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Antibody response of endangered riparian brush rabbits to vaccination against rabbit hemorrhagic disease virus 2

Megan E. Moriarty,[1] Jaime L. Rudd, Fumika Takahashi, Eric Hopson, Colleen Kinzley, Darren Minier, Alex Herman, Mary Lou Berninger, Fawzi Mohamed, Muzafar Makhdoomi, Leslie W. Woods, Hon S. Ip, Deana L. Clifford

Abstract. Rabbit hemorrhagic disease virus 2 (RHDV2; *Caliciviridae*, *Lagovirus europaeus*), the cause of a highly transmissible and fatal lagomorph disease, has spread rapidly through the western United States and Mexico, resulting in substantial mortality in domestic and wild rabbits. The disease was first detected in California in May 2020, prompting an interagency/zoo/academia/nonprofit team to implement emergency conservation actions to protect endangered riparian brush rabbits (*Sylvilagus bachmani riparius*) from RHDV2. Prior to vaccinating wild rabbits, we conducted a vaccine safety trial by giving a single SC dose of Filavac VHD K C+V (Filavie) vaccine to 19 adult wild riparian brush rabbits captured and temporarily held in captivity. Rabbits were monitored for adverse effects, and serum was collected before vaccination, and at 7–10, 14–20, and 60 d post-vaccination. Sera were tested using an ELISA to determine antibody response and timing of seroconversion. Reverse-transcription quantitative real-time PCR (RT-qPCR) was performed on rectal swabs to evaluate infection status. No adverse effects from the vaccine were observed. Before vaccination, 18 of 19 rabbits were seronegative, and RHDV2 was not detected by RT-qPCR on any rectal swabs. After vaccination, all rabbits developed an antibody response, with titers of 1:10–1:160. Seroconversion generally occurred at 7–10 d. The duration of antibody response was ≥60 d in 12 of 13 rabbits. Sixteen animals were released and 4 were recaptured several months later, offering a glimpse into longer duration immune response. Our study has informed vaccination strategies for this species and serves as a model for protecting other vulnerable lagomorphs against RHDV2.

Keywords: endangered species; rabbit hemorrhagic disease; serology; vaccination.

The riparian brush rabbit (RBR; *Sylvilagus bachmani riparius*) is a federal- and state-listed endangered subspecies of brush rabbit, endemic to the northern San Joaquin Valley of central California.[32] This subspecies occurs in dense oak forests with successional shrub habitat along riparian corridors.[15] Profound habitat loss, degradation, and fragmentation have restricted their current range and distribution, making the RBR vulnerable to extinction.[15] The largest known population of RBR is located at the San Joaquin River National Wildlife Refuge (SJRNWR, https://www.fws.gov/refuge/san-joaquin-river), with an estimated population of 2,200–3,500.[16,33] In addition to habitat loss, degradation, and fragmentation, RBRs face many other threats including flooding, wildfire, drought, invasive species, and more recently, disease.[5]

Rabbit hemorrhagic disease virus (RHDV; *Caliciviridae*, *Lagovirus europaeus*) is the cause of a highly infectious and often fatal lagomorph disease characterized by acute hepatitis, hemorrhage, and disseminated intravascular coagulation.[30] Two pathogenic genotypes are recognized: RHDV1 (GI.1; classical form) and RHDV2 (GI.2).[17] A new nomenclature based on phylogenetic relationships has been proposed, in which the species is identified, followed by the genogroup, genotype, and variant (e.g., the phylogenetically derived *Lagovirus europaeus/GI.2/. . .* and its common

Wildlife Health Laboratory, California Department of Fish and Wildlife, Rancho Cordova, CA, USA (Moriarty, Rudd, Clifford); Department of Medicine and Epidemiology, Karen C. Drayer Wildlife Health Center, School of Veterinary Medicine (Moriarty, Clifford), California Animal Health and Food Safety Laboratory (Woods), University of California–Davis, Davis, CA, USA; San Luis National Wildlife Refuge Complex, U.S. Fish and Wildlife Service, Los Banos, CA, USA (Takahashi, Hopson); Department of Animal Care, Conservation and Research, Oakland Zoo–Conservation Society of California, Oakland, CA, USA (Kinzley, Minier, Herman); Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, Greenport, NY, USA (Berninger, Mohamed, Makhdoomi); U.S. Geological Survey, National Wildlife Health Center, Madison, WI, USA (Ip). Current affiliations: University of California – Santa Cruz, Santa Cruz, CA, USA (Moriarty); Endangered Species Recovery Program, Stanislaus State University, Turlock, CA, USA (Rudd).

[1]Corresponding author: Megan E. Moriarty, University of California–Santa Cruz, 115 McAllister Way, Santa Cruz, CA 95060, USA.
mmoriart@ucsc.edu

name GI.2/RHDV2/b).[20] Rabbit hemorrhagic disease (RHD) results in high morbidity and mortality, with fatality rates of 70–90% for RHDV1 and 5–70% for RHDV2.[1,8,17,18] This virus has the potential to cause substantial mortality in wild rabbit and hare populations, particularly when it is first introduced.[13,23] Both RHDV1 and RHDV2 are extremely persistent in the environment and can be transmitted through direct contact with infected rabbits and indirectly by means of fomites or mechanical vectors.[1,14]

The first report of RHDV1 was in China in 1984[21]; the virus now has a worldwide distribution and is considered endemic in many countries.[1] A new viral genotype, RHDV2, emerged in France in 2010[17] and quickly spread around the world.[28] RHDV2 spread from France to North America,[32] and progressively disseminated throughout the United States, fatally infecting domestic and wild rabbits and hares within 4 y of its introduction in 2018. To date, RHDV2 has been identified in 29 U.S. states, 19 Mexican states, and 4 Canadian provinces. In the United States, confirmed cases in wild rabbits have been concentrated in western states.[31] In May 2020, the first confirmed RHDV2 mortality in California occurred in a wild black-tailed jackrabbit (*Lepus californicus*) from the Colorado Desert in Riverside County.[2] Soon after, the California Department of Fish and Wildlife (CDFW) initiated a statewide RHDV2 surveillance system and established a website for the public to report sightings of dead lagomorphs to track the geographic spread of the virus and species affected. Between May 2020 and January 2021, RHDV2-related deaths in wild rabbits and hares had been documented in several California counties and had spread over 400 km from the index case, or could have spread from multiple introductions, rapidly moving northward towards RBR habitat.[3]

Filavac VHD K C+V vaccine (Filavie) is registered for the active immunization of domestic rabbits from 10-wk-old to reduce mortality due to RHD caused by RHDV1 and RHDV2. This commercial vaccine has proven effective at protecting farmed and pet rabbits, but wild rabbit vaccination campaigns are lacking, due to many challenges. To date, free-ranging lagomorph populations in North America have not been vaccinated against RHDV1 or RHDV2. However, various commercial vaccines against myxoma virus and RHDV1 were used in free-ranging European rabbits (*Oryctolagus cuniculus*) in Spain 30 y ago to promote species recovery following widespread population declines.[6,7]

In response to the RHDV2 incursion into California, and the subsequent threat that the virus posed to the small population of RBRs, an ad hoc team of experts from federal and state agencies and non-agency partners from zoos, academia, and non-governmental organizations was created to strategize an intervention and protect RBR from the serious risk of disease-induced extinction. After consultation with subject matter experts about the risks and benefits of a population-level intervention, it was determined that vaccinating RBRs against RHDV2 would have the greatest benefit. However, more information was needed about the safety and efficacy of RHDV2 vaccination in wild North American lagomorphs, and whether the vaccine could produce an antibody response. To address these needs, we 1) evaluated the safety of a single SC dose of Filavac in wild RBRs that were temporarily held in captivity; 2) collected serum at specified time intervals to determine if antibodies to RHDV2 could be detected post-vaccination; and 3) evaluated the timing of seroconversion.

Materials and methods

Capture and captive care of riparian brush rabbits

In August and September 2020, 21 free-ranging RBRs were captured from the SJRNWR and brought into temporary captivity to evaluate vaccine safety. Rabbits were captured in double-door, wire-mesh live traps (61 cm long ×15.2 cm high and wide; Tomahawk model 203, Tomahawk Live Traps). Traps were modified from the standard model 203 by construction with a smaller 1.3 × 2.5-cm mesh that acts as predator proofing, and plexiglass was affixed to the inner side of the doors to prevent animals from injuring themselves against the doors.

Trapping was opportunistic and depended on the presence of RBR sign (e.g., scat, runways, browse cuts on vegetation). Traps were placed in bushes whenever possible to protect the rabbits from inclement weather, direct sun, and to avoid predators; traps were opened before sunset and checked the next morning after sunrise. Captured rabbits were handled using a pillowcase, examined for injury and parasites, species recorded, and demographic data collected, including weight, morphometrics (right ear and right rear foot length), sex, reproductive status, and age class (adult vs. juvenile). Rabbits were identified with a unique ear tag (Style 893; National Wing Bands). Biosecurity practices included cleaning traps with 10% bleach, wearing disposable nitrile gloves during animal handling, and disinfecting equipment (1% Virkon S; Lanxess) between individuals.

Live-captured RBRs were transported to the Oakland Zoo (Oakland, CA, USA) where they were temporarily held in captivity for the vaccine safety trial and serial blood sample collections. Rabbits were housed individually in steel laboratory cages 41 cm tall × 61 cm deep × 76 cm wide in a semi–climate-controlled indoor–outdoor quarantine space of Oakland Zoo's veterinary hospital. This area had little external disturbance, skylights for natural light cycles, and video monitoring of each cage. Temperatures were maintained at 4.4–26.7°C (40–80°F). Timothy hay was provided as substrate, and each rabbit received an 18 cm high × 30 cm wide hide box for shelter and perching that had a sliding door and hinged top to decrease animal stress. Cages were spot cleaned as needed during daily feeding, and received a deep clean while rabbits were out of the enclosures for handling and examination. Diet included native browse (leaves, woody

stems) from locations where the rabbits were collected, as well as mixed leafy greens (kale, Italian parsley, dandelion leaves, red chard, mustard leaves) and ad libitum commercial rabbit pellets (Oxbow Animal Health). Water was provided ad libitum in water bottles (Lixit Animal Products), manufactured specifically for rabbits, hung outside the cage, and supplemented with 6 romaine lettuce leaves per d in case rabbits did not take to water bottles. Food intake was recorded, and behavior was monitored via remote cameras daily as indicators of RBR acclimation, health, and comfort level. All rabbits that died during the study received a postmortem examination.

All field work, sample collection, and vaccination activities were coordinated by U.S. Fish and Wildlife Service (USFWS) and CDFW agency staff, as authorized under USFWS Biological Opinion 08ESMF00-2020-F-2562, dated 2020 Aug 7. Handling protocols in captivity underwent additional review by CDFW and Oakland Zoo veterinarians.

Sample collection for antibody determination and vaccine safety evaluation

After an acclimation period of ~1 wk in captivity, RBRs were examined and sampled by veterinary and animal care staff under general anesthesia (Isoflurane 1–4% via vaporizer, with oxygen flow of 1–3 L/min). Blood samples were collected from each rabbit just before vaccination (day 0), then again at 7–10, 14–20, and ~60 d post-vaccination (dpv). Heart rate, respiratory rate, and respiratory effort were monitored, and a physical examination was performed by a veterinarian to assess general health, body condition, and reproductive status. Rabbits deemed healthy were then positioned in lateral or dorsal recumbency and 0.4–1.0 mL of blood was collected from a jugular or lateral saphenous vein using a 27-ga, 1.3-cm (0.5-in) needle attached to a 1-mL syringe.

Whole blood was placed into a serum separator tube (MiniCollect; Greiner Bio-One), centrifuged within 6–8 h at $1{,}000 \times g$ for 15 min, serum decanted, then frozen at –80°C until testing. In addition to sera, rectal swabs were collected for viral RNA detection to evaluate an individual's RHDV2 infection status. Rectal swab sampling occurred close to the time of vaccination (from 5 d before to 7 d after). A sterile polyester-tipped applicator was gently introduced no more than 1 cm into the rabbit's rectum, rotated, and placed into a cryovial with 1.5 mL of viral transport medium and stored at –80°C until reverse-transcription quantitative real-time PCR (RT-qPCR) testing.

Filavac is an inactivated polyvalent RHDV1/RHDV2 vaccine with aluminum hydroxide as an adjuvant that contains inactivated RHDV strain LP.SV.2012 (variant strain 2010, RHDV2) and RHDV strain IM507.SC.2011 (classical strain, RHDV1). The vaccine is filled in glass type I containers, closed with a nitrile rubber stopper, and sealed with an aluminum cap. On day 0, a 0.5-mL dose of Filavac was administered to each RBR via SC interscapular injection while the rabbit was under general anesthesia. Each rabbit was closely monitored during anesthetic recovery for at least one hour to detect possible immediate hypersensitivity reactions including anaphylaxis, severe swelling or erythema, or respiratory distress. Additionally, food intake, attitude, and activity were monitored daily via visual observations and remote cameras to detect more subtle changes that could be due to vaccination, including lethargy, inappetence, and swelling. Although transient fever has been reported in domestic rabbits vaccinated with Filavac,[25] body temperature was not monitored due to the stress that daily handling would cause for the wild rabbits. At each subsequent examination and sample collection event, rabbits were examined for any swelling or nodule development at the injection site.

Vaccinated rabbits were released into the wild in the SJRNWR. Several rabbits were recaptured in spring 2021, fall 2021, and spring 2022, at which time booster Filavac vaccinations were administered.

Laboratory testing

ELISA is commonly used for serologic detection of vaccine-induced anti-RHDV antibodies in domestic and wild rabbits.[10,27,29] Serum samples were tested using the RHDV2 antibody ELISA kit from the OIE Reference Laboratory for Rabbit Hemorrhagic Disease following the kit's instructions.[17] This blocking ELISA detects RHDV2-specific antibodies in test sera. Testing was performed at the United States Department of Agriculture–Foreign Animal Disease Diagnostic Laboratory (Greenport, NY, USA). Briefly, the test and controls were serially diluted 4-fold in duplicate using dilutions 1:10, 1:40, 1:160, and 1:640 directly in plates coated overnight with RHDV2 hyperimmune serum. RHDV2 antigen was immediately added, and the plates were incubated for one hour. Next, a diluted enzyme-conjugated antibody (mAb-HRP) was added, and the plates were incubated for one hour. Washing steps were performed following each incubation. Lastly, an OPD (o-phenylenediamine dihydrochloride) substrate was added, and plates were incubated for 5 min. The reaction was stopped, and color development was recorded at 490 nm. The purpose of the mAb-HRP is to measure whether RHDV2 antigen was captured on the plate in the initial incubation of serum and antigen. A color change indicates a negative test, meaning that the enzyme-conjugated antibody bound the RHDV2 antigens. The absence of color indicates a positive test, meaning that the RHDV2 antigen was bound to specific antibodies in the test serum and the RHDV2 antigen was not available to bind to the coated plate. The serum titer corresponds to the dilution of the serum that inhibits absorbance at 490 nm of the negative control serum by >25%. According to the ELISA kit instructions, intermediate values are considered to be doubtful and should be reported as inconclusive; we interpreted inconclusive results as negative.

Table 1. Titers of a blocking ELISA for rabbit hemorrhagic disease virus 2–specific antibodies in the serum of 19 vaccinated riparian brush rabbits (*Sylvilagus bachmani riparius*). Blood samples were collected just before vaccination (day 0), then at 7–10, 14–20, and ~60 d post-vaccination (dpv). In some cases, rabbits were subsequently recaptured and revaccinated, at least twice over the next 1.5 y (spring 2021, fall 2021, spring 2022).

Rabbit	Day 0	7–10 dpv	14–20 dpv	60 dpv	Spring 2021 (7–8 mo post-initial vaccination)	Fall 2021 (5 mo post-booster vaccination)	Spring 2022 (7 mo post-booster vaccination
1	Neg	1:10	1:10	1:40	—	—	—
2	Neg	1:40	1:10	1:40	—	—	—
3	Neg	1:10	1:40	—	—	—	—
4	Neg	Neg	1:10	1:10	—	—	—
5	Neg	1:10	Neg	—	—	—	—
6	1:40	1:160	1:160	1:160	—	—	—
7	Neg	1:40	1:40	1:40	—	—	—
8	Neg	1:40	1:40	1:10	—	—	—
9	Neg	1:10	1:10	1:10	—	—	—
10	Neg	1:10	Neg	Neg	—	—	—
11	Neg	1:40	1:40	1:40	—	—	—
12	Neg	1:160	1:40	1:10	—	—	—
13	Neg	1:10	1:10	—	—	—	—
14	Neg	1:40	1:40	—	—	—	—
15	Neg	1:40	1:10	1:160	—	—	—
16	Neg	1:10	1:40	1:40	Neg	Neg	—
17	Neg	1:10	1:40	1:40	Neg	1:40	—
18	Neg	1:40	1:40	—	Neg	1:10	1:10
19	Neg	1:10	1:10	1:10	1:10	1:10	—

Neg = negative. Dash (—) indicates no available titer at a given time.

RT-qPCR was used to detect RHDV2 in rectal swabs.[11] Although the primary sample type used for RHDV2 PCR is liver, nasal and rectal swabs can also be used to detect RNA viral shedding.[4,19] Testing was performed at the United States Geological Survey–National Wildlife Health Center (Madison, WI, USA). Swab samples were briefly vortexed and centrifuged at 1,000 × *g* for 10 min; 200 µL of supernatant was extracted (Kingfisher Flex robotic platform, MagMax nucleic extraction kit; Thermo Fisher) according to the manufacturer's instructions. Samples were eluted in 90 µL of the elution buffer provided by the kit manufacturer (https://www.thermofisher.com/order/catalog/product/AM1830). RT-qPCR was performed (TaqMan fast prep virus kit; Thermo Fisher) on 5 µL of the extracted RNA.

Postmortem examination

Any rabbits that died during our study were submitted to the California Animal Health and Food Safety Laboratory (Davis, CA, USA) for postmortem examination, which included gross and microscopic evaluation of tissues, as well as bacterial culture and/or analysis of tissue minerals. Ancillary testing was based on individual clinical history and gross and microscopic findings. Tissues were collected and fixed in 10% neutral-buffered formalin from each carcass, including portions of lungs, heart, trachea, liver, kidneys, spleen, skeletal muscle, esophagus, reproductive tissues, tonsils, thyroid glands, adrenal glands, tongue, stomach, small and large intestines, brain, and skin. Three- to 4-µm sections of tissues were stained with H&E.

Data analysis

Serum titer data were summarized into tables and graphed for visual display of changing antibody levels over time and individual variation in immune response. The date of the first positive antibody detection was interpreted as the time of seroconversion. The proportion of rabbits that seroconverted and the duration of antibody response were recorded.

Results

Of the 21 free-ranging RBRs that were brought into captivity, 19 RBRs were vaccinated and participated in the safety trial (Table 1). All 19 vaccinated rabbits remained healthy, and no adverse effects of the vaccine were observed over a 2-mo period. Serum was collected from 14 individuals at all 4 times (0, 7–10, 14–20, 60 dpv); 5 animals had sera from 3 times (0, 7–10, 14–20 dpv). Four of the rabbits without the 60-dpv titer were released back into the wild early due to

captivity-related stress, and one adult male died (rabbit 14; Suppl. Table 1).

All 18 rectal swabs collected from individuals at baseline tested negative on the rectal RT-qPCR, indicating no RHDV2 infection. In total, 80 sera were tested. Titers were 1:10–1:160, with most titers low (1:10) to moderate (1:40; Table 1, Fig. 1). Eighteen of 19 rabbits had no detectable RHDV2 antibody response before vaccination. Rabbit 6 was seropositive at T0 before being vaccinated; it was excluded from further analyses and is discussed separately. Of the 18 rabbits seronegative at the time of vaccination, 17 of 18 seroconverted at 7–10 dpv; rabbit 4 seroconverted at 14–20 dpv (Table 1). Most initial titers at 7–10 dpv were 1:10 ($n=9$), followed by 1:40 ($n=7$), and 1:160 ($n=1$); rabbit 4 had an initial titer of 1:10. Titers generally remained constant or increased over time ($n=12$), but 5 rabbits had titer fluctuations over time, and the titer of rabbit 12 consistently decreased over time; of note, this rabbit's initial titer at 7–10 dpv was the highest of the group at 1:160, then waned to 1:40 at 14–20 dpv, and then to 1:10 at 60 dpv.

The duration of antibody response lasted at least 60 d in 12 of 13 rabbits with sera collected at all 4 times (0, 7–10, 14–20, 60 dpv). Most rabbits had a 60-d titer of 1:10 ($n=5$) or 1:40 ($n=6$), with rabbit 6 strongly seropositive at 1:160. Rabbit 10 did not have an immune response at 60 d, but had a single detectable titer of 1:10 at 7–10 dpv and was subsequently seronegative. Due to treatment for an unrelated medical issue (Suppl. Table 1), the final blood sample for rabbit 8 was collected at 91, rather than 60 dpv; the initial titer was 1:40 at 7–10 dpv and 14–20 dpv, whereas the final titer decreased to 1:10 at 91 dpv.

Rabbit 6 had a moderately high titer at 1:40 at day 0. This rabbit also had a consistently detectable and high titer of 1:160 at all 3 subsequent times. The RHDV2 rectal swab collected at day 0 was negative. There were no clinical or behavioral differences observed in this animal.

Sixteen rabbits were successfully released back to the SJRNWR near their original capture locations. Rabbits 16–19 were subsequently recaptured at least twice over the next 1.5 y (Table 1; Fig. 2). All 4 rabbits were recaptured in spring 2021, 7–8 mo after their initial vaccine, and blood was collected before administering a booster vaccine. Three of the 4 rabbits, all of whom had detectable antibodies during their final sampling event in captivity, had no detectable antibodies at this recapture (Table 1; Fig. 2). Rabbit 19 had a titer of 1:10, which was the same as its titer at 60 dpv. All 4 rabbits were recaptured in fall 2021, 5 mo after their first booster vaccine; 3 rabbits now had a detectable titer of 1:10–1:40; rabbit 16 was seronegative when recaptured in fall 2021. Rabbit 18, which was captured for a fourth time in spring 2022, 7 mo after its third vaccination, had a persistent titer of 1:10.

Five deaths occurred during our study. Two of the 21 rabbits originally caught were excluded because they died shortly after entering captivity. An adult female escaped her enclosure, sustained life-threatening injuries, and was euthanized 4 d after admission. An adult male died during initial examination, and the only significant finding from the postmortem examination was pulmonary atelectasis, a relatively common post-anesthetic complication.[12] The other 3 deaths occurred among vaccinated rabbits before they could be released (Suppl. Table 1).

Discussion

We found that a single SC dose of Filavac could be safely administered to RBRs without any observed adverse effects. Most rabbits had an immune response to vaccination, evidenced by antibody titer development within 7–10 dpv. However, the magnitude of vaccine-induced antibody titers was lower than anticipated. One explanation could be species-specific differences in response. Domestic rabbits (*Oryctolagus cuniculus*) vaccinated with Filavac had antibody titers of 1:40–1:640, with seroconversion occurring within 7–15 d.[17,19] In an experimental challenge study of wild eastern cottontails (*Sylvilagus floridanus*), RHDV2-specific antibody titers were 1:10–1:2,560.[22] Another explanation for low titers might be ELISA performance on a non-target species; the test was validated for domestic *Oryctolagus*, not free-ranging *Sylvilagus*.

Data from naturally infected and vaccinated domestic rabbits and wild eastern cottontails indicate that RHDV2 antibody titers are generally low, but even low levels of specific anti-RHDV2 antibodies offer protection from disease because humoral immunity is the primary defense against RHDV2.[17] Protective antibody titers against RHDV2 (≥1:10) have been determined in laboratory rabbits during experimental studies in which vaccination prevented clinical signs, viral shedding, and death.[17,19] It remains unknown what degree of antibody response may protect RBRs against RHDV2 given that vaccine challenge studies cannot be conducted in this endangered species.

To determine result reproducibility and further examine the inconclusive titers identified in our study, we retested a subset of 20 serum samples from 5 RBRs (results not shown). To increase discernment in the range of potential titers, we performed 2-fold serial dilutions from 1:10 to 1:1,280. Inconclusive results were negative upon 2-fold testing of the sera, and titer results were very similar to the same pattern observed within individuals, indicating excellent reproducibility. Titers occasionally increased 2-fold in 7 of 20 samples; however, inconclusive titers remained inconclusive in 6 of 7 samples. Given that titers in vaccinated RBRs tend to be lower than in vaccinated domestic

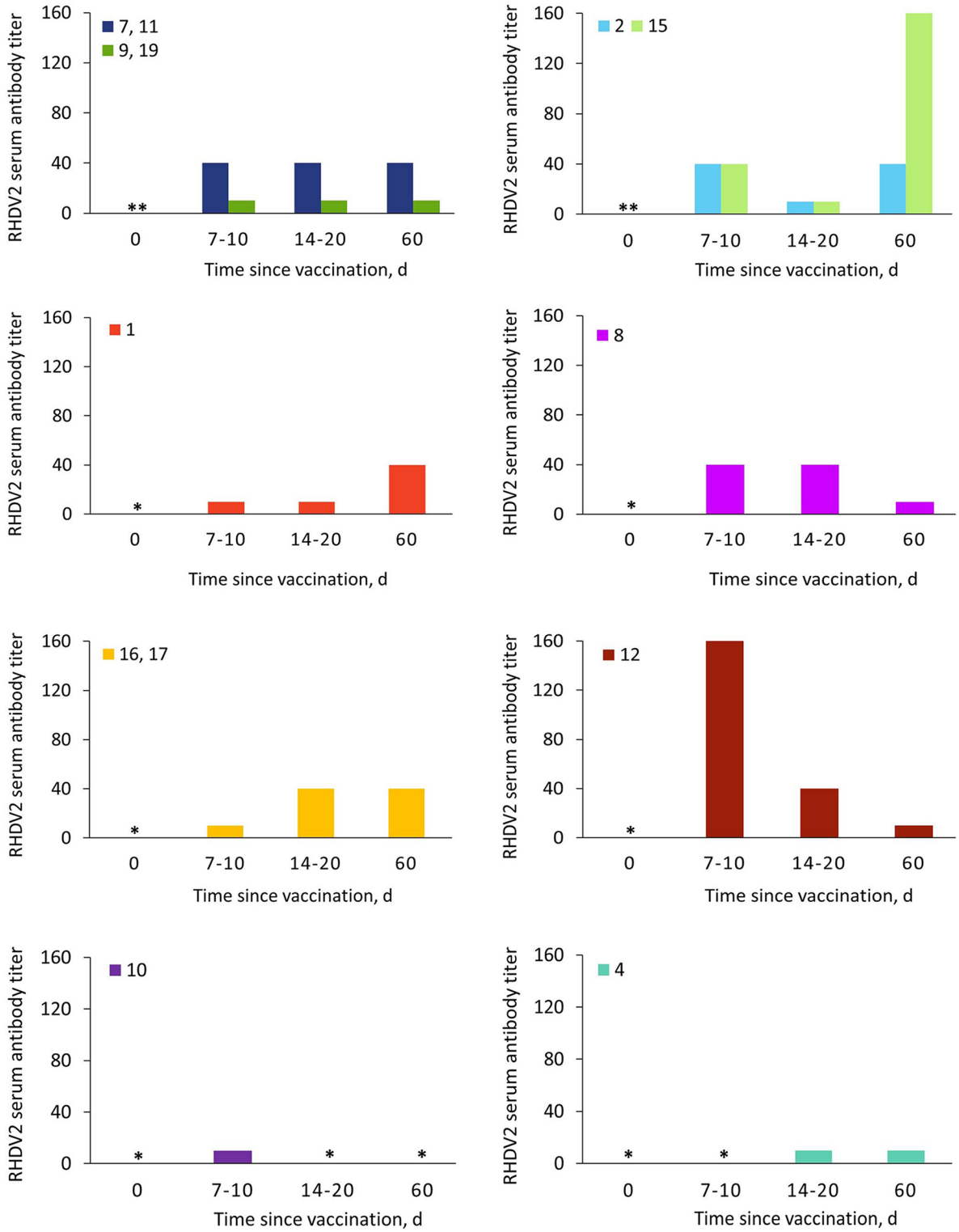

Figure 1. Rabbit hemorrhagic disease virus 2 (RHDV2)-specific antibody titers in the serum of 13 vaccinated riparian brush rabbits (*Sylvilagus bachmani riparius*). Blood samples were collected at day 0, then at 7–10, 14–20, and ~60 d post-vaccination (dpv). The rabbit sera were tested using four 4-fold serial dilutions of 1:10, 1:40, 1:160, and 1:640. Rabbit 6 is not shown because it was seropositive before being vaccinated. Rabbits 3, 5, 13, 14, and 18 are not shown because they were not sampled at 60 dpv; however, rabbit 3 followed a pattern similar to rabbits 16 and 17; rabbit 5 followed a pattern similar to rabbit 10; rabbit 13 followed a similar pattern to rabbits 1, 9, and 19; rabbits 14 and 18 followed a pattern similar to rabbits 7, 8, and 11. An asterisk indicates no detectable RHDV2 antibody response at a given time.

rabbits, additional discrimination in the range of 1:10–1:160 using 2-fold serial dilutions may be warranted for future studies.

Although we assessed antibody responses to RHDV2 vaccination within 60 d, opportunistic follow-up data offered a glimpse into longer-duration immune responses. The low positive titer for rabbit 8 at 91 dpv demonstrated that a detectable but waning antibody response persisted at 3 mo. Titers were undetectable in 4 RBRs recaptured 7–8 mo after initial vaccination; however, an antibody response was usually detectable at 5–7 mo after a booster vaccination was administered. Even if low titers are assumed to be protective, the waning antibody response in some rabbits within the first 60 d and the lack of detectable antibody titers in 3 of 4 of rabbits recaptured 7–8 mo later indicates that booster vaccination should occur before 12 mo to maintain detectable titers. Duration of Filavac immunity in domestic rabbits is 12–18 mo. Yearly revaccination is standard; however, booster vaccination is recommended every 6 mo in at-risk animals or in areas where disease is present.[17,24,26] Duration of immunity may be species-dependent, and additional research may inform the optimal vaccine frequency and booster interval for RBRs.

The only RBR that was seropositive before vaccination had reliably high and internally consistent titers, making field collection or laboratory error unlikely. This rabbit's titers were notably higher than others; only 2 other RBRs achieved titers of 1:160, one at 7–10 dpv and one at 60 dpv. The most probable explanation is nonspecific antibody cross-reactivity, such as another virus in the *Caliciviridae* family or *Lagovirus* genus, as was proposed in an eastern cottontail RHDV2 experimental challenge study in which 2 animals developed unexpected antibody titers (1:10, 1:40).[22] Active natural infection with RHDV2 at the time of sampling is highly unlikely given that the rabbit appeared healthy, RHDV2 was not detected by RT-qPCR on rectal swab, and no RHDV2-associated deaths were reported within 400 km of our study area. Seropositivity could indicate previous virus exposure and survival; however, the first confirmed RHDV2 detection at our study site occurred in May 2022, when 4 unvaccinated RBRs died from RHDV2 infection, indicating that RBR infection may be fatal.[9] Although we cannot conclusively rule out the possibility of natural RHDV2 infection or exposure, given the totality of evidence regarding species susceptibility, negative infection and exposure data from all other study animals, and virus spatial distribution, it is highly unlikely that rabbit 6 had been exposed to RHDV2.

Despite substantial efforts to minimize stress and create a semi-natural environment for wild RBRs in temporary captivity, 5 deaths occurred, and we suspect that stress was a major contributing factor. Four RBRs experiencing inappetence and weight loss were released early to ensure their well-being and reduce mortality risk. One of these RBRs was recaptured in the wild on 3 occasions several months after release and appeared healthy.

Based on our findings, a large-scale field vaccination program to protect RBRs from RHDV2 was immediately initiated and is ongoing, with the goal of keeping 15% of the population vaccinated. This vaccination effort is being monitored with serologic testing to determine the duration of antibody response to vaccination and optimal booster frequency and interval in free-ranging RBRs.

As RHDV2 continues to spread, our study provides critical information to inform vaccination efforts aimed at reducing extinction risk in other vulnerable lagomorphs, such as the pygmy rabbit (*Brachylagus idahoensis*), American pika (*Ochotona princeps*), snowshoe hare (*Lepus americanus*), and New England cottontail (*Sylvilagus transitionalis*). Although vaccination campaigns of free-ranging populations are challenging, vaccination may be the most effective management tool to protect threatened and endangered lagomorphs against RHDV2, at least until long-term conservation efforts improve resiliency in these small, geographically restricted species.

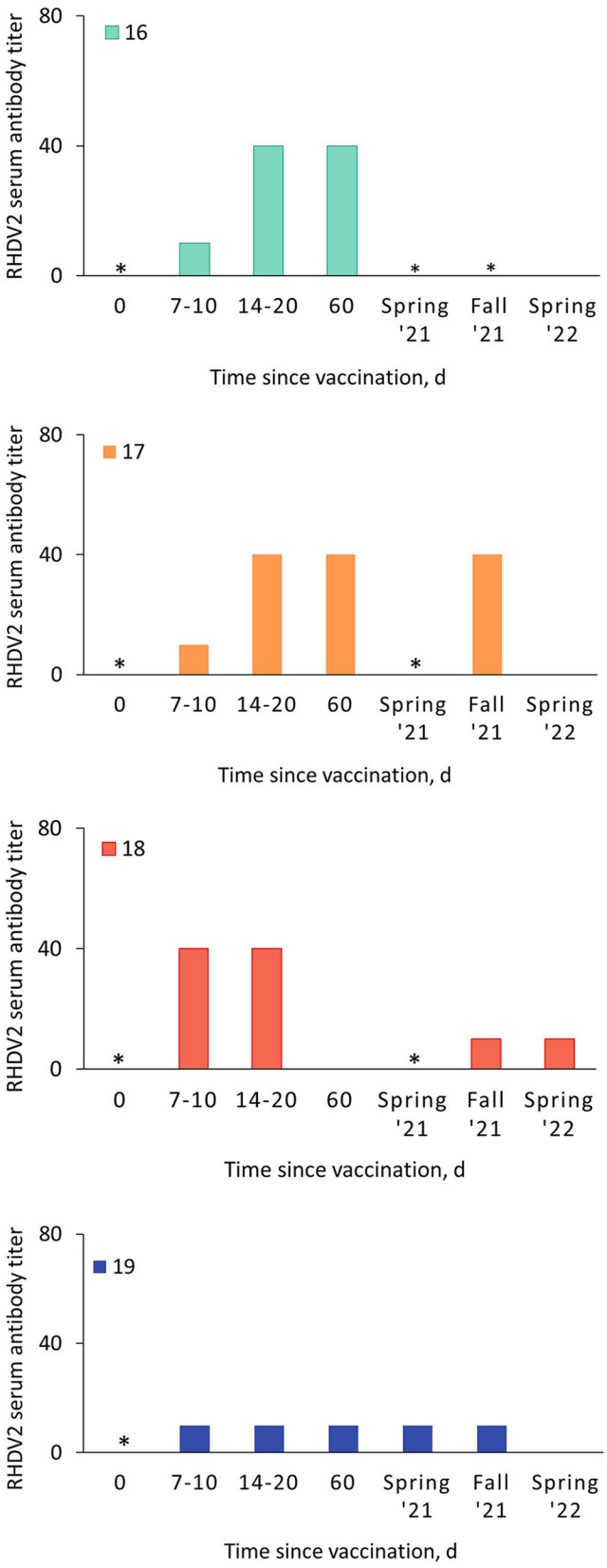

Figure 2. Rabbit hemorrhagic disease virus 2 (RHDV2)-specific antibody titers in the serum of 4 vaccinated riparian brush rabbits (*Sylvilagus bachmani riparius*). Blood samples were collected at day 0, then at 7–10, 14–20, and ~60 days post-vaccination. In addition, these rabbits were released into the wild and subsequently recaptured 2–3 times over the next 1.5 y (spring 2021, fall 2021, spring 2022). At each capture event, blood samples were collected, and a booster vaccine was administered. Rabbits 16, 17, and 19 were vaccinated 3 times (2 booster vaccines), and rabbit 18 was vaccinated 4 times (3 booster vaccines). The time between vaccinations ranged from 5–8 mo. An asterisk indicates no detectable RHDV2 antibody response at a given time.

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Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service or the official policy of the USDA, DOE, ORAU/ORISE, but do represent the views of the U.S. Geological Survey. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Declaration of conflicting interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ORCID iD

Megan E. Moriarty https://orcid.org/0000-0003-0379-2499

Supplemental material

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References

1. Abrantes J, et al. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet Res 2012;43:12.
2. Asin J, et al. Outbreak of rabbit hemorrhagic disease virus 2 in the southwestern United States: first detections in southern California. J Vet Diagn Invest 2021;33:728–731.
3. Asin J, et al. Early circulation of rabbit haemorrhagic disease virus type 2 in domestic and wild lagomorphs in southern California, USA (2020–2021). Transbound Emerg Dis 2022;69:e394–e405.
4. Asin J, et al. An RT-qPCR assay from rectal swabs for the detection of rabbit hemorrhagic disease virus 2 in natural cases. Transbound Emerg Dis 2023;2023:1869692.
5. California Department of Fish and Wildlife. Five-year status review of riparian brush rabbit (*Sylvilagus bachmani riparius*). Version 4. Report to the Fish and Game Commission. 2020. https://ecos.fws.gov/ServCat/DownloadFile/171805
6. Calvete C, et al. Short-term negative effects of vaccination campaigns against myxomatosis and viral hemorrhagic disease (VHD) on the survival of European wild rabbits. J Wildl Manage 2004;68:198–205.
7. Calvete C, et al. Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits. Vet Rec 2004;155:388–392.
8. Capucci L, et al. Increased pathogenicity in rabbit haemorrhagic disease virus type 2 (RHDV2). Vet Rec 2017;180:426.
9. Clifford DL, et al. RHDV2 in endangered riparian brush rabbits—surveillance, vaccination, and diagnostic test validation [abstract]. In: Wildl Soc Ann Conf; Louisville, KY, USA; November 2023: 5–9.
10. Cooke BD, et al. Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiol Infect 2000;124:563–576.
11. Duarte MD, et al. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J Virol Methods 2015;219:90–95.
12. Duggan M, Kavanagh BP. Pulmonary atelectasis: a pathogenic perioperative entity. Anesthesiology 2005;102:838–854.
13. Guerrero-Casado J, et al. Recent negative trends of wild rabbit populations in southern Spain after the arrival of the new variant of the rabbit hemorrhagic disease virus RHDV2. Mamm Biol 2016;81:361–364.
14. Henning J, et al. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol Infect 2005;133:719–730.
15. Kelly PA. Reintroduction of the riparian brush rabbit in the San Joaquin Valley, California, USA. In: Soorae PS, ed. Global Reintroduction Perspectives: 2018. Case Studies from Around the Globe. IUCN, 2018:210–215.
16. Landers R, et al. Population estimation of riparian brush rabbit using camera traps at San Joaquin River National Wildlife Refuge. Technical report. U.S. Fish and Wildlife Service, 2021. Cited in: Russell RE, et al. Modeling the response of an endangered rabbit population to RHDV2 and vaccination. Conserv Sci Pract 2024;6:e13072.
17. Lavazza A, Capucci L. Chapter 3.7.2. Rabbit haemorrhagic disease. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 12th ed. WOAH, 2023. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/A_summry.htm
18. Le Gall-Reculé G, et al. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. Vet Res 2013;44:81.
19. Le Minor O, et al. Rabbit haemorrhagic disease: experimental study of a recent highly pathogenic GI.2/RHDV2/b strain and evaluation of vaccine efficacy. World Rabbit Sci 2019;27:143–156.
20. Le Pendu J, et al. Proposal for a unified classification system and nomenclature of lagoviruses. J Gen Virol 2017;98:1658–1666.
21. Liu SJ, et al. A new viral disease in rabbits. Anim Husb Vet Med (Xumu yu Shouyi) 1984;16:253–255.
22. Mohamed F, et al. Comparative susceptibility of eastern cottontails and New Zealand white rabbits to classical rabbit haemorrhagic disease virus (RHDV) and RHDV2. Transbound Emerg Dis 2022;69:e968–e978.
23. Monterroso P, et al. Disease-mediated bottom-up regulation: an emergent virus affects a keystone prey, and alters the dynamics of trophic webs. Sci Rep 2016;6:36072.
24. Morin H, et al. Durée d'immunité conférée par un vaccin vis-à-vis des calicivirus classique et variant de la maladie virale hémorragique [Duration of immunity conferred by a vaccine against classical caliciviruses and hemorrhagic viral disease variants]. J Recherche Cunicole 2015. French. http://www.cuniculture.info/Docs/Magazine/Magazine2015/Fichiers-pdf-JRC/06-Morin.pdf
25. Müller C, et al. Characterization of protective humoral and cellular immune responses against RHDV2 induced by a new vaccine based on recombinant baculovirus. Vaccine 2019;37:4195–4203.
26. Rocchi MS, Dagleish MP. Diagnosis and prevention of rabbit viral haemorrhagic disease 2. In Pract 2018;40:11–16.
27. Rouco C, et al. Epidemiology of RHDV2 (*Lagovirus europaeus*/GI.2) in free-living wild European rabbits in Portugal. Transbound Emerg Dis 2018;65:e373–e382.
28. Rouco C, et al. Worldwide rapid spread of the novel rabbit haemorrhagic disease virus (GI.2/RHDV2/b). Transbound Emerg Dis 2019;66:1762–1764.
29. Strive T, et al. Retrospective serological analysis reveals presence of the emerging lagovirus RHDV2 in Australia in

wild rabbits at least five months prior to its first detection. Transbound Emerg Dis 2020;67:822–833.
30. Ueda K, et al. Disseminated intravascular coagulation (DIC) in rabbit haemorrhagic disease. Jpn J Vet Res 1992;40:133–141.
31. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Rabbit hemorrhagic disease map application [Internet]. 2023. [cited 2023 Oct 30]. https://www.aphis.usda.gov/aphis/maps/animal-health/rhd
32. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Emerging risk notice—animal health: rabbit hemorrhagic disease in British Columbia, Canada, 2018. https://www.aphis.usda.gov/animal_health/downloads/Rabbit-Hemorrhagic-Disease_062018.pdf
33. U.S. Fish and Wildlife Service. Endangered and Threatened Wildlife and Plants; Final Rule to List the Riparian Brush Rabbit and the Riparian, or San Joaquin Valley, Woodrat as Endangered. Fed Reg 2000;65 FR 8881.