A Field-Test of Rhodamine B as a Biomarker in Raccoons

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ABSTRACT Rhodamine B is a dye that when ingested results in fluorescent bands in growing hair and whiskers of many mammals. Previous research at Wildlife Services' (WS) National Wildlife Research Center (NWRC) found that rhodamine B is a successful biomarker in raccoon whiskers and that raccoons do not have a taste aversion to the dye when it comprises ≤ 3% of a bait. Our study assessed the ease of bait distribution, whisker collection, and evaluation of the biomarker for potential use in the Oral Rabies Vaccination (ORV) program administered by the WS National Rabies Management Program (NRMP). In collaboration with WS operations personnel from Ohio and Michigan, 750 fishmeal polymer baits each containing 150 mg of rhodamine B were hand distributed at NASA's Plum Brook Station, Sandusky, Ohio in the summer of 2008. Four weeks after baits were distributed whiskers from 162 raccoons were collected. Wildlife Services biologists and technicians evaluated the whiskers for fluorescence using a handheld UV magnifying lamp. Biologists then sent the whiskers to the NWRC, Ft. Collins, Colorado for confirmation of fluorescence under a UV microscope. Results suggest a high level of agreement between the two methods of evaluation. Surveys completed by biologists confirmed that the ease of use, less invasive sampling techniques and promptness of results obtained through the use of rhodamine B are advantageous to the tetracycline biomarker presently used by the ORV program. All participants recommended further evaluation of rhodamine B for its inclusion in future efforts requiring biomarker evaluation.

KEY WORDS biomarker, *Procyon lotor*, rabies, raccoon, rhodamine B

Biomarkers are tools that use cellular, biochemical, or molecular characteristics to identify, often through indirect means, when an event or physiological process of interest has occurred in an individual. There are many uses for biomarkers in wildlife disease and damage management fields (Fry and Dunbar 2007). Vaccination programs have used biomarkers to provide evidence of an animal's exposure to vaccine baits and to develop contingency strategies to augment insufficient bait coverage. Additionally, biomarkers are used to identify nontarget species in lethal control operations. Biomarkers have also been used for a variety of other research applications including population, density studies, foraging and movement studies. There is also an increased interest in using biomarkers for wildlife contraception programs. Our research focused on improving the National Rabies Management Program's (NRMP) Oral Rabies Vaccination (ORV) Program administered by USDA, APHIS, Wildlife Services (WS).

Presently, the ORV program uses tetracycline as a biomarker to determine whether raccoons, coyotes, and fox have encountered and eaten rabies vaccine-filled baits. Tetracycline is both affordable and well understood. Its deposition in teeth and bones allows for temporal data to be gathered, thus multiple exposures over years can be understood from a single tooth. One disadvantage of tetracycline is that it requires invasive sampling methods via either tooth extraction or destructive sampling. Additionally, the deposition of tetracycline in teeth is not ubiquitous. Older animals whose teeth are growing at a much

slower rate may not efficiently take up the antibiotic; thus, incorrectly suggesting lower uptake in mature animals (Johnston et al. 1999). There is also some debate on the long term implications of releasing tetracycline into the environment (Levy 1998). For these reasons we were interested in assessing the feasibility of rhodamine B as a biomarker for field applications.

Rhodamine B is a xanthene dye and is listed by the Environmental Protection Agency (EPA) as a class 4B inert substance (EPA Reg. CAS No. 81-88-9). It is a relatively benign dye that has been used extensively in the cosmetics industry, pharmaceuticals, and microscopy and is a tracing agent used to understand water flow. Rhodamine B is a green powder that stains a bright pink color. Rhodamine B is both a physical and systemic biomarker and is absorbed "instantaneously" when ingested. Rhodamine B produces both short- and longer-term effects. Short-term effects last for up to a week and include dying of the fur, mouth, feces, urine, and may be also revealed in the blood sera for a few days. Longer-term effects include the manifestation of fluorescent bands in growing tissue such as fur, feathers, and whiskers visible under UV light. These results are similar to tetracycline fluorescent bands observed in teeth. The utility of rhodamine B has been tested in a number of mammal species as well as birds (Johns and Pans 1981, Lindsey 1983, Knowlton et al. 1987, Fisher et al. 1999). In each of the mammal species studied the results and persistence of the dye was similar.

Our research on the usefulness of rhodamine B as a biomarker for the ORV program involved three phases. First, we evaluated rhodamine B as a potential biomarker through captive animal studies that utilized varying doses of rhodamine B. In this phase we confirmed that doses as low as 25 mg resulted in fluorescent bands in raccoon whiskers. During this phase we also looked at the persistence of the dye and which hair types were best for detecting the biomarker. Next, we confirmed that raccoons did not exhibit a taste aversion to rhodamine B; again this occurred in a pen study. In the final phase and the focus of this report, we tested rhodamine B in a field setting. Our goal was not only to monitor the uptake of rhodamine B by free-ranging raccoons but also to attain feedback from biologists and technicians who distributed the baits, collected samples, and finally evaluated the results of uptake by raccoons through examination of whiskers.

METHODS

Our field evaluation of rhodamine B was conducted in conjunction with a previously scheduled NRMP raccoon density survey at NASA's Plum Brook Station, Sandusky, Ohio. We distributed 750 fishmeal polymer baits containing 150 mg of rhodamine B along transects that would also be used in the raccoon density study (Fig. 1). Baits were distributed across a 3 square-kilometer sampling area.

Figure 1. Fishmeal polymer bait containing 150 mg of rhodamine B.

Four weeks after baits were distributed we began trapping raccoons. We sampled 162 raccoons during the two week density

study. We collected 6–10 whiskers from each individual. When possible, we requested that biologists select light-colored whiskers to make evaluation more straightforward. We stored whiskers in clear zip lock bags and marked them with a unique ID number. We sampled recaptured raccoons once for whiskers. We stored whiskers in a dark location, a refrigerator or freezer, until they could be evaluated by biologists.

Biologists received varying training in how to distribute baits and evaluate whiskers. We provided each biologist with training materials including an instructional handout on safety precautions related to handling rhodamine B and whisker evaluation, which included photographs of whiskers positive for rhodamine B exposure. In addition, we consulted with the biologist by phone in 2 cases, trained 2 additional participants directly and the fifth individual was trained by one of the individuals previously trained by the researcher.

Five biologists viewed whiskers in a dark room using a handheld UV lamp with 3x magnification and 2 long-wave UV bulbs that emitted a wavelength of 365 nm at 20.3 cm (8 inches; Q-22B, Spectroline, Westbury, NY). Biologists then recorded the number of whiskers per individual and the number of whiskers fluorescing from that individual. Each individual raccoon was identified by a unique ID number and metadata including trapping location. We requested each of the biologists evaluate the whiskers independently. The primary investigator collected whiskers and data sheets for confirmation and analysis. After the evaluation of whiskers was completed we sent biologists a survey to evaluate their perception of rhodamine B as a biomarker and the ease of whisker evaluation (Fig. 2).

To confirm rhodamine B exposure in whiskers, we prepared samples according to procedures described by Fisher (1998). We fixed whiskers to a standard microscope slide using Fluoromount-G (Southern Biotech, Birmingham, AL) a water-soluble, non-fluorescing compound for mounting slides. We viewed slides under 2.5x magnification using a fluorescent microscope comprised of a 100W high pressure mercury bulb and a rhodamine B filter block (TRITC, Leica, Germany).

RESULTS AND DISCUSSION

Our earlier pen studies confirmed that rhodamine B marked the whiskers of raccoons at doses between 25–250 mg. We also demonstrated that raccoons would eat food containing the dye at concentrations of 1% and 3% (Fry et al. in press). Of the 162 raccoons sampled, 57 were positive for rhodamine B exposure using the UV microscope, which is considered 100% accurate (Fisher 1998). Biologists who used the handheld UV lamp were also quite accurate in detecting the fluorescence (Table 1). Biologists identified 47 individuals that were false positive and 15 unique individuals who consumed rhodamine B were not identified as positive by biologists.

Table 1. Results of whisker evaluation preformed by biologists. 57 animals were confirmed positive for rhodamine B via UV microscope.

Biologist	% correct	False Positives	False Negatives
1	53	33	6
2	72	17	11
3	68	20	9
4	82	0	11
5	86	0	12
$\bar{x}$ (±SD)	73 (13)	14 (14)	10 (2)

The greatest discrepancy between the results obtained by biologists and the UV microscope results were the number of false positive results; 47 unique animals were

Rhodamine B Survey

Part 1: Trapping and Collecting Whiskers

Please rate on a scale of 1 to 5

	Poor				Excellent
1. Ease of Whisker Collection	1	2	3	4	5
2. Was training appropriate –collection only	1	2	3	4	5

If collecting only for presence or absence of a biomarker would you prefer to pull whiskers or teeth?

Do you think it is feasible to collect whiskers from a raccoon that was not anesthetized? Why or why not?

Part II: Evaluating whiskers

1. Was training adequate for you to evaluate whiskers? If not, how could this be improved?

2. Did you expect that it would take a longer or shorter time to go through the 160 bags of samples? Be specific, what did you expect.

3. How confident were you that you correctly observed a fluorescent band?
   1 – Very confident >80%
   2 – Confident >60%
   3 - Not very confident > 40%
   4- Not very confident < 40 %
   5 – I guessed every time

4. What did you like about this biomarker and its analysis?

5. What did you dislike about the procedure?

6. Would you support the idea of using Rhodamine B as biomarker for the ORV program? Please tell me why.

Additional comments/concerns/suggestions:

Part III: Distribution of RB baits (do not answer if you did not distribute baits)

Please comment on the messiness of the baits and suggests improvements that you would like to see made.

Figure 2. Survey completed by biologists involved in rhodamine B bait distribution, sample collection and whisker evaluation.

considered false positive. We believe this is a result of the varying intensity of fluorescence exhibited in whiskers and could be remedied by increasing the number of photo examples provided to individuals evaluating whiskers. Having photos depicting varying intensities of fluorescence may have alleviated or reduced the occurrence of these false negatives. Fifteen rhodamine B positive individuals were not detected by biologists. Of the 15 undetected individuals, 6 animals were missed by all 5 biologists, 1 was missed by 4 biologists, and 3 were missed by 3 biologists. It is likely that the faint fluorescence, and thus undetected positive samples, results when an animal only ingests a fraction of the bait, and hence received a very low dose of rhodamine B. This concern is not unique to rhodamine B, similar ambiguity arises with other biomarkers including tetracycline.

We asked biologists to complete a survey regarding their experience with the whisker collection and evaluation. We used a 5-point scale to address many of the questions, with 5 out of 5 representing complete agreement with the statement. The first part of the survey accessed the biologists' responses to collecting whiskers for rhodamine B evaluation. When asked to rate the ease of whisker collection, the average response was 4.5 out of 5. Biologists agreed (4 out of 5 points) that the training provided on whisker collection was sufficient. All biologists agreed that whisker extraction was preferable to tooth extraction

when collecting samples to evaluate for biomarkers. Lastly, biologists concurred that sampling whiskers on non-anesthetized raccoons was a feasible option. We were very pleased with the results of the first part of the survey which suggested that biologists approved of the techniques used to collect whiskers for biomarker evaluation.

The next part of the survey assessed the biologists' responses to evaluating whiskers for rhodamine B. When asked if training was appropriate for evaluating rhodamine B in whiskers most agreed it was appropriate, but suggested that additional photo references be made available that show varying intensities of fluorescence. This suggestion will be incorporated into future rhodamine B evaluation. Biologists were prepared for the amount of time it took to evaluate whisker samples (2–2.5 hours for 162 raccoons) and appreciated determining the results of their work rather than simply sending samples to the laboratory for evaluation. All biologists believed their results using the handheld UV monitor to be at least 60% accurate, which is supported by the UV microscope evaluation and resulting comparison.

A third part of the survey requested input on the ease and cleanliness of rhodamine B bait distribution. Only one biologist participated in bait distribution and responded affirmatively that baits were not difficult, nor excessively messy to handle, when proper guidelines were followed.

Results from this study are promising. We are pleased to report that wild raccoons not only accepted rhodamine B but also were successfully marked by the biomarker. The biologists involved in this study unanimously approved the use of the biomarker and provided consistent results. We suggest that if rhodamine B is used for future rabies vaccine baiting campaigns that additional training materials be provided and that a sub-sample of whiskers collected in the field be evaluated using a UV microscope to accurately determine an error rate among whiskers and between biologists. The ease of use makes rhodamine B an ideal biomarker for inclusion in large scale vaccine distribution programs.

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