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Paul Ramey
Ohio State University

Bradley F. Blackwell
USDA/APHIS/WS National Wildlife Research Center, bradley.f.blackwell@aphis.usda.gov

Robert Gates
Ohio State University

Richard Slemons
Ohio State University, Veterinary Preventive Medicine

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ORAL RABIES VACCINATION OF A NORTHERN OHIO RACCOON POPULATION: RELEVANCE OF POPULATION DENSITY AND PREBAIT SEROLOGY

Paul C. Ramey,[1] Bradley F. Blackwell,[2] Robert J. Gates,[1,4] and Richard D. Slemons[3]

[1] Ohio State University, School of Environment and Natural Resources, 2021 Coffey Road, Columbus, OH 43210, USA
[2] United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Ohio Field Station, 6100 Columbus Avenue, Sandusky, OH 44870, USA
[3] Ohio State University, Veterinary Preventive Medicine, 1920 Coffey Road, Columbus, OH 43210, USA
[4] Corresponding author (email: gates.77@osu.edu)

ABSTRACT: Ohio's oral rabies vaccination (ORV) program was established to prevent the westward spread of the raccoon (*Procyon lotor*) rabies virus (*Lyssavirus, Rhabdoviridae*) in Ohio, USA. The program, which targets raccoons, distributes vaccine-bait units (VBU) at a target density of 75 units/km^2. Few studies have examined the relationship of VBU density and target population density to the prevalence of rabies virus-neutralizing antibodies (RVNA). We conducted experimental VBU distributions in August 2003 and August 2004, 150 km west of the ORV zone where there was no history of raccoon rabies. We measured change in RVNA titers in blood collected from live-trapped raccoons before and after VBU distributions. A closed population mark–recapture estimate of the size of the target population was 91 raccoons/km^2, compared to the realized VBU distribution density of 70 units/km^2. Surprisingly, 41% of 37 serum samples were RVNA-positive (≥0.05 IU/ml) before VBU distribution in 2003, but all titers were <0.25 IU/ml. Although viable VBUs were distributed in August 2003, only 21% of 315 samples were RVNA-positive before VBU distribution in 2004, but 9% had titers ≥0.25 IU/ml. Tetracycline (biomarker in bait) prevalence in teeth indicated that 57% of raccoons ingested VBUs after distribution in 2003, and 54% ingested VBUs after distribution in 2004. However, only 8% and 11% of sera were positive for RVNA (≥0.05 IU/ml) after VBU distribution in 2003 and 2004, respectively. Only 4–5% of sera collected after bait distribution had titers ≥0.25 IU/ml each year. The standard distribution density of 75 VBUs/km^2 was insufficient to produce a population-wide immunoprotective response against rabies infection in our high-density target population. Presence of RVNA in a presumed naïve population before baiting demonstrates that estimating prevalence of RVNA after oral rabies vaccination can be problematic without knowledge of background titers and seasonal changes in prevalence of RVNA before and after baiting.

Key words: Oral rabies vaccination, population density, *Procyon lotor*, rabies virus, virus-neutralizing antibodies, raccoon, vaccination, V-RG.

INTRODUCTION

Large-scale distribution of oral rabies vaccine-bait units (VBUs) was prompted by the mid-Atlantic epizootic of raccoon (*Procyon lotor*) variant rabies in the eastern United States in the 1980s (Rupprecht et al., 1995). A vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine that produces a detectable rabies virus-neutralizing antibody (RVNA) titer (≥0.05 IU/ml) in laboratory animals (Rupprecht et al., 1988; Hanlon et al., 2002) has subsequently been utilized in control programs (Slate et al., 2005). Aerial distribution of VBUs has been ongoing throughout the eastern United States since the early 1990s, and in Ohio, USA, since 1997. The Ohio Department of Health (ODH) began distributing oral rabies vaccines (ORV) after an epizootic produced 62 cases of raccoon rabies in eastern Ohio during 1997 (ODH, 2002). There were no reported raccoon-variant rabies cases in eastern Ohio by 2000 and only one to two cases per year thereafter (ODH, 2004); however, in 2004, 45 confirmed case of raccoon rabies were observed in northeastern Ohio (Fig. 1; ODH, 2006a). The 2004 outbreak was contained with intensive aerial and ground baiting with ORV. The Ohio ORV zone is part of a larger ORV program that follows natural land features along the Appalachian Ridge, creating a barrier westward of raccoon-variant rabies (Fig. 2).

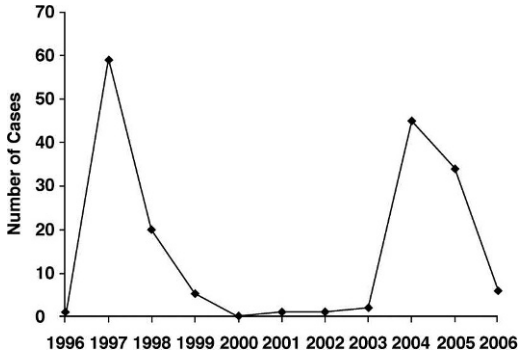

FIGURE 1. Annual numbers of reported raccoon rabies cases in Ohio during 1996–2006 (as of 5 October 2006; ODH 2006b).

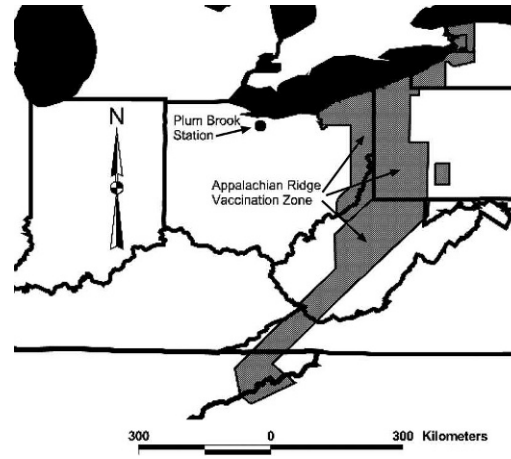

FIGURE 2. Location of Plum Brook Station relative to the 2004 eastern Ohio and Appalachian Ridge Oral Rabies Vaccination (ORV) zones.

The standard operational baiting protocol distributes 75 VBU/km^2 over large areas where raccoon population densities are highly variable. Baits are distributed at higher densities in suburban interface areas where high raccoon densities are known or expected to occur. The level of herd immunity achieved by baiting at varying densities of VBU and target population densities is not well understood. However, RVNA prevalence in raccoons increased when VBU density was increased from 75 to 300 baits/km^2 (ODH, 2001). Prevalence of RVNA is also measured by US Department of Agriculture–Wildlife Services (WS) after VBUs are distributed during the operational baiting program. No study or evaluation to date has measured host density or prevalence of RVNA in a naïve target population before VBUs were distributed. Prevalence of RVNA due to VBU distribution could be overestimated if RVNA was present in the population before VBU distribution. Further, without estimates of raccoon population density it is unclear whether the standard target VBU distribution density (75 units/km^2; ODH, 2001; WS, 2004) is sufficient to vaccinate all target populations. Knowledge of target population densities and prevaccination serology are needed to plan and evaluate the effectiveness of ORV distribution programs.

This study measured change in RVNA prevalence among raccoons after distributing VBUs (target density 75 units/km^2) in an area with no known history of raccoon rabies and where raccoon population density was estimated at the time of baiting. Our objectives were to 1) estimate raccoon population density within the baited area and 2) determine RVNA prevalence and presence of a VBU biomarker (tetracycline) in teeth in raccoons before and after distribution of VBUs following the Ohio operational protocol. We expected that RVNA would not be present in the population before we distributed baits and that RVNA prevalence would increase in the population after distribution of VBUs at 75 baits/km^2. We also predicted that post-bait prevalence and RVNA titers would increase between an initial baiting in 2003 and a second baiting in 2004.

MATERIALS AND METHODS

Study area

The study was conducted on the National Aeronautics and Space Administration (NASA) Plum Brook Station (hereafter, Plum Brook). Plum Brook includes 22 km^2 and is located within the Lake Erie coastal plane in northern Ohio, $<$4 km south of Sandusky in Erie

County (41°27′N, 82°42′W) and 150 km west of the Appalachian Ridge oral rabies vaccination zone (Fig. 2). The site encompasses active research facilities, abandoned warehouses, barns, trailers, and outbuildings. Vegetation communities present on Plum Brook consist of 40% herbaceous field, 30% shrubland (*Cornus* spp.), and 30% oak-dominated (*Quercus* spp. and *Populus* spp.) hardwood forest (Linhart et al., 2002; NASA, 2002). Human access to Plum Brook is controlled through a 2-m-high chain-link fence topped with barbed wire that encloses the 22-km perimeter and a guard-house centrally located on the northern boundary. Restricted-access roads traverse the interior of Plum Brook and a paved patrol road parallels the entire perimeter fence. Creeks and ponds provide permanent sources of water throughout Plum Brook. Areas south and east of Plum Brook are mostly cropland, including corn (*Zea mays*), soybean (*Glycine max*), and wheat (*Triticum aestivum*). The northern boundary abuts suburban residential property, whereas the western boundary adjoins a mixture of residential and agricultural areas. There were no physical boundaries to prevent movement of raccoons between Plum Brook and the surrounding area.

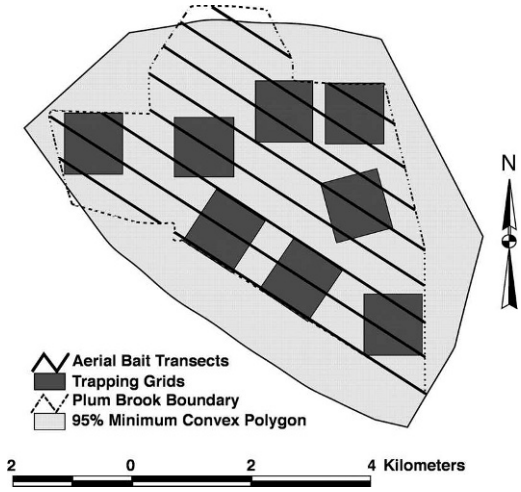

FIGURE 3. Minimum convex polygon containing 95% of locations of radio-marked raccoons, boundaries of Plum Brook Station, 1-km^2 trapping grids, and flight lines used to distribution oral rabies vaccine bait units during 2003 and 2004 in Erie County, Ohio, USA.

Oral rabies vaccine

The Raboral V-RG® (Merial, Duluth, Georgia, USA) vaccine used in this study is licensed for oral vaccination of raccoons (Hanlon et al., 2002), and is currently used in state and federal rabies-control programs (Slate et al., 2005). The bait is a hollow cube of fish-meal polymer and wax that seals a plastic sachet that contains the V-RG vaccine. Tetracycline is mixed with the fish-meal polymer to serve as a biomarker of bait ingestion (Linhart and Kennelly, 1967; Nunan et al., 1994). Orally administered tetracycline can be detected in calcific tissues of mammals after 2 days post-consumption (Hanlon et al., 1989). Raccoons are exposed to the vaccine only if the sachet is punctured and a sufficient vaccine dose is ingested to elicit serologic response. Because the vaccinia virus vector also replicates in the infected host, detection of vaccinia virus antibodies (VVA) may be used as an indicator of serologic response to the vaccine.

Trapping

Raccoons were live-trapped and ear-tagged from 6 May 2003 to 16 October 2003 and from 30 March 2004 to 21 October 2004 within eight 1-km^2 grids that were representative of habitats on Plum Brook (Fig. 3). The eight grids were grouped into four pairs such that each pair was separated by ≥1 km at the closest point; each grid included 30 possible trapping points, spaced 250 m apart. Trapping was done throughout the season on one randomly selected half of each grid (north, south, east, or west). The 15 trapping points in selected grid halves were divided into three subsets. Two of three subsets were trapped in each grid half during each week. We rotated trap placement in a fixed order between the three subsets of possible locations every 4 wk so that every point within the grid half was trapped at least twice.

Each grid pair (i.e., 20 traps/night) was trapped for four nights; and grid pairs were rotated each week so that all eight grids were trapped once every 4 wk. One rotation through all eight grids was considered a single trapping period. Six rotations (four prebait and two post-bait) were completed during 2003 and seven (5 prebait and two post-bait) during 2004.

Trapping effort varied from 10 traps/night during the first week of trapping to 40 traps/night during the last week ($\bar{x}$=20 traps/night) in 2003. Trapping effort in 2004 was constant throughout the season (20 traps/night). All traps that captured nontarget species or where bait was missing were counted as 0.5 trap-nights (Beauvais and Buskirk, 1999). Young-of-the-year raccoons were too small to tag and were released after capture.

Two previous placebo VBU studies were conducted on Plum Brook (Linhart et al., 2002; Blackwell et al., 2004). We sampled a reference raccoon population in 2004 at Old Woman Creek (OWC) National Estuarine Research Reserve 15 km east of Plum Brook (41°22′N, 82°31′W) after unexpectedly detecting RVNA before VBU distribution in 2003. Five traps were placed opportunistically at OWC one night per week during the 2004 trapping season.

Single-door, live-catch cage traps (Tomahawk 108.5, 107×30×30 cm; Tomahawk, Wisconsin, USA) baited with marshmallows and a 4:3:1 vanilla extract:honey:anise extract mixture were used for capture. Raccoons were anesthetized with a 5:1 ketamine:xylazine solution of 100 mg/ml each administered at a dosage of 12 mg/kg as described (ODH, 2002). Sedated animals were removed from traps, checked for presence of ear tags, and assessed for overall condition; body weight, sex, and age (adult, subadult, juvenile) were recorded. Age was estimated by tooth development and wear, the presence/absence of the penile frenulum for males, and mammary gland development for females (Lotze and Anderson, 1979). Animals were marked with one tag (Hasco 1005-3; Dayton, Kentucky, USA) in each ear.

Blood collection and analysis

Blood samples (approximately 10 ml) were collected from the jugular vein with a 21-gauge 4-cm needle attached to a vacuum tube after shaving and cleansing the ventral portion of the neck with isopropyl alcohol. Blood samples were stored (<6 hr) in a cooler until centrifuging (Clay Adams Dynac Centrifuge 420101; Franklin Lakes, New Jersey, USA) for 20 min at 800 × G. Serum was divided among three cryovials, each containing ≥0.5 ml and stored at −20 C. Two serum samples were kept in reserve and one was shipped to the Centers for Disease Control and Prevention (CDC) to be analyzed for RVNA titer, via the rapid fluorescent focus inhibition test (Reagan et al., 1983). Vaccinia virus antibody titers were determined (2003 only) by the CDC using the enzyme-linked immunosorbent assay (Marennikova et al., 1981).

Tooth extraction and tetracycline analysis

The first premolar was extracted for aging and biomarker analysis while the animal was sedated. The premolar is a single-rooted tooth (absent in some individuals) that is more easily extracted than a canine; premolar extraction is reported not to affect recapture rates of raccoons (Beasley and Rhodes, 2007). The extracted premolar was placed in an envelope labeled with the animal's identification number and collection date; teeth were not extracted from individuals without first premolars.

Teeth were collected before and after ORV distribution to establish a baseline to distinguish tetracycline deposited during our study from past placebo ORV experiments on Plum Brook (Linhart et al., 2002; Blackwell et al., 2004). Age, presence of tetracycline, and year of tetracycline deposition were determined by cross-sectioning the teeth and examining tooth annuli (Matson's Laboratory, LLC, Milltown, Montana, USA).

Radiotelemetry

Some captured adult (>1 yr) raccoons were fitted with 130-g radio collars equipped with mortality switches (Advanced Telemetry Systems, Isanti, Minnesota, USA). Eight males and nine females were radio-collared from 10 September 2003 to 24 September 2003; 22 males and 22 females were radio-collared from 22 June 2004 to 24 September 2004; these raccoons were monitored from from 23 September 2003 to 31 October 2003 and from 22 June 2004 to 23 November 2004. All animals were checked once per week for changes in signal pulse that indicated mortality (after 8 hr of inactivity). Raccoons were located by triangulation using handheld Yagi antennas (Ellis, 1964) three to five times per night between sunset and sunrise, and over one to three nights every 1–2 wk. A set of three or more bearings was obtained on each animal, typically within 10- to 15-min periods. Triangulation bearings were plotted on a computer in the field (LOCATE II; Nams 2000) and inconsistent bearings were discarded. We used the animal movement extension operating with Spatial Analyst in ARCVIEW 3.2 (Hooge and Eichenlaub, 2000) to obtain the minimum convex polygon (MCP) area that encompassed all locations of radio-collared raccoons. This MCP, the spatial "footprint" of our study population, was used to calculate density from abundance estimates based on mark–recapture data. The Kaplan–Meier method was used to estimate weekly survival rates of radio-marked raccoons (Heisey and Fuller, 1985). All animal handling procedures followed protocol 2003A0119, approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee and reviewed by the Institutional Animal Care and Use Committee convened by the WS National Wildlife Research Center.

Density estimation

The raccoon population was estimated by mark–recapture. Individual raccoons typically have unique capture probabilities (Gehrt and Fritzell, 1998) that change over time due to trap experience and recruitment of trap-naïve juveniles into the population (Moore and Kennedy, 1985). Population estimates from closed-population models that relax assumptions about effects of capture heterogeneity, trap response, and time were evaluated (Pollock et al., 1990). Program CAPTURE (Pollock et al., 1990) was used to estimate adult population size (±95% confidence interval).

Only adult raccoons were used in population estimates to meet the population closure assumption and the adult raccoon density estimate was doubled to account for juveniles present in the population at the time of baiting. We assumed an average fecundity of two kits per female per year (Ritke, 1990) and a 1:1 sex ratio, which is typical for wild raccoons (Broadfoot et al., 2001). Departure from an even sex ratio in the trapped sample was evaluated with chi-square tests ($\alpha=0.05$).

We simulated the method used by WS to estimate relative densities of raccoons in baited areas (WS, 2004). The WS relative-density estimator is based on the minimum-number-known-alive (MNKA) method, the simplest mark–recapture estimator, but with the most restrictive assumptions (population closure, constant homogeneous capture probabilities; Pollock et al., 1990). The MNKA method assumes that all animals present are captured with a sufficient level of trapping effort. The WS protocol uses a rectangular or circular 3-km^2 area, selected to represent surrounding habitat (WS, unpubl.). Fifty live-traps, baited with anise/vanilla and marshmallows, are placed opportunistically without clumping throughout the area to maximize captures. If 50 live traps capture ≤2 unique raccoons over four consecutive nights, then trapping ceases on the fifth day and MNKA estimates of population density are based on 250 trap-nights. Trapping continues for five more nights and MNKA estimates are based on 500 trap-nights if <75 unique raccoons are captured over the 10-night period. If ≥75 individual raccoons are captured after 10 nights and the proportion of unique individuals captured is ≥0.05, then trapping continues for another five nights and MNKA estimates are based on 750 trap-nights.

The WS criteria was met for the third level of trapping effort, so the number of unique individuals captured during ≥700 trap-nights each year was determined. We accumulated 700 (2003) and 720 (2004) trap-nights from three of the grid pairs over three four-consecutive-night trapping periods separated by 4 wk for each grid (10 traps/grid×four nights×three periods×six grids=720 trap-nights). Thus, our simulation of the WS protocol began when trapping commenced in May and ended in August. We averaged MNKA estimates over four simulations in which one unique grid pair was withheld.

Catch-per-unit effort (CPUE) is another population estimator that accounts for untrapped individuals remaining after completing the WS relative density protocol. Consequently, capture data also were analyzed using a CPUE removal model. In contrast to MNKA, CPUE does not require that all animals are captured, but does assume population closure and constant and homogeneous capture probabilities. The CPUE model also assumes that every individual in the population is marked when no new animals are captured. Population estimates and 95% prediction intervals were obtained by regressing the number of new (untagged) individuals captured/trap-night on total cumulative captures of novel individuals (White et al., 1982). The population estimate is the x-intercept ($y=0$) of the least-squares line.

Population density was estimated by dividing population estimates obtained from CPUE and Program CAPTURE by area of the MCP that encompassed all radio-locations of radio-marked raccoons during 2003 and 2004. Population density from MNKA was estimated in a similar manner except that MCP areas were calculated separately for each simulation of the WS protocol. Specifically, we used MCP areas derived only from locations of radio-marked raccoons that were initially captured on the same trapping grids used in each of the four simulations. Capture records were pooled across trapping grids to obtain CPUE estimates in 2003 and 2004.

Vaccine bait unit distribution and viability test

Vaccine bait units were distributed on 26 August 2003 and 19 August 2004 following the operational bait distribution protocol used in Ohio (WS, 2004; ODH, 2006a, b); however, VBUs were distributed by helicopter (Bell Jet Ranger®; Ft Worth, Texas, USA) rather than fixed-wing aircraft. Baits were not distributed over water or buildings and unlike the WS (2004) protocol, additional VBUs were not hand-distributed around ponds or buildings.

We distributed VBUs over 14 southeast–northwest oriented flight lines (30–227 baits/

line), with an estimated 27-m spacing of VBUs along the line (Fig. 3). Flight lines were spaced 0.5 km apart and the helicopter traveled at a speed of 80 km/hr, and altitude of 150 m above ground. Trapping was suspended for 1 wk after bait distribution to allow time for animals to contact VBUs.

Vaccine viability was tested with a subsample of VBUs placed in two cage traps (10 VBUs/trap) immediately after bait distribution in 2003. Both traps were exposed to ambient conditions but one was exposed to direct sunlight, whereas the other was shaded. A third group of 10 VBUs was refrigerated at 3 C as a control. All VBUs were collected at the end of trapping (7 wk) and sent to the CDC for viral vaccine titer measurement using cell culture (Rupprecht et al., 1988).

Statistical analyses

Chi-square tests of independence (Sokal and Rohlf, 1995) were used to compare RVNA and VVA prevalence (titers $\geq$0.05 IU/ml) in individual raccoons (α=0.05). Chi-square was also used to test independence of RVNA and tetracycline prevalence. We calculated odds ratios (Sokal and Rohlf, 1995) to determine the likelihood that a VVA-positive raccoon was also RVNA-positive in 2003. Odds ratios also expressed the likelihood that a tetracycline-positive raccoon was also RVNA-positive in 2003 and 2004.

RESULTS

Radiotelemetry

None of 17 radio-collared raccoons died or dispersed from Plum Brook during 2003. We detected 12 deaths among 52 radio-collared raccoons in 2004. The Kaplan–Meier survival rate estimate was 75% when trapping ceased in 2004. Raccoons moved outside of Plum Brook to forage at night but nearly always returned to Plum Brook before dawn. Only one of 52 (2%) raccoons was known to disperse beyond Plum Brook in 2004. However, we lost contact with eight (15%) raccoons before trapping ceased in 2004. The MCP (32.8 km^2) encompassing all locations of radio-collared raccoons obtained during 2003 and 2004 extended beyond the boundaries of Plum Brook (Fig. 3). The MCP areas used with MNKA estimates of population size averaged 30.3 km^2 (range 26.2–32.3 km^2).

TABLE 1. Adult population (N) with 95% confidence interval (CI) and density estimates from minimum-number-known-alive (MNKA), catch-per-unit effort (CPUE) and closed-population mark-recapture model (M_{th}) for raccoons on Plum Brook Station, Erie County, Ohio, USA, during May 2003 to October 2003 and March 2004 to October 2004.

Method	N	95%CI	No./ km^2
MNKA			
2003	134	NA	4.4
2004	192	NA	6.4
CPUE			
2003	438	182[a]	13.4
2004	527	208[a]	16.1
M_{th}			
2003	1,753	587	53.4
2004	1,231	248	37.5

[a] 95% Confidence interval at x intercept (Sokal and Rohlf, 1995).

Target population and baiting densities

We accumulated 1,784 and 1,745 effective trap nights during 2003 and 2004, respectively. Trap success rate was 22% during 2003, and recapture rate (recaptured individuals/total captures) was 12%, compared to 31% and 30%, respectively, during 2004. Sex ratios (1.2 male:1 female each year) in our trapped sample did not differ from unity each year ($\chi^2 \leq 2.23$, df=1, $P \geq 0.14$). Age ratios (juvenile:adult) of raccoons captured after August were 0.79:1 in 2003 and 2004. Adult females captured with dependent young were most frequently accompanied by two juveniles at trap sites, supporting the 1:1 age ratio we used to account for juveniles in our estimates of total population size.

The closed-population capture model (M_{th}) produced adult population estimates that were 8.2 and 2.1 times higher than the MNKA and CPUE estimates, respectively, during 2003 and 2004 (Table 1). The CPUE estimate of adult population size was nearly two times higher than MNKA, but only 32% of the M_{th} estimate. After doubling adult M_{th} population estimates (to account for juveniles) and then dividing by the effective trapping area (32.8 km^2), we estimated that raccoon

TABLE 2. Prevalence of tetracycline by year of deposition in live-trapped raccoons before and after distribution of oral rabies vaccine-bait units (VBUs) on Plum Brook Station, Erie County, Ohio, USA, May 2003 to October 2003 and March 2004 to October 2004.

	2003 Pre-VBU		2003 Post-VBU		2004 Pre-VBU		2004 Post-VBU	
	n	%	*n*	%	*n*	%	*n*	%
Year of deposition								
2002[a]	0	0	1	2	14	9	1	1
2003	0	0	11	17	93	58	20	26
2004	0	0	0	0	11	7	21	27
No deposition	19	100	53	81	43	27	36	46
Total	19	100	65	100	161	100	78	100

[a] Likely resulting from placebo ORV bait study (Blackwell et al., 2004).

population densities at the time of baiting were 107/km^2 in 2003 and 75/km^2 in 2004. Unbaited areas (buildings, water bodies, etc.) comprised only 6% of Plum Brook so the realized VBU density was 70 baits/km^2 (1,544 VBUs distributed each year).

Prevalence of tetracycline, VVA, and RVNA

All teeth collected during the pre-VBU distribution (May–August) period in 2003 were tetracycline-negative (Table 2). However, tetracycline was detected in the 2002 annulus of 16 of 172 (9%) tetracycline-positive tooth sections collected after baits were distributed in 2003. Biomarker was detected in the 2003 annulus of 17% of teeth collected after VBU distribution in 2003. Tetracycline was detected in the 2003 or 2004 annuli in 26–27% of tooth sections collected after VBU distribution in 2003. Interestingly, biomarker was detected in 73% of teeth collected during April–August 2004, prior to the second baiting. Most (79%) of tetracycline-positive teeth collected before baiting in 2004 were attributed to deposition in the 2003 annulus, but biomarker also was detected in the 2002 and 2004 annuli (9–12% of positive tooth sections).

Nearly half of serum samples obtained before VBU distribution in 2003 had low positive RVNA titers ($0.05 \leq \text{titer} < 0.25$ IU/ml before VBU distribution (Table 3). Only 8% of serum samples were RVNA-positive after VBU distribution in 2003, but 50% of positive samples had titers ≥ 0.25 IU/ml (Table 3 and Fig. 4). Tetracycline-positive individuals were 2.58 times more likely to be RVNA-positive than tetracycline-negative raccoons after VBU distribution in 2003 ($\chi^2 = 7.79$, df=1, $P < 0.01$; Table 4).

Vaccinia virus antibodies (measured only in 2003) showed a similar pattern to that of RVNA titers, with 41% prevalence before VBU distribution falling to 20% after VBU distribution in 2003. Prevalence of RVNA did not differ between VVA-positive and VVA-negative serum samples (odds ratio =1.13) before VBU distribution ($\chi^2 = 0.43$, df=1, $P = 0.51$), but VVA-positive raccoons were 2.6 times more likely to be RVNA-positive than VVA-negative raccoons after baits were distributed in 2003 ($\chi^2 = 10.15$, df=1, $P < 0.01$; Table 5).

RVNA was present (≥ 0.05 IU/ml) in 21% of sera collected before VBU distribution (April–August) in 2004; 9% of titers were ≥ 0.25 IU/ml (Table 3) compared to 0% in 2003. As in 2003, the proportion of RVNA-positive animals declined after VBU distribution (September–October) in 2004. Only 18% of samples were RVNA-positive (≥ 0.05 IU/ml) and 44% of positives had titers ≥ 0.25 IU/ml (Table 3 and Fig. 4). Tetracycline-positive individuals were 1.7 times more likely to be RVNA-positive than tetracycline-negative raccoons after VBU distribution in 2004 ($\chi^2 = 3.03$, df=1, $P = 0.08$; Table 4).

Five of 36 (14%) serum samples col-

TABLE 3. Prevalence of rabies virus-neutralizing antibody (RVNA) by titer level (IU/ml) in live-trapped raccoons before and after distribution of oral rabies vaccine-bait units on Plum Brook Station, Erie County, Ohio, USA, May 2003 to October 2003 and March 2004 to October 2004.

	Prevaccine		Postvaccine		Total	
	n	%	*n*	%	*n*	%
2003 RVNA titer (IU/ml)						
≥0.25	0	0	4	4	4	3.0
0.12–<0.25	0	0	0	0	0	0.0
0.05–<0.12	15	41	4	4	19	14.3
<0.05	22	59	88	92	110	82.7
Total	37	100	96	100	133	100.0
2004 RVNA titer (IU/ml)						
≥0.25	28	8.9	4	5	32	8.0
0.12–<0.25	3	0.9	1	1	4	1.0
0.05–<0.12	34	10.8	4	5	38	9.5
<0.05	250	79.4	78	90	328	81.6
Total	315	100.0	87	100	402	100.1

lected at OWC during 2004 were positive for RVNA (≥0.05 IU/ml), but all titers were <0.12 IU/ml. We observed the highest percentage of positive samples in May (38%, $n=8$), followed by June (17%, $n=6$) and July (13%, $n=8$). We collected no positive sera from OWC during April ($n=6$), August ($n=5$), or September ($n=3$).

We collected 17 pairs and three triads of matched (two to three samples from the same individual) sera from 17 individual raccoons in 2003 and 2004 (Table 6). Over

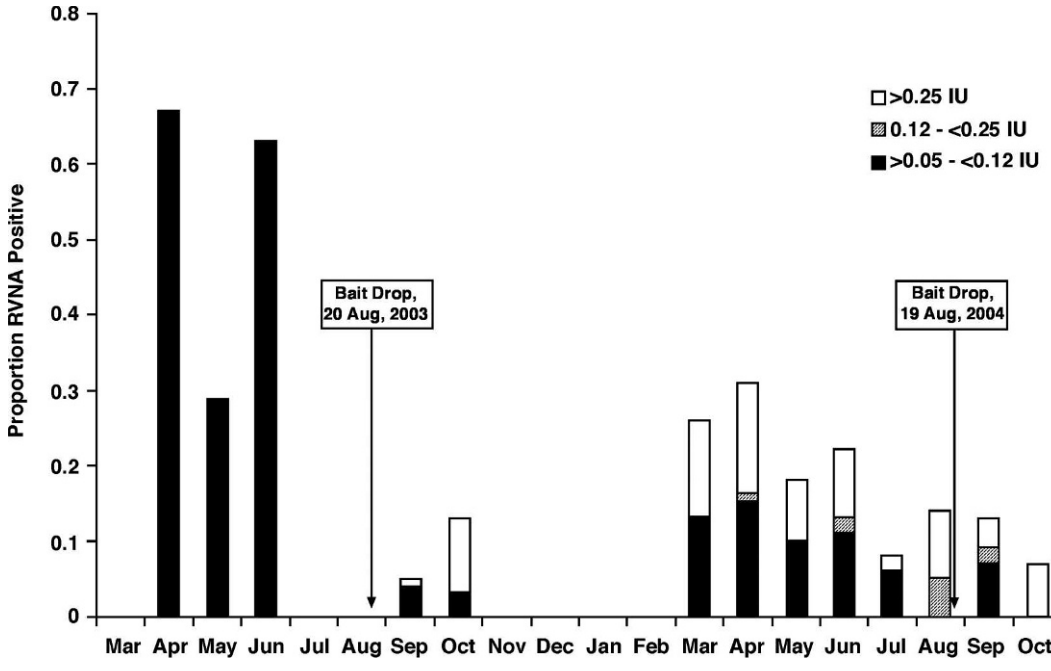

FIGURE 4. Proportion of live-trapped raccoons that were rabies virus-neutralizing antibody–positive, by titer and month, before and after distribution of oral rabies vaccines on Plum Brook Station, Erie County, Ohio, USA, May–October 2003 and March–October 2004.

TABLE 4. Cross tabulations of prevalence of rabies virus-neutralizing antibody (RVNA) and tetracycline in sera and teeth collected live-trapped raccoons after distribution of oral rabies vaccine-bait units on Plum Brook Station, Erie County, Ohio, USA, September 2003 to October 2003 and September 2004 to October 2004.

	RVNA											
	2003						2004					
	Positive		Negative		Total		Positive		Negative		Total	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Tetracycline												
Positive	2	25	9	10	11	12	3	33	17	22	20	23
Negative	6	75	79	90	85	89	6	68	61	78	67	77
Total	8	100	88	100	96	100	9	100	78	100	87	100

the 2 yr, eight pairs of matched sera were obtained within the prebait period, seven pairs were obtained before and after baiting, two pairs were obtained within the post-bait period, and three pairs were obtained during the post-bait period in 2003 and the prebait period in 2004. Titers of RVNA did not change in only one matched pair that was collected during the postbait period. Otherwise, RVNA titers increased in seven of 20 (35%) matched pairs and decreased in 12 of 20 (60%) matched pairs. Three of seven matched pairs with increasing RVNA titers were collected before and after baiting. Each of these three raccoons had no detectable titers before baiting but were RVNA-positive after baiting in 2004. Three raccoons had antibody titers that increased between the post-bait period in 2003 and the prebait period in 2004. Only one of these three individuals had detectable RVNA titer in the initial sample. Seven of twelve matched sera with RVNA titers that declined were collected within the prebait period. Titer levels of RVNA declined in four of twelve matched sera that were collected during the pre- and postbait periods. One of 12 matched pairs where RVNA titers declined was collected within the postbait period.

Median tissue culture infectious dose of vaccine ($TCID_{50}$)

The geometric mean titer (GMT) of V-RG virus in the VBUs after 51 days of refrigeration was 9.0 $\log_{10}$ $TCID_{50}$/ml (8.2–9.2 $\log_{10}$ $TCID_{50}$/ml). The GMT for shaded VBUs was 7.2 $\log_{10}$ $TCID_{50}$/ml ($<$5.2–9.2 $\log_{10}$ $TCID_{50}$/ml). No virus was detected after 51 days in any of the VBUs exposed to sunlight. Mean daily temperature during the VBU exposure experiment was 16 C with a mean daily maximum of

TABLE 5. Cross-tabulations of the prevalence of rabies virus-neutralizing antibody (RVNA) and vaccinia virus vector antibody (VVA) in sera collected from live-trapped raccoons before and after distribution of oral rabies vaccines on Plum Brook Station, Erie County, Ohio, USA, April 2003 to October 2003.

	RVNA											
	Prevaccine						Post-vaccine					
	Positive		Negative		Total		Positive		Negative		Total	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
VVA												
Positive	4	27	5	237	9	24	3	37	16	17	19	19
Negative	11	73	17	77	28	76	5	63	77	83	82	81
Total	15	100	22	100	37	100	8	100	93	100	101	100

TABLE 6. Change in RVNA titers (IU/ml) of matched sera collected from individual raccoons live-trapped on Plum Brook Station, Erie County, Ohio, USA, May 2003 to October 2003 and March 2004 to October 2004.

	Prevaccine		Pre- to post-vaccine[a]		Post-vaccine		Post- to prevaccine[b]		Total	
Period	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Change										
Increase	1	12	3	43	0	0	3	100	7	35
None	0	0	0	0	1	50	0	0	1	5
Decrease	7	88	4	57	1	50	0	0	12	60
Total	8	100	7	100	2	100	3	100	20	100

[a] Within a single year.

[b] Between 2003 and 2004.

22 C and mean daily minimum of 11 C (ranging from 33 C on 25 August 2003 to 0 C on 6 October 2003).

DISCUSSION

Target population density

Doubling (to account for juveniles) the M_{th} estimate of adult population size and dividing by area used by radio-marked raccoons, we estimated that density of our target population averaged 91 raccoons/km^2 in 2003 and 2004. With a realized baiting density of 70 VBU/km^2 both years, 0.6–0.9 baits were distributed per raccoon in 2003 and 2004. Raccoon densities vary from 6–11/km^2 in rural areas to 40–125/km^2 in urban areas (Table 7). The predominantly native vegetation of Plum Brook, bounded on one side by residential development and three sides by cropland, resembles the suburban–rural interface of the midwestern United States. Den sites in buildings, absence of hunting, and close proximity to anthropogenic food sources are features that typically support high-density raccoon populations (Prange et al., 2003, 2004).

Target population response to VBU distribution

Presence of bait biomarker within specific annuli of teeth collected during September–October indicated that 17–27% (mean=23%) of raccoons ingested baits after VBU distribution in 2003 and 2004. However, biomarker was present in 73% of teeth collected during April 2004 to August 2004, with 58% prevalence in the 2003 annulus. Bait biomarker was not detected before baits were first distributed in 2003, yet tetracycline was detected in the 2002 annulus of 1–8% of teeth collected thereafter. Furthermore, tetracycline was detected in the 2004 annulus in 11% of teeth collected before the second baiting. Blackwell et al. (2004) found that 83% of VBUs were removed by raccoons and nontarget species within 1 wk of hand-placement (at 75 VBU/km^2) on five of our trapping grids in 2002, so it is unlikely that any baits remained for consumption by raccoons after winter 2003. Pooling all teeth collected after baiting in 2003 and before baiting in 2004 and disregarding the putative year of biomarker deposition, 58% of our target population ingested baits after VBUs were distributed in 2003; 54% ingested baits after VBUs were distributed a second time in 2004. Tetracycline is not always detected in calcific tissue after raccoons ingest ORV baits (Johnston et al., 2005) so we may have underestimated bait contact.

Although background titers of RVNA and VVA were present in our population before baiting, bait distribution affected prevalence of RVNA in the target population. Prevalence of RVNA was associated with bait ingestion in 2003 and 2004 because tetracycline-positive raccoons were 2.6 and 1.7 times more likely to be RVNA-positive than were tetracycline-

TABLE 7. Published estimates of raccoon population densities in eastern North American by landscape type and method of estimation.

Population density (No./km^2)	Landscape type	Estimation method	Literature source
125	Urban	Mark–recapture	Riley et al., 1998
100	Urban	Mark–recapture	Rosatte, 2000
94	Urban	Mark–recapture	Schinner and Cauley, 1974
40	Urban	Mark–recapture	Gehrt, 2002
91	Suburban/rural	Mark–recapture	This study
68	Suburban	Mark–recapture	Hoffmann and Gottschang, 1977
37	Suburban	Mark–recapture	Gehrt, 2002
33	Suburban/rural	Line transect	Blackwell et al., 2004
11	Rural	Mark–recapture	Gehrt, 2002

negative raccoons after baiting each year. The diminished strength of association between biomarker and RVNA prevalence in 2004 would be expected if some raccoons that were tetracycline- and RVNA-positive in 2003 did not ingest baits in 2004. Raccoons that were VVA-positive also were 2.6 times more likely to be RVNA-positive than VVA-negative raccoons after baiting in 2003, indicating that V-RG vaccine contained in baits stimulated production of RVNA in some individuals.

Prevalence of RVNA titers >0.25 IU/ml increased after baiting in 2003, providing additional evidence that VBU distribution influenced RVNA titers in our target population. We found no raccoons with RVNA titers >0.12 IU/ml before VBUs were first distributed in 2003, but titers ≥0.25 were detected before (9%) and after (4%) bait distribution in 2004. With elevated RVNA titers present before baiting in 2003 and 2004, we expected to observe a strong anamnestic response if a large fraction of our population had previously been exposed to rabies virus or consumed the V-RG vaccine, but this was not the case. However, we did observe modest increases in the proportions of RVNA-positive raccoons with titers ≥25 IU/ml after baiting in 2003 and 2004, and an overall increase in prevalence of titers ≥25 IU/ml 7–15 mo after initial baiting in 2003.

Averaging years, 9.8% of our target population had elevated (>0.05 IU/ml) RVNA titers after baiting. This was not substantially higher than 8–9% prevalence of RVNA-positive (≥0.05 IU/ml) raccoons observed in two ORV-naïve areas near the Ohio ORV zone in 2005 (WS, 2005). In contrast, RVNA prevalence (≥0.05 IU/ml) was 33% after ~65 VBU/km^2 were first distributed in the northeastern Ohio ORV zone where rabies was epizootic (ODH, 2002). Prevalence of RVNA was nearly doubled when ODH (2001) increased bait densities from 75 VBU/km^2 (22% antibody prevalence) to 300 VBU/km^2 (41% antibody prevalence). ODH (2001) distributed baits and sampled raccoons over a larger area that included a wider range of habitats than Plum Brook. Because the eastern Ohio ORV zone is predominantly rural, ODH (2001) probably distributed baits to a target population with lower overall density than we estimated on Plum Brook (Table 7).

The low prevalence of RVNA that we observed after baiting can be attributed in part to distributing <1 VBU per raccoon. However, with at least 56% of our target population consuming baits and 9.8% prevalence of RVNA in sera after VBU distribution each year, only 17% of raccoons developed elevated RVNA titers after ingesting baits. The vaccine contained in VBUs exposed to ambient conditions remained viable for several weeks after distribution, so defective vaccine cannot explain the low prevalence

of RVNA after baiting in 2003. Blackwell et al. (2004) found that nearly 90% of vaccine sachets recovered from baits that disappeared 1 wk after hand-placement on five of our trapping grids in 2002 were perforated, suggesting oral contact with vaccine. Applying our estimate of bait ingestion rate to the estimate of oral contact rate made by Blackwell et al. (2004), half (50.6%) of our population may have ingested vaccine without acquiring sufficient quantities of vaccine to elicit serologic response.

A high prevalence of RVNA (41%) was observed before baits were distributed on a naïve site. With positive RVNA titers ($\geq$0.05 IU/ml) detected before VBUs were distributed in 2003, we cannot definitively separate background RVNA titers from those produced by ingesting infective doses of vaccine after baiting. Surprisingly, RVNA titers declined between pre- and post-bait sampling periods in 2003 and 2004, suggesting an independent seasonal cycle of elevated RVNA in our population. The same trend also was evident on a nearby control site in 2004. A seasonal decline in prevalence of positive antibody titers ($<$0.05 IU/ml) to the vaccinia virus in the same sera tested for RVNA also was observed. If all background RVNA titers disappeared from our population before baiting, then prevalence of RVNA as a result of baiting was correctly expressed as the proportions of sera with elevated RVNA titers ($>$0.05 IU/ml) during post-bait sampling periods (8.4–11.3%). However, if we disregard antibody titers that were considered background (0.05–0.12 IU/ml) during the pre-bait period in 2003, only 4.2–5.7% of our target population developed elevated RVNA titers after baiting in 2003 and 2004.

Jenkins et al. (1988) also found RVNA titers of $\geq$0.05 IU/ml to $<$0.25 IU/ml in raccoon populations naïve to rabies epizootics. Jenkins et al. (1988) attributed titers $<$0.25 IU/ml to nonspecific antibodies and considered such levels to be insufficient to produce immune protection to rabies challenge (see LaFon, 2002). The CDC reports titers $>$0.05 IU/ml as positive (Rupprecht et al., 1988) and animals with titers $>$0.05 IU/ml have been found to be protected from fatal rabies virus infections following challenge in laboratory experiments (Rupprecht et al., 1988). Although populations of RVNA-positive wild raccoons have been regularly monitored after distribution of VBUs since inception of the ORV program, there is disagreement about the minimum antibody titer that prevents fatal rabies infections in free-ranging populations. Regardless of what minimum antibody titer confers immunoprotection, $<$12% of our target population could have been protected from rabies infection after baiting in 2003 and 2004.

A vaccination rate of 63% was sufficient to stop the spread of rabies on the Cape Cod peninsula (Robbins et al., 1998) where natural land features and a narrow ORV zone may have facilitated success. This rate is well above the prevalence of RVNA observed before or after baiting in our study. Although the level of population immunity necessary to provide a barrier to rabies outbreaks remains controversial (Rupprecht et al., 1995; World Health Organization, 2005), rates $>$80% have been suggested in some modeling studies (Coyne et al., 1989; Bruggemann, 1992). Regardless, the westward spread of raccoon rabies seems to have been achieved in Ohio with considerably lower prevalence of elevated RVNA titers after baiting.

Sources of background RVNA

Possible explanations for the presence of background RVNA include previous vaccination, translocation of infected raccoons, and exposure to a nonraccoon variant of the rabies virus or other viral infections that induce production of nonspecific antibodies. High prebait prevalence of RVNA might be expected after a large-scale trap–vaccinate–release pro-

gram (Broadfoot et al., 2001). However, no local veterinarians or rehabilitators that were contacted had knowledge that vaccinated raccoons were released in the area. Furthermore, RVNA-positive raccoons were widely distributed across the study area, an unlikely occurrence if vaccinated animals had been released, especially on a highly secure area such as Plum Brook.

Although translocation of raccoons infected with rabies is known to occur (Dobson, 2000), we discount this explanation because all raccoons that we captured appeared healthy; moribund raccoons were not reported on Plum Brook during our study. Translocation of an infected raccoon into a naïve area with high raccoon density would likely have caused a local outbreak of rabies that would have been detected by Plum Brook personnel or local residents.

Detection of RVNA after a raccoon-rabies epizootic is not uncommon (Carey and McClean, 1983). Prevalence of RVNA in a raccoon-rabies endemic area has been reported to be 10–28% (Bigler et al., 1973; Jenkins et al., 1988), considerably less than we observed before baiting in 2003. The detection of RVNA-positive animals also has been reported for skunks (*Mephitis mephitis*) by Rosatte and Gunson (1984; 21%) and raccoons (Hill et al., 1992; 5%) that were sampled outside of enzootic areas. Further, nonfatal exposure to rabies has been documented in spotted hyenas (*Crocuta crocuta*) in the Serengeti and in an oncilla (*Leopardus tigrinus*) in Bolivia (East et al., 2001; Deem et al., 2004).

Nonfatal infection of individuals exposed to a nonraccoon variant of rabies virus is another explanation for presence of RVNA in a wild raccoon population outside of an enzootic area. Bat (Chiroptera) and skunk variants of rabies virus have been identified in Ohio (ODH, 2006a). We found strong evidence of a seasonal cycle of RVNA prevalence in our population. Prevalence of RVNA was high during May–June and diminished by August in both years of our study. A similar trend also was found at our control site. These results support the possibility of exposure to a nonraccoon variant of rabies virus during winter or early spring.

Hill et al. (1993) demonstrated that raccoons can develop RVNA titers after exposure to the skunk strain of rabies virus. The relatively low number of skunks captured (only three over ~4,000 trap-nights) during both years of our study suggests that RVNA-positive raccoons were not likely exposed to a skunk variant of rabies virus in our study. Also, no cases of skunk rabies have been documented in Erie County, Ohio, since 1989, whereas bat strain rabies cases are sporadic but geographically widespread in Ohio (ODH, 2007).

Rabies infection can occur from exposure to air in caves with rabid bats as well as from ingestion of rabies-infected tissues (Constantine, 1967; East et al., 2001). Raccoons used abandoned structures on Plum Brook that also provide suitable roosting sites for bats. Although little is known regarding bat populations on Plum Brook, we suggest that use of man-made structures by both raccoons and bats on the area could provide venues for transmission of the rabies virus. Although we recognize that exposure of raccoons to the bat variants of rabies virus may not completely account for the high RVNA prevalence in the 2003 predistribution sample, exposure to a nonraccoon variant of rabies virus is a plausible explanation for the presence of RVNA in areas where rabies is not enzootic.

Although we distributed less than one VBU per raccoon to a high-density target population, over half of the raccoons ingested baits. Nevertheless, <12% of raccoons developed RVNA titers that could be considered immunoprotective to rabies infection. Distributing more baits to high-density raccoon populations in semiurban areas would not only expose bait to more individuals, but also provide more opportunities for individuals that encounter baits to consume an infective

dose of vaccine. Consequently, accurate estimates of target population density are needed to assure that adequate densities of VBUs are distributed to successfully deliver a cost-effective ORV program. There is a need to estimate target population densities in areas close to dense human populations where targeted VBU distribution may be an alternative method of distribution. The MNKA method severely underestimated raccoon density in our population. Density estimates increased with a removal model (CPUE), but were still well below those obtained with more robust methods based on capture histories. Finally, if naturally occurring background RVNA titers occur in naïve populations, then estimates of prevalence of RVNA obtained during operational baiting programs might incorrectly attribute those titers to ORV distribution, especially in spring or early summer. Development of nonspecific RVNAs without exposure to vaccine may explain why RVNAs are found in raccoons that are tetracycline-negative in bone or tooth sections (Johnston et al., 2005). Serologic surveys should be conducted if possible before distributing oral vaccine baits to thoroughly evaluate efficacy of an ORV program. Finally, the source and seasonal changes in expression of RVNA in naïve area requires further investigation, as does the level of immunoprotection afforded by low antibody titers against infection with rabies virus.

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