

Rabies vaccine vehicle optimization for orotopical administration to wild vampire bats

(Desmodus rotundus)

by

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A thesis submitted in partial fulfillment of

the requirements for the degree of

Master of Science

(Comparative Biomedical Sciences)

At the UNIVERSITY OF WISCONSIN, MADISON

2025

Acknowledgements

Ever since reading David Quammen's *Spillover* back in middle school, I knew I wanted to be a virus hunter. I was captivated by tales of jungle expeditions seeking to trace and prevent the emergence of deadly pathogens. Over a decade later, I have already fulfilled that dream. Getting to work with vampire bats in remote regions of Mexico has been an exceptional experience in my journey.

I am extremely grateful for the opportunity to participate in this project for the past two years. I firstly thank my major co-advisors Mostafa Zamanian, who guided me seamlessly through my degree despite having expertise in parasitology rather than bats, and Tonie Rocke, who oversaw much of this research and welcomed me to collaborate with her team at the National Wildlife Health Center (NWHC). I am also fortunate for the guidance of Wendy Turner with her vast knowledge of disease ecology in contributing to my committee. I want to especially thank Jorge Osorio, who brought me into his lab as an undergraduate student five years ago and has fostered my growth as a researcher, giving me numerous opportunities and experiences over these years. This work likewise would not have been possible without the leadership of Elsa Cardenas Canales in growing collaborations and facilitating field work in Mexico. I am also grateful for the mentorship of Daniel Limonta who took me under his wing to grow me from a technician into a scientist in a few short, but intense, months. Many others from NWHC, UW-Madison, and elsewhere contributed to, supported and guided me in this work, including Lisa Powers, Merrie Urban, Charlotte Chorzempa, the NWHC Animal Services Team (especially Ryan Dashek), Danielle Maldonado, Saminathan Mani, Maria Stella Lopez Carvajal, Maleni Ramirez Martinez, and Rita Dixon.

Lastly, I thank all my friends and family for providing an escape from the rigors of academia. My parents, Rick and Rachel, supported me with their unwavering love and welcomed me home for many weekends away from Madison. I also thank the iron-sharpeners in my life for walking alongside and encouraging me, especially Caleb, Changwon, Isaac, Sam, Brian, David, and Nick. Above all, I thank my Lord and Savior Jesus Christ, for the completion of this work was only possible not by might nor by power, but by His Spirit (Zech. 4:6).

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Abstract

Rabies vaccination of vampire bats (*Desmodus rotundus*) has been proposed as a superior control method to culling but has yet to be implemented. Success of rabies vaccination depends on a topical vehicle that spreads through a bat colony via allogrooming while additionally preserving vaccine immunogenicity. This work describes the *in vitro* and *in vivo* optimization of a new orotopical gel made with carboxymethyl cellulose (CMC) for rabies vaccine delivery to vampire bats.

Physical properties three CMC formulations were compared to petroleum jelly, a commercial ointment, and glycerin jelly at 40 °C, 20 °C, and 0 °C using rheological tests. These tests indicate that CMC exhibits optimal properties for topical application to bats even at extreme temperatures possible during field vaccination.

The *in vitro* stability of our raccoon poxviral-vectored rabies vaccine candidate within CMC was measured at time points up to 6 months at 40 °C, 23 °C, and 4 °C. CMC preserved vaccine titers after extended storage of CMC at 4 °C and short exposure at higher temperatures of potential vampire bat environments.

Autonomous transferability of our CMC vaccine delivery formulation was tested in three microchipped vampire bat colonies in rural Jalisco, Mexico. Intra-colony gel uptake using the fluorescent biomarkers rhodamine B and rhodamine 110 allowed differentiation of topical spread by adult females and adult males. In all three colonies, application of topical treatment of ~15-20% of the bat colony resulted in estimated uptake by 70-90% of the colony by Bayesian modeling. This study advances our rabies vaccination strategy for vampire bats by providing a topical vehicle suitable for field application that may additionally be employed for other significant bat diseases.

Introduction

Bats (Order *Chiroptera*) serve as reservoirs of several of the most lethal emerging viral diseases in humans including Ebola, Nipah, Hendra, coronaviruses and rabies virus (1). While much remains to be understood about the emergence patterns of these viruses, preventing outbreaks of these diseases before spillovers even occur presents an ambitious goal and ultimate challenge. Rather than emphasizing prompt responses to outbreaks of disease, primary prevention strategies, such as ecological interventions and reservoir immunizations, attempt to intervene at the source of infection (2,3).

Rabies virus represents one of the greatest successes of primary prevention, yet further work remains. While eradicated in some regions, rabies continues to be a menace in other regions where poor infrastructure impedes enactment of prevention measures (4). Not only in humans, but in most mammalian species including bats, untreated rabies is virtually 100% fatal (5). Annually, 20,000 human rabies deaths occur in India alone, representing ~35% of deaths worldwide (6). Wildlife reservoirs of rabies virus include raccoons, foxes, skunks, and bats, while the majority of human cases globally are attributed to rabid dogs (7). Vaccination is the main tool to fight against rabies, both in humans and these other hosts. The impracticality of large-scale parenteral vaccination for wildlife species led to the development of flavored baits containing rabies vaccine to orally inoculate many wildlife reservoirs, combating the viral disease at its source (8). These baits are widely distributed off airplanes into the habitat range of wild animals who find and consume the baits. This strategy contributed to rabies eradication or control in large regions of Europe and the United States (8–10). Parenteral rabies vaccination campaigns in stray animals are also effective (11).

In Latin America, the success of such mass vaccination campaigns of dogs (12,13) led bats to surpass dogs as the primary rabies reservoir in the early 2000s. This is especially due to one species: the common vampire bat (*Desmodus rotundus*) (14,15). First reported in the early 1900's, vampire bat-transmitted rabies (VBR) poses significant health risk to humans (15,16) and, especially, to livestock (17). Each year, an estimated 450 independent outbreaks are mediated by the common vampire bat leading to major damages to the livestock industry and infrequent human fatalities (17). VBR causes an estimated economic burden of \$50 million USD to the livestock industry (18). Additionally, the suitable habitat range for vampire bats is expected to spread due to changing climates (19,20), and annual reports of VBR are trending upwards in several countries since the year 2000 (14,21). Recent models predict future expansion of the bats into the southern United States (20,22) posing risk of extensive damages in this region with high populations of livestock (23). The blood feeding behavior of these aptly named "vampires" plays into the hands the rabies virus, which is transmitted through saliva. Each feeding session, which the bats must do nearly every night to survive, potentially exposes their prey to the deadly virus. As agricultural practices exploded over the past century (24,25), so too has the bats' reliance on livestock as a food source increased (26).

Given the major impact of vampire bats on this key industry of Latin America, various methods were developed to fight back. These strategies include vaccination of livestock, which though effective, can be expensive and not practiced ubiquitously (14). Bat populations may also be controlled through roost destruction with explosives, fire, toxic gas, sealing off caves, or cutting down hollow trees. Most commonly, populations are controlled by application of a topical poison (27). These topical poisons, called "vampiricides", are spread onto the back of captured bats then released back to their colony. The socially adept bats groom the poison off

each other, leading to die-offs within the colony. Vampiricide was first developed in the 1970s as a method of culling vampire bats, especially when rabies is suspected within the colony (28).

One study demonstrated that a single treated bat could transfer vampiricide to up to 15 individuals (29). Contrarily, more recent work debates the utility of this culling practice in slowing down rabies transmission (30). Since its inception, these methods have been insufficient in preventing the expansion of VBR. Reactive application of vampiricide has also been shown to exacerbate rabies virus transmission by provoking dispersal of infected bats to new roosts (30,31). Furthermore, the poison may transfer to multiple non-target species in the wild, causing unintended mortality (14,28).

The ingestion of topically applied (orotopical) vaccines has been previously proposed as a superior method to bolster current control strategies (32,33). Mathematical simulations predict that transferrable vaccines that similarly utilize bat allogrooming behavior outperform culling practices in reducing the probability, size, and duration of rabies outbreaks (34). In earlier work, members of our group developed and tested a recombinant raccoon poxvirus (Poxviridae: *Orthopoxvirus*) expressing a mosaic lyssavirus glycoprotein (RCN-MoG) (35,36). This vaccine produced protective immunity intradermally in A/J *Mus musculus* mice (35), and orotopically in big brown bats (*Eptesicus fuscus*) (35). Notably, orotopical vaccination blocked shedding of rabies virus in vampire bats (36). Researchers in our team investigated glycerin jelly (GJ) as a topical vehicle to deliver the RCN-MoG vaccine in these captive colonies (35,36) and to assess topical vehicle uptake in the wild (34) with the biomarker rhodamine B (RB). However, the highly temperature dependent physical properties of GJ preclude its practicality for field-use in Latin America, where tropical and subtropical climates commonly exceed its melting point (37,38). Additionally, crystallization of GJ was noted in a separate trial in Jalisco, Mexico (39).

In this work, we describe the optimization of a novel carboxymethyl cellulose (CMC) gel vehicle for orotopical administration of the RCN-MoG vaccine to vampire bats. Beginning in chapter 1, we compared physical properties of CMC gel to GJ, petroleum jelly, and a commercial veterinary ointment using rheological tests. These tests emulated application at environmentally relevant temperatures. This characterization supports our understanding of how different topical materials will behave during and after application. We also briefly contextualize the significance of these results in relation to the development of a sprayable vaccine formulation.

In chapter 2, we further characterize CMC as a vaccine vehicle. RCN-MoG vaccine preservation was assessed by tracking viral titers within CMC and GJ over six months across a range of temperatures. A second stability test was performed up to three months to determine the impact of the RB biomarker, and the use of sucrose as a stabilizer to enhance virus preservation. We also assessed viral shedding and antibody development after orally vaccinating big brown bats (*Eptesicus fuscus*) with a single dose of RCN-MoG using the CMC vehicle.

In chapter 3, we test the ability of CMC to spread within wild colonies of vampire bats in Mexico. Supported by our understanding of its physical properties, we hypothesized that CMC would demonstrate high rates of transfer. In October-November 2024, we applied CMC gel mixed with the fluorescent biomarker RB to a wild colony of microchipped vampire bats in rural Jalisco, Mexico to investigate gel transfer and uptake of CMC in a field setting. A second field study was performed in June 2025 using two fluorescent biomarkers – RB and rhodamine 110 (R110) - to determine gel transfer rates of adult male and adult female cohorts within two additional microchipped colonies in Jalisco. Results validate CMC as a promising topical vehicle for vaccine delivery to vampire bats.

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Chapter 1

**RHEOLOGICAL CHARACTERIZATION OF GEL AND PASTE-LIKE MATERIALS
FOR TOPICAL APPLICATION TO BATS**

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Introduction

Vaccination presents an appealing alternative to practices of culling vampire bats. Like culling, vaccination would reduce the number of animals susceptible to rabies virus, without the unintended ecological effects or risk to off-target species. However, a major challenge impedes the implementation of such a strategy. Bats do not consume the oral baits used for other wild animals, raising the question of how to practically and efficiently deliver a vaccine. The method of vampiricide application may hold the key. Around the turn of the millennia, research began into administering an oral vaccine to the bats through a similar topical paste. Rather than mixing in a poison, a vaccine could in theory similarly propagate through a vampire bat colony by the social grooming behavior. Past studies performed both in captivity and in the field began investigating this proposed idea to develop a formulation for the topical delivery of a vaccine (1–4).

These studies primarily tested two topical vehicles: petroleum jelly (PJ) and glycerin jelly (GJ). PJ, a common base used in vampiricide pastes, proved challenging to work with for vaccination. The vaccine cannot be prepared directly in PJ and must be mixed in, resulting in dilution of the vaccine. It was also described that substantial paste was found falling off the bats in these studies (1,4). Meanwhile, previous field trials using glycerin jelly as the topical vehicle proved inconsistent, likewise because of its suboptimal physical properties. Its low melting temperature brings into question widespread use of GJ in the subtropical and tropical climates found in Latin America. Additionally, the GJ preparation used during a second field trial in Mexico crystallized and became difficult to apply (5). These past studies emphasize that in selecting a novel topical vehicle, it is imperative to identify a material with optimal physical properties for application, especially at warmer temperatures.

In this chapter, the physical properties of multiple gel and paste-like materials were measured with rheological testing. Rheology, the study of how a material responds to deformation, is a commonly used method for the characterization of gel-like materials (6). Six different substances were characterized by a steady rate sweep test to measure viscosity and a dynamic strain sweep test to measure viscoelastic properties. In addition to GJ and PJ, 10% solution of carboxymethyl cellulose (CMC), 12.5% CMC, 15% CMC, and Silverquine® (SQ) – a commercial veterinary ointment - were characterized.

Methods

Characterization of Physical Properties

Sample Preparation

Precise weights of CMC powder (Sigma-Aldrich, cat. no. 419281; St. Louis, Missouri, USA) were added to ultrapure water (Modulab® High Flow, Evoqua, Pittsburgh, Pennsylvania, USA) to prepare bulk solutions of 10%, 12.5%, and 15% (w/v). CMC gels were dissolved at room temperature overnight.

GJ (Carolina Biological Supply Co, cat. no. 865495; Burlington, North Carolina, USA) was melted at 37 °C and combined with ultrapure water at the ratio of 55.5:44.5 previously used in field studies (2). GJ solidified while kept at room temperature overnight.

PJ (Vi-Jon Inc, St. Louis, Missouri, USA) and SQ (Silverquine LLC, SW Ranches, Florida, USA) are commercial products.

Rheological Measurements

For characterization of rheological properties, an ARES LS2 Advanced research grade rheometer (TA Instruments, New Castle, Delaware, USA) was fitted with 50 mm cone and plate

geometry. An FS18-HD Refrigerated/Heating Circulator (Julabo, Allentown, Pennsylvania, USA) and Peltier Plate were used for temperature control. Two different rheological tests were performed at 0 °C, 20 °C, and 40 °C with at least 5 min allowed for temperature equilibration. Testing was performed the day after gel preparation.

The first test, a steady rate sweep test, was used to record viscosity over a shear ramp from 0.05 s^{-1} to 200 s^{-1} . Low shear rates characterize the near-zero shear rate and mimic behavior at rest. The range of 20 s^{-1} to 200 s^{-1} has previously been described as the shear rate to investigate spreading properties (7–9).

Secondly, a dynamic strain sweep test characterized viscoelastic properties. The elastic (storage) modulus G' and viscous (loss) modulus G'' were measured at a frequency of 1 hz with an initial strain of 0.015% up to 1000%.

Results

CMC-based vaccine formulation exhibits optimal properties for topical application

A steady rate sweep test measured viscosity across a range of shear rates, or rates of deformation, from 0.05 s^{-1} to 200 s^{-1} . Lower shear rates reflect how a substance behaves at rest (10) and is termed the near-zero shear rate, while high shear rates ($20 - 200 \text{ s}^{-1}$) relate to spreading applications (7–9). The Newtonian plateau describes the region where viscosity remains constant over changes in shear rate.

All tested materials exhibited properties of non-Newtonian substances, where changes in shear rate result in changes in viscosity (Figure 1). Likewise in all substances, the viscosity decreased with increasing shear rates, indicating shear-thinning behavior. However, trends in viscosity varied greatly between each substance. Generally, an increase in temperature led to a

decrease in near-zero viscosity except in SQ. Surprisingly, the viscosity of SQ changed minimally with temperature unlike all other materials. PJ and GJ melted at 40 °C and thus their viscosities measured near 0 Pa*s regardless of shear rate at this temperature. At 0 and 20 °C, PJ was the only substance that did not approach a Newtonian plateau at the lower limit of shear rates in this study. In contrast to PJ, CMC, SQ and GJ all exhibited a Newtonian plateau, whereby viscosity reaches a constant value with changes in shear rate. All CMC solutions plateaued at shear rates between 0.05 s⁻¹ and 0.8 s⁻¹. SQ and GJ only exhibited plateaus at shear rates from 0.05 s⁻¹ to 0.1 s⁻¹. Altering the CMC concentration modulates the viscosity of the solution. An increase in the concentration of CMC resulted in an increase in the viscosity. The apparent zero-shear viscosity of 10%, 12.5%, and 15% CMC, ranged from approximately 40-170, 110-400, and 190-680 Pa*s from 0 to 40 °C respectively. At all temperatures, SQ plateaued around 200-250 Pa*s.

At shear rates within the range of 20-200 s⁻¹, shear rates associated with spreading (7,8), viscosity curves generally converged from the Newtonian plateaus regardless of changes in temperature in all substances (Figure 2). CMC solutions displayed the highest viscosities in this range, yet this was still a drastic reduction compared to the viscosity at low shear rates. Similarly, the viscosity of CMC increased with increase in concentration. Over this range of shear rates, viscosity decreased from approximately 18 (at 20 s⁻¹) to 2.5 (at 200 s⁻¹), 25 to 5, and 35 to 5 Pa*s in 10%, 12.5%, and 15% CMC, respectively. In contrast, SQ ranged from 4 to 1, GJ from 5 to 0.8, and PJ from 6.5 to 0.1 Pa*s.

The dynamic strain sweep test differentiated substances as either having properties of viscoelastic liquids or elastic solids. CMC, GJ, and SQ, all exhibited a linear viscoelastic region at low strain (Figure 3). In these substances, a slight increase in G' and G'' was noted as strain %

approached the yield strain. At the yield strain, G' and G'' begin to decrease sharply and where deformation becomes irreversible. In contrast, PJ did not have a linear region - both G' and G'' decreased steadily. Initially, $G' > G''$, with G'' eventually having a larger magnitude than G' at a strain between 0.1 and 10%. The magnitude decreased from 10,000-15,000 Pa to 100-200 Pa at 40 °C, signifying a dramatic reduction in structural integrity and melting. Likewise, GJ also melted at 40 °C, where G' and G'' were near 0 Pa across all strain percentages. The G' and G'' of CMC solutions were comparable in magnitude. G' ranged from 160 Pa at 0 °C and 40 Pa at 40 °C in 10% CMC, from 350 to 100 Pa in 12.5% CMC, and 525 to 160 Pa in 15% CMC. G'' ranged from 165-75 Pa, 300-150 Pa, and 410-215 Pa, respectively. At 0 °C, $G' > G''$ in 12.5% and 15% CMC. At all other conditions, CMC acted as a viscoelastic liquid with $G'' > G'$, exhibiting viscoelastic properties even at 40 °C. As in the viscosity measurement, SQ varied little with change in temperature, and like GJ, the structure was primarily G' (~50-60 Pa) with minimal G'' (~5 Pa), signifying an elastic solid.

Discussion

Characterization of physical properties of a topical vehicle under environmental temperatures aids in identifying a vehicle proper for application, as highlighted by the shortcomings of glycerin jelly (GJ) and petroleum jelly (PJ). Rheology, measuring how a material sample responds to deformation, presents a useful method for understanding physical properties of gel-like materials (6). The temperatures tested (0 °C – 40 °C) represent extreme conditions under which a vaccine gel might be applied to vampire bats for rabies vaccination or potentially to other bat species in temperate climates. A topical vehicle should have low viscosity, measured by steady rate sweep test, at high shear rates to facilitate spreading, but high

viscosity at low shear rates to limit flowing and loss of the topical (11,12). This is known as shear thinning behavior.

When characterizing viscoelastic properties of materials, measured by dynamic strain sweep test, the storage modulus G' characterizes the elastic component (energy stored during deformation) and the loss modulus G'' characterizes the viscous component (energy dissipated during deformation). The magnitude of measurements of these moduli describe the solid-like and liquid-like component, structural rigidity of a material, and the amount of strain applied when deformation becomes irreversible (13).

In the initial 1970s study developing vampiricide, PJ stood apart from nine other candidate vehicles as suitable for topical application. It was demonstrated to adhere well to bat fur and transfer within a captive colony of vampire bats (14). However, studies using PJ as a vaccine vehicle were not as promising because it led to “intense” loss of paste falling off of the bats (4) and dilution of the vaccine (1). Another previous work attempted to measure vaccine stability within PJ; however, the recombinant vaccine virus could not be recovered after mixing with PJ, making it difficult to measure stability of the vaccine (15). In the present study, PJ demonstrated shear thinning behavior without a Newtonian plateau in accordance with previous rheological characterizations (9). PJ began to melt at the 40 °C condition as evidenced by the near zero viscosity, and dramatic decrease in magnitude of G' and G'' in the steady rate sweep test. At 0 °C and 20 °C there was no linear viscoelastic region in the dynamic strain sweep test – G' and G'' continually decreased – indicating a weak microstructure that is easily deformed when spreading.

GJ, another previously tested topical vaccine vehicle (2,3,5), proved unsuccessful because of inconsistent physical properties, especially under varied temperatures. Our study

highlighted the strong dependence of the consistency of GJ on temperature. We found the dilution of GJ in water previously used (2) has a melting temperature around 30 °C. Thus, in the rheological measurements taken at 40 °C, the viscosity was 0 Pa*s and G' and G'' were likewise near 0 Pa, exemplifying that GJ had no structural integrity at this temperature. When not melted, GJ is primarily solid-like with elastic behavior as demonstrated by $G' > G''$ at 0 °C and 20 °C. While vampire bats typically roost where temperatures remain below 37 °C (16) and do not well tolerate temperatures above 33 °C (17), environmental temperatures may exceed 40 °C during heat waves in some regions (18).

SQ – made of a hydrogel base of carbomer and triethylamine (TEA) – may present the best properties for a sprayable vaccine formulation. Hydrogels are composed of a 3D structure of cross-linked polymer chains that can absorb significant amounts of liquid while still maintaining structure (19). A spray application avoids the need to capture the bats, reducing stress and labor intensiveness. To adequately spray a substance, low viscosity under high shear rates is critical. The minimal variation in properties across the temperatures tested enforces the reliability of SQ for application in a wide range of environments. Like GJ, SQ is primarily solid-like with $G' > G''$ and a low magnitude of G'' (~5 Pa). This may result in SQ being less sticky when compared to substances like CMC. Additional work is needed to determine preparation of the vaccine within a hydrogel. As carbomer creates an acidic solution prior to the addition of TEA, the vaccine must be added after gelation. This results in dilution of the vaccine similar to trials using PJ.

The rheological properties of CMC solutions are well studied and known to vary with the molecular weight and degree of substitution of the CMC powder used (20–22). In our study, the CMC gel preparations of 10%, 12.5%, and 15% with 1.2 degree of substitution and average molecular weight of ~250,000 g/mol demonstrates shear thinning behavior and viscoelastic

properties corresponding with these previous reports. In the steady rate sweep test, CMC demonstrates less variation than GJ and PJ in zero-shear viscosity over the studied range of temperature. At 40 °C, the near-zero viscosities of 10%, 12.5%, and 15% CMC were approximately 35, 110, and 190 Pa*s, respectively, demonstrating high viscosity at high temperatures. This improves on the GJ formulation which has a near-zero shear rate approaching 0 Pa*s at 30 °C when it becomes completely liquified. Its viscosity is around five-fold higher than SQ at high shear rates, meaning CMC may prove much more difficult to spray (see Appendix A) compared to SQ. In CMC, G'' is greater than G' in the linear viscoelastic region, suggesting that CMC acts as a viscoelastic liquid that flows slowly over time. This may contribute to improved surface wettability and to the enhanced stickiness of CMC that we observed compared to GJ and SQ. Notably at 0 °C, G' becomes greater than G'' in 12.5% and 15% CMC within the linear viscoelastic region, indicating trends toward solidification at temperatures near freezing, while still exhibiting viscoelastic properties.

Additional described properties of CMC, a polymer melt, support its use as a topical vaccine vehicle. The muco-adhesion of CMC (23) may enhance viral vaccine entry into mucosal tissue. Mucoadhesive cellulose-based films have been previously used to facilitate entry of Modified Vaccinia Ankara – a commonly used poxviral vaccine vector - into the buccal mucosa of mice (24). CMC is also commonly used in drug delivery (25), is non-toxic at high quantities (26), and was found to mix easily with buffer containing RB without crystallization. CMC is easy to prepare in two steps: first the vaccine buffer is prepared and then CMC powder is mixed in. As detailed in chapter 2, we recovered a high yield of vaccine virus after mixing with CMC. Considering these results, we decided to proceed with 12.5% CMC as the topical formulation for vaccination studies and field trials.

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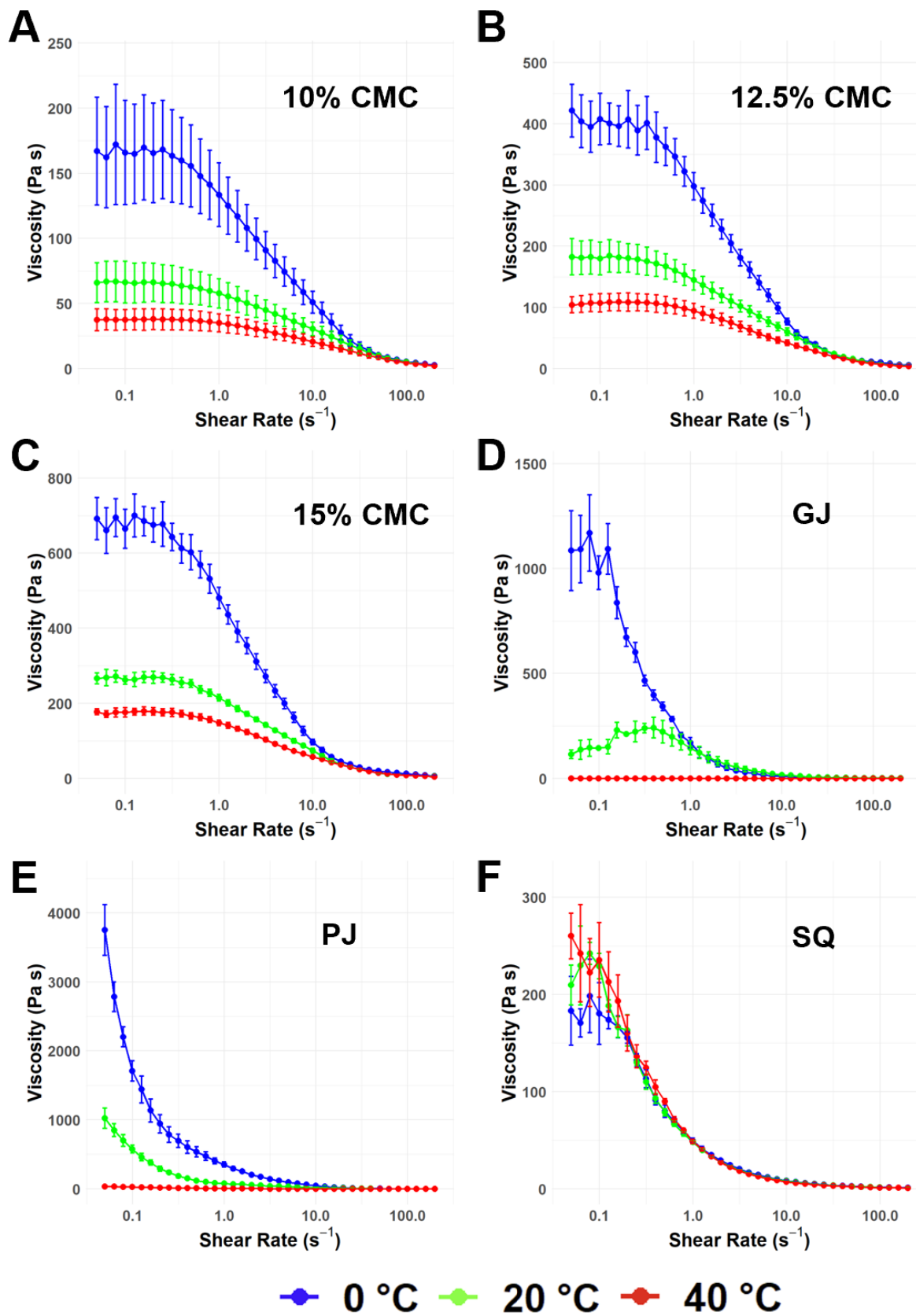


Figure 1. Viscosity measurements of topical gels and pastes by steady rate sweep test with an ARES LS2 Rheometer. The viscosities of **A)** 10%, **B)** 12.5%, and **C)** 15% solutions of carboxymethyl cellulose (CMC), **D)** glycerin jelly (GJ), **E)** petroleum jelly (PJ), and **F)** Silverquine hydrogel (SQ) were measured over shear rates from 0.05 to 200 (s^{-1}). Five minutes of temperature equilibration was allowed between temperatures. Error bars represent standard error of three experimental replicates.

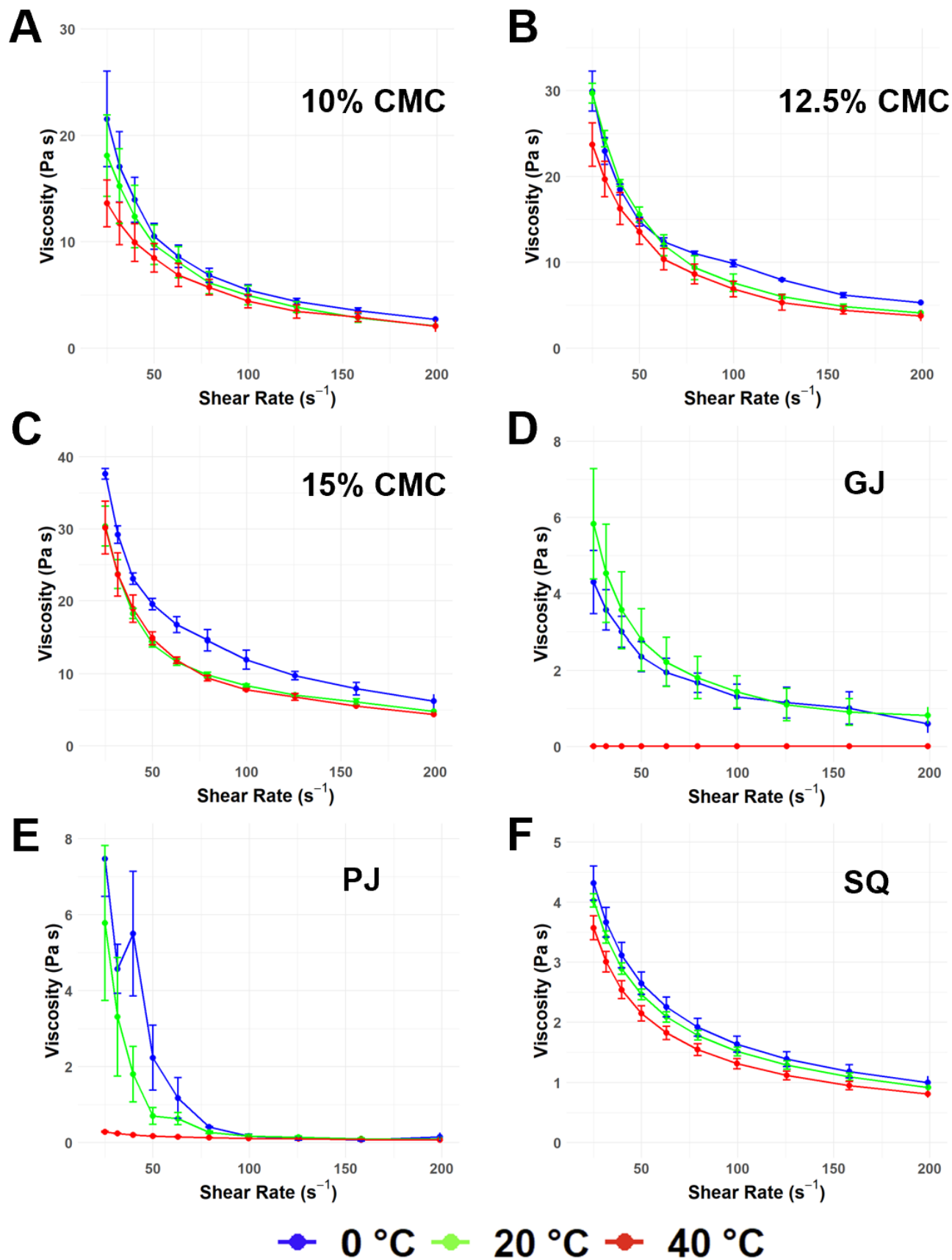


Figure 2. Viscosity measurements at shear rates associated with spreading (20-200 s⁻¹) of topical gels and pastes by steady rate sweep test with an ARES LS2 Rheometer. The viscosities of **A**) 10%, **B**) 12.5%, and **C**) 15% solutions of carboxymethyl cellulose (CMC), **D**) glycerin jelly (GJ), **E**) petroleum jelly (PJ), and **F**) Silverquine hydrogel (SQ) are depicted at shear rates from 20 to 200 s⁻¹. Five minutes of temperature equilibration was allowed between temperatures. Error bars represent standard error of three experimental replicates.

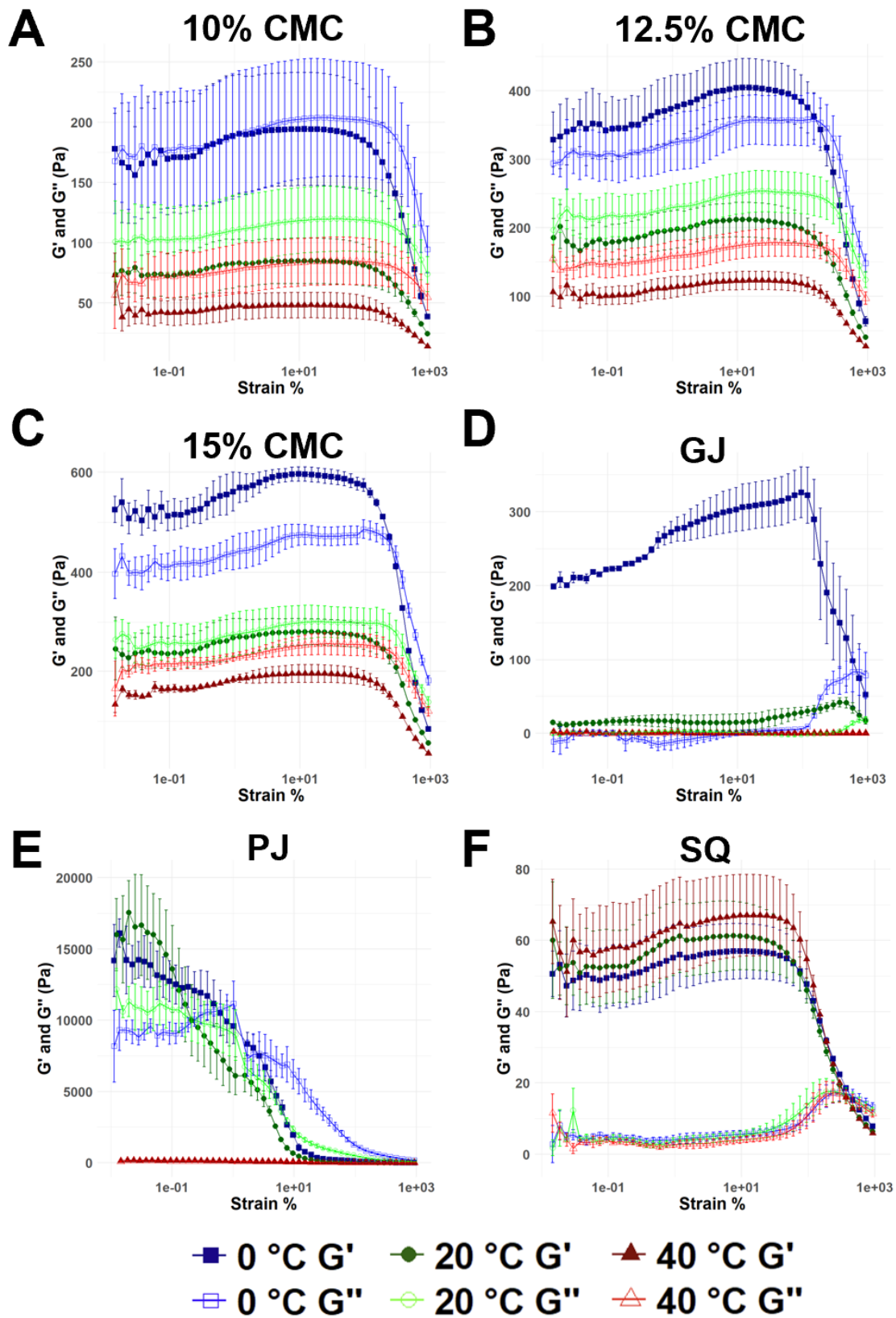


Figure 3. Characterization of viscoelastic properties of topical gels and pastes by dynamic strain sweep test using an ARES LS2 Rheometer. The viscoelastic properties of **A)** 10%, **B)** 12.5%, and **C)** 15% solutions of carboxymethyl cellulose (CMC), **D)** glycerin jelly (GJ), **E)** petroleum jelly (PJ), and **F)** Silverquine hydrogel (SQ) were measured over a strain sweep from 0.015% to 1000%. G' represents the elastic modulus – energy stored during deformation, while G'' represents the loss modulus – energy dissipated during deformation. Error bars represent standard error of three experimental replicates. Five minutes of temperature equilibration was allowed between temperatures.

Chapter 2

ASSESSMENT OF CARBOXYMETHYL CELLULOSE AS A VEHICLE FOR RCN- MOG VIRAL STABILITY AND VACCINATION OF BIG BROWN BATS (*EPTESICUS FUSCUS*)

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Introduction

Vaccines prevent rabies disease. The development of the rabies vaccine was a major milestone in combatting the invariably fatal disease. Prophylaxis can be administered both pre-exposure – recommended only for high-risk individuals - and post-exposure prior to symptom onset. Exposures arise from bites of infected animals shedding rabies virus in their saliva. Before the 1960s, dozens of people in the US died of rabies each year (1). Now, around 100,000 people in the US receive post-exposure prophylaxis annually and deaths are rare (1). Despite effective vaccines, it is estimated that globally there are 59,000-70,000 human rabies fatalities each year (2,3). The majority of these deaths occur in Asia (~60%) and Africa (~35%) (4). In many regions where rabies is still endemic, poor infrastructure for surveillance and diagnosis complicate access to vaccines (4) and vaccination may pose high costs to the patient or the government (5). Worldwide economic costs attributed to rabies prevention, prophylaxis and mortality are estimated at \$8.5 billion annually (2).

Along with 16 other viral species, rabies virus belongs to the genus *Lyssavirus*. It is the most common lyssavirus worldwide, and the only lyssavirus found in the Americas and thus in vampire bats. All lyssavirus species cause rabies-like disease, and all but two have been linked to bat reservoirs. Rabies disease usually presents as a “furious” form manifested by neurological symptoms and encephalitis. The “dumb” form, characterized by progressive paralysis rather than encephalitis, occurs in about 1 in 5 cases. Lyssaviruses are dsRNA viruses that produce bullet-shaped virions with five structural proteins: glycoprotein (G), large polymerase protein (L), matrix protein (M), nucleoprotein (N), and phosphoprotein (P). The G protein, embedded in the viral envelope, facilitates viral entry and interacts directly with the host cell membrane receptors,

thereby playing a central role in the pathogenicity and virulence of lyssaviruses (6). As such, vaccines primarily target priming the immune system against the G protein (7).

Over the past century and a half, several types of rabies vaccines have been developed. Louis Pasteur developed the first in 1885. He attenuated rabies virus through serial passaging in rabbits and then partially inactivated the virus by desiccating extracted spinal cord tissue of the infected rabbits to varying degrees (8). This vaccine series required as many as 13 doses (9). Live-attenuated rabies vaccines are rarely used to immunize people today due to risk of reversion into a virulent strain, although they are still used in baits for wild animals (10). Heat inactivated vaccines are much safer and commonly used in people, livestock, and domestic animals. However, the efficacy of these vaccines suffers from decreased immunogenicity, requiring boosting every 1-5 years (11). This prompted ongoing experimentation with next-generation vaccines such as genetically modified attenuated rabies viruses, recombinant viral vector vaccines, and mRNA vaccines (10). While none are approved for human use, a recombinant vaccinia viral vector vaccine – Raboral V-RG® – is widely used in animal baits (12).

Previously, our lab group developed a raccoonpox viral-vectored rabies vaccine (RCN-MoG). Rather than solely expressing the G protein of the rabies virus, the recombinant virus expresses a mosaic lyssavirus glycoprotein (MoG) with antigenic coverage of 664 lyssavirus sequences belonging to nine of the viral species within lyssavirus phylogroup I (12). Although this remains to be tested, this vaccine could provide broader protection against other lyssavirus strains compared to vaccines based solely off the classical rabies virus. This vaccine produced protective immunity in mice (*Mus musculus*) and big brown bats (*Eptesicus fuscus*) after challenge with the CVS-11 rabies virus strain (12). Notably, the vaccine blocked viral shedding

in saliva of vaccinated vampire bats despite some vaccinated bats still succumbing to challenge (13). Currently there is no commercial rabies vaccine for bats.

In developing a topical vaccine formulation for rabies vaccination of vampire bats, a material that has optimal physical properties must also maintain immunogenicity of the vaccine. To assess this, we performed two viral stability assays to measure the viral titer of our RCN-MoG vaccine candidate over time within the selected 12.5% CMC formulation. The first assay compared viral stability within CMC to the previously used glycerin jelly (GJ) vehicle. In the second stability assay, the effect of the additional components rhodamine B (RB) – the biomarker used in field studies (14) – and sucrose – a known stabilizer of poxvirus vectors (15) - was investigated. Additionally, we performed a vaccination study with big brown bats to assess antibody production and viral shedding after a single dose oral inoculation with the RCN-MoG vaccine mixed in 12.5% CMC.

Methods

Vaccine Stability Assays

Cells and Viruses

Vero cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle Media (DMEM; Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 5% fetal bovine serum and 1x antibiotics-antimycotics and incubated at 37°C with 5% CO₂. The previously described RCN-MoG vaccine (12) was used.

Vaccine Gel Preparation

To prepare RCN-MoG vaccine in CMC gel, RCN-MoG virus was homogenized in sterile 10mM Tris hydrochloride (Tris-HCl) buffer (pH 9.0) at a final concentration of 10⁸ PFU/mL in 4

mL total volume. CMC powder (Sigma-Aldrich, cat. no. 419281; St. Louis, Missouri, USA) was then added at a final concentration of 12.5% (0.5 g) and stirred with a sterile microspatula.

For the GJ preparation, RCN-MoG virus was added to sterile 10mM Tris-HCl buffer (pH 9.0). Then melted glycerin jelly (GJ) was added such that the ratio of Tris buffer to GJ (Carolina Biological Supply Co, cat. no. 865495; Burlington, North Carolina, USA) was 55.5:44.5. The final concentration of RCN-MoG was 10^8 plaque-forming units (PFU)/mL in 4 mL total volume. After homogenizing, 100 μ L aliquots of the GJ preparation were transferred with a needle-free syringe into sterile 1.5 mL Eppendorf tubes.

Both the GJ aliquots and CMC gel were stored at 4°C overnight to allow the CMC powder to dissolve completely. Next, the CMC gel was briefly mixed and likewise aliquoted (100 μ L) into sterile 1.5 mL Eppendorf tubes with a needle-free syringe. Aliquots were then placed at their respective storage temperatures (4 °C, 23 °C, or 40 °C). At each timepoint (0 hr, 24 hr, 48 hr, 96 hr, 1 wk, 2 wk, 1 mo, 3 mo), two aliquots were moved from each temperature and stored at -80 °C until titration by viral plaque assay.

In a second stability assay, CMC formulations were prepared as above except using 10mM Tris-HCl with 0.3% RB (formulation R), and 10mM Tris-HCl with 0.3% RB and 20% sucrose (formulation S) as buffers. Vaccine titers were measured at 0 h, 1 wk, 2 wk, 1 mo, and 3 mo.

Titration of RCN-MoG Vaccine by Viral Plaque Assay

Viral plaque assays were performed with Vero cells in 24-well plates (TPP, Trasadingen, Switzerland). Gel samples were thawed at 4 °C and dissolved by adding 900 μ L of 1x DMEM to each tube with 1 h incubation at 37 °C. Samples were mixed by pipetting every 15 min to facilitate dissolution. Cells were inoculated with 100 μ L of ten-fold dilutions of the dissolved

gels. After 1 h of infection at 37 °C with 5% CO₂, the inoculum was removed and 1 mL of plaque assay media (1x DMEM, 1.5% CMC, 2% FBS, 1x antibiotics-antimycotics) was added. Plates were fixed with 10% formaldehyde after 3 days of infection and stained with 1% crystal violet in 20% ethanol.

Statistical Modeling

A linear regression model was fitted to log₁₀-transformed viral titers to quantify viral decay. Covariates include time, formulation, and temperature. Pairwise comparisons using the pairs function was performed of slope estimates of each formulation-temperature combination generated by the emtrends function of the emmeans R package (16). Log fold change was calculated relative to the initial time point (T=0).

Vaccine Study

Ethics Statement

This study was carried out in accordance with protocol #EP200127 approved by the Institutional Animal Care and Use Committee at the USGS National Wildlife Health Center (Madison, WI, USA).

Cells and Viruses

The RCN-MoG virus was used for inoculating the bats (12,17). CVS-11 rabies virus (Genbank accession # AB069973) was provided by the Centers for Disease Control (CDC) for performing antibody testing. Baby hamster kidney cells (BHK-21, ATCC #CRL12072) were grown in Modified Eagle's Medium (MEM) supplemented with 10% FBS and 1x antibiotics-antimycotics and incubated at 37 °C with 5% CO₂.

Study Design

Twenty-one adult *Eptesicus fuscus* bats (16 female, 5 male) were obtained in collaboration with the Wisconsin Department of Natural Resources in the fall of 2024. Bats were kept in torpor in an environmental hibernation chamber at the National Wildlife Health Center (Madison, WI). In January 2025, bats were aroused and housed in mesh screen cages (Reptarium, Apogee, Dallas, TX, USA). These bats were initially used for biomarker development study described in Appendix B. In March, eighteen bat pups (8 males, 10 females) were born in captivity. Bats were fed mealworms (*Tenebrio molitor*) supplemented with vitamin-rich substrate with water provided *ad libitum*.

For vaccination, bats were randomly assigned to three treatment groups: A (Tris) – 1×10^8 PFU RCN-MoG in Tris buffer (pH 9.0); B (CMC) - 1×10^8 PFU RCN-MoG in Tris buffer (pH 9.0) with 12.5% CMC; and C (Control) – Tris buffer control (Table 1). The pups were approximately 2-2.5 months old at the time of vaccination. One hundred microliters of inoculum were delivered orally with a needle free syringe. Plasma samples were collected at baseline, 14-days post-vaccination (dpv) from the uropatagial vein. A terminal serum sample was collected at 40 dpv following euthanasia. Weights were recorded weekly and 1- and 3- dpv.

RCN-MoG Quantitative Polymerase Chain Reaction (qPCR) of Oral Swabs

Oral swabs were collected on days 0, 1, 3, 5, and 7 post-vaccination and stored in tubes with 200 μ L RNeasy Lysis Solution (Qiagen, Waltham, Massachusetts, USA). DNA was extracted from 50 μ L of rigorously vortexed swab suspension using the Zymo Quick DNA Mini Prep kit (Zymo Research, Tustin, California, USA) with 30 μ L of elution buffer. Master mix was prepared according to Table 2 and thermocycle conditions were programmed as in Table 3. The MoG-F (5'-GGAAGAGTAATATCTTCTTGGGAATC-3') primer targets the MoG protein; RCN-R (5'CTATAACTATTTTTCCATTGTTTGCCATG-3') targets the raccoonpox vector.

Micro Rapid Fluorescent Focus Inhibition Test (Micro-RFFIT)

Rabies virus neutralizing antibodies (RVNA) were measured in plasma (baseline and 14 dpv) or serum (40 dpv) samples by the previously described micro-RFFIT (18). Briefly, samples were heat inactivated at 56 °C for 30 min. Then, four-fold dilutions of serum were prepared in MEM supplemented with 10% FBS and antibiotics-antimycotics in 4-well Teflon-coated slides. CVS-11 rabies virus was added to each well and incubated for 90 minutes at 37 C and 5% CO₂. After incubation, BHK cells were added to each well and again incubated for 20 hours. Slides were fixed for in acetone and then stained with FITC rabies conjugate (Fujirebio U.S Inc., Malvern, PA) and Evan's blue counterstain. Slides were visualized with a SMZ1270 stereomicroscope (Nikon Instruments, Melville, New York, USA) using a SMZ1270/800N P-EFLC GFP BP AT (480 nm / 535/40 nm) filter set (Nikon Instruments, Melville, New York, USA).

Results

Preservation of viral vaccine titers in CMC and GJ

We measured viral titers at time intervals up to six months to assess preservation of the RCN-MoG vaccine within CMC and GJ under different temperature conditions. Slightly more virus was recovered from dissolved GJ (Log₁₀ 8.02 +/- 0.12 PFU/mL) than CMC (7.64 +/- 0.14) at the initial time point (Figure 1A). Within the first week, titers notably decreased only at 40 °C (Figure 1B). At 4 °C, high titers were preserved up to 3 months in CMC and 6 months in GJ (Figure 1C) with a negligible decay rate of RCN-MoG in both formulations (Figure 1D; CMC: Log₁₀ -0.0339 ± 0.018 PFU/mL per week; GJ: -0.00079 ± 0.018). At 23 °C, viral titer decreased in CMC at a moderate rate (-0.463 ± 0.038), while in GJ decay was slower (-0.181 ± 0.018).

RCN-MoG viral titer rapidly decreased at 40 °C in both CMC (-3.50 ± 0.264) and GJ (-1.50 ± 0.12). RCN-MoG was no longer detectable at 40 °C after 30 days in CMC, 90 days in GJ, and 180 days at 23 °C in CMC. All statistical comparisons in decay rates were significant except for GJ and CMC at 4 °C, and CMC at 4 °C with GJ at 23 °C (Figure 1D).

In the second stability assay, slightly less virus was recovered from formulation R (Log₁₀ 7.26 +/- 0.078 PFU/mL), while the same titer was recovered from C (7.50 +/- 0.074) and S (7.49 +/- 0.132) at T=0 h (Figure 2A). At 23 °C, the addition of RB accelerated viral decay, while the titer within formulation S followed a similar trend to the base formulation C (Figure 2B). At 23 °C, the decay rate of R (Log₁₀ -0.937 +/- 0.114 PFU/mL per week) was more than double formulations C (-0.411 +/- 0.114) and S (-0.404 +/- 0.0348) shown in Figure 2C. Notably, when stored at 4 °C, decay was virtually identical up to one month between all formulations, but R began to diverge at the three-month measurement. The decay rates 4 °C storage were estimated as follows: formulation C: -0.0686 +/- 0.0404; R: -0.130 +/- 0.0348; and S: -0.0773 +/- .0348. Most significant differences comparing decay rates were found between 4 °C and 23 °C, while R decayed significantly faster than C and S at 23 °C.

Limited Viral Shedding and Neutralizing Antibodies Following RCN-MoG Prime Vaccination

In the big brown bat vaccination study, positive qPCR samples of DNA extracted from oral swabs collected to detect viral vaccine shedding in saliva were only detected 1 dpv. No samples on other days of collection had a CT < 35. Only two of the pups in group B (CMC) were positive with CT values of 34 and 31.2 (Figure 3A). Other 1 dpv samples with amplification fell into an indeterminate range of CTs between 35 and 38 (A – 4/13, B – 3/13, C – 2/13). The amplification product denatured at 81-82 °C in the melt curve (Figure 3B).

No samples developed measurable neutralizing antibodies by micro-RFFIT post-vaccination. One bat was found to be seropositive at baseline. No negative effects were noted post-vaccination, and bat weights in all treatment groups increased (Figure 4).

Discussion

An optimal treatment vehicle must preserve the vaccine, in addition to having physical properties suitable for topical administration to vampire bats. To facilitate vaccine deployment, the vaccine should be readily shipped and stored without dependence on cold-chains in regions where reliability of infrastructure may be unpredictable (19). Our assessment of viral stability in CMC under different temperature conditions provides insight into vaccine preservation when stored and persistence in field-relevant temperatures.

Minimal decay of viral titer within CMC was evident at refrigeration temperatures (4 °C) with only a 0.63 log fold decrease in viral titer after 3 months of storage. This will enable shipment and storage of the vaccine when challenges in infrastructure prevent maintaining temperatures below freezing. If refrigeration is lost, vaccine titer may also be preserved up to 1 week at 23 °C with only a 0.46 log fold decrease in titer and 24 hrs at 40 °C with 0.54 log fold decrease. Although virus stability was generally better preserved in GJ, the viral titer initially decreased more rapidly in GJ than in CMC at 40 °C with a log fold decrease of 0.82 after 24 hrs. The implications of these vaccine preservation data depend on the determination of the minimum effective dose of RCN-MoG in vampire bats; however, during field application, we expect gel to be consumed within hours after application. Importantly, other environmental factors may impact viral persistence that were not tested in this study, such as humidity (20) and its effect on gel evaporation, and exposure to solar radiation (21).

In the second stability assay, the addition of RB to the gel formulation accelerated viral decay at 23 °C, while the addition of 20% sucrose - a known stabilizer of poxviral vaccine vectors (15) – negated this effect. This effect of RB was not noted after 1 month at 4 °C, which may suggest a temperature-influenced mechanism by which RB accelerates viral particle inactivation. The titer of formulation R began to diverge at three months leading to a weekly decay rate nearly double that of formulations C and S. As most statistically significant differences compared formulations between 4 °C and 23 °C rather than at the same temperature, these results suggest storage temperature had a greater influence on viral decay rates than formulation. Other additives could also be considered to improve stability within the CMC gel formulation such as albumin or amino acids (15).

In the vaccination study, no neutralizing antibodies developed post-vaccination. In previous studies, low levels of neutralizing antibodies were detected, and antibody levels did not correspond directly with survival post-challenge (12). This study shows that serology is not an effective measure of immune response post-vaccination after a single dose of the RCN-MoG vaccine in juvenile and adult big brown bats. A future study could be performed with rabies challenge to determine if a single dose of RCN-MoG is protective within the CMC formulation. Despite not producing neutralizing antibodies, vaccination could still be protective against rabies challenge. Viral vector vaccines also confer immunity through development of effector T cells (22).

Limited viral shedding of the RCN-MoG was detected in oral swabs only 1 dpv. Interestingly, viral shedding was detected up to 9 days with an RCN construct in Brazilian free-tailed bats (*Tadarida brasiliensis*) (23), suggesting possible differences in susceptibility to RCN between different bat species. Given that the only definitively positive swab samples belonged

to the CMC group, CMC likely does not inhibit the virus's ability to infect the oral mucosal tissue of the bats. Validation of the qPCR limit of detection is needed to differentiate the indeterminate samples. Regardless, this range of samples represents minimal viral shedding.

Overall, these studies suggest that CMC is able to sufficiently preserve RCN-MoG vaccine titers. However, further work is needed to determine effects of the CMC vehicle in immune response development. Viral shedding should also be measured in the target species (vampire bats) given the disparity in shedding by big brown bats and Brazilian free-tailed bats.

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Table 1. Distribution of big brown bats (*Eptesicus fuscus*) by vaccination treatment group.

Bats were given 100 μ L of treatment orally with a needle-free syringe. Treatment group A: Tris - 1×10^8 PFU in Tris-HCl buffer; treatment group B: CMC – 1×10^8 PFU in Tris-HCl buffer with 12.5% CMC; control group C – Tris-HCl buffer.

| | A – Tris | B - CMC | C - Control |
|------------------|-----------------|----------------|--------------------|
| Adult Males | 2 | 2 | 1 |
| Adult Females | 5 | 6 | 5 |
| Juvenile Males | 3 | 2 | 3 |
| Juvenile Females | 3 | 3 | 4 |

Table 2. SYBR green quantitative polymerase chain reaction (qPCR) master mix.

| Reagent | Volume (μL) |
|--|-----------------------------------|
| Sso Advanced Universal SYBR Green Master Mix (Bio-Rad, Hercules, California, USA) | 10 |
| MoG-F primer | 0.3 |
| RCN-R primer | 0.3 |
| Nuclease free water | 5.4 |
| Sample DNA | 4 |
| Total | 20 |

Table 3. SYBR green quantitative polymerase chain reaction (qPCR) thermocycle conditions. PCR was run on a CFX Opus 96 real-time PCR system (Bio-Rad, Hercules, California, USA). Amplification products were analyzed with a melt curve.

| Step | Temperature (°C) | Time | # of Cycles |
|------------------------------------|-------------------------|-------------|--------------------|
| Initial Denaturation | 98 | 3 min | 1 |
| Denaturation | 98 | 30 s | 40 |
| Annealing and Extension | 60 | 20 s | |

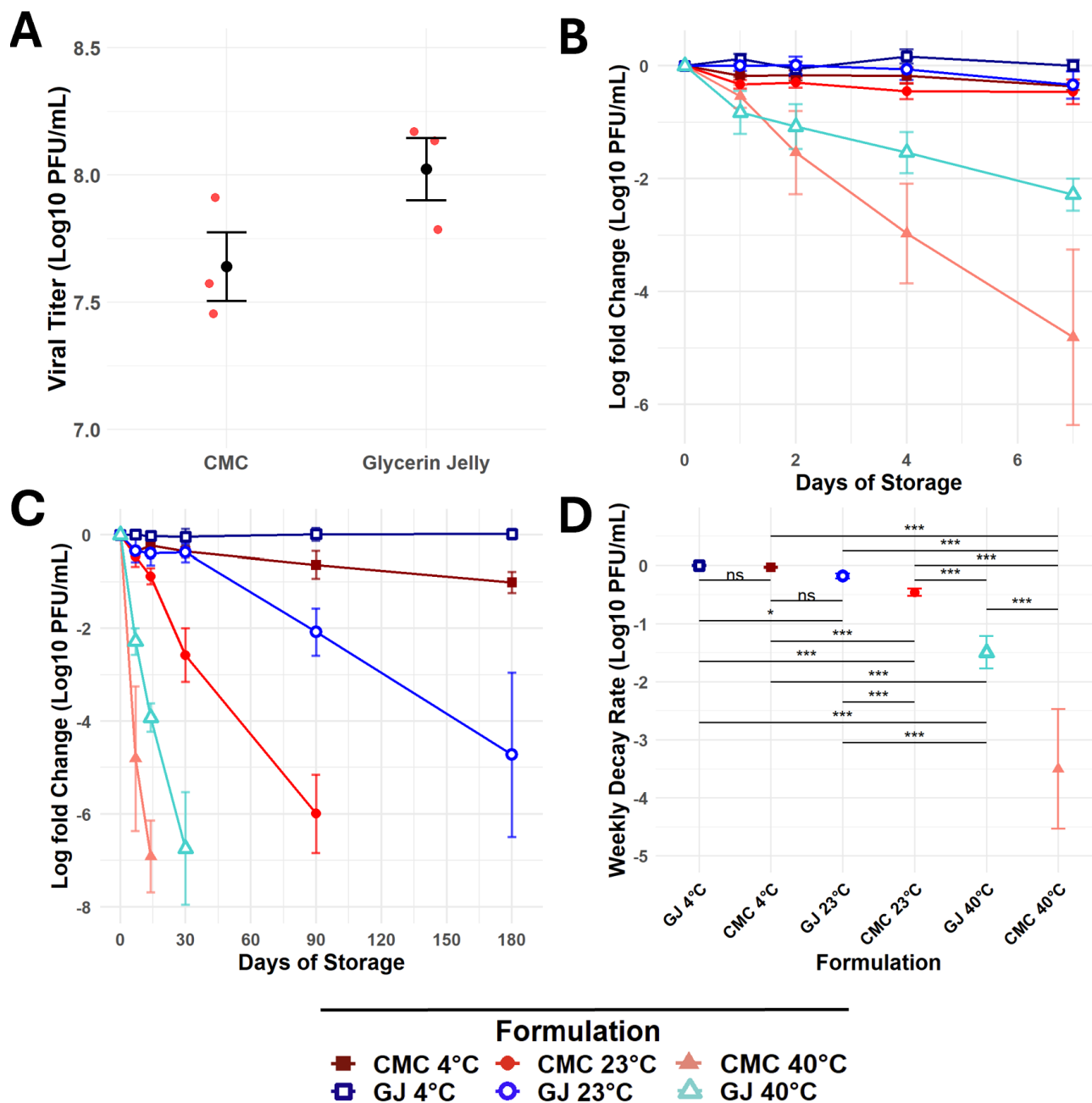


Figure 1. Viral stability of a recombinant raccoonpox vaccine expressing a mosaic lyssavirus glycoprotein (RCN-MoG) stored within carboxymethyl cellulose (CMC) and glycerin jelly (GJ) formulations at 4 °C, 23 °C, and 40 °C. A concentration of 10⁸ plaque forming units (PFU)/mL was mixed into CMC and GJ. A) A baseline titer was recovered and measured by plaque assay after dissolving aliquots of CMC and GJ that had immediately been

frozen (T=0 hr). Red dots represent experimental replicates and error bars represent standard error. Log fold change in vaccine titer at each storage temperature was measured periodically over **B)** seven days and up to **C)** six months of storage. Error bars depict the standard error of three experimental replicates. **D)** Weekly decay rates of RCN-MoG titer within CMC and GJ at each storage condition were determined by a linear regression model. Statistical comparisons were made using emtrends (ns = no significance; * = $p < 0.05$; *** = $p < 0.001$).

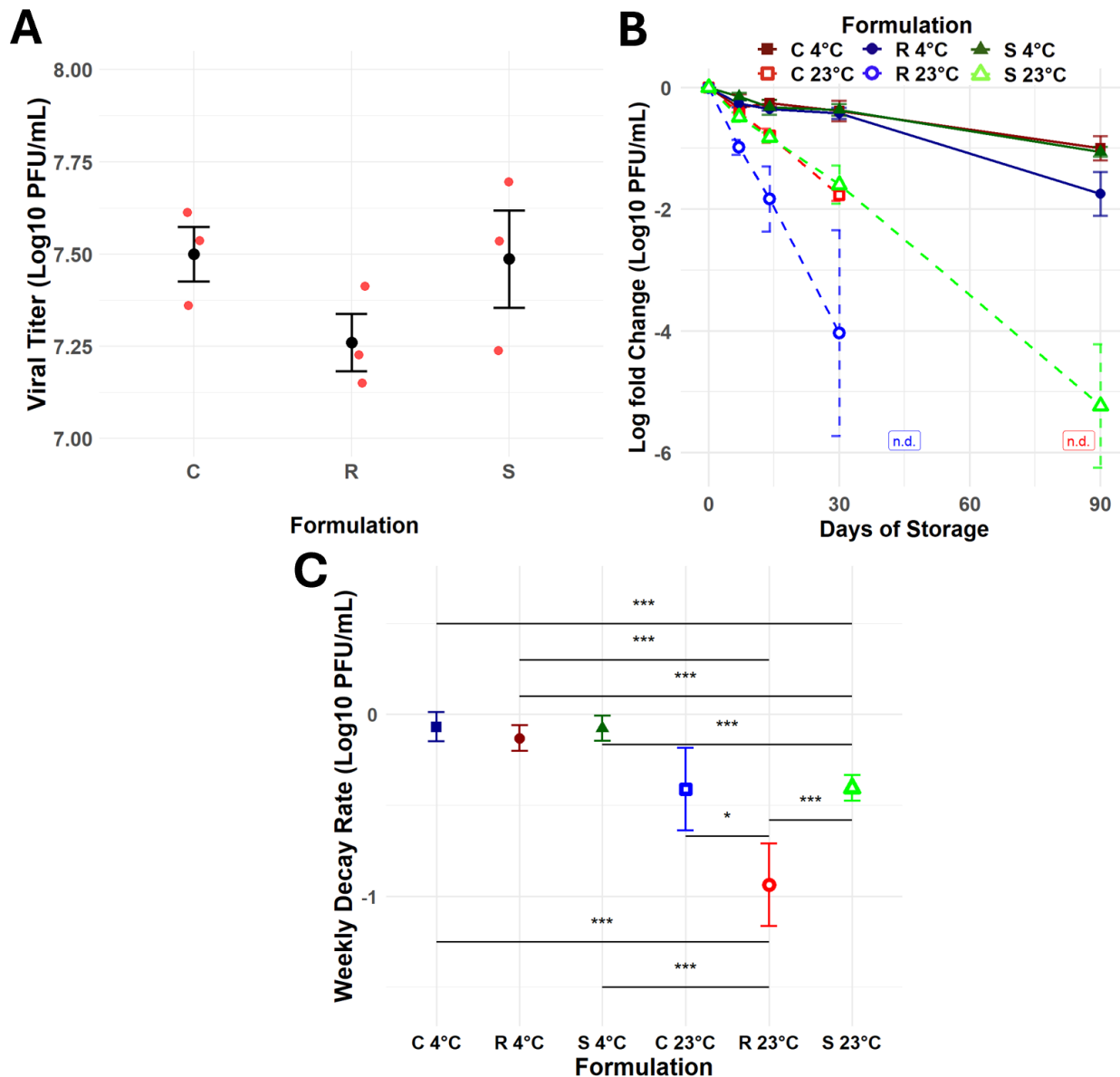


Figure 2. Viral stability of a recombinant raccoonpox vaccine expressing a mosaic lyssavirus glycoprotein (RCN-MoG) stored within three carboxymethyl cellulose (CMC) formulations at 4 °C, and 23 °C. A concentration of 10^8 plaque forming units (PFU)/mL was mixed into formulations C - 12.5% CMC gel, R - 12.5% CMC gel with 0.3% rhodamine B (RB), and S – 12.5% CMC gel with 0.3% RB and 20% sucrose A) A baseline titer was recovered and measured by plaque assay after dissolving aliquots of C, R, and S formulations that had immediately been frozen (T=0 hr). Red dots represent experimental replicates and error bars

represent standard error. **B)** Log fold change in vaccine titer at each storage temperature was measured periodically up to three months. Error bars depict the standard error of three experimental replicates; n.d. – not detected. **C)** Weekly decay rates of RCN-MoG titer within C, R, and S formulations were determined by a linear regression model. All statistical comparisons were made using emtrends (* = $p < 0.05$; *** = $p < 0.001$). Comparisons not shown were not significant.

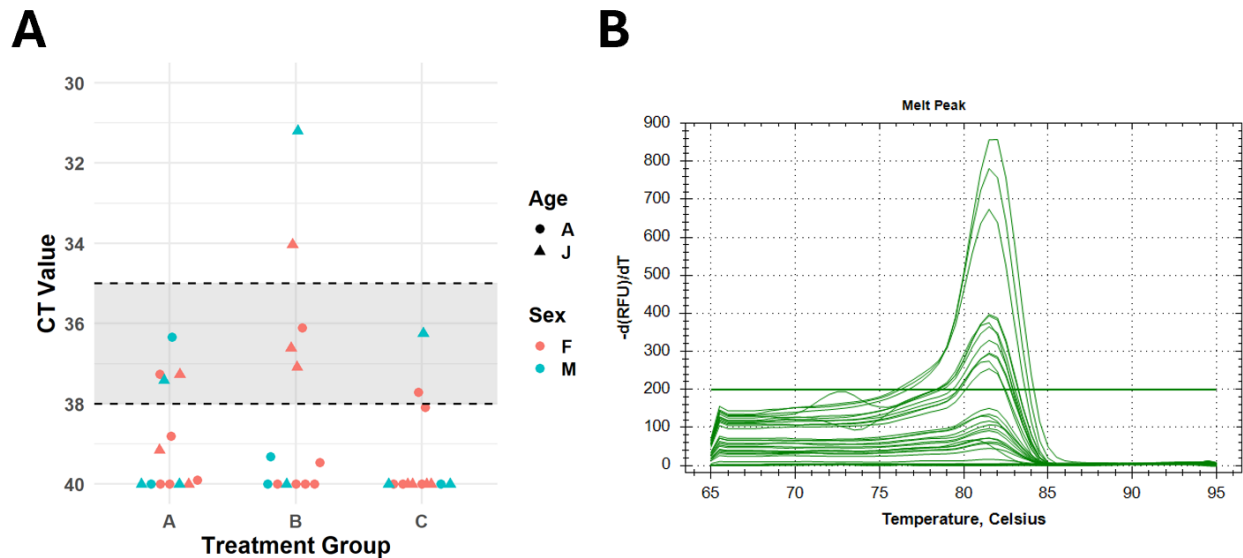


Figure 3. SYBR green qPCR of oral swabs collected 1-day post-vaccination (dpv). A)

Samples with CT-values < 35 were considered positive; CT 35-38 were indeterminate. Treatment group A: Tris - 1×10^8 PFU in Tris-HCl buffer; treatment group B: CMC - 1×10^8 PFU in Tris-HCl buffer with 12.5% CMC; treatment group C - Tris-HCl buffer only. Bats were given 100 μ L of treatment orally with a needle-free syringe. **B)** Amplification products were assessed by melt curve.

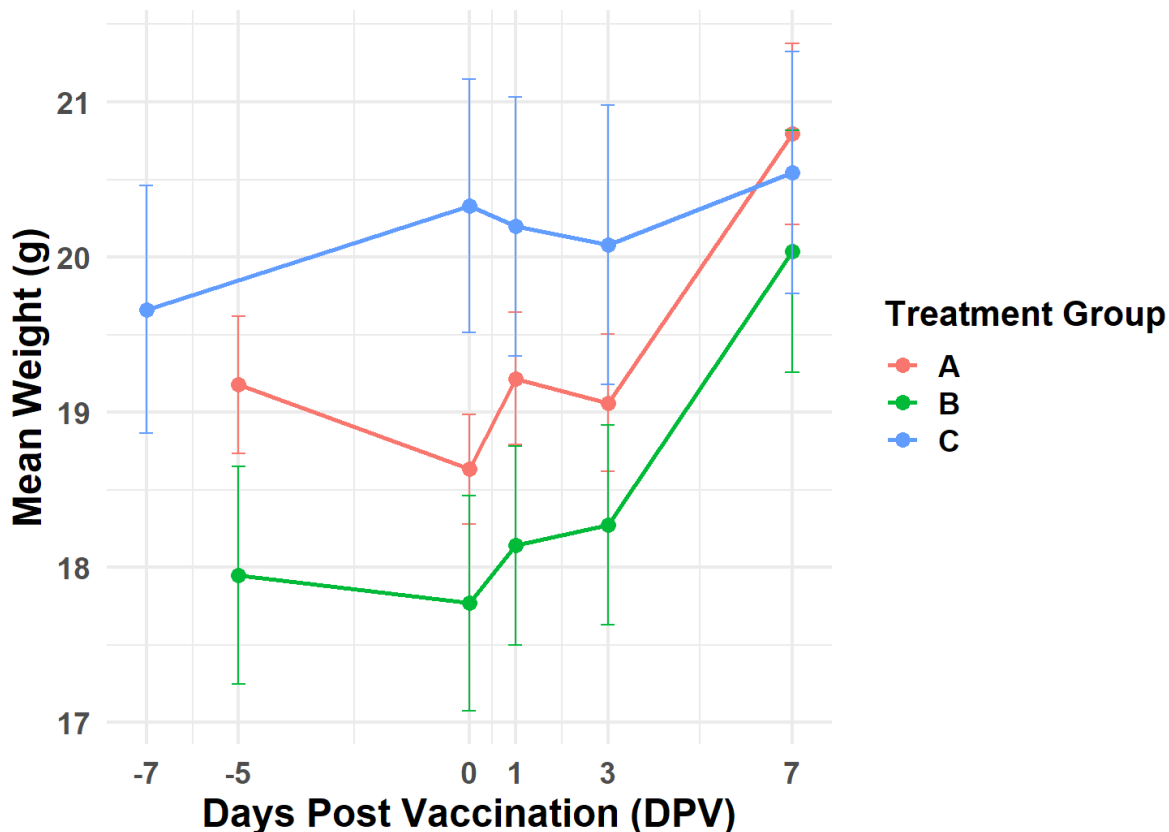


Figure 4. Bats Weights over time. Bats were weighed 5 or 7 days before treatment and on days 1, 3, and 7 post-treatment. Bats were given 100 μL of treatment orally with a needle-free syringe. Treatment group A: Tris - 1×10^8 PFU in Tris-HCl buffer; treatment group B: CMC - 1×10^8 PFU in Tris-HCl buffer with 12.5% CMC; treatment group C - Tris-HCl buffer only. Error bars represent standard error.

Chapter 3

**TOPICAL TRANSFER AND UPTAKE OF CARBOXYMETHYL CELLULOSE GEL
WITHIN WILD COLONIES OF VAMPIRE BATS (*DESMODUS ROTUNDUS*) IN
JALISCO, MEXICO**

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Introduction

Vampire bats rely on social grooming, a behavior critical for survival. They groom socially significantly more than other bat species (1). It is hypothesized that allogrooming strengthens social bonds to encourage sharing of blood meals (2). The high metabolism of the bats requires that they consume blood meals on a nearly nightly basis, or they will starve (3). If any bat fails to feed on a given night, other bats regurgitate part of their bloodmeal to share with the hungry bat (4,5).

The age and sex of a bat influence its social dynamics within a colony. Vampire bat colonies are typically characterized as mostly male bachelor roosts or maternity roosts (6). In addition to the harem of adult females that raise juveniles, some dominant adult males reside in maternity roosts (7). Juvenile males typically disperse from their natal roost at the age of 12-18 months (8). Adult males switch roosts more frequently, while females maintain higher roost fidelity (9–11). It has also been observed that social grooming occurs more frequently between adult females and juveniles - both male and female – while adult males allogroom less (2).

The successful delivery of topically applied treatment to vampire bats exploits this allogrooming behavior. Topical application to a single individual can lead to treatment of numerous bats through these social interactions. In one study during the development of vampiricide, a single bat transferred the poison to as many as 15 others (12). Another study in Brazil identified greater mortality when vampiricide was applied only to adult females compared to when it was applied to adult males, suggesting that social dynamics have significant implications on dissemination of treatment within a colony (11).

As vaccination provides the ecological benefit of not causing mortality in vampire bats – and importantly in off-target species (13) - a different measurement must be used to trace uptake.

The fluorescent xanthene dye rhodamine B (RB) is a common proxy in field studies to measure bait consumption in multiple species including dogs (14), raccoons (15), badgers (16,17), prairie dogs (18). RB, a systemic biomarker, deposits in growing tissues including hair, nails, and whiskers. While other common biomarkers like tetracycline (19) or ethyl-iophenoxic acid (20) require more invasive sampling of teeth or blood, and measurement by mass spectrometry, the hair of captured bats is readily collected and visualized by fluorescence microscopy.

In 2017, Bakker et al (21) performed a field trial in Peru to investigate the feasibility of orotopal vaccination of vampire bats. RB was mixed into the glycerin jelly vehicle detailed in chapter 1 and applied to captured bats. After releasing the bats back into their roost, the glycerin jelly was consumed. RB fluorescence in hair samples collected during follow-up captures revealed uptake of the experimental topical vehicle. This work postulated that vaccination outperforms culling practices at reducing frequency, intensity, and duration of rabies outbreaks. Additionally, culling has been associated with possible acceleration of rabies virus transmission (22,23).

In this chapter, we measure transfer and uptake of the novel carboxymethyl cellulose (CMC) vehicle within three wild vampire bat colonies. We performed these field trials in rural Jalisco, Mexico. Solar powered radio frequency identification (RFID) systems were installed at each location. Bats were microchipped with passive integrated transponder (PIT) tags during previous captures at the site, facilitating robust population analysis.

Results from the first colony tested in October-November 2024 demonstrated that high rates of CMC transfer, but notably, less to adult males than to adult females and juveniles. To investigate this dimorphism further, we developed rhodamine 110 (R110) as a second rhodamine-based biomarker (Appendix B). R110, a metabolite of RB and a structurally similar

xanthene dye (24), emits green fluorescence rather than orange. In June 2025, we applied CMC in two additional colonies, applying RB to adults of one sex and R110 to the other. This second study highlighted the differences in gel transfer and uptake between age and sex cohorts and validated CMC as a delivery vehicle that propagates within wild vampire bat colonies.

Methods

Ethics Statement

The Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin – Madison, WI, USA approved all animal work (Protocol #V006701-A02). Permit #SPARN/DGVS/05928/24 was obtained from Secretaria de Medio Ambiente y Recursos Naturales (SEMARNAT, Mexico City, Mexico) for capturing and collecting hair samples from vampire bats.

Field Study 1

The first field study was performed at a vampire bat colony roosting in a long-abandoned ranch-house ~10 km outside the Casimiro Castillo township, Jalisco, Mexico (19.580572, -104.534957). This colony had been monitored since October 2023 with follow-up captures every six months. During these previous captures, subcutaneous passive integrated transponder (PIT) tags were implanted in all captured bats to track bat movement at the roost. A solar powered electromagnetic antenna and radio frequency identification (RFID) system (Biomark, Boise, Idaho, USA) detected PIT tags.

On October 31 and November 3 and 7, 2024, we performed diurnal captures. Two personnel entering the roost captured bats using butterfly nets, and a mist net was installed at the lone exit to ensnare bats attempting to fly out of the roost. Captured bats were immediately

removed from the nets and kept in wire cages until processing. On each day of capturing, three capture sessions of 10 min spaced 1.5 h apart were performed. Captured bats were sexed and aged, with age assessed by fusion of the phalangeal epiphyses (25). For analysis, subadults were grouped as juveniles.

The day prior to the first bat capture, 0.225 g (final concentration 0.3% w/v) RB was dissolved in 75 mL water. Then, 9.375 g CMC was added and manually stirred for a final concentration of 12.5%.

During bat capture 1, 1 mL of the gel was topically applied to the dorsal fur of 24 bats (13 females, 11 males) with a needle free syringe. These bats were also given 100 μ L orally to act as gel consumption controls for hair sampling. Treated bats were then released into the roost. This dose of RB was previously determined to clearly mark vampire bat hair samples.

Twenty total bats were euthanized during captures 2 and 3 by local authorities as part of the activities of the Mexican rabies campaign for rabies prevention in livestock species due to equine rabies cases reported nearby.

Field Study 2

The second field study was performed at two vampire bat colonies roosting in unused mines outside the Jirosto (19.753056, -104.733056) and Ahuacapan (19.651389, -104.33) townships in Jalisco, Mexico. These colonies had been monitored by RFID since January 2025 with this study marking the first follow-up capture.

Captures at Ahuacapan were performed on June 11 and 16, 2025; while captures at Jirosto were performed on June 14 and 19, 2025. One personnel member entering the roost captured bats using a butterfly net. A harp net was installed at the lone exit to ensnare bats attempting to fly out of the roost during capture efforts. Captured bats were immediately

removed from the nets and kept in cages until processing. On each day of capturing, a single capture session of 10 min was performed.

The day prior to the first bat capture, two CMC preparations were made with either RB or R110. For RB, 0.225 g (final concentration 0.3% w/v) RB was dissolved in 75 mL water. Then, 9.375 g CMC was added and stirred (final concentration of 12.5% w/v). For R110, 750 μ L of 100% ethanol followed by 7.5 mL of 10x PBS were added to 1.125 g R110 (1.5%) facilitate dissolution of the biomarker. Then, 66.75 mL water was added, and 9.375 g CMC was mixed in. Dosing of R110 was based on a previous test in big brown bats (*Eptesicus fuscus*) as a proxy for vampire bats (Appendix B). An aliquot of each gel was stored at 4 °C for four days for use at the Jirosto location.

During bat capture 1, 1 mL of the RB gel was topically applied to the dorsal fur of 4 adult males at Ahuacapan and 24 adult females at Jirosto with a needle free syringe. Inversely, gel with R110 was applied to 4 adult females at Ahuacapan and 22 adult males at Jirosto. These bats were also given 100 μ L orally of their respective gels to act as controls for hair sampling. The treated bats were then released back to the colony.

RB and R110 Visualization in Bat Hair

On captures 2 (Casimiro: November 3, 2024; Ahuacapan: June 16, 2025; Jirosto: June 19, 2025) and 3 (Casimiro: November 7, 2024), hair samples were plucked with tweezers from captured bats and stored in mini resealable plastic bags. Bags were protected from light for later visualization with a fluorescence microscope. For Field Study 1, uptake of RB within the hair samples was qualitatively assessed with a Zeiss Imager A2 microscope (Oberkochen, Baden-Württemberg, Germany) with a Zeiss 424931 Microscope Fluorescence Filter Cube (excitation 546 nm / emission 590 nm). For Field Study 2, RB and R110 fluorescence was visualized with

an SMZ1270 stereomicroscope (Nikon Instruments, Melville, New York, USA) using SMZ1270/800N P-EFLC TRITC AT (540/25 nm / 605/55 nm), SMZ1270/800N P-EFLC GFP LP AT (480/30 nm / 515 nm), and SMZ1270/800N P-EFLC GFP BP AT (480 nm / 535/40 nm) filter sets (Nikon Instruments, Melville, New York, USA). Two observers independently assessed samples, which were only recorded as positive if both noted fluorescence.

Bat Colony Size Estimation

The colony size was estimated during capture 1 by using the Lincoln-Petersen method (26) with the microchipped bats detected in the colony the night prior as the “marked” population. Each captured bat was scanned with an RFID scanner (AVID, Norco, California, USA) for microchip presence. This method was used to estimate the number of bats to topically treat.

$$Colony\ size = \frac{(\#\ of\ captured\ bats) * (\#\ of\ RFIDs\ detected\ previous\ night)}{(\#\ of\ captured\ bats\ with\ RFID)}$$

Statistical Analysis

To assess variation in biomarker coverage across sex and age cohorts in Field Study 1, we used a generalized linear model with a binomial distribution and a logit link function within a Bayesian framework. For our response variable we used the presence of the biomarker (0/1) in the hair samples collected in unique bats from capture sessions 2 and 3. As predictor variables, we included a grouping variable combining sex and age to produce four distinct cohort effects: adult female, adult male, juvenile female and juvenile male. For our covariate estimates, we used diffuse Gaussian priors with a mean of 0 and standard deviation of 2. The model was fit using the *brms* package(27) in the R statistical language(28).

The *hypothesis()* function in *brms*(27) was used to compare rates of uptake between cohort pairs of non-treated bats. This test evaluated the posterior distribution of the difference between pairs of cohort uptake estimates. We interpreted comparisons whose 95% credible interval for this difference excluded 0 as evidence for differences in uptake rates between cohorts.

In Field Study 2, we implemented a multinomial Bayesian model using Stan(29) to predict colony-wide uptake specific to each cohort. A contingency matrix was made of collected hair samples categorized as (1) RB positive, (2) R110 positive, (3) positive for both markers, or (4) negative. An implicit flat Dirichlet prior was used, representing a uniform probability for each outcome. Using a Hamiltonian Monte Carlo (HMC) algorithm(30), the multinomial model produced posterior distributions of uptake probabilities for each cohort.

For both Field Study 1 and 2, each posterior draw of predicted probabilities of cohort-specific uptake from the respective model was combined with a simulated distribution of uncaptured bats in each cohort. These distributions were generated by sampling a normal distribution truncated at the lower bound by number of known captured bats, using the calculated colony size estimate as the mean and its standard error as the standard deviation of the distribution. The age and sex cohort was assigned for uncaptured bats in each simulation by generating these characteristics from binomial distributions parameterized based on the observed probabilities of sex and age from known captures. The uptake status of uncaptured bats was generated from a multinomial distribution using the posterior draw of predicted probabilities of cohort-specific uptake.. These outcomes were combined with observed counts to generate full posterior distributions of colony-level biomarker uptake.

The resulting posteriors of predicted uptake within each cohort were summarized as the median and 95% credible intervals for reporting and visualization.

In both field studies, we also derived other summary statistics. For each pair of the posterior draw of uptake probabilities and cohort distribution, the total number of treated bats (included topically treated) was divided by the total colony size for that draw. The posterior of the proportion of total colony uptake was then summarized as the median and 95% credible interval of these draws. Uptake was also summarized at the cohort level by calculating the predicted proportion of treated bats (both including and excluding topically treated) relative to the total cohort size. These cohort-specific uptake rates were likewise summarized as medians with 95% credible intervals for each cohort. Similarly, in Field Study 1, using the posterior draws of uptake probabilities, the colony-wide transfer rate was calculated as the total number of transfer-treated bats (excluding topically treated) divided by the total number of topically treated bats. This rate was also summarized using the median and 95% credible intervals.

Likewise, for Field Study 2, transfer rates were calculated from “donor” to “recipient” cohort pairings for uptake of each specific biomarker. This was estimated by summing the posterior draws of transfer-positive bats (RB only plus both positive, or R110 only plus both positive) in the “recipient” cohort for each simulation. This was then divided by the total bats in the “donor” cohort that were topically treated with RB or R110. The resulting cohort-level transfer rate distributions were summarized as medians and 95% credible intervals for each biomarker.

For both models, four Markov chains were run for 8,000 iterations with the first 2,000 iterations removed for burn-in. To assess model convergence, we used the Gelman-Rubin test

statistic, with a value <1.2 indicating a lack of evidence of nonconvergence of MCMC chains(31) and visually inspected the trace plots for chain mixing.

A Chi-squared test was used to analyze the age and sex distribution of bats across the three days of captures in Field Study 1.

Results

High uptake rate of CMC-based delivery formulation within wild vampire bat colonies

In our first trial, CMC gel uptake by wild vampire bats was measured using the fluorescent biomarker rhodamine B (RB) within a microchipped colony in Casimiro, Jalisco, Mexico (Figure 1A). The bats inhabited an abandoned ranch-house (Figure 1B) in which we previously installed an RFID system for microchip detection (Figure 1C). Over three capture sessions (Figure 1D), 156 total *Desmodus rotundus* bats were captured (Figure 1E), representing 95 unique bats (Table 1). Of these unique bats, 57/95 (60.00%) were female, and 66/95 (69.47%) were adults. The age and sex distribution did not change significantly across captures (Chi-squared test, $p = 0.165$). No bats of other species were captured.

During capture 1, 47/89 (52.8%) bats had microchips from previous trips to the site (Figure 1E), while 62 microchipped bats were detected the night before capturing (Figure 1F). This resulted in an estimated colony size of 117 (± 5.625 SE) by the Lincoln-Petersen method. Forty-two new PIT tags were implanted resulting in the observed increase in bats detected by RFID. One milliliter of 12.5% CMC with 0.3% RB was topically applied to the dorsal fur of 24 bats (13 females and 11 males), representing an estimated 20.5% of the colony. RFID data shows a decline in the number of detected bats each night within the colony beginning after capture 1.

During bat capture sessions 2 and 3, 34 and 27 hair samples were collected, respectively. Accounting for recaptures, 48 unique bats were sampled, including 13 topically treated with

CMC gel (Figure 2A-D). Hair samples were visualized with a fluorescent microscope (Figure 2E-F), and all 13 recaptured treated bats recaptured were RB-positive. Of the nontreated bats, 29/35 (82.86%) were RB positive. Notably, the only negative hair samples belonged to adult males (AM). Three AMs had positive hair samples on capture 2 but negative samples on capture 3 (Figure 2A). These individuals were considered RB positive for analysis.

Based on the positivity counts hair samples by cohort and colony demographic estimates, gel uptake within the entire colony was modeled (Figure 2G) using binomial Bayesian regression modelling with brms (27). All Gelman–Rubin (\hat{R}) values equaled 1.00, indicating no evidence for model nonconvergence. Near 100% rates of uptake were predicted in non-treated adult female (AF, 98.3%; 95% Credible Interval (CrI): 87.0% - 99.9%), juvenile female (JF, 95.6; CrI: 77.1% - 99.6%) and juvenile male (JM, 99.3%; CrI: 93.7% - 100%) cohorts. Notably, an estimated uptake of 47.9% (CrI: 33.8%-62.1%) was found in non-treated AMs. Bayesian hypothesis testing with brms(27) indicated that AMs have a lower proportion of uptake compared to other cohorts. Colony-wide uptake was estimated at 88.0% (CrI: 82.1% - 91.7%). Based on the median projection in population size and uptake, 103 bats consumed the gel, with each bat consuming on average ~200 μ L of the total 24 mL of topically applied gel (1 mL applied to 24 bats), assuming all the gel was consumed.

In Field Study 2, two additional colonies were studied within the same region as the Casimiro location (Figure 3A). These colonies roosted inside unused mines near Ahuacapan (Figure 3B) and Jirosto (Figure 3C). Over two capture sessions for treatment application and hair collection (Figure 3D), 57 (Figure 3E) and 262 (Figure 3F) total *Desmodus rotundus* bats were captured at each site, respectively. This represents 35 and 217 unique bats (Table 2). No bats of other species were captured. At Ahuacapan, 11/35 (31.43%) bats were female, and no volant

juveniles were captured (100% adults). In contrast, 137/217 (63.13%) bats were female, with 202/217 (93.09%) adults at Jirosto.

During capture 1, 26/32 (81.25%; Ahuacapan) and 52/140 (37.14%; Jirosto) bats had PIT microchips from previous captures. Thirty-five and 122 microchipped bats, respectively, were detected the night before capturing (Figure 3G-H). This results in an estimated colony size of 43 (± 1.77 SE) at Ahuacapan. Despite mitigation efforts, 17 (14 adult females, 3 adult males) mortalities were recorded at Jirosto attributed to overheating and capture stress. This results in an adjusted colony estimate at Jirosto of 311 (± 26.54 SE). One milliliter of 12.5% CMC with 0.3% rhodamine B (RB) was topically applied to the dorsal fur of 4 adult males in Ahuacapan and 24 adult females in Jirosto. Gel preparation with 1.5% R110 was applied to 4 adult females in Ahuacapan and 22 adult males in Jirosto. Based on population estimates, the total percentage of the colony treated was 18.6% at Ahuacapan and 14.8% at Jirosto.

Between both colonies, 13 RB-treated bats and five R110-treated bats were recaptured. The hair sample of one RB-treated bat was recorded as R110 positive, and another was positive for both markers. All other samples matched the biomarker with which they were treated. Two recaptured AMs at Ahuacapan showed signs of hair loss due to unconsumed dried gel.

Six and 84 bats were microchipped during capture 1 and two and 48 during capture 2, causing the observed increase in RFID-detected bats. RFID data at Ahuacapan was limited by inconsistent solar power (Figure 3G), while bat counts were stable at Jirosto before and after captures (Figure 3H).

Combining counts of hair samples positive for RB, R110, or both, and negative hair samples, and colony demographic estimates, a multinomial Bayesian model predicted RB and R110 gel uptake within the entire colony. All Gelman–Rubin (\hat{R}) values equaled 1.00, again

indicating no evidence for model nonconvergence. In Jirosto, 117 hair samples were collected (Figure 4A). Combining results of both biomarkers, high rates of uptake were predicted in non-treated AF (79.1%; CrI: 72.1% - 84.5%), JF (79.9%; CrI: 62.5% - 89.2%) and JM (64.6%; CrI: 45.5% - 78.9%) cohorts (Figure 4B). Markedly, an estimated uptake of 44.0% (CrI: 35.0%-53.7%) was found in non-treated AM. The model estimated that each treated AFs transferred CMC gel to 5.23 (CrI 4.15 - 6.48) additional bats, while AMs transferred to 2.95 (2.21 - 3.92) bats. As shown in Figure 4C, AFs primarily transferred to other AFs (4.37; CrI: 3.43 - 5.48) with low transfer to AMs (0.36; CrI: 0.20 - 0.66). AMs more evenly transferred to both AFs (1.55; CrI: 1.08 - 2.22) and other AMs (1.10; CrI: 0.76 - 1.61). Including topically treated bats, colony-wide uptake was estimated at 73.3% (CrI: 68.6% - 77.3%).

In Ahuacapan, only one of the 25 collected hair samples demonstrated cross-sex transfer, and only four samples were obtained from adult females (Figure 4D). High rates of uptake were predicted in non-treated AFs (92.6%; CrI: 68.7% - 99.7%; Figure 4E). Again, the model estimated a lower uptake of 51.3% (CrI: 45.2%-57.4%) in non-treated AMs. Each treated AF transferred CMC gel to 2.07 (CrI: 1.40 - 2.89) additional bats, while AMs transferred to 4.13 (CrI: 3.39 - 5.00) bats. As shown in Figure 4F, AFs primarily transferred to other AFs (1.90; CrI: 1.26, 2.69) with minimal predicted transfer to AMs (0.14; CrI: 0.02 - 0.49). Likewise, AMs transferred more to other AMs (3.16; CrI: 2.68 - 3.85) compared to AFs (0.91; CrI: 0.43 - 1.69). Including topically treated bats, colony-wide uptake of 69.2% (CrI: 62.8% - 73.9%) was projected.

Discussion

Like vampiricide, the proposed orotopical vaccination leverages the natural allogrooming propensity of vampire bats. Grooming facilitates transfer of the topical vehicle between treated and untreated bats. Ideally, treatment would be applied to a small proportion of a colony for effective rabies management due to challenges in locating and capturing entire roosts. Captures for vampiricide application are commonly performed at ranches where bats are known to be feeding, rather than at the roost itself (12). This highlights the importance of understanding vaccine transfer and uptake within colonies and between cohorts at low rates of application.

In our initial field trial, an estimated gel uptake of 88.0% (CrI: 82.1% - 91.7%) of the colony was measured after treatment of 20.5% of the bats, considering assumptions of population size and demographic distribution. This corresponds to gel uptake by 3.29 bats (CrI: 2.85 – 3.75) per initial bat treated, improving on the transfer of glycerin jelly reported in Bakker et al. (2019) of 1.45-2.11 (21). Field Study 2 corroborated with this higher transfer rate. Topical application to 14.8% (46/311 bats) of the Jirosto colony led to predicted uptake of 73.3% (68.6% - 77.3%). Similarly, after treatment of 18.6% (8/43 bats) of the Ahuacapan colony, we predicted total uptake of 69.2% (62.8% - 73.9%). This higher transfer is likely related to increasing the volume of gel applied per bat from 0.45 mL to 1 mL and the higher proportion of non-treated bats remaining for gel uptake.

The use of rhodamine 110 (R110) alongside rhodamine B (RB) enhanced understanding of topical transfer dynamics. The visualized biomarkers of treated recaptures matched the treatment applied with high accuracy (94.4%, 17/18), providing confidence in distinguishing the two markers. This novel application of R110 allowed differentiation of transfer by adult female (AFs) and adult male (AMs) vampire bats.

Vampire bat colonies are categorized as either maternity colonies with some resident and non-resident males or primarily-male bachelor roosts (9,32). Social grooming interactions and thus topical vehicle uptake may vary between different sites with different structures of sex and age ratios (33). Analysis of the 117 hair samples collected in Jirosto, a maternity colony, revealed a significantly higher transfer rate by AFs of 5.22 (4.15, 6.48) compared to 2.95 (2.21, 3.92) by AMs. It has also previously been documented that males may be less effective than females at spreading vampiricide within a colony (11). Contrarily at Ahuacapan, AMs transferred at a higher rate of 4.13 (3.39 – 5.00) compared to 2.07 (1.40 - 2.89) for AFs. The Ahuacapan colony structure resembles a bachelor roost, having few AFs, although the site was initially a maternity roost when established in January 2025. The lower AF transfer rate is likely the result of a colony structure with fewer AFs.

Strikingly, uptake in other AFs accounted for over 80% of the transfer by treated AFs at both sites. Transfer from AM to AM (1.10; 0.76, 1.61) was more than double AF to AM transfer (0.36; 0.20, 0.66) at Jirosto. Similarly, in the primarily male colony in Ahuacapan, no samples were collected demonstrating AF to AM transfer resulting in a near-zero transfer rate in the multinomial model (0.14; 0.02 - 0.49). Only one hair was sample collected demonstrating transfer from AM to AF; however, we only obtained four AF samples. At Ahuacapan, AMs transferred at a high rate (3.16; 2.68 – 3.85) to other AMs. These results suggest that AMs are more effective than AFs at treating other AMs.

AMs are disproportionately implicated in the dispersal of vampire bat rabies (VBR) (10). The results at all three field sites suggest that AMs are less likely to consume orotopically applied vaccines. We still predicted moderate uptake in non-treated adult male bats at each site: 47.9% (33.8% - 62.1%) at Casimiro, 44.0% (35.0% - 53.7%) at Jirosto, and 51.3% (45.2% -

57.4%) at Ahuacapan. However, this result differs from Bakker et al. (2019) which found a nonsignificant difference in the proportion of males that showed uptake of RB compared to females (21). This previous finding could be associated with a high proportion of treatment application up to 50% in the colonies reported. AMs have been observed to spend significantly less time grooming than other cohorts (2), which could explain this lower proportion of uptake.

As previously reported, vampire bats tend to abandon roosts when disturbed, relocating to nearby manmade and natural roosts (21,22). While RFID-detected bat counts were stable in the Jirosto location, the number detected declined by around 50% following captures in Casimiro. Additional disturbance was caused by the euthanasia of 20 of the bats from our colony carried out by authorities of the Mexican Rabies Campaign as equine rabies cases were reported near the roost site. While RFID data was unreliable at Ahuacapan due to poor solar power, many bats abandoned the Ahuacapan roost prior to this study during captures in January 2025. In May 2024, we visited a large colony of over 100 bats within an abandoned facility 10 km away as a potential study site. However, before captures could be initiated, the majority of the colony also relocated.

Epidemiological models indicate that vaccination significantly reduces duration and frequency of rabies outbreaks compared to culling even at low rates of vaccination (21). The high estimated gel consumption rates in AFs in all sites, and JMs and JFs in Casimiro found in this study will likely aid in preventing rabies virus introduction to naive colonies. As a lower proportion of AMs consumed the gel, targeting males as juveniles may result in higher rates of vaccination; however, development and duration of the immune response in vaccinated juvenile bats must be assessed. Updating models to incorporate this effect of differing rates of vaccine uptake between males and females will improve understanding of the efficacy of vaccination

compared to culling. Given that VBR has been known to spread among colonies in a slow wavelike fashion (34–36), strategies of ring vaccination around areas of predicted spread may be effective at containing expansion (21).

Samples varied in fluorescence intensity, and in some cases, classifying positivity was challenging. Fluorescence observation is not a quantitative measure of uptake volume, and it is unknown if faint samples correspond to consumption of an immunogenic vaccine dose. To maintain conservative estimates, we only considered samples positive when detected by both observers. The results assume accurate detection of biomarkers, despite the possibility that strong fluorescence of one marker may mask the signs of the other marker. However, we did identify a few samples that were positive for both biomarkers. We recorded the hair sample of one treated AF at Jirosto as positive for the opposite biomarker than the one applied. This discrepancy could have been a misapplication or misidentification of its sex, although, trends of biomarker transfer patterns would minimally be altered.

CMC gel as a topical vehicle for vaccine delivery may be proposed for treating other bat species. In the United States, an estimated 40,000 individuals receive rabies prophylaxis each year after exposure to multiple bat species (37). Furthermore, 36 of 40 (90%) fatal rabies cases acquired in the United States between 2000 and 2022 were acquired from bats (38). CMC could additionally be used to deliver a white nose syndrome (WNS) vaccine (39) or to scale up deployment of a herpesvirus-vectored rabies vaccine (40). As WNS-affected bats often inhabit colder regions in the United States and Canada, it is notable that CMC additionally maintains its physical properties at colder temperatures. However, vampire bats participate in allogrooming at higher rates than other bat species (1), and field studies would be needed to determine gel transfer within colonies of other targeted bat species.

The identification of vampire bats within 50 km of the southern border of the United States (41) and the expectation that their geographic range will continue to expand (42) emphasizes the need for superior control methods. While topical application of vaccines presents an appealing alternative to culling practices that may exacerbate rabies transmission, expanding studies to more sites and regions will improve understanding in the dimorphism of uptake rates in AFs and AMs observed in this study. Likewise, additional studies are needed to determine the impact of vaccination on vampire bat colony sizes, although it is proposed to include contraceptives as a method for population control while minimizing unintended ecological impacts of anticoagulant poisons (43).

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Table 1. Age and sex distribution of unique captured vampire bats (*Desmodus rotundus*) over three capture sessions of Field Study 1 in Casimiro, Jalisco, Mexico. Age was determined by fusion of the phalangeal epiphyses. Subadults were grouped with juveniles.

| Cohort | Unique Bats Captured | Percentage of Captures (%) |
|----------------------|-----------------------------|-----------------------------------|
| Adult Female (AF) | 40 | 42.11 |
| Adult Male (AM) | 26 | 27.37 |
| Juvenile Female (JF) | 17 | 17.89 |
| Juvenile Male (JM) | 12 | 12.63 |
| Total | 95 | 100 |

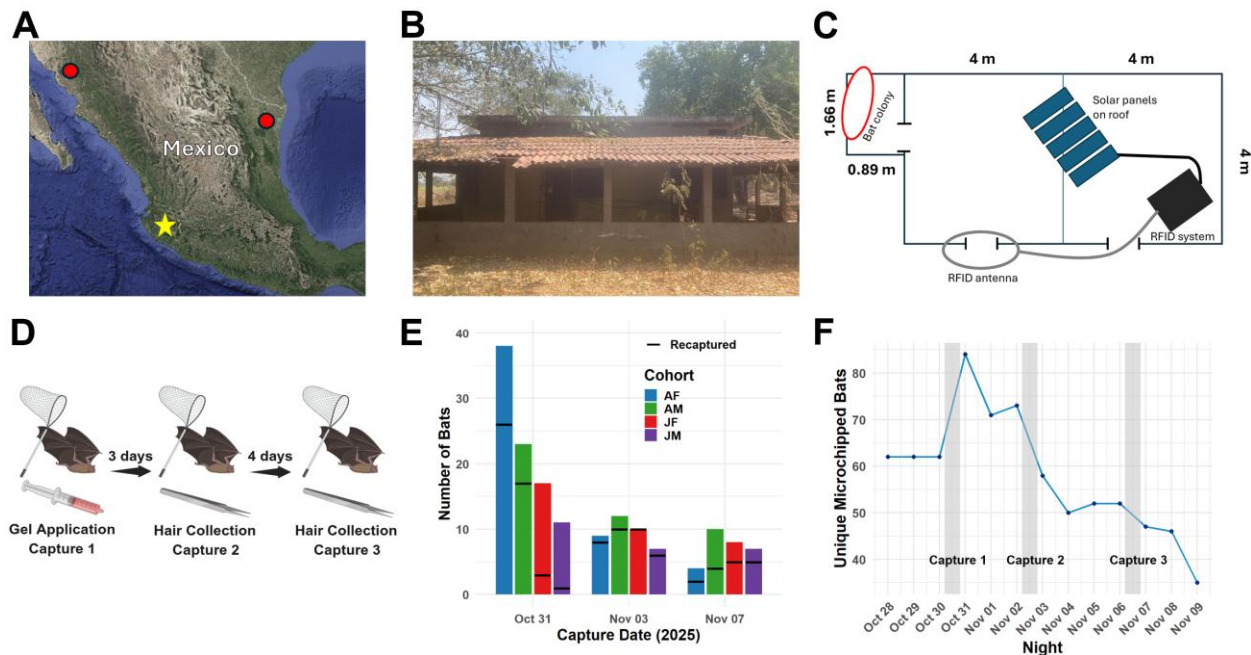


Figure 1. Field Study 1 design and demographics of captured vampire bats (*Desmodus rotundus*) over three capture sessions in Casimiro, Jalisco, Mexico. A) The field study region in Mexico is marked with the yellow star, while regions with the northernmost sightings of vampire bats are labeled with red circles (map retrieved from Google Earth v 10.80.0.1 on May 15, 2025). **B)** The vampire bat colony inhabited the photographed abandoned ranch-house. Photograph by C.K. on May 28, 2024. **C)** In the ranch-house, a radio frequency identification (RFID) system was installed (overhead schematic) to detect movement of microchipped bats. **D)** Three captures were performed for gel application with rhodamine B biomarker during capture 1 and hair collection during captures 2 and 3. **E)** During each capture, bats were sexed and aged. Black lines represent the number of recaptures from the previous capture session, or in the case of capture 1, bats with RFID microchips from previous captures at this site. **F)** Each night, the RFID system detected the movement of microchipped bats into and out of the roost. RFID data was filtered to count each unique bat detected.

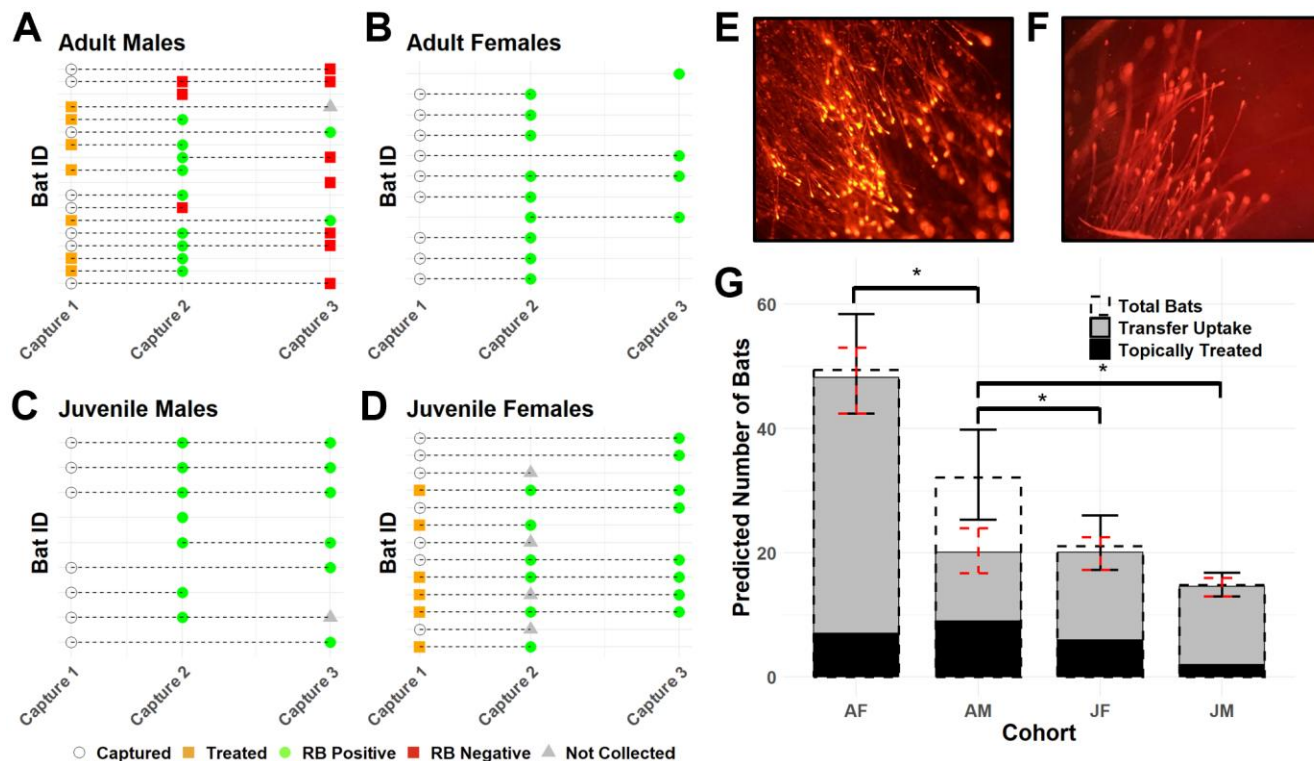


Figure 2. Estimated gel uptake within a vampire bat (*Desmodus rotundus*) colony in Casimiro, Jalisco, Mexico, after approximately 20.5% of the colony was treated topically with 1 mL of carboxymethyl cellulose (CMC) gel containing 0.3% rhodamine B (RB). Panels **A** (adult males - AM), **B** (adult females - AF), **C** (juvenile males - JM), and **D** (juvenile females - JF) depict collection and positivity of hair samples of bats captured during captures 2 and 3, and capture and treatment status of these bats during capture 1. On capture 1, orange squares represent topically treated bats, while open circles were captured, but not treated. On captures 2 and 3, green circles are RB positive, red squares are RB negative, and non-sampled bats are gray triangles. The presence of orange fluorescence distinguished between **E**) positive and **F**) negative hair samples. Samples were visualized with a Zeiss Imager A2 microscope (Oberkochen, Baden-Württemberg, Germany) and a Zeiss 424931 Microscope Fluorescence Filter Cube (excitation 546 nm / emission 590 nm). **G**) A Bayesian regression model estimated uptake within each cohort of bats. Statistical significance indicates difference in coverage

proportions of non-treated bats determined by brms hypothesis testing. Error bars represent propagation of uncertainty in total bats within each cohort (black) and uptake of treatment (red).

Table 2. Age and sex distribution of unique captured vampire bats (*Desmodus rotundus*) over two capture sessions of Field Study 2 in Jalisco, Mexico. Age was determined by fusion of the phalangeal epiphyses. Subadults were grouped with juveniles.

| Cohort | Unique Captured Bats | Percentage of Captures (%) |
|-------------------------|-----------------------------|-----------------------------------|
| Jirosto Colony | | |
| Adult Female (AF) | 137 | 63.13 |
| Adult Male (AM) | 65 | 29.95 |
| Juvenile Female (JF) | 7 | 3.23 |
| Juvenile Male (JM) | 8 | 3.69 |
| Total | 217 | 100 |
| Ahuacapan Colony | | |
| Adult Female (AF) | 11 | 29.73 |
| Adult Male (AM) | 24 | 64.86 |
| Total | 35 | 100 |

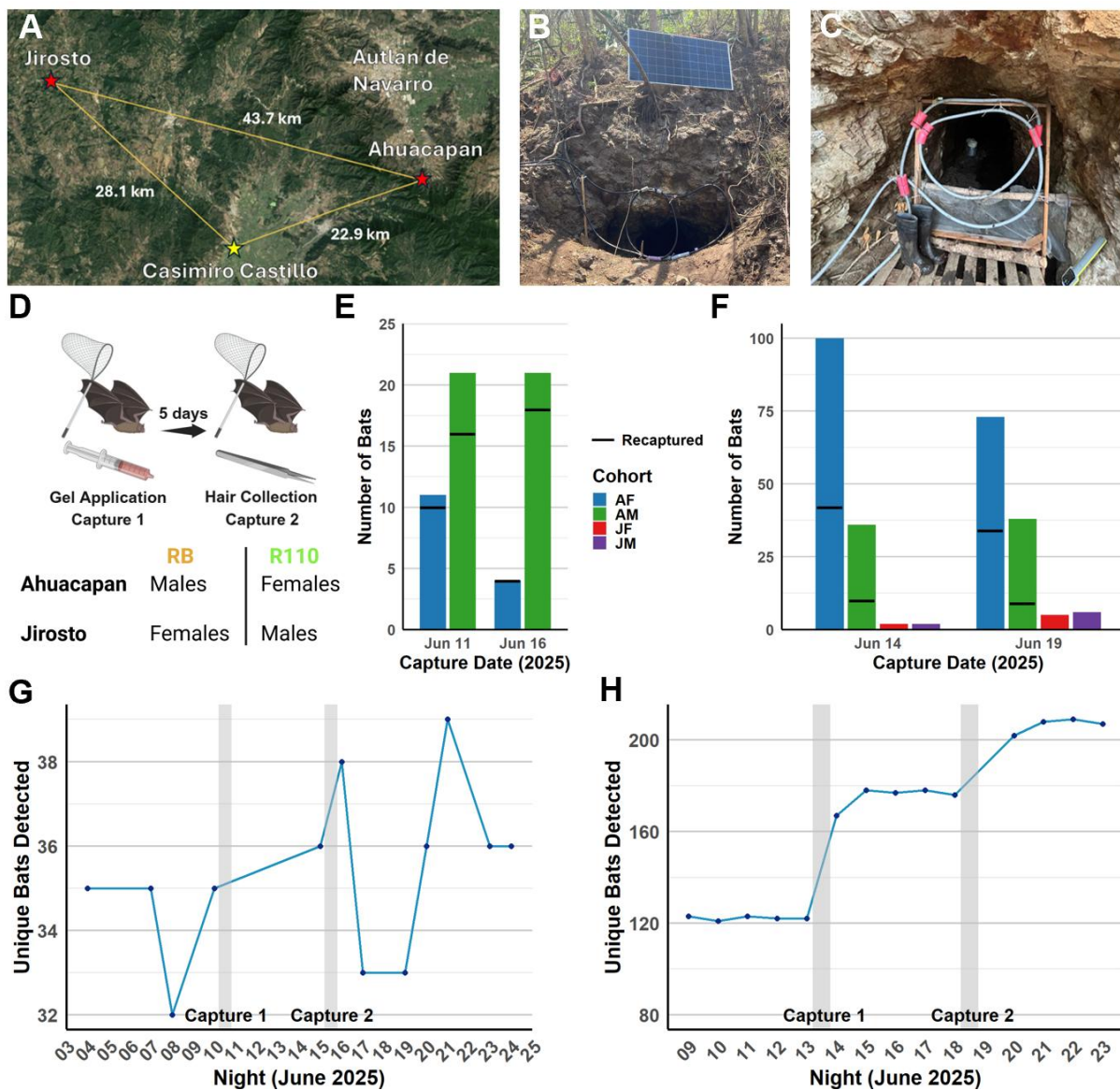


Figure 3. Field Study 2 design and demographics of captured vampire bats (*Desmodus rotundus*) during two capture sessions at two sites in Jalisco, Mexico. A) This study was performed at colonies near the Jirosto and Ahuacapan townships (red stars) in the same region as study 1 (yellow star). The map was retrieved from Google Earth v 10.80.0.1 on September 2, 2025. The colonies roosted in a cave at **B)** Ahuacapan and an inactive mine at **C)** Jirosto. **D)** At each site, two captures were performed for application of topical gel with biomarkers rhodamine B (RB) and rhodamine 110 (R110), and hair collection. Created in BioRender. C.K. (2025). At

E) Ahuacapan and **F)** Jirosto, bats were sexed and aged during each capture. Black lines represent the number of recaptures from the previous capture session, or in the case of capture 1, bats with microchips from previous captures at each site. A radio frequency identification (RFID) system nightly detected unique bats at **G)** Ahuacapan and **H)** Jirosto. Six and 84 bats were microchipped during the first capture session and two and 48 during the second session, causing the observed increases in RFID-detected bats. RFID recording was interrupted during nights that are not plotted, resulting in incomplete data for those nights. Panels E-H were generated with R v4.2.2⁴⁰.

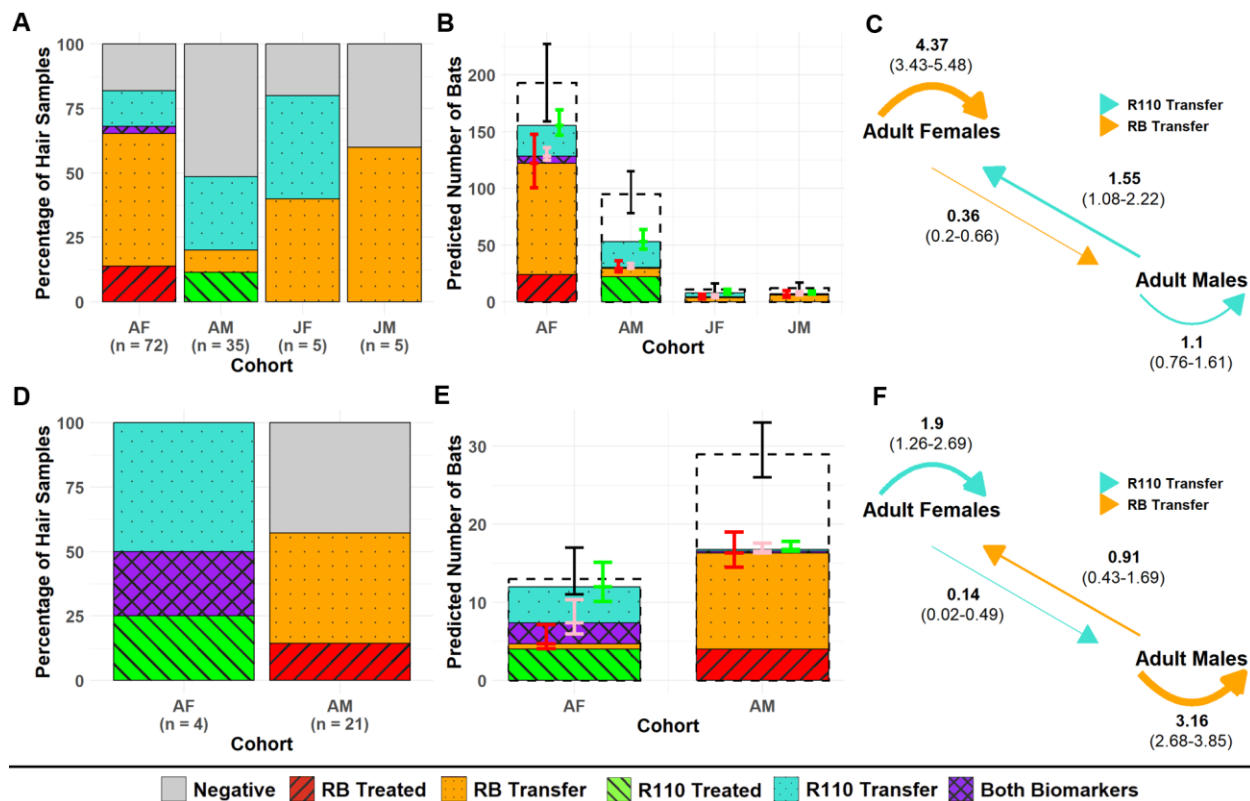


Figure 4. Rhodamine B (RB) and Rhodamine 110 (R110) uptake within two colonies of vampire bats (*Desmodus rotundus*) in Jalisco, Mexico. Fluorescence in hair samples collected from each colony was visualized with a Nikon SMZ1270 stereomicroscope. Using **A)** percentages of biomarker positivity from the Jirosto colony samples, **B)** a multinomial Bayesian model projected uptake within the entire colony. Dashed outlines of the demographic distribution were estimated from mark-recapture calculations and demographics of unique bats captured over the course of the study. Black error bars show the 95% confidence interval in these estimates. **C)** By dividing the predicted total bats of bats with biomarker uptake by the number of bats topically treated, transfer rates between adult females and adult males were estimated. Arrow thickness is proportional to transfer rate. This analysis was replicated for data from the Ahuacapan colony with panels depicting **D)** percentages of biomarker visualization in hair samples, **E)** model predicted uptake within the colony, and **F)** estimated transfer rates. AF - adult

female; AM – adult male; JF – juvenile female; JM – juvenile male. Error bars for uptake display the 95% credible intervals derived from the multinomial model. This figure was generated with R v4.2.2⁴⁰.

Appendix A

Spray application of 4% CMC to a Yuma bat (*Myotis yumanensis*) colony in Idaho

Cole Knuese, Rita Dixon, Charlotte Chorzempa, Tonie Rocke

Introduction

A sprayable vaccine formulation could provide significant advantages over the manual application of a thick paste. Without the need to capture bats, spraying a vaccine would require less labor and resources and minimize stress on the bats. Vampiricide application relies on capturing bats and applying the topical paste by hand, and we similarly demonstrated the dissemination of 12.5% carboxymethyl cellulose (CMC) after applying on vampire bats. In this work, we test uptake of a sprayable, less viscous 4% carboxymethyl cellulose (CMC) solution in a colony of Yuma bats.

While there are currently no vampire bats in the US, there are 47 other species of mostly insectivorous bats (1). Many of these species have been severely impacted by white nose syndrome (WNS), which has caused devastatingly high mortality to bats in the northern US and Canada (2,3). WNS first entered the Americas in 2006 and has swept across the country westward. A raccoonpox vectored vaccine (RCN-Cal/SP) has also been developed for WNS (4). A sprayable formulation for delivering the RCN-CAL/SP and RCN-MoG vaccines would accelerate their utility and deployment. Across the US, an estimated 40,000 individuals annually receive rabies prophylaxis post-exposure to natively found bat species (5), and 90% of fatal rabies cases acquired from 2000-2022 were of bat origin (6).

Methods

Ethics Statement

This study was performed in accordance with protocol #EP210621 approved by the Institutional Animal Care and Use Committee (IACUC) at the USGS National Wildlife Health Center (Madison, WI, USA).

Study Site

This study was performed in a colony of Yuma bats (*Myotis yumanensis*) inhabiting the basement of the Swan Falls dam in Swan Falls, Idaho, in August 2025. Several clusters of approximately 50-100 bats congregate on the walls inside the structure and are easily accessible. While population calculations were not performed during this study, previous estimates from emergence counts at the site suggest a colony of over 2,000 bats.

Formulation preparation

The day prior to spray application, 1 g of rhodamine B (RB) was dissolved in 200 mL of distilled water for a concentration of 0.5% RB. Then 8 g of CMC was stirred in for a final concentration of 4%. A concentration of 0.5% RB was previously found to mark hair follicles after consumption of ~100 μ L.

Spray application

Around 10 am, three researchers entered the chamber where the bats were roosting and sprayed the 200 mL of formulation using industrial spray bottles. Bats were sprayed directly at close range, targeting their back and chest.

Hair collection

Two days after the spray application, bats were collected from the walls by hand and placed together into laundry hampers until processing. Bats were removed one by one from the

hampers, and age and sex were recorded. Hair samples including the follicle were plucked with tweezers from the dorsal fur of the bats, which were then released. After roughly 1 h of hair sampling, remaining bats were released.

However, it was noted that samples from few juvenile females were obtained during this period. As remaining bats were being released at the end of sampling, we identified an additional seven juvenile females from which we collected hair samples.

Results

Moderate Uptake of Sprayable Formulation of Carboxymethyl Cellulose

The 4% CMC solution was very viscous and led to substantial loss of formulation either from dripping out of the spray bottles or not sticking to the bats' fur. After being sprayed, bats almost immediately began to fly away. It was difficult to avoid spraying the head of the bats.

Two days after spray application, hair samples were collected from 142 bats (Figure 1; 101 adult females, 1 adult male, 18 juvenile females, and 22 juvenile males). Of the hair samples, 18 were deemed indeterminate due to lack of hair follicles for RB visualization. Of the remaining 124 samples, 28 were RB positive (22.6%). Interestingly, juvenile males (8 of 20) were 2–3 times more likely to be positive than juvenile females (2 of 15) and adult females (18 of 88). Comparing juvenile females and adult females to juvenile males, differences in proportions were not statistically significant (Fisher's exact test: JF $p = 0.134$; AF $p = 0.0833$).

Excluding the seven juvenile females identified when releasing bats, we sampled 22 juvenile males and only 11 juvenile females. Under the null hypothesis of a 50:50 sex ratio, a binomial test gave $p = 0.0801$.

Discussion

This study demonstrated uptake of a sprayable topical formulation in Yuma bats without the need to capture them. However, uptakes levels were low compared to the volume sprayed, emphasizing the need for improvements in the formulation or spray method used. We sprayed approximately 2,000 doses of formulation but only observed rhodamine B (RB) fluorescence in 22.6% of hair samples collected. Substantial loss of formulation was noted either dripping out of the spray bottle or not sticking to the bats' fur.

Juvenile males were 2-3 times more likely to be positive than adult females and juvenile females. Of juvenile male hair samples, 8/20 (40%) were RB positive, compared to just 2/15 (13.3%) juvenile females, although this difference was not significantly different ($p = 0.134$). In adult females, 18/88 samples were positive (20.45%, $p = 0.0833$). It was noted that when the outermost row of bats from a cluster was sprayed, they would leave, exposing the next row to spray. Perhaps juvenile males were more likely on the outer edges of the clusters as August is approaching the season when they disperse from the maternity roost. This could also explain why more juvenile males were captured than juvenile females, although this variance was likewise not statistically significant ($p = 0.0801$). As this was a maternity roost, it was not unexpected that only one adult male was captured.

These results may also not be representative of the entire colony if the sampling method introduced any bias. On each day of the study, the bats that were easiest to access were more likely to be sprayed and captured for sampling. As only one day of capturing was performed, bats were not marked, preventing calculations to estimate population size.

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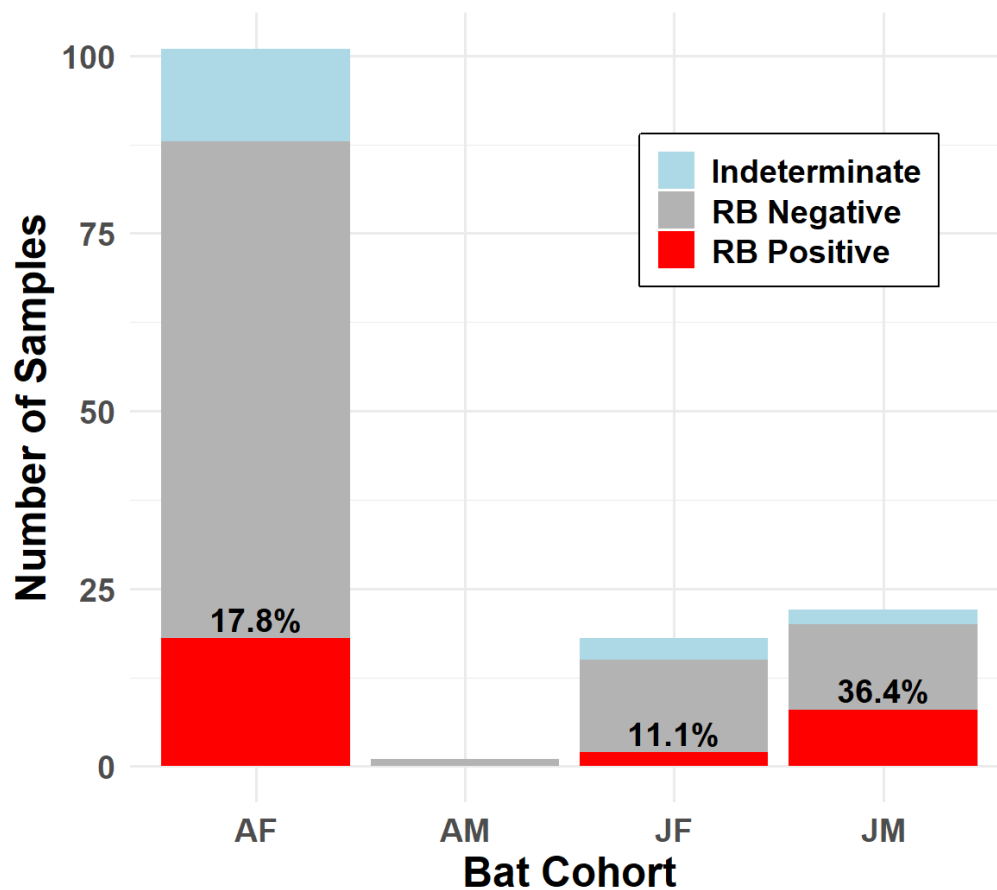


Figure 1. Rhodamine B (RB) positivity in hair samples collected from Yuma bats (*Myotis yumanensis*) two days after spray application with 4% carboxymethyl cellulose (CMC) solution containing 0.5% RB. Bat were aged and sexed into four cohorts: AF - adult female; AM – adult male; JF – juvenile female; JM – juvenile male.

Appendix B: Biomarker dosing study

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Introduction

Prior to the vaccination study in chapter 2, we conducted preliminary trials with the big brown bats (*Eptesicus fuscus*) brought into captivity to evaluate other rhodamine dyes as potential biomarkers. The goal was to develop a second marker to use in concordance with rhodamine B (RB). This would allow us to differentiate topical transfer by two cohorts in the same field trial. Initially we tested rhodamine 110 (R110), which emits green fluorescence rather than orange like RB. Similarly to RB (1), R110 has been shown to distribute systemically within rats, mainly to the liver and kidney (2). We later tested dosing of three other rhodamine dye variants: rhodamine 101 (R101), sulforhodamine B (SRB), and rhodamine WT (RWT). The fluorescence spectrum and manufacturer's information of all rhodamines are found in Table 1.

Methods

Due to the limited number of available bats, we treated groups of three bats at a time, and no bat received more than three rounds of treatment. Bats were given at least 10 days between dosing if no fluorescence was seen. For each marker, we tested doses of 50 μL (~25 mg/kg) and 100 μL (~50 mg/kg). A dose of 150 μL (~75 mg/kg) was additionally tested for R110 and R101. Individual bats were not fixed into treatment groups of the same marker, rather they could receive doses of different markers.

A 1% buffer of rhodamine dye was prepared first by dissolving 0.03g of each rhodamine powder in 30 μL food-grade ethanol to facilitate dissolution. Then 2.97 mL of Tris buffer pH 9.0 was added. Lastly, 0.375 g CMC stirred in to form a 12.5% gel. RWT is already a liquid, so it was diluted to a final concentration of 1% in Tris buffer.

We collected hair samples at baseline, and 4 h, 3 days, and 7 days post-dosing. Visualization was performed with an SMZ1270 stereomicroscope (Nikon Instruments, Melville, New York, USA) using SMZ1270/800N P-EFLC TRITC AT (540/25 nm / 605/55 nm), SMZ1270/800N P-EFLC GFP LP AT (480/30 nm / 515 nm), and SMZ1270/800N P-EFLC GFP BP AT (480 nm / 535/40 nm) filter sets (Nikon Instruments, Melville, New York, USA).

Results and Discussion

Most bats treated with a high dose (100 – 150 μ L) of R110 showed fluorescence in hair samples (Figure 1A-B). However, fluorescence was brightest at the 4-h timepoint and progressively dimmed at later timepoints. This suggested that R110 could potentially be used as a biomarker, although the fluorescence signal appeared less distinguishable compared to RB.

It was noted that samples from bats that had ingested RB also fluoresced under the GFP filter (Figure 1C-D). R110 is a metabolite of RB (2), so the bats that ingested RB could have also had R110 present in the hair. The RB gel itself did not fluoresce in the green channel, suggesting that this effect is not the result of spectral overlap. For this reason, we attempted dosing with other rhodamine dye variants: rhodamine 101 (R101), sulforhodamine B (SRB), and rhodamine WT (RWT). This overlap effect was not as pronounced in the vampire bat hair samples (Figure 2).

These other rhodamines (R101, SRB, and RWT) have similar fluorescence spectra to RB. We hypothesized that their molecular structures could be less likely metabolized into R110, perhaps leading to less overlap in fluorescence with R110. However, little research has been published using these molecules *in vivo*. No fluorescence was noted in the hair follicles of bats treated with these three rhodamines at any tested concentration nor timepoint (Figure 3). We

discontinued the dosing regimen before the high dose for SRB and RWT due to this absence of fluorescence.

No adverse effects were noted from treatment with any marker. Notably, no signs of taste aversion for R110, R101, SRB, nor RWT were noted at the tested concentration. Aversion to RB has been reported at a concentration as low as 0.2% in black rats (*Rattus rattus*) (3). Slight aversion to 1% RB in this study was noted.

References

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Table 1. Fluorescence spectra and manufacturer's information of all rhodamine dyes evaluated for use as biomarkers.

| Dye Name | Excitation (nm) / Emission (nm) | Catalog Number | Manufacturer |
|---------------------------|--|-----------------------|---------------------------------|
| Rhodamine B (RB) | 543/569 | R6626 | Sigma-Aldrich |
| Rhodamine 110 (R110) | 504/520 | 419070025 | Thermo Fisher Scientific Inc |
| Rhodamine 101 (R101) | 567/588 | 419060010 | Thermo Fisher Scientific Inc |
| Sulforhodamine B (SRB) | 565/586 | A14769.06 | Thermo Fisher Scientific Inc |
| Rhodamine WT (RWT) | 550/588 | 106023 | Kingscote Chemicals |

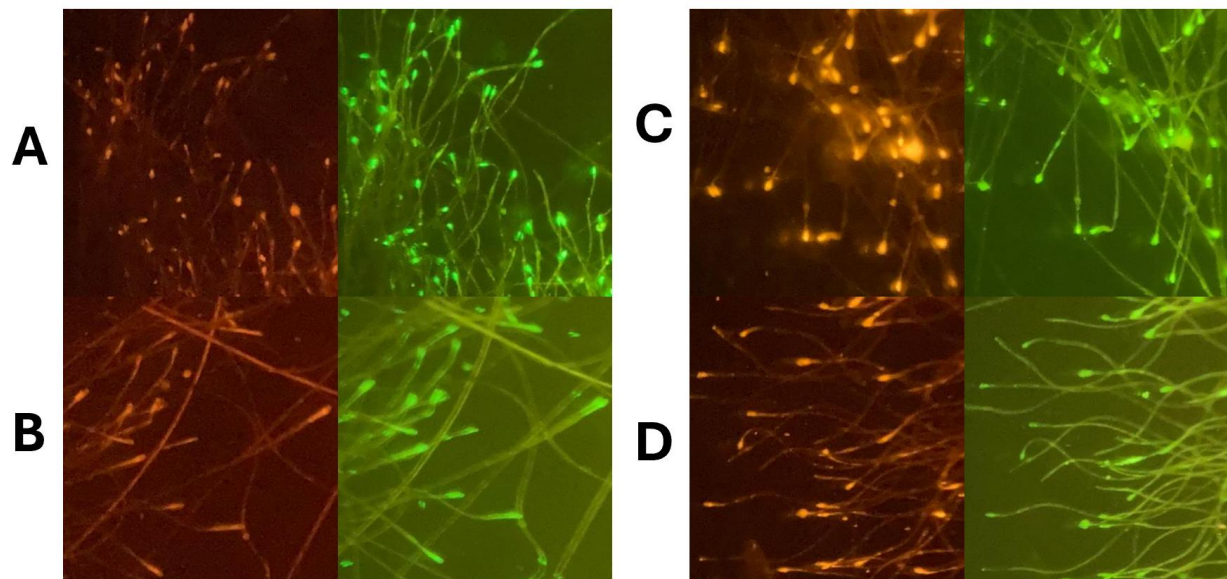


Figure 1. Hair samples of big brown bats (*Eptesicus fuscus*) treated with rhodamine 110 (R110) and rhodamine B (RB). Hair samples of bats treated with **A)** a high dose (150 μ L) of R110 collected at 4 h and **B)** 3 days, and **C)** a low dose (50 μ L) of RB collected at 4 h, and **D)** 3 days. For each panel, the left image was captured using the SMZ1270/800N P-EFLC TRITC AT (540/25 nm / 605/55 nm) filter and the right image with the SMZ1270/800N P-EFLC GFP LP AT (480/30 nm / 515 nm) filter.

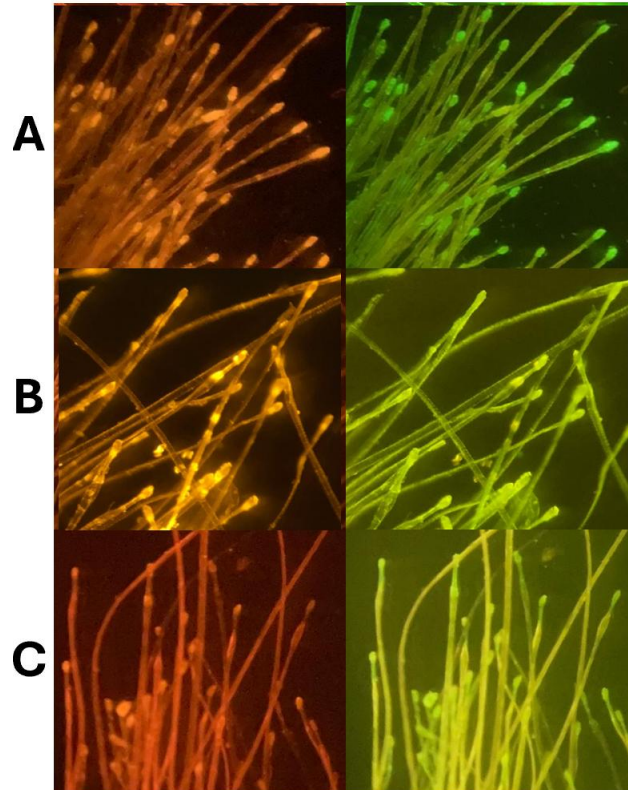


Figure 2. Hair samples of vampire bats (*Desmodus rotundus*) collected in the field five days after topical application of carboxymethyl cellulose gel containing rhodamine 110 (R110) or rhodamine B (RB) (see Chapter 3 for study details). Panels depict A) a sample positive for R110, B) a sample positive for RB, and C) a negative sample. For each panel, the left image was captured using the SMZ1270/800N P-EFLC TRITC AT (540/25 nm / 605/55 nm) filter and the right image with the SMZ1270/800N P-EFLC GFP LP AT (480/30 nm / 515 nm) filter.

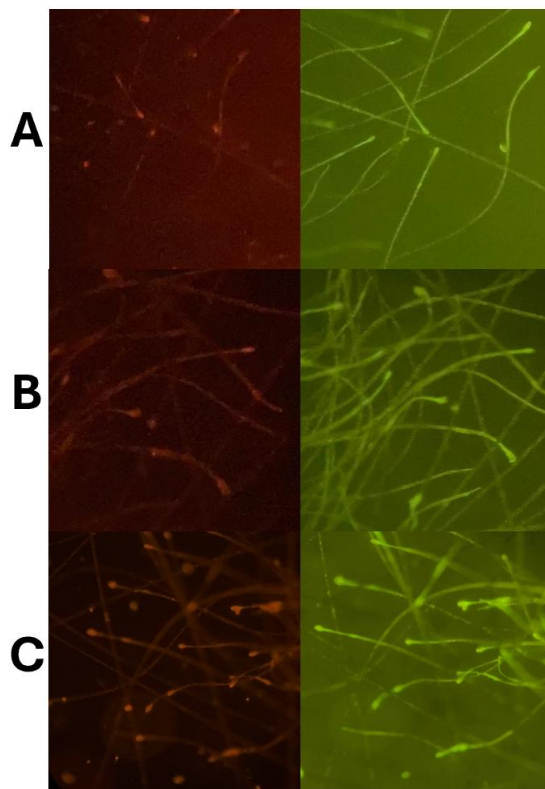


Figure 3. Hair samples of big brown bats (*Eptesicus fuscus*) taken 4 h after treatment with rhodamine 101 (R101), sulforhodamine B (SRB) and rhodamine WT (RWT): Bats were treated with **A**) a high dose (150 μ L) of R101, **B**) a medium dose (100 μ L) of SRB, and **C**) a medium dose (100 μ L) of RWT. For each panel, the left image was captured using the SMZ1270/800N P-EFLC TRITC AT (540/25 nm / 605/55 nm) filter and the right image with the SMZ1270/800N P-EFLC GFP LP AT (480/30 nm / 515 nm) filter.

Conclusion

In this work we characterize carboxymethyl cellulose (CMC) as a topical vehicle for rabies vaccine delivery to vampire bats. Firstly, we determined the physical properties of CMC improved on those of previous formulations and should facilitate application in both warm and cold environments. The formulation additionally preserves RCN-MoG vaccine for adequate periods of time. While a sprayable vaccine presents advantages over a manually applied paste, work remains to be done to implement this approach. CMC is difficult to spray without specialized equipment even at a diluted concentration of 4%. At high shear rates CMC displayed higher viscosity than other tested substances. A hydrogel showed the most promising physical properties for spraying yet may present challenges in mixing and recovering the vaccine. A sprayable vaccine may not be practical in many situations, in which case there remains significant benefit to a thicker paste like CMC. Due to challenges in locating vampire bat roosts, CMC paste could be applied to bats captured at the ranches when feeding.

Most importantly, CMC demonstrated high rates of spread in wild bat colonies in Mexico. In three distinct colonies, we estimated uptake by ~70-90% of bats after application of just 15-20% of the colony. Treated bats transferred the vaccine vehicle to 2-5 other bats, depending on its age and sex and the colony structure. Interestingly, adult males exhibited lower uptake than adult females and juveniles at all sites.

Overall, this study provides a framework for the characterization of novel topical vaccine vehicles for vaccination of bats. Future work should prioritize confirming immunogenicity of the vaccine within CMC and expanding understanding of gel uptake across ecological, seasonal, and regional variables.