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PROPOSED FIELD EVALUATION OF A RABIES RECOMBINANT VACCINE FOR RACCOONS (*PROCYON LOTOR*): SITE SELECTION, TARGET SPECIES CHARACTERISTICS, AND PLACEBO BAITING TRIALS

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ABSTRACT: Prior to a limited field application of an orally-administered vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine for wildlife, background data were obtained for the proposed site on Parramore Island, Virginia (USA). Mammalian target and nontarget species, potentially at risk for exposure to vaccine were inventoried. Placebo baiting trials with a fishmeal polymer bait resulted in high bait disturbance (88 to 100%), primarily by raccoons (*Procyon lotor*), with infrequent visitation and no evidence of bait consumption by deer (*Odocoileus virginianus*), small mammals or avian species. Definitive bait acceptance rates by raccoons (indicative of bait ingestion) were difficult to accurately determine based exclusively on premolar and vibrissae samples collected antemortem from live-trapped raccoons for tetracycline and rhodamine B biomarker analyses, respectively. Bait acceptance rate was more accurately determined during a pilot baiting trial conducted on North Island, South Carolina, when mandibles (postmortem samples) were examined for tetracycline incorporation. Parasitologic findings in raccoons on Parramore Island included *Hepatozoan procyonis*, *Phagicola angrense* and *Physaloptera rara* and a variety of incidental microscopic lesions, and provided baseline pathological data for comparison subsequent to V-RG vaccine application. A population density estimate of one raccoon/2.7 ha was calculated using mark–recapture data for comparison after vaccine deployment. Limited reproductive data, including estimates of pregnancy rates by palpation, the number of live kits/litter live-trapped with previously pregnant raccoons or observed in the dens of radio-collared raccoons, was gathered to assess the effect of proposed oral vaccination with V-RG vaccine. Home ranges were assessed by radio-telemetry of 15 raccoons; all radio-collared raccoons currently reside on Parramore Island. Longest straight line distance travelled by raccoons was <2 km, except when animals were translocated and were found to return to their original range.

Key words: Rabies, recombinant vaccine, raccoon, *Procyon lotor*, bait, oral vaccination, biomarker, parasitologic survey.

INTRODUCTION

An ongoing rabies epizootic among mid-Atlantic raccoon (*Procyon lotor*) populations (Centers for Disease Control, 1988) coupled with the laboratory development of a safe, orally-efficacious, vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine for raccoons (Rupprecht et al., 1986) have led to intensified applied raccoon ecological research and baiting trials in preparation for field application of an oral rabies vaccine. Following extensive laboratory evaluation, a limited field trial of a recombinant vaccine at a biosecure site is a logical prerequisite to widespread mainland application in endemic rabies areas. Raccoon baiting studies in Pennsylvania and Virginia have shown that distribution of placebo baits containing tetracycline as a biomarker, at an approximate density of 1.2 baits/ha, achieved bait acceptance levels as high as 76%, based on tetracycline positive teeth and bones from live-trapped and hunter-collected raccoons (Rupprecht et al., 1987; Perry et al., 1989). Given these encouraging preliminary observations, and pending federal,

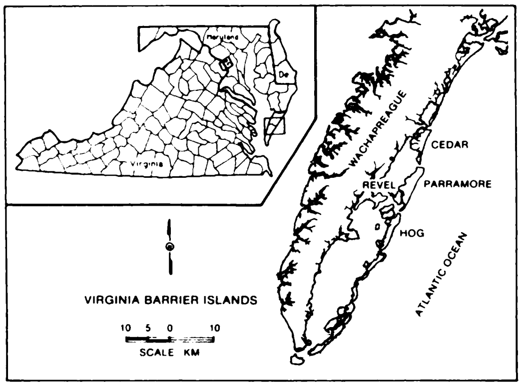

FIGURE 1. Eastern shore of Virginia (USA) and Parramore Island.

state, and local approval for a limited vaccine field trial in the USA, several potential field sites are under consideration. One such candidate study area is Parramore Island, Virginia, due to its geographical isolation, diverse habitat, relatively large and stable raccoon population, and lack of known endangered or threatened species (Dueser et al., 1979). Primary objectives of this study were to describe mammalian species diversity, relative population parameters of island raccoons, the interaction and exchange of island raccoons with those of the mainland or nearby islands, baseline rabies and raccoon poxvirus antibody titers, and raccoon bait preferences in an effort to further improve bait delivery techniques. The findings of this pre-immunization study provided background data for comparison during the proposed vaccine field trial, in order to assess the potential ecological effect of a V-RG recombinant vaccine resulting from intentional environmental release.

MATERIALS AND METHODS

Study site description

Parramore Island (USA; 37°11'N, 75°38'W) is the largest and most biologically diverse of the Virginia barrier islands comprising the Virginia Coast Reserve, a reserve of approximately 14,000 ha of barrier islands, salt marsh, mainland fields and forests along the Atlantic Coast of the Eastern Shore of Virginia (MacLeod and Hennessey, 1981). Parramore Island contains 3,440 ha (880 upland ha), is 12.8 km long and 1.2 to 2.0 km wide, and is 7.7 km from the mainland and the nearest town of Wachapreague, Virginia (USA; Fig. 1). Except for a U.S. Coast Guard Station with a crew of 10 to 12 located on the north end of Parramore Island, there are no permanent human inhabitants. Parramore Island is completely surrounded by water; the Atlantic Ocean defines its eastern edge. The two nearest barrier islands, Cedar Island to the north and Hogg Island to the south, are separated from Parramore Island by ocean inlets 10 to 15 m deep and 1.5 km wide. The western side of Parramore Island consists of salt marsh ranging in width from 0.2 to 2.0 km, terminating against Swash Bay or broad tidal channels. On the west side of the southern third of Parramore Island is Revel's Island, a small bayside island. There is physiogeographic evidence that part of the upland areas of these two islands once comprised a common ancient sand dune (MacLeod and Hennessey, 1981). These islands are currently separated by a tidal gut called "The Swash," less than 0.3 km wide and 2.0 to 4.0 m deep at mean low tide. Parramore Island and Revel's Island are separated from the mainland by extensive expanses of salt marsh and four major bays: Bradford Bay, Revel's Island Bay, Swash Bay and Upshur Bay. Additional biotic information includes a census of herpetofauna, avifauna and mammalian species, in addition to a description of flora and a detailed vegetation map (McCaffrey, 1975; Dueser et al., 1976, 1979; Dueser and Brown, 1980; MacLeod and Hennessey, 1981). Typical upland vegetation includes loblolly pine (*Pinus taeda*), wax myrtle (*Myrica cerifera*), red cedar (*Juniperus virginiana*), red bay (*Persea palustris*), yaupon holly (*Ilex vomitoria*), black cherry (*Prunus serotina*), and greenbriar (*Smilax* sp.) (McCaffrey, 1975).

Besides placebo bait evaluation on Parramore Island, an additional baiting trial was conducted on North Island, South Carolina (USA; 33°13'N, 79°11'W). North Island is part of the Tom Yawkey Wildlife Reserve; it has 834 ha of upland, 1,703 ha of wetland, and 576 ha of beaches and dunes. Habitats are representative of the South Carolina barrier island systems including beaches, sand dunes, shrub thickets (primarily *Myrtle* spp.), fresh and saltwater marshes, loblolly pine forest and tidal mud flats (Sandifer et al., 1980).

Identification of target and nontarget species

Mammalian species composition on Parramore Island was inventoried by direct observations, the use of Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin 54487, USA) baited with fresh fish and limited small mammal trapping in upland areas using Sherman live traps (H. B. Sherman Traps Inc., Tallahassee, Florida 32301, USA) baited with oatmeal and peanut butter as described by Dueser et al. (1979). Live-trapped furbearers were sedated with a mixture of 10 mg/kg ketamine (Veterinary Products, Bristol Laboratories, Division of Bristol-Meyers Co., Syracuse, New York 13220, USA) and 0.4 mg/kg xylazine (Haver, Bayvet Division, Miles Laboratory, Inc., Shawnee, Kansas 66203, USA) administered intramuscularly. After recording the sex, age (Sanderson, 1950) and weight, the animals were ear-tagged (National Band and Tag Co., Newport, Kentucky 41072, USA), and in some cases, radio-collared (Lotek Engineering Inc., Aurora, Ontario, Canada L4G 4J9), blood samples were collected, as described below and the animals were then released. During baiting trials, the right first upper premolar and several vibrissae were collected for biomarker analyses from live-trapped furbearers. Live-trapping was used to estimate the number of raccoons/unit area with calculation of the Peterson-Lincoln population estimate and standard error: $SE(N) = (((M^2(n + 1)(n - m))/((m + 1)^2(m + 2)))^{1/2}$, using Bailey's formula for a closed population ($N = M(n + 1)/(M + 1)$; where M is the number of raccoons marked, n is the total number of raccoons observed, and m is the number observed that were marked) (Bailey, 1952; McCullough and Hirth, 1988).

Rabies and raccoon poxvirus seroprevalence

Blood samples were collected with a 5 cc syringe and a 20 ga needle via the jugular or anterior vena cava from all live-trapped raccoons and red foxes (*Vulpes vulpes*), while they were sedated for physical examination and ear-tagging. Serum samples were removed from clotted blood samples and frozen at −20 C for subsequent determination of pre-immunization rabies neutralizing antibody titers, by a modification of the rapid fluorescent focus inhibition test (Reagan et al., 1983). In addition, raccoon poxvirus antibody titers were determined by a virus neutralization test (Wiktor et al., 1984). The sample size (95% confidence level) required for a serological survey at or above a 20% prevalence level (Alexander et al., 1972) was derived from Cannon and Roe (1982) using the following formula: $n = [1 - (1 - a)^{1/D}][N - (D - 1)/2]$, where n is the required sample size, a is the probability (confidence level) of observing at least one diseased animal in sample when the disease affects at least D/N in population, D equals the number of diseased animals in population, and N is the population size. The effective raccoon population size was calculated as described above.

Parasitologic and pathologic survey

Field postmortem examination and histopathology was conducted on any furbearers found dead in the field. In addition, a sample of the raccoon population (N = 23) on Parramore Island was live-trapped for incidental pathological findings. Animals were euthanized by the intravenous administration of sodium pentabarbital (Euthanasia-6 Solution, Vet Labs Limited, Lenexa, Kansas 66215, USA). Tissues collected for routine field necropsy included brain and representatives of all major organs, and were processed for routine microscopic examinations as previously described (Rupprecht et al., 1986).

Radiotelemetry

Fifteen adult raccoons of both sexes (nine females, six males) on Parramore Island were fitted with radio-collars transmitting at 150.0 to 150.9 kHz with activity and mortality modes. Animals were monitored twice daily for 3 wk, once daily for 45 days and twice weekly thereafter from January to September 1988 with a telemetry receiver (Wildlife Materials Inc., Carbondale, Illinois 62901, USA). Locations were determined by triangulation of telemetry fixes and plotted on U.S. Geological Survey topographical maps. Because one Parramore Island raccoon was occasionally located on Revel's Island, a translocation study was initiated to determine how readily raccoons would cross a small tidal gut (0.3 km wide, 2 to 4 m deep at mean low tide) separating Parramore Island and Revel's Island to return to their normal range. A Parramore Island adult male raccoon was radio-collared and released on Revel's Island, approximately 9.5 km from the original trap site. Two Revel's Island residents, an adult male and a pregnant female, were radio-collared, transferred to Parramore Island, and released 1.6 km from their original trap sites.

Bait preference studies

Raccoon bait preferences were determined using direct observation trials conducted from a blind in an abandoned building on Parramore Island from March through September 1988. The baits were placed in a cleared area 20 m from the observation point. Observers and baits were in place 1 hour before sunset. After dark, illumination was provided by a 300,000 candle-power floodlight connected to a 12 V automobile battery. Direct observation was coupled with videotaping using a telescopic lens for greater detail. Smorgasbord bait trials were utilized for initial qualitative assessment of raccoon preferences. Fifteen individual repetitions of a smorgasbord test were conducted that included the presentation of eight different varieties of experimental fishmeal polymer baits (Smith and Daigle, 1988) and one each of Canadian polyurethane sponge (Johnston et al., 1988), Canadian sachet (K. Lawson, Connaught Laboratories Limited, Willowdale, Ontario, Canada M4R 3T4) and German sachet (Schneider et al., 1988) baits. The latter three baits were developed by Canadian and European researchers for red fox rabies control applications. The experimental fishmeal polymer baits were cylindrical (length 4 cm, diameter 3 cm) and consisted of various proportions of fish oil (5 to 20% by weight), fish meal (75 to 95%) and a synthetic polymer binder (0.5 to 10%) with an empty gelatin capsule (Bell-Tex Laboratories, Little River, Texas 76554, USA) in the center representing a vaccine chamber. Baits were placed in a grid pattern, 0.3 m apart, with the order changed randomly. A successful trial consisted of an individual raccoon entering the smorgasbord area, making its choices and leaving the test site without interference from other raccoons. Qualitative differences in bait handling and amount consumed were recorded. In addition, paired direct observation trials were conducted to determine if placement of the bait in a polyethylene bag (clear, sandwich bag) affected bait choice. Two identical fishmeal polymer baits were offered 0.3 m apart, one inside a bag and one without a bag. Paired direct observation trials were also used to determine if molasses was necessary to overcome a potentially adverse reaction (taste) when tetracycline (150 mg) was incorporated in the matrix. Two tetracycline-laden fishmeal polymer baits, one with molasses in its matrix and one without, were offered 0.3 m apart. A trial was considered successful, as mentioned above. Paired direct observation trials were statistically evaluated by the Chi-square test with significance determined at $P \leq 0.05$.

Placebo baiting trials

Bait acceptance trials on Parramore Island were conducted on four study sites ranging in area from 2 to 51 ha. All fishmeal polymer baits were placed in polyethylene bags, overlayed with a fish-based slurry and hand-distributed along wildlife trails and at the mouth of ground dens within each study site to achieve predetermined baiting densities. The first bait acceptance trial was conducted on a 13 ha isolated upland area (Little Beach), entirely surrounded by salt water marsh consisting primarily of salt-water cordgrass (*Spartina alterniflora*) and glasswort (*Salicornia virginica*) where 117 baits were distributed resulting in a density of 8.7 baits per ha. Twenty-six baits (13 baits/ha) were distributed on the southern-most study site (South Tip), a 2 ha area of brackish and salt marsh including five small upland hummocks described in the literature as "Parramore Pimples" (Dueser et al., 1976) with one to two raccoon ground dens on each. One hundred forty baits were distributed on the third study site (51 ha), at the southern end of an ancient sand dune ridge (Italian Ridge) with elevations of 4 to 6 m above sea level, to achieve a baiting density of 2.7/ha. The fourth study site (Bayside) included 7.4 ha of salt marsh and thicket interface on the bayside of the island, on which 25 baits were distributed along two parallel transect lines resulting in a bait density of 3.4/ha.

Two biomarkers were used in placebo bait acceptance trials conducted on Parramore Island: rhodamine B (Sigma Chemical Co., St. Louis, Missouri 63178, USA) was included in the potential vaccine compartment and tetracycline (Upjohn Co., Kalamazoo, Michigan 49001, USA) was incorporated in the fish-based slurry. Gelatin capsules mimicking a sterile vaccine chamber were filled with 100 mg of rhodamine B and placed in the center of cylindrical fishmeal polymer baits. Baits were placed in small polyethylene bags (clear sandwich bags) and overlaid with 15 ml of a fish-based slurry (vegetable oil and canned salmon) containing 100 mg of tetracycline per dose. Bait disturbance/contact was assessed along each bait transect line daily. Sixty percent of the baits on each study site were placed on substrate (i.e., sand or mud) that allowed determination of species (based on tracks) contacting the baits (Linhart and Knowlton, 1975). Bait acceptance was assessed by examination of first premolars (antemortem samples) collected from captured, sedated and subsequently released animals, under a Leitz ultraviolet illumination microscope for tetracycline deposition within cementum and dentine (Johnston and Voight, 1982), and by visible rhodamine B staining on live-trapped furbearers, by fluorescent bands on vibrissae due to rhodamine B ingestion, as described (Lindsey, 1983) and by rhodamine B-stained scat. Raccoon and fox rhodamine positive scat indices were determined by removal of scat along the bait lines one day prior to distribution of baits. During the first and second day after bait distribution, fresh scat were counted and characterized as normal or discolored pink by rhodamine ingestion. The index was recorded as number positive over total number of observed scat.

An additional pilot baiting trial was conducted on a 30 ha upland area of North Island, South Carolina. Cylindrical wax ampules (W. and F. Manufacturing Co., Inc., Buffalo, New York 14240, USA), 3.4 cm × 1.0 cm diameter, mimicking sterile, water-resistant vaccine chambers and containing 100 mg of tetracycline were inserted into the fishmeal polymer baits (Fig. 2) without tetracycline in the matrix. The baits were placed in small plastic bags and overlayed with a fish-based slurry as previously described, except that no tetracycline was included in the slurry.Two hundred seventy-five baits were distributed, one every 30 m, along flagged transects running east–west from the bayside to the ocean to achieve a baiting density of 9.2 baits/ha. One hundred baits were placed on sand for species visitation documentation. Tomahawk live-traps were used to collect raccoons immediately prior to the baiting trial and subsequent to distribution of the baits. Ten live-traps were set one night prior to bait distribution; 220 trap nights were conducted on days two to seven postbait distribution. Live-trapped raccoons were sedated and euthanized as described above. A field necropsy was performed noting gastrointestinal contents and mandibular bone samples were collected for tetracycline analysis.

FIGURE 2. Fishmeal polymer bait and wax ampule.

RESULTS

Identification of target and nontarget species

Mammalian species composition on Parramore Island included both target and nontarget species: raccoons (*Procyon lotor*); red foxes (*Vulpes vulpes*); white-tailed deer; river otters (*Lutra canadensis*); grey squirrels (*Sciurus carolinensis*); and eastern cottontail rabbits (*Sylvilagus floridanus*). Raccoon tracks and scat were abundant on Parramore Island; red foxes were occasionally seen (approximately once a week) and scats and tracks were easily found. Sightings of white-tailed deer were frequent (approximately one every 2 to 3 days). River otter sign was present only at two sites on Parramore Island. Grey squirrels were observed only on the north end of Parramore Island near the Coast Guard station. There were three sightings of eastern cottontail rabbits on the island. Three hundred small mammal trap nights (Sherman live-traps) yielded only one marsh rice rat (*Oryzomys palustris*) and two house mice (*Mus musculus*). The other small mammals previously reported on Parramore Island (Dueser et al., 1976, 1979), muskrats (*Ondatra zibethicus*), meadow voles (*Microtus pennsylvanicus*), and Norway rats (*Rattus norvegicus*), were not trapped in the upland area sampled. From October 1987 through June 1988, 2,140 trap nights resulted in the initial capture of 118 raccoons and two red foxes, and cumulative recaptures of 243 raccoons. An estimate of raccoon density, based on mark–recapture (October through December 1987), was $N = M(n + 1)/(M + 1)$; where M = number of raccoons marked (81), n = total number of raccoons observed (161), m = number observed that were marked (80). Thus, N = 162 raccoons on the 427 ha area sampled by trapping or 1/2.7 ha, yielding a population estimate of 326 (SE ± 12.6) raccoons per 880 ha of upland area on Parramore Island (Bailey, 1952; McCullough and Hirth, 1988) (Table 1). The age distribution of live-trapped raccoons was 80% adults versus 20% juveniles and subadults (Table 2). The sex ratio of Parramore Island raccoons was 1.3, male to female. Thirteen of 15 (87%) adult females live-trapped from February through April 1988 were assessed as pregnant by abdominal palpation. Additionally, eight adult female raccoons live-trapped May through June 1988 were either lactating or pregnant. Size of raccoon litters ranged from one to four with

TABLE 1. Parramore Island raccoon (*Procyon lotor*) live-trapping data by month, October through December 1987.

Month	Trapnights	New captures	Cumulative recaptures
October	239	33	14
November	621	39	37
December	336	9	29
Total	1,196	81	80

TABLE 2. Age[a] and sex distribution of live-trapped raccoons (*Procyon lotor*) on Parramore Island, Virginia, October through July 1988.

	Male	Female	Total	Male : female	%
Juvenile	9	3	12	3.0	9
Subadult	4	10	14	0.4	11
Adult	60	45	105	1.3	80
Total	73	58	131	1.3	

[a] At initial capture.

a mean of 2.4 (SE = 0.34) based on 10 litters.

Seroprevalence data

A subsample (N = 20) of raccoon sera was screened for rabies virus neutralizing antibodies; all were seronegative (≤0.2 IU/ml). Additionally, there was no evidence of inhibition of in vitro cytopathic effect in the subsample (N = 20) screened for raccoon poxvirus antibody. The number of samples that must be screened for 95% confidence level detection of raccoon poxvirus antibody if 20% seroprevalence is assumed is 13(n), where n is the required sample size, $n = [1 - (1 - a)^{1/D}][N - (D - 1)/2]$, a = 95% confidence level when the disease affects at least 20% (D/N) of the population, D = 65, N = 326. The prevalence of raccoon pox antibody must be below 20%, as no titers were detectable from 20 samples screened.

Parasitologic and pathological findings

Background pathologic and parasitologic findings included multifocal granulomatous myocarditis due to *Hepatozoan procyonis* present in 100% of 23 raccoons. In addition, multifocal granulomatous myositis was present in 14 of 23 raccoons, also due to *H. procyonis*. Other findings included an 83% point prevalence of intestinal digenean infection (*Phagicola angrense*) previously reported by Snyder et al. (1989a). Gastrointestinal parasites identified as *Physaloptera rara* were present in 15 of 23 animals (65%). Multifocal granulomatous lung lesions consisting of accumulations of alveolar foamy macrophages and cholesterol clefts, with evidence of lipid deposition due to chronic parenchymal insult of unknown etiology were present in 13 of 23 raccoons (57%). Digeneans (*Eurytrema* sp.) were observed from histological sections of the pancreatic ducts in six of 23 raccoons examined (26%) and have been reported previously in raccoons with no associated morbidity or mortality (Denton, 1942; Penner et al., 1954). Other incidental lesions included a single case of heartworm (*Dirofilaria immitis*); the infection was clinically inapparent with two immature parasites in the right ventricle as previously reported by Snyder et al. (1989a). There was one case each of mild infections of *Macracanthorhynchus ingens* and *Gnathostoma* sp. Specimens of *M. ingens*, *Gnathostoma* sp. and *P. rara* are deposited in the U.S. National Parasite Collection (Beltsville, Maryland 20705, USA; accession numbers 80814, 80813, and 80812, respectively). Apparent raccoon mortality factors included circumstantial evidence of raccoon kit predation by adult foxes. On several occasions, beach tracks indicated that a fox and a small raccoon (apparently foraging with its mother), had interacted. Fox tracks leading away from the site suggested that the fox was dragging an object away, presumably a juvenile raccoon. One raccoon neonate died within 24 hr of birth, unable to nurse due to a severe cleft palate. One radio-collared, adult male raccoon died from trauma compatible with a bite wound. Also, one radio-collared, lactating, adult female raccoon was found dead in the field with gross lesions limited to serosanguinous fluid in the pleural cavity; these lesions were of unknown etiology.

Radiotelemetry

Fifteen adult Parramore Island resident raccoons of both sexes were radio-collared and determined to regularly remain on Parramore Island. One adult male Parramore Island raccoon was found on Revel's Island, a small, adjacent bayside island; on three occasions (May, June and September) it remained there for <36 hr. Including these outlying points on Revel's Island across The Swash (0.3 km wide, 2 to 4 m deep at mean low tide), this raccoon had the largest home range of 143 ha, consisting primarily of salt marsh and a few Parramore mounds. The average home range of radio-collared raccoons on Parramore Island was 34 ha. Juvenile raccoon dispersal, based on live-trapping and recapture data, was limited to areas adjacent to and overlapping with their mothers home range. There was no evidence of exchange or interaction of raccoons between Parramore Island and raccoons on the two nearest barrier islands (1.5 km across ocean inlets) or the mainland (7.7 km across salt marsh and bays).

During the translocation study, the adult male Revel's Island resident returned to the area of its original capture by crossing The Swash within 20 hr. The pregnant female from Revel's Island remained on or near a small upland hummock where it was released on Parramore Island for 18 days but subsequently returned to Revel's Island. The translocated Parramore Island adult male raccoon remained on Revel's Island for 14 days with the exception of days 3 and 8 when it was located on Parramore Island. However, on both occasions it was located on Revel's Island the following day. On day 15, this raccoon left Revel's Island by crossing The Swash for the third time, and remained in a new area on Parramore Island, 2 km north of Revel's Island but 7 km south of its original capture site for several weeks. It returned to its original capture site within 7 wk of its translocation.

Bait preference studies

During each of the 15 pilot smorgasbord trials, raccoons always chose one of the fishmeal polymer baits first, each time selecting this type of bait over the Canadian sponge, Canadian sachet and German sachet baits, although this was not statistically significant due to the high number of bait types offered and the limited number of valid repetitions. However, bait handling differences were noted between the fishmeal polymer baits and the other bait types. When fishmeal polymer baits were chosen, they were always >50% consumed and the soft gelatin placebo vaccine chambers were chewed or consumed. Whereas when the other bait types were contacted, they were <25% consumed and the sachet or placebo vaccine compartments were rarely penetrated. In paired bag/no bag preference tests, raccoons preferentially chose the bait in the bag first, 20 of 22 times (91%). During 17 paired direct observation trials used to determine raccoon preference between fishmeal polymer baits with and without molasses (when tetracycline was incorporated in the matrix), raccoons chose the polymer bait without molasses 65% of the time (11/17), not significantly different from uptake of the fishmeal polymer bait with molasses (6/17).

Placebo baiting trials

The first placebo bait trial resulted in high bait disturbance with 96% of the baits contacted during the first 24 hr; all of the 117 baits were contacted within 48 hr. Of 70 baits placed on substrate allowing identification of species based on tracks, 62 (89%) showed evidence of contact and consumption of baits by raccoons, with evidence of fox activity and bait consumption at 8 (11%) of the bait stations by day 2. Fourteen raccoons were live-trapped on the study site within 2 wk (490 trap nights).

Only one (7%) of these antemortem premolar tooth samples were positive for tetracycline. During the first day of live-trapping, 48 hr after baits were distributed, only two raccoons were visibly marked with rhodamine B. By this index alone, bait acceptance by raccoons was only 14% (2/14). However, six of 10 (60%) fresh raccoon scats in the study area were discolored by rhodamine B. The smallest study site (South Tip) also resulted in high bait disturbance rates, primarily by raccoons, where 23 of 26 baits (88%) were contacted within 48 hr. In this area, four raccoons were live-trapped with a trapping effort of 44 trap nights, but no premolars were positive for tetracycline deposition. Similarly, only one of four animals (25%) exhibited visible staining with rhodamine B, but scat indices suggested a higher acceptance rate with two of four (50%) fresh raccoon scats stained with rhodamine B. At the third placebo study site (Italian Ridge), 91% of baits were contacted within 48 hr. During 108 trap nights, 16 individual raccoons were captured. All premolar tooth samples collected for tetracycline analysis were negative. None of the raccoons on this site were visibly stained with rhodamine B. However, two of 16 vibrissae samples (12%) were positive. Scat indices were suggestive of a higher bait acceptance rate; two of five (40%) fresh scats in the area were positive for rhodamine B ingestion. The bait disturbance rate on the fourth placebo study site (Bayside) was 100% within 48 hr. However, none of the live-trapped raccoons on this study site had visible rhodamine B staining. Fifty-two trap nights resulted in the capture of nine individuals. One of nine premolar samples (11%) was positive for tetracycline ingestion. Three of nine vibrissae samples (33%) were positive under ultraviolet light for rhodamine B ingestion. In addition, three of five fresh scats in the study area were also positive, suggesting bait ingestion as high as 60%.

On the North Island study site, four raccoons were obtained for negative control mandibular bone tetracycline analysis prior to bait distribution. These four samples were tetracycline-negative. Bait disturbance rates on the North Island study site were 38% on the first night, a cumulative 78% disturbance over the first and second nights and a cumulative 93% disturbance by the third night. Sixty-nine percent of the baits disturbed at the 100 stations on sand were entirely consumed leaving only the polyethylene bags. The remaining 31% were partially consumed. There was evidence of raccoon tracks at all of the bait stations where baits were partially or totally consumed. Eight raccoons were collected from days 2 to 5 postbait distribution. Fishmeal polymer was found in the stomachs of three raccoons collected on day 2. The intestinal contents of the raccoons collected on days 3 to 5 included fishmeal polymer and wax indicative of vaccine chamber consumption. All eight mandibular bone samples (100%) were positive for tetracycline, indicating consumption of the vaccine chamber.

DISCUSSION

On 14 April 1989, the United States Department of Agriculture, Animal and Plant Health Inspection Service granted approval for limited field trials of V-RG recombinant vaccine against rabies. In anticipation of state and local approval for such field trials and preparatory to monitoring its proposed effect, it was necessary to verify applied ecological components of mammalian target and nontarget species populations. Ideally, species composition should be as diverse as possible, mimicking mainland biota so as to simulate conditions of future, wide-scale mainland field application. Due to the obvious geographic isolation of Parramore Island, diversity of nontarget species was limited. Mammalian species composition on Parramore Island included raccoons, red foxes, white-tailed deer and infrequent evidence of river otters, grey squirrels and eastern cottontail rabbits. These findings were partially in agreement with previous mammalian species census studies on Parramore Island (Dueser et al., 1976, 1979; MacLeod and Hennessey, 1981) with the exception that the presence of meadow voles, Norway rats and muskrats was not confirmed. However, there were sufficient populations of raccoons, red foxes and deer inventoried suitable for a vaccine safety trial to assess the potential extent and effect of contact with placebo or vaccine-laden baits.

A population density estimate of one raccoon/2.7 ha was calculated for comparison with population estimates to be generated at the same time of year after the initiation of the vaccine trial to evaluate the potential impact of the vaccine on relative raccoon abundance. The estimate was markedly higher than raccoon population densities reported elsewhere, such as the one/7.2 ha in Shelby County, Tennessee (Moore and Kennedy, 1985), or the one/17.4 ha in Stewart County, Tennessee (Kennedy et al., 1986). However, the Parramore Island estimate was consistent with a trend toward higher raccoon densities in areas with permanent water sources where an abundance of water-associated foods can be found (Moore and Kennedy, 1985) and where up to one/2 ha have been reported (Kaufman, 1982). Additionally, on Parramore Island there are no significant predators of adult raccoons including the absence of predation by hunting or trapping pressure, stray dogs or death by automobile. The age distribution of live-trapped raccoons on Parramore Island was compatible with a nonharvested population; the preponderance (80%) of adults (Table 2) was similar to those values obtained in Tennessee and Florida, where populations consisted of 67 and 69% adult raccoons, respectively (Bigler et al., 1981; Smith and Kennedy, 1985). In contrast, the age distribution of an exploited population in York County, Pennsylvania (USA) consisted of only 12% adults, the majority of raccoons being young of the year (50%) and subadults (38%) (Kirkland and Gillman, 1984). The sex ratio of Parramore raccoons (1.3 males to each female) was comparable to reported ratios of approximately 1:1 from other raccoon demographic studies (Bigler et al., 1981; Kirkland and Gillman, 1984; Smith and Kennedy, 1985). Limited reproductive data, including estimation of pregnancy rates by abdominal palpation, the number of live kits seen with previously pregnant raccoons and radio-tracking of known breeders, were gathered for comparison to reproductive parameters to be collected during the vaccine trial.

Establishment of baseline health parameters of raccoons was necessary in this preimmunization phase to prevent incidental lesions from being ascribed to the vaccine and to document these histopathological lesions and parasites common to the Parramore raccoon population to determine the potential impact of oral immunization with recombinant vaccine in the face of underlying internal and external parasite burdens. Background findings included relatively high point prevalences of incidental lesions due to internal parasites, yet cases were clinically inapparent in the majority of raccoons in this study. However, one juvenile raccoon death was due to an overwhelming infection of *Phagicola angrense* resulting in a severe hemorrhagic gastroenteritis, the first reported mortality attributable to such infection (Snyder et al., 1989b). Multifocal granulomatous lung lesions could not be identified as to source or relative importance. One radio-collared, lactating, adult raccoon found dead in the field had approximately 150 ml of serosanguinous fluid in its chest. It is unknown if this exudate was related to the lung lesions described in the other cases. Clinical or histopathological observations suggestive of viral infections, such as rabies, canine distemper or orthopox virus were not made, although other surveys suggest a more common occurrence of these infectious agents in raccoons (Alexander et al., 1972; Hoff et al., 1974). The raccoon rabies epizootic has not yet reached the eastern shore of Virginia (Centers for Disease Control, 1988).

Monitoring raccoon movement by ear-tagging and radiotelemetry yielded no evidence of exchange or interaction of raccoons from Parramore Island with those of the two nearest barrier islands or the mainland. All radio-collared Parramore Island raccoons remained on Parramore Island with the exception of one adult male raccoon which was occasionally found on Revel's Island, a small, adjacent bayside island. While physically capable of dispersal between Parramore Island and Revel's Island, only infrequent visitation by one radio-collared raccoon was observed. These visitations were limited to three 36 hr periods. The specific habitat of Revel's Island was similar to the area on Parramore Island where this animal was normally found; food and water did not appear to be significant motivational factors for visitation. Other potential factors may include breeding, although the raccoon's crossings occurred in May, June and September, after the most active breeding period of February and March. There was no evidence suggesting spontaneous raccoon dispersal by swimming across ocean inlets or other large bodies of water, or travel across expanses of salt marsh for more than 2.0 km.

Although animal sign (scat and tracks) indicates the existence of two river otters on Parramore Island, there was no change, increase, decrease, or shift in areas utilized by these individuals during the past 12 mo. While river otters are excellent swimmers, there is no evidence that individuals on Parramore Island represent potential vectors for vaccine dispersal from the field trial site, because their high usage areas indicated by scat and tracks have remained confined and predictable. The small mammal population of Parramore Island included at least one accomplished swimmer, the marsh rice rat; low trap success indicative of limited numbers and lack of observed contact with placebo baits during the placebo baiting trials and at the direct observation station indicated that the small mammal population of Parramore Island would also not compromise biosecurity of the proposed field trial site.

Initial oral wildlife baiting strategies were designed for the red fox (Baer et al., 1971; Schneider, 1985), a major rabies vector in Europe and Canada, but the emergence of the mid-Atlantic raccoon rabies epizootic necessitated tailoring vaccine and baiting strategies to raccoons. Raccoons are similar to red foxes in that they are opportunistic omnivores but have considerably more dexterity than foxes enabling them to discriminate between edible portions and inedible vaccine-laden components of a given bait. Smorgasbord trials showed that when Canadian sponge, Canadian sachet and German sachet baits were contacted, $<25\%$ was consumed, and the sachet or placebo vaccine compartment was rarely penetrated, suggesting considerable vaccine wastage. The results of the North Island baiting trial indicated that the wax ampule was a readily accepted vaccine chamber, since all biomarker-positive animals must have penetrated and consumed the contents. Bait placement within polyethylene bags may enhance visual attractiveness for foxes (Johnston and Voight, 1982). Our results concur that baits placed in bags result in enhanced uptake, as ascertained by direct observation of raccoons in paired baiting trials.

Placebo bait acceptance trials on Parramore Island included rhodamine B within the bait to evaluate its potential use as a visible antemortem biomarker, readily assessed in the field by discoloration of fur, skin or of fluorescence under ultraviolet light (Lindsey, 1983; Perry et al., 1989). Its performance in previous cage trials was characterized by pink discoloration of contacted fur or skin lasting 7 to 10 days, easily viewed under normal light, and pink fluorescence of the discolored area under ultraviolet light. In contrast, the utility of rhodamine B in the field was disappointing. Only three of 41 raccoons were weakly stained on the muzzle during the first day of live-trapping, 48 hr after baits were distributed. A heavy rainfall on the first day of trapping at two study sites may have reduced external marking by rhodamine B; none of the raccoons on these sites were visibly stained. However, 20% of vibrissae collected from raccoons live-trapped on these two sites were positive due to rhodamine B ingestion. Subsequent direct observation revealed an aversive reaction when raccoons punctured the rhodamine B-filled gelatin capsule within the fishmeal polymer bait. This rejection of the biomarker was reduced by incorporation in a fish-based slurry. Nevertheless, even small amounts of rhodamine B cause marked, pink discoloration of fecal material 1 to 2 days following ingestion. In these placebo baiting studies, fresh scat discolored by rhodamine B suggested bait acceptance as high as 60%. Antemortem analysis of tetracycline deposition in premolar tooth samples was low at 5% (2/41). Subsequent study has demonstrated that premolar samples are poor indicators of tetracycline ingestion and that harvesting the mandible yields a superior sample for tetracycline analysis, especially in aged animals (data not shown). Due to the lack of a suitable antemortem biomarker, bait acceptance rates on Parramore Island, as compared to disturbance, were difficult to determine accurately in these studies. High bait disturbance rates were recorded during the placebo baiting trials (88 to 100% by 48 hr), primarily by raccoons and secondarily by red foxes, with no evidence of bait contact by deer, small mammals or birds, when disturbance was assessed by animal sign at bait placed on mud or sand or by rhodamine-positive scat identification. Moreover, a small scale baiting trial on North Island achieved 100% bait acceptance, readily determined from postmortem mandibular bone samples. Although based on a small scale trial yielding a limited sample size (eight raccoons), this supported direct observational studies and bait disturbance trends in the field on Parramore Island indicating that the fishmeal polymer bait was readily accepted by raccoons.

From these pre-vaccination studies, Parramore Island meets basic requirements as a suitable biosecure site for a proposed limited field trial to assess the safety, efficacy and stability of an orally-administered, rabies recombinant vaccine in wildlife; mammalian species composition is reasonably diverse and population indices of the target species exceed expectations necessary in selective harvesting to monitor the environmental consequences of such a release. The proposed study area on Parramore Island to be baited with vaccine-laden baits is 6 km from the nearest island, Revel's Island, and at least 2 km from a manned Coast Guard station. Intensive surveillance throughout the baited area and on both edges of the tidal gut separating the southern part of Parramore Island from Revel's Island will be maintained throughout future study. The baiting density of the proposed vaccine trial will be high at 10 baits/ha.

An overabundance of baits in the field will favor multiple bait consumption by target and nontarget species enhancing documentation of the safety of the vaccine under field conditions. Baiting densities during future mainland applications will vary according to raccoon density. An estimate of the baiting density necessary to vaccinate a substantial portion (i.e., 70%) of the raccoon population is approximately 3 baits/ha in areas of high raccoon density, i.e., 30 raccoons/km^2. Bait acceptance determinations based upon a reliable antemortem biomarker will be optimal to determine extent of target and nontarget species exposure to vaccine-laden baits. However, until such an antemortem biomarker has proven reliable prior to the proposed field trial, mandibular bone samples from raccoons, red foxes, deer and small mammals will be analyzed during routine surveillance for a reliable postmortem biomarker (tetracycline) indicative of bait consumption. In addition, the induction of rabies virus neutralizing antibody and observation of gross or histopathological lesions will be used to assess extent of contact with and safety of the V-RG recombinant vaccine. Given that more than 30 animal species tested to date with V-RG recombinant virus in captivity have produced no adverse effects (Rupprecht and Kieny, 1988) and that field trials with recombinant rabies vaccine are now under way in Europe (Pastoret et al., 1988) with no adverse effects, similar evaluation is critically needed in North America in order to practically and safely control sylvatic rabies.

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