B, because our primary objective was to confirm if specific doses of rhodamine B marked raccoons. No raccoons were observed to regurgitate the rhodamine B solution.

Following the one-time dosage event, we restrained raccoons in a squeeze trap or other appropriate device, and collected an average of 3 whiskers (range 1–5) by plucking from the root using forceps or needle-nose pliers. Plucking guaranteed that the entire whisker was collected, a preferable option to cutting which may result in the portion of the whisker expressing fluorescent bands being retained by the raccoon. We also collected a small

tuft of guard and body hairs to determine if fluorescence was detectable in these hair types. Our sampling began immediately prior to rhodamine B dosage (day 0) and continued once a week for 13 weeks, a duration consistent with WS post oral rabies vaccination sampling procedures. Because rhodamine B is detectable only in hair and whiskers that are growing at the time of biomarker ingestion, we minimized the number of whiskers collected on each sampling interval to ensure that enough whiskers were available to last the duration of the study. For each individual sampled, we examined all whisker and guard and body hairs collected for fluorescence. We prepared samples according to procedures described by Fischer (1998). We fixed cleaned hair and whiskers to a standard microscope slide using Fluoromount-G™ (SouthernBiotech, Birmingham, Ala.), a water-soluble, non-fluorescing compound for mounting slides. We viewed slides using 2 methods: a handheld UV lamp with 3× magnification and 2 long-wave UV bulbs that emitted a wavelength of 365 nm at 20.3 cm (Q-22B, Spectroline, Westbury, N.Y.); and under 2.5× magnification using a fluorescent microscope comprised of a 100W high-pressure mercury bulb and a rhodamine filter block, TRITC (Leica, Germany). We used the handheld UV lamp in a dark, windowless room. A single observer used both methods to examine all samples for evidence of fluorescence. We calculated the proportion of individuals whose whisker and hair samples exhibited a fluorescent band, and considered an individual marked when we detected a band on ≥1 whisker or hair.

Proportional data were not normally distributed, so we used an arcsine-root transformation to induce homoscedasticity (Smith 1976). We then used general linear mixed–models ANOVA to characterize factors influencing the proportion of individuals exhibiting fluorescent bands in whiskers and hairs. This method enabled the fitting of random terms and therefore accounted for repeated sampling across error terms. We used restricted maximum likelihood (REML) methods for model estimation, Satterthwaite's F–tests to gauge effects (McCullagh and Nelder 1989), and first-order autocorrelation as a covariance structure. In all models, the proportion of marked individuals was the response variable, and individual nested within dosage level was a random effect. For our analysis of fluorescence in whiskers, dosage level (100, 150, or 200 mg of rhodamine B), sampling interval, and detection method (UV lamp or fluorescent microscope) were fixed effects. For our analyses of guard and body hairs, detection method was omitted as a fixed effect; we were unable to detect fluorescing guard and body hairs using the UV lamp. We tested all 2-way interactions of fixed factors and made *a posteriori* pairwise comparisons using least squares means tests (Zar 1999). Significance for all tests was set at an alpha of 0.05. We conducted a post-hoc power analysis using data from the study to assess the optimal number of whiskers needed to detect exposure to rhodamine B. Using the conservative estimate that 58% (SD = 0.357) of whiskers from rhodamine-B-positive animals showed fluorescence, we used the following equation to determine sample size:

$$n = \log(\alpha) \div \log(\beta) \quad (1)$$

where α = 0.01, and β = 1- probability of detecting fluorescence (0.58).

At the conclusion of this component of the study, raccoons from the 2006 cohort were immobilized with a mixture of ketamine

Table 1: Rhodamine B dosage rates for raccoons. All doses marked raccoons.

Rhodamine B dose	*n*	Minimum (mg/kg)	Maximum (mg/kg)	Average (mg/kg)
Control	2			
200 mg[a]	6	18.1	32.2	22.9
150 mg[a]	6	14.7	25.6	20.0
100 mg[a]	6	9.2	16.7	12.7
75 mg	8	6.3	13.6	11.4
50 mg	8	3.8	7.46	6.2
25 mg	8	2.1	4.6	3.8
15 mg	8	1.5	2.25	2.2
10 mg	8	1.0	1.4	1.2

[a]Rhodamine B dose volume was 23.3 mg/mL.

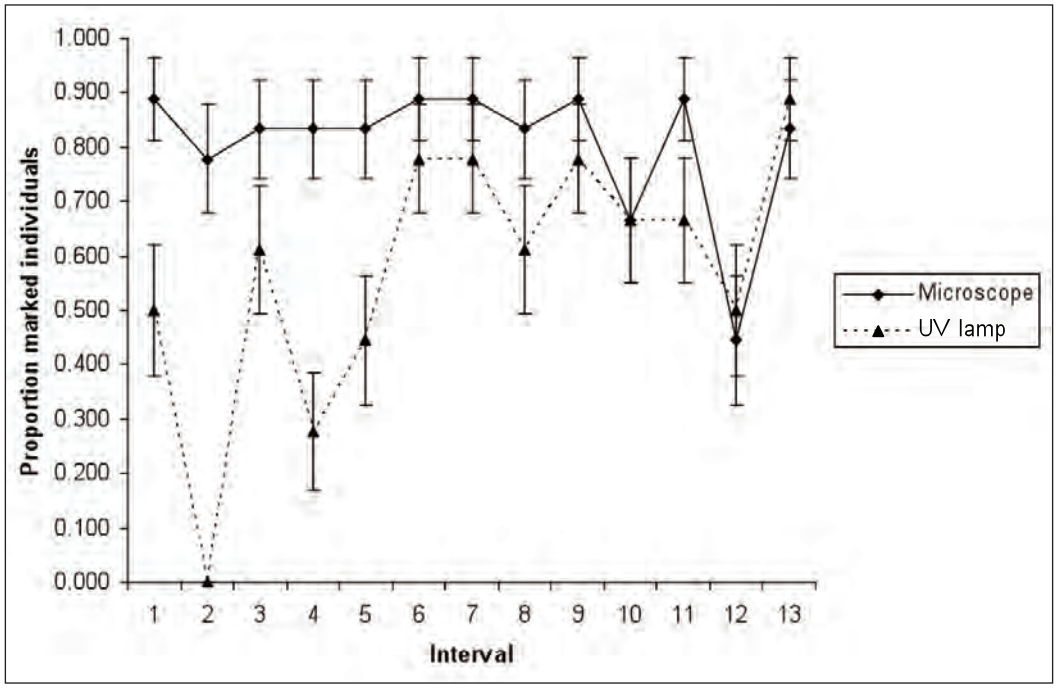

Figure 1: Proportion of racoons whose whiskers were marked by rhodamine B using both the UV microscope and a hand-held UV lamp over 13 weeks of sampling.

hydrochloride (10 mg/kg) and xylazine hydrochloride (2 mg/kg), and euthanized with an injection of Beuthanasia-D Special (0.25 m/kg; Schering-Plough, Union, N.J.). A veterinary pathologist at the Colorado State University Veterinary Diagnostic Laboratory necropsied all raccoons to assess possible toxicological effects of rhodamine B. Gross and histopathological examination was conducted on tissues, including, but not limited to, brain, liver, thyroid, lung, lymph nodes, heart, kidney, spleen, intestine, and stomach. We did not sample blood, urine, and feces due to the reported lack of persistence of rhodamine B in these substances (Fisher 1998).

We used the 2007 cohort of 8 raccoons (2 females, 6 males) to identify a minimum dosage effective in marking raccoons, and whether raccoons displayed a taste aversion to rhodamine B. We added rhodamine B at a concentration of 3% to the raccoon's daily kibble (Mazuri Omnivore diet A, PMI Nutrition International, Shoreview, Minn.) by adding 3 g of rhodamine B to every 100 g of ground kibble, and reconstituted the mixture using water, corn oil, and a minimal amount of flour. We reformed the kibble, dried it at room temperature for 3 days, and stored it under refrigeration until used. We gave each raccoon the appropriate mass of kibble to ensure that dosages of 75 mg, 50 mg, 25 mg, 15 mg, and 10 mg of rhodamine B were ingested. Dosages were given a minimum of 15 days apart, and all raccoons received the same dosage level on the same day. Dosage level was reduced sequentially to determine a minimal effective dose. We fed the rhodamine B food between 0700–0900 hours and recorded whether food had been consumed at 3-hour intervals for the first 9 hours, and then at 24 hours.

We extracted 1 to 8 whiskers from each raccoon 10 to 14 days after they had eaten food containing rhodamine B. Allowing 10 to 14 days between rhodamine B consumption and sampling was sufficient to create independent bands in whiskers; thus, by the time raccoons were offered 10 mg of rhodamine B, 5 bands were observable. We stored whiskers in plastic zip-lock bags, and prepared and evaluated them as described above. We recorded data on the number of positive whiskers using both the hand-held UV lamp and fluorescent microscope.

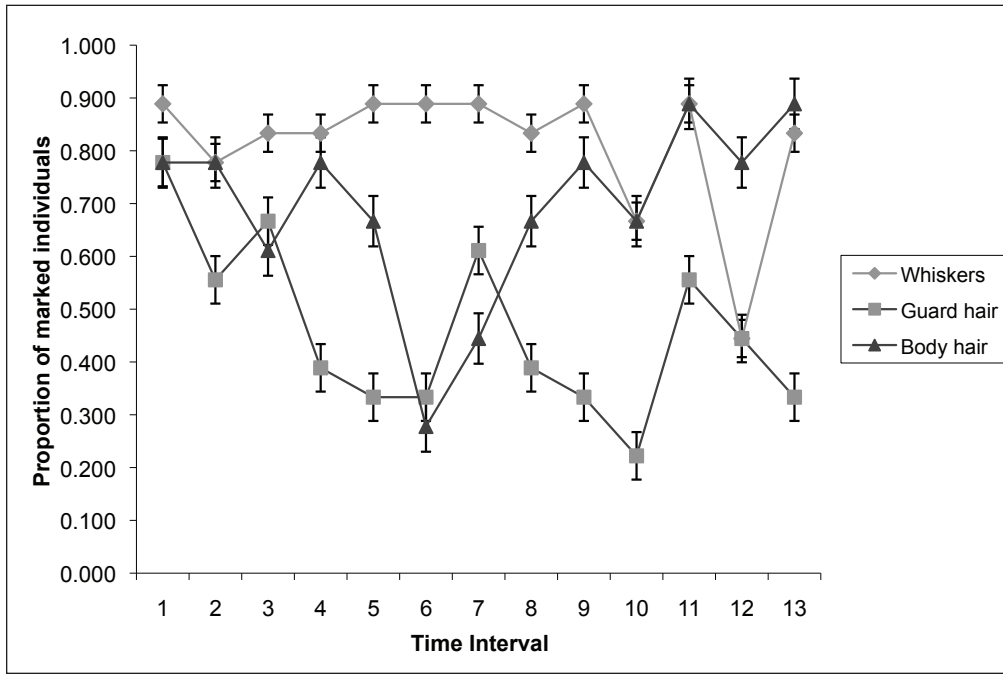

Figure 2: Proportion of raccoons whose whiskers, guard hair, and body hair were marked by rhodamine B using the UV microscope over 13 weeks of sampling.

Results

All raccoons fed rhodamine B ($n = 18$) showed evidence (i.e., bands) of fluorescence in their whiskers, whereas the 2 control animals did not. Our ability to detect fluorescing whiskers differed relative to method of detection and time interval (method × interval: $F_{10,172} = 3.69$, $P < 0.002$; Figure 1), but not with dosage level ($F_{2,15.5} = 0.50$, $P = 0.61$). On average, we were able to detect fluorescing whiskers in 81% and 58% of individuals sampled at each interval when using the fluorescent microscope and UV lamp, respectively. The proportion of marked individuals detected using the fluorescent microscope was consistently greater than with the UV lamp, with the exception of weeks 12 and 13. At week 12, the proportion of marked individuals detected using the microscope declined by 50% from the previous interval, but then increased by 47% during the next week (Figure 1). When pooled across intervals and among individuals, 50% of all individual whiskers collected from treatment raccoons were positive for rhodamine B.

We were able to detect fluorescence in guard and body hairs with the microscope but not with the UV lamp; thus, we report fluorescence observed using the UV microscope (Figure 2). For guard hairs, our ability to detect fluorescent bands differed with dosage ($F_{2,72.4} = 13.39$, $P < 0.001$) and week ($F_{12,192} = 2.27$, $P = 0.01$). Least squares means tests indicated that the proportion of fluorescing samples from animals receiving either 150 or 200 mg of rhodamine B was greater than for those receiving a dose of 100 mg. For body hairs, our ability to detect fluorescent bands differed with week ($F_{12,193} = 4.03$, $P < 0.001$) but not with dosage ($F_{2,72.4} = 0.59$, $P = 0.68$).

All raccoons offered varying doses of food containing rhodamine B ingested the food within 24 hours except in 2 cases. In those 2 situations, individuals did not consume food containing 25 mg and 10 mg doses of rhodamine B, and we did not see fluorescent bands in the whiskers in each of these 2 circumstances. These 2 individuals consumed the food and exhibited fluorescence in their whiskers at all other doses.

Discussion

All raccoons that ingested rhodamine B were marked by the dye through fluorescence in their whiskers, regardless of dosage, and the mark persisted for at least 13 weeks. Our

results indicate that, as a biomarker, rhodamine B has several advantages over tetracycline, including ease in evaluating presence, long-term persistence, detectability using minimally invasive sampling methods, palatability, and affordability.

Analysis of methods used to evaluate whiskers and hair for fluorescence suggested that fluorescent microscopy was more successful than the hand-held UV lamp. Reliance solely on a hand-held UV lamp may result in underestimating bait uptake, and trigger the costly and unnecessary implementation of additional baiting and vaccination campaigns. Both methods, UV lamp and fluorescent microscope, have value and, when used in concert, represent an improvement over methods used to detect tetracycline uptake. The cost and ease of using the hand-held UV lamp makes it an appropriate option for biologists and managers in most field applications, whereas the precision of the fluorescent microscope allows for validation of samples when necessary.

Although fluorescent bands were detectable in both body and guard hairs, whiskers appear to be the better indicators of exposure to rhodamine B and offer several advantages over hair samples. First, unlike body and guard hairs, where growth is seasonal, whiskers grow year-round (Ling 1970), allowing biomarker uptake to be determined over a continuous temporal scale. Second, whiskers often are white and, thus, easier to evaluate than the bicolor body and guard hairs (Fisher 1998). Finally, individual whiskers are larger than hairs and easier to handle and prepare for microscopy.

A complicating factor in our study was that sampling occurred weekly for 13 weeks, and we collected a varying number of whiskers resulting in varying sample effort and repeated sampling of the same individuals. This intensive sampling increased the likelihood that whiskers extracted from a given individual were not growing when the animal ingested rhodamine B, and, therefore, were not marked. Our data indicate that it is necessary to collect 6 whiskers from an individual to determine with 99% confidence whether a raccoon has been exposed to rhodamine B.

Using a hand-held UV lamp, we were able to detect rhodamine B in samples for all animals that consumed food containing as little as 10 mg of the dye. The ability of low doses (e.g., 10 mg) to consistently mark raccoons makes the dye cost-effective and provides great potential for inclusion in small baits and with medications or vaccines. We did not randomly assign individuals from the 2007 cohort to dosage levels. Rather, individuals received each dosage sequentially, to ensure that every dosage level generated a fluorescent band and that serial exposure to baits was detectable. Rhodamine B is absorbed instantaneously and, in turn, flushed from the system within 48 hours (Fisher 1998). As a result, a fluorescent band should represent a discrete marker of a single dosing event. Our results confirm this notion: the 6 raccoons that ate all 5 dosages had 5 fluorescent bands visible while the 2 raccoons that only ate 4 dosages had 4 bands visible at the conclusion of the study.

Unlike Hanlon et al. (1989), we found that raccoons did not exhibit a taste aversion to food containing rhodamine B. We believe the divergent findings can be attributed to the fact that the rhodamine B concentration in baits used by Hanlon et al. (1989) was greater than the 3% concentration recommend to avoid taste aversion (Fisher 1998). However, Hanlon et al. (1989) reported that only 100 mg of rhodamine B solution was offered to raccoons in plastic sachets, and no concentration was noted. Our use of a 3% concentration was palatable to raccoons and likely had the added benefit of minimizing adverse effects on the environment, as well as target and nontarget animals.

Rhodamine B is classified by the U.S. Environmental Protection Agency as an inert Class 4B substance, and concerns related to its safety appear to be minimal (Clark 1953, Gangolli et al. 1971, Elliott et al. 1990, Fisher 1999). Research by Umeda (1956) suggests that rhodamine B is carcinogenic. However, that conclusion has been disputed (Goldberg 1967, Gangolli et al. 1971, International Agency for the Research of Cancer 1978). Pathology reports from the raccoons given 100 to 200 mg of rhodamine B were comparable to control raccoons, suggesting no deleterious effects at the dosages tested. All gross and histological findings of raccoons were consistent with diseases often found in free-ranging raccoons (Hamir et al. 1996, Mikaelian et al. 1999, Yoshikawa et al. 1999), with no indication

of pathological effects due to exposure to rhodamine B. Our finding of no immediate pathological effects is supported by others; Elliott et al. (1990) found no evidence that rhodamine B was a genotoxic threat, and others have found little indication that rhodamine B is mutagenic (Clark 1953, Fisher 1998). Finally, the oral lethal dose (LD50) of rhodamine B is >500 mg/kg in rats (Smart 1984) and 890 mg/kg in mice (Rochat et al. 1979, Fisher 1998), well below the dosage necessary to mark raccoons as part of the oral vaccination program.

Management implications

The use of biomarkers in wildlife vaccination programs is germane when information on population-level vaccine exposure is needed for timely assessment of program success. Since 1995, the WS oral rabies vaccination (ORV) program has used tetracycline as a biomarker to evaluate vaccination bait uptake. However, due to the logistics and cost of incorporating tetracycline into baits, the ORV program has been relegated to using tetracycline-marked baits in <50% of the ORV zones. Where tetracycline has been used, the time lag between laboratory submission and dissemination of results (up to 18 months) prohibits timely implementation of management actions. We estimate the replacement of tetracycline with rhodamine B can save the ORV program >$5 each time they attempt to detect the biomarker. Additionally, the use of hand-held UV lamps to measure population bait uptake will allow for rapid evaluation and timely implementation of supplemental baiting or other management interventions.

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