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3 An optimized rabies vaccine vehicle for

4 orotopical administration to wild vampire bats

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22

Abstract

23

24 Rabies vaccination of vampire bats (*Desmodus rotundus*) has been proposed as a

25 superior control method to culling but has yet to be implemented. Success of rabies

26 vaccination depends on a topical vehicle that spreads through a bat colony via

27 allogrooming while additionally preserving vaccine immunogenicity. This work describes

28 the *in vitro* and *in vivo* optimization of a new orotopical gel for rabies vaccine delivery to

29 vampire bats. Autonomous transferability of our carboxymethyl cellulose (CMC) gel

30 vaccine delivery formulation was tested in a microchipped vampire bat colony in rural

31 Jalisco, Mexico. Intra-colony gel uptake was traced using the fluorescent biomarker

32 rhodamine B. Importantly, application of topical treatment of ~20% of the bat colony

33 resulted in estimated uptake by over 85% of the colony. The *in vitro* stability of our

34 raccoon poxviral-vectored rabies vaccine candidate within CMC was measured at time

35 points up to 3 months at 40 °C, 23 °C, and 4 °C. Extended storage at 4 °C and short

36 exposure at higher temperatures of potential vampire bat environments preserved

37 vaccine titers within CMC. Furthermore, physical properties of our CMC formulation

38 were compared to the previously used glycerin jelly at 40 °C, 20 °C, and 0 °C using

39 rheological tests. These tests indicate that CMC exhibits optimal properties for topical

40 application to bats even at extreme temperatures possible during field vaccination. This

41 study advances our rabies vaccination strategy for vampire bats by providing a topical

42 vehicle suitable for field application that may additionally be employed for other

43 significant bat diseases.

44

45 **Key words:** rabies, vaccine, vampire bat, topical, carboxymethyl cellulose, biomarker,

46 rhodamine B

47

48 **1. Introduction**

49 In Latin America, the vampire bat (*Desmodus rotundus*) is the primary reservoir of the

50 deadly rabies virus (Rhabdoviridae, Lyssavirus)[1]. First reported in the early 1900's,

51 vampire bat-transmitted rabies (VBR) poses significant health risk to humans[2,3] and,

52 especially, to livestock[4]. Each year, VBR causes an estimated economic burden of $50

53 million USD to the livestock industry[5]. Additionally, the suitable habitat range for vampire

54 bats is expected to spread due to changing climates[6,7], and annual reports of VBR are

55 trending upwards in several countries[1,8]. Recent models predict future expansion of the

56 bats into the southern United States[7] posing risk of extensive damages in this region

57 with high populations of livestock[9]. Across the US, an estimated 40,000 individuals

58 annually receive rabies prophylaxis post-exposure to natively found bat species[10], and

59 90% of fatal rabies cases acquired from 2000-2022 were of bat origin[11].

60

61 Attempts to mitigate VBR rely on vaccination of livestock and proactive or reactive

62 culling of bat colonies via application of "vampiricide" – a topical paste containing

63 poisons that spread by the allogrooming behavior of the bats[12]. However, since their

64 inception in the 1970's, these methods have been insufficient in preventing the

65 expansion of VBR. Reactive application of vampiricide has been shown to exacerbate

66 rabies virus transmission by provoking dispersal of infected bats to new roosts[13,14].

67   Furthermore, the poison may transfer to multiple non-target species in the wild, causing

68   unintended mortality[1,12].

69

70   The ingestion of topically applied (orotopical) vaccines has been previously proposed as

71   a superior method for bolstering current control strategies[15,16]. Mathematical simulations

72   predict that transferrable vaccines that similarly leverage bat allogrooming behavior

73   outperform culling practices in reducing the probability, size, and duration of rabies

74   outbreaks[17]. In earlier work, members of our group developed and tested a recombinant

75   raccoon poxvirus (Poxviridae: Orthopoxvirus) expressing a mosaic lyssavirus

76   glycoprotein (RCN-MoG)[18,19]. This vaccine was shown to produce protective immunity

77   intradermally in A/J mice (*Mus musculus*)[18], and orotopically in big brown bats

78   (*Eptesicus fuscus*)[18]. Notably, orotopical vaccination blocked shedding of rabies virus in

79   vampire bats[19]. Our team investigated glycerin jelly (GJ) as a topical vehicle to deliver

80   the RCN-MoG vaccine in these captive bat colonies[18,19] and to assess topical vehicle

81   uptake in the wild[17]. However, the highly temperature dependent physical properties of

82   GJ preclude its practicality for use in the field throughout Latin America, where tropical

83   and subtropical climates commonly exceed its melting point[20,21]. Additionally,

84   crystallization of GJ was noted in a separate field trial in Jalisco, Mexico[22].

85

86   In this work, we describe the optimization of a novel carboxymethyl cellulose (CMC) gel

87   vehicle for orotopical administration of the RCN-MoG vaccine to vampire bats. We

88   compared physical properties of CMC gel and GJ with rheological tests to emulate

89   application at environmentally relevant temperatures. Vaccine preservation was

90   assessed by tracking viral titers over three-months across a range of temperatures.

91   Lastly, we applied CMC gel mixed with the fluorescent biomarker rhodamine B (RB) to a

92   wild colony of microchipped vampire bats in rural Jalisco, Mexico to investigate gel

93   transfer and uptake of CMC in a field setting.

94

95   **2. Methods**

96   **Characterization of Physical Properties**

97   **Gel Preparation**

98   Precise weights of CMC powder (Sigma-Aldrich, cat. no. 419281; St. Louis, Missouri,

99   USA) were added to ultrapure water (Modulab® High Flow, Evoqua, Pittsburgh,

100   Pennsylvania, USA) to prepare bulk solutions of 10%, 12.5%, and 15% (w/v). CMC gels

101   were dissolved at room temperature overnight.

102

103   GJ (Carolina Biological Supply Co, cat. no. 865495; Burlington, North Carolina, USA)

104   was melted at 37 °C and combined with ultrapure water at a ratio of 55.5:44.5 as was

105   previously described[17]. GJ was solidified and kept at room temperature overnight.

106

107   **Rheological Measurements**

108   For characterization of rheological properties, an ARES LS2 Advanced research grade

109   rheometer (TA Instruments, New Castle, Delaware, USA) was fitted with 50 mm cone

110   and plate geometry. An FS18-HD Refrigerated/Heating Circulator (Julabo, Allentown,

111   Pennsylvania, USA) and Peltier Plate were used for temperature control. Two different

112   rheological tests were performed at 0 °C, 20 °C, and 40 °C with at least 5 min allowed

113   for temperature equilibration. Testing was performed the day after gel preparation.

114   The first test, a steady rate sweep test, was used to record viscosity over a shear ramp

115   from 0.05 s^{-1} to 200 s^{-1}. Low shear rates characterize the near-zero shear rate and

116   mimic behavior at rest. The range of 20 s^{-1} to 200 s^{-1} has previously been described as

117   the shear rate to investigate spreading properties[23,24].

118   Secondly, a dynamic strain sweep test characterized the viscoelastic properties of CMC

119   and GJ. The elastic (storage) modulus G' and viscous (loss) modulus G" were

120   measured at a frequency of 1 hz with an initial strain of 0.015% up to 1000%.

121

122   **RCN-MoG Vaccine Stability Assay**

123   **Cells and Viruses**

124   Vero cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle Media (DMEM;

125   Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 5% fetal bovine serum and

126   1x antibiotics-antimycotics and incubated at 37°C with 5% CO_2. The previously

127   described RCN-MoG vaccine[18] was used in the viral stability assay.

128

129   **Vaccine Gel Preparation**

130   To prepare RCN-MoG vaccine in CMC gel, RCN-MoG virus was homogenized in sterile

131   10mM Tris hydrochloride (Tris-HCl) buffer (pH 9.0) at a final concentration of 10^8

132   PFU/mL in 4 mL total volume. CMC powder was then added at a final concentration of

133   12.5% (0.5 g) and stirred with a sterile microspatula.

134

135   For GJ preparation, RCN-MoG virus was added to sterile 10mM Tris-HCl buffer (pH

136   9.0). Then melted glycerin jelly was added such that the ratio of Tris buffer to glycerin

137   jelly was 55.5:44.5 and the final concentration of RCN-MoG was 10^8 plaque-forming

138   units (PFU)/mL in 4 mL total volume. After homogenizing, 100 µL aliquots of the GJ

139   preparation were transferred with a needle-free syringe into sterile 1.5 mL Eppendorf

140   tubes.

141

142   Both the GJ aliquots and CMC gel were stored at 4°C overnight to allow the CMC

143   powder to dissolve completely. Next, the CMC gel was briefly mixed and likewise

144   aliquoted (100 µL) into sterile 1.5 mL Eppendorf tubes with a needle-free syringe.

145   Aliquots were then placed at their respective storage temperatures (4 °C, 23 °C, or 40

146   °C). At each timepoint (24 hr, 48 hr, 96 hr, 1 wk, 2 wk, 1 mo, 3 mo), two aliquots were

147   moved from each temperature and stored at -80 °C until titration by viral plaque assay.

148

149   **Titration of RCN-MoG Vaccine by Viral Plaque Assay**

150   Viral plaque assays were performed with Vero cells in 24-well plates (TPP, Trasadingen,

151   Switzerland). Gel samples were thawed at 4 °C and dissolved by adding 900 µL of 1x

152   DMEM to each tube with 1 hr incubation at 37 °C. Samples were mixed by pipetting

153   every 15 min to facilitate dissolution. Cells were inoculated with 100 µL of ten-fold

154   dilutions of the dissolved gels. After 1 hr of infection at 37 °C with 5% CO_2, the inoculum

155   was removed and 1 mL of plaque assay media (1x DMEM, 1.5% CMC, 2% FBS, 1x

156   antibiotics-antimycotics) was added. Plates were fixed with 10% formaldehyde after 3

7

157   days of infection and stained with 1% crystal violet and 20% ethanol. Log fold change in

158   viral titer was standardized to the initial time point (T=0).

159

160   **Western Blot**

161   Six well plates of Vero cells were infected with 100 µL of dissolved CMC gel. After 18

162   hours, when >80% CPE was present, the supernatant and cells were harvested and

163   centrifuged at 3,000 g for 10 minutes. After decanting, the cell pellet was resuspended

164   in 100 µL of 4X Laemmli Sample Buffer (Bio-Rad, Richmond, California, USA)

165   containing 10% 2-mercaptoethanol (Sigma Aldrich, Germany). Samples were denatured

166   at 95°C for 5 min. SDS-PAGE resolved samples were then transferred to a

167   nitrocellulose membrane (Bio-Rad, Richmond, California, USA). The membrane blocked

168   was at 23 °C in 5% milk in PBS-T (blocking buffer) for 2 hours. Rabies virus glycoprotein

169   polyclonal antibody (Invitrogen, Waltham, Massachusetts, USA) was used at 1:1000 in

170   blocking buffer with 5% rabbit serum for 90 minutes at 23 °C. After three washes with

171   PBS-T, Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (Invitrogen, Waltham,

172   Massachusetts, USA) was used at 1:4000 in blocking buffer with 5% goat serum for 90

173   minutes at 23 °C. After a final three washes, the membrane was developed with Pierce

174   1-step Ultra TMB Blotting solution (Thermo Fisher Scientific, Waltham, Massachusetts,

175   USA). Membranes were scanned with an Epson Scanner (EPSON Perfection 4490

176   Photo) using the Epson Scan Utility v3.24 software.

177

178   **Bat Field Study**

179   **Ethics Statement**

8

180   The Institutional Animal Care and Use Committee (IACUC) at the University of

181   Wisconsin – Madison, WI, USA approved all animal work (Protocol No. V006701-A02).

182   Permit number SPARN/DGVS/05928/24 was obtained from Secretaria de Medio

183   Ambiente y Recursos Naturales (SEMARNAT, Mexico City, Mexico) for capturing and

184   collecting hair samples from vampire bats.

185

186   **Study Site**

187   The field study was performed at a vampire bat colony roosting in a long-abandoned

188   ranch-house ~10 km outside the Casimiro Castillo township, Jalisco, Mexico

189   (19.580572, -104.534957). This colony had been monitored since October 2023 with

190   follow-up captures every six months. During these previous captures, subcutaneous

191   radio frequency identification (RFID) microchips were implanted in all captured bats to

192   track bat movement at the roost through detections by a solar powered electromagnetic

193   antenna and RFID detection system (Biomark, Boise, ID).

194   On October 31 and November 3 and 7, 2024, we performed diurnal captures. Two

195   personnel entering the roost captured bats using butterfly nets, and a mist net was

196   installed at the lone exit of the roost to ensnare bats attempting to fly out of the roost

197   during capture efforts. Captured bats were immediately removed from the nets and kept

198   in cages until processing. On each day of capturing, three capture sessions of 10 min

199   spaced 1.5 hr apart were performed. Each captured bat was sexed and aged, with age

200   determined by assessing fusion of the phalangeal epiphyses[25]. For analysis, subadults

201   were grouped as juveniles.

202 Twenty of the bats were euthanized during captures 2 and 3 by local authorities as part

203 of the activities of the Mexican rabies campaign for rabies prevention in livestock

204 species due to equine rabies cases reported nearby.

205

206 **Gel Application and Sample Collection**

207 The day prior to the first bat capture, 0.225 g (final concentration 0.3% w/v) RB was

208 dissolved in 75 mL water. Then, 9.375 g CMC was added and manually stirred for a final

209 concentration of 12.5%. The gel dissolved overnight.

210 During bat capture 1 (October 31, 2024), 1 mL of the gel was topically applied to the

211 dorsal fur of bats with a needle free syringe. These bats were also given 100 µL orally to

212 act as gel consumption controls for hair sampling. This dose of RB was previously

213 determined to clearly mark vampire bat hair samples. The bats were then released back

214 to the colony. On captures 2 and 3 (November 3 and 7, 2024), hair samples were

215 plucked with tweezers from captured bats and stored in envelopes protected from light

216 for later visualization with a fluorescence microscope.

217

218 **RB Visualization in Bat Hair**

219 Uptake of RB within the hair samples was qualitatively assessed with a Zeiss Imager A2

220 microscope (Oberkochen, Baden-Württemberg, Germany) with a Zeiss 424931

221 Microscope Fluorescence Filter Cube (excitation 546 nm / emission 590 nm).

222 Observations were performed and assessed separately by two individuals. Samples

223 were only recorded as positive if fluorescence was noted by both observers.

224

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 estimation method was used to determine the number of bats to topically treat during capture 1.

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

Statistical Analysis

To assess variation in biomarker coverage across sex and age cohorts in our field trail we used a generalized linear model with a binomial distribution and a logit link function. 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 our predictor variables, we included a grouping variable for both sex and age resulting in four distinct bat cohorts: adult female, adult male, juvenile female and juvenile male. The model was fit using the BRMS package[22] in the R statistical language (R Core Team 2024). R version 4.4.2 was used to generate estimated biomarker coverage within the colony based on the positivity of collected hair samples. A hypothesis test built into the BRMS package was used to determine significant differences in the rates of coverage within the cohorts of non-treated bats.

For our covariate estimates, we used weakly informative priors with a normal distribution with a mean of 0 and standard deviation of 2. The model was run for 8,000 iterations with 2,000 iterations for warmup. To assess model convergence, we used the

11

247 Gelman-Rubin test statistic, with a value <1.2 indicating convergence[27] and visually

248 inspected the trace plots for signs of divergent transitions.

249 Additionally, viral titer decay rates were modeled by linear regression and statistical

250 comparisons were made using emtrends function of the emmeans R package[28]. A Chi-

251 squared test was used to analyze the age and sex distribution of bats across the three

252 days of captures. Fisher's exact test was used to analyze differences in RB positivity of

253 collected hair samples of bat cohorts stratified by the combination of age and sex.

254

255

256 **3. Results**

257 **CMC-based vaccine formulation exhibits optimal properties for topical application**

258 To compare the consistency of CMC and GJ across a range of temperatures,

259 rheological testing was performed. The temperatures tested (0 °C – 40 °C) represent

260 extreme conditions under which a vaccine gel might be applied to vampire bats for

261 rabies vaccination or to other bat species in temperate climates.

262 A steady rate sweep test was performed to measure viscosity across a range of shear

263 rates, or rates of deformation. As the viscosity of CMC solutions is modulated by

264 concentration (Figure 1A; Figure S1), a concentration of 12.5% was considered to have

265 the optimal consistency. The apparent zero-shear viscosity of 12.5% CMC, estimated by

266 the Newtonian plateau and an approximation of gel behavior at rest, exhibited much

267 variation with changes in temperature compared to GJ (Figure 1B). Zero-shear viscosity

268 for 12.5% CMC ranged from approximately 110-400 Pa*s from 0 to 40 °C compared to

269 0.05 – 1,070 Pa*s for GJ. At all temperatures, the viscosity of CMC and GJ decreased

270   as shear rate increased, indicating shear-thinning behavior where viscosity decreases

271   as deformation rates increase.

272   At shear rates within the range of 20-200 s^{-1} (Figure 1C), shear rates associated with

273   spreading[23,24], CMC was more viscous than GJ and varied minimally with changes in

274   temperature. In the dynamic strain sweep test, CMC gel exhibited viscoelastic

275   properties and acted as a viscoelastic liquid even at 40 °C (Figure 1D). GJ which

276   displayed properties of an elastic solid at 0 °C and 20 °C (Figure S2) but became

277   liquified at 40 °C (Figure 1D).

278

279   **[Figure 1]**

280

281   **CMC gel preserves viral vaccine titers**

282   We measured viral titers at time intervals up to three months to assess preservation of

283   our RCN-MoG vaccine within CMC and GJ under different temperature conditions.

284   Stability of the RCN-MoG vaccine titers was highly temperature dependent within both

285   CMC and GJ formulations (Figure 2A-C). High titers were preserved up to 3 months at 4

286   °C (Figure 2B) with minimal decay of RCN-MoG in both formulations (CMC: -0.0385 $\pm$

287   .0221 PFU/mL per week; GJ: -0.00311 $\pm$ 0.0191 PFU/mL per week; Figure 2C). At 23

288   °C, viral titer decreased in CMC at a moderate rate (-0.463 $\pm$ 0.062 Log10 PFU/mL per

289   week), while in GJ decay was slower (-0.157 $\pm$ 0.041 Log10 PFU/mL per week). RCN-

290   MoG viral titer rapidly decreased at 40 °C in both CMC (-3.50 $\pm$ 1.03 Log10 PFU/mL per

291   week) and GJ (-1.50 $\pm$ 0.28 Log10 PFU/mL per week). RCN-MoG was no longer

292   detectable at 40 °C after 30 days in CMC and after 90 days in GJ. Of note, slightly more

293   virus was recovered from dissolved GJ (Log10 8.02 +/- 0.123 PFU/mL) than CMC

294   (Log10 7.64 +/- 0.136 PFU/mL) at the initial time point (Figure S3).

295   Cells infected with RCN-MoG stored in CMC at 4 °C for three months still produced

296   antigen detected by western blot (Figure 2D). While replication levels of samples stored

297   at 23 °C reduced drastically over time, vaccine poxviral DNA was preserved throughout

298   the study (Table S1-S2; Figure S4).

299

300   **[Figure 2]**

301

302   **High uptake rate of CMC-based delivery formulation within a wild vampire bat**

303   **colony**

304   CMC gel uptake by wild vampire bats was measured using the fluorescent biomarker

305   rhodamine B (RB) within a microchipped colony in Jalisco, Mexico (Figure 3A). The bats

306   inhabited an abandoned ranch-house (Figure 3B) in which we previously installed an

307   RFID system for microchip detection (Figure 3C). Over three capture sessions (Figure

308   3D), 156 total *Desmodus rotundus* bats were captured (Figure 3E), representing 95

309   unique bats. No bats of other species were captured.

310

311   During capture 1, 47/89 (52.8%) bats had RFID microchips from previous trips to the

312   site (Figure 3E), while 62 microchipped bats were detected the night before capturing

313   (Figure 3F). This results in an estimated colony size of 117.4 ($\pm$ 5.625 SE) by the

314   Lincoln-Petersen method. One milliliter of 12.5% CMC with 0.3% rhodamine B (RB) was

315   topically applied to the dorsal fur of 24 bats (13 females and 11 males), representing an

14

316   estimated 20.5% of the colony. Forty-two new microchips were implanted during capture

317   1, resulting in the observed increase in detected RFIDs (Figure 3F).

318

319   Age and sex demographics within the entire colony were determined from the

320   proportions of unique bats captured belonging to each subpopulation across the three

321   captures. Of the bats in the colony, 57/95 were female (60.00%), and 66/95 were adults

322   (69.47%) (Table 1). RFID data shows a decline in the number of microchipped bats

323   detected each night within the colony beginning after capture 2 (Figure 3F). The age

324   and sex distribution of bats captured during captures 2 and 3 were not statistically

325   different from capture 1 (Chi-squared test, p = 0.165).

326   **[Table 1]**

327

328   **[Figure 3]**

329

330   During bat capture sessions 2 and 3, 34 and 27 hair samples were collected,

331   respectively. Accounting for recaptures, hair samples were obtained from 48 unique

332   bats, including 13 that were initially topically treated with CMC gel (Figure 4A-D). Hair

333   samples were visualized with a fluorescent microscope (Figure 4E-F), and all 13 treated

334   bats that were recaptured were RB-positive. Of the nontreated bats, 29/35 (82.86%)

335   were RB positive. Notably, the only negative hair samples belonged to adult male bats

336   resulting in a statistically different proportion of positive samples compared to the other

337   cohorts (Fisher's exact test, p = 0.002). Three adult male bats that were captured during

338   both captures for hair sample collection had positive hair samples on capture 2 but

15

339   negative samples on capture 3 (Figure 4A). These individuals were still considered RB

340   positive for analysis.

341

342   Based on the proportion of positive and negative hair samples and colony demographic

343   estimates, total gel uptake by the colony was modeled using Bayesian regression

344   modelling with the BRMS package[29]. Gel uptake within non-treated and non-captured

345   bats was predicted using the brm model (Figure 4G). Near 100% rates of uptake were

346   predicted in non-treated adult female (97.4%; confidence interval (CI): 85.7% - 100%),

347   juvenile female (94.5%; CI: 73.3% - 100%) and juvenile male (98.8%; CI: 92.3% -

348   100%) cohorts. Notably, an estimated uptake of 48.4% (CI: 30.4%-69.6%) was found in

349   non-treated adult males. Across the entire colony, uptake was estimated at 88.0%

350   (103/117; 90 - 110) (CI: 76.9% - 94.0%). Based on this projection, 103 bats consumed

351   the gel, with each bat consuming on average ~200 µL of the total 24 mL of topically

352   applied gel (1 mL applied to 24 bats), assuming all the gel was consumed.

353

354   **[Figure 4]**

355

356   **4. Discussion**

357   While rabies vaccination of vampire bats in captivity has been shown to reduce rabies

358   virus shedding[19] the delivery of the vaccine to wild vampire bats presents a major

359   barrier to the implementation of this strategy as a rabies control measure. Although

360   effective in humans and domesticated animals, parenteral vaccination is impractical for

361   bats. Likewise, oral baits successfully used for other wildlife species[30] are not suitable

16

362   for bats due to differing feeding behavior. Because topically administered poisons

363   demonstrate high rates of oral uptake within bat colonies[31,32], orotopical vaccination of

364   vampire bats has been proposed but lacks an optimal delivery vehicle. In this work, we

365   describe the development of a topical vehicle that maintains optimal physical properties

366   even at extreme temperatures, preserves our viral vector vaccine candidate, and

367   exhibits high rates of transfer and oral uptake between treated and untreated bats in a

368   wild bat colony.

369

370   Previous bat studies tested petroleum jelly[33] – a paste commonly used for anticoagulant

371   poison delivery – or glycerin jelly (GJ)[17–19] as the topical vaccine delivery vehicles.

372   Petroleum jelly proved difficult to work with resulting in dilution of the vaccine and

373   substantial loss of vaccine paste[33]. Meanwhile, the consistency of GJ is highly

374   temperature dependent as demonstrated by our study. We found the mixture of GJ in

375   water previously used[17] has a melting temperature around 30 °C. While vampire bats

376   typically roost where temperatures remain below 37 °C[34] and do not well tolerate

377   temperatures above 33 °C[35], environmental temperatures may exceed 40 °C during

378   heat waves in some regions[20].

379

380   Characterization of physical properties of a topical vehicle under environmental

381   temperatures is necessary for proper application as revealed by the shortcomings of

382   GJ. Rheology, measuring how a material sample responds to deformation, presents a

383   useful method for measuring physical properties of gel-like materials[36]. The rheological

384   properties of CMC solutions are well studied and vary with the molecular weight and

17

385   degree of substitution of the CMC powder used[37–39]. The CMC gel preparation used in

386   our study demonstrates shear thinning behavior and viscoelastic properties

387   corresponding with these previous reports. In a steady rate sweep test, CMC

388   demonstrates less variation than GJ in zero-shear viscosity over the studied range of

389   temperature. At 40 °C, the near-zero shear rate of CMC was approximately 110 Pa*s,

390   demonstrating high viscosity at high temperatures. This improves on the GJ formulation

391   which has a near-zero shear rate approaching 0 Pa*s at 30 °C when it becomes

392   completely liquified. In the characterization of viscoelastic materials, the storage

393   modulus G' characterizes the elastic component (energy stored during deformation) and

394   the loss modulus G" characterizes the viscous component (energy dissipated during

395   deformation). In CMC, G" is greater than G' in the linear viscoelastic region which

396   suggests CMC acts as a viscoelastic liquid that flows slowly over time. This may

397   contribute to improved surface wettability and to the enhanced stickiness of CMC that

398   we observed compared to GJ. In GJ, G' > G", indicating primarily elastic solid-like

399   behavior. Notably at 0 °C, G' becomes greater than G" in CMC indicating trends toward

400   solidification at temperatures near freezing, while still exhibiting viscoelastic properties.

401

402   Additional described properties of CMC support its use as a topical vaccine vehicle. The

403   muco-adhesion of CMC[40] may enhance viral entry into mucosal tissue. Mucoadhesive

404   cellulose-based films have been previously used to facilitate entry of Modified Vaccinia

405   Ankara – a commonly used poxviral vaccine vector - into the buccal mucosa of mice[41].

406   CMC is also commonly used in drug delivery[42], is non-toxic at high quantities[43], and was

407   found to mix easily with buffer containing RB.

408

409   To facilitate vaccination strategies, the vaccine must be readily shipped and stored

410   without dependence on cold-chains in regions where reliability of infrastructure may be

411   unpredictable[44]. Our assessment of viral stability in CMC under different temperature

412   conditions provides insight into vaccine preservation under storage conditions and

413   persistence in field-relevant conditions. Minimal decay of viral titer within CMC was

414   evident at refrigeration temperatures (4 °C) with only a 0.63 log fold decrease in viral

415   titer after 3 months of storage. This will enable shipment and storage of the vaccine

416   when challenges in infrastructure prevent maintaining temperatures below freezing. If

417   refrigeration is lost, vaccine titer may also be preserved up to 1 week at 23 °C with only

418   a 0.46 log fold decrease in titer and 24 hrs at 40 °C with 0.54 log fold decrease.

419   Although virus stability was generally better preserved in GJ;, the viral titer initially

420   decreased more rapidly in GJ than in CMC at 40 °C with a log fold decrease of 0.82

421   after 24 hours. Other additives could be considered to improve stability within the CMC

422   gel formulation such as sucrose, which has been shown to enhance poxviral vaccine

423   vector stability[45]. The implications of these vaccine preservation data depend on the

424   determination of the minimum effective dose of RCN-MoG in vampire bats; however,

425   during field application, we expect gel to be consumed within hours after application.

426

427   Like applications of vampiricide, orotopical vaccination utilizes the natural propensity of

428   vampire bats to allogroom to facilitate the transfer of vaccine between treated and

429   untreated bats. Ideally, only a small portion of a colony would need to be treated to

430   effectively manage rabies due to difficulties in locating and treating entire roosts.

19

431   Vampire bat captures are commonly performed at ranches where bats are known to be

432   feeding to apply vampiricide, rather than at the roost istelf[31]. This highlights the

433   importance of understanding the rates of vaccine transfer and uptake within colonies

434   and between cohorts at low rates of application. The RB biomarker used in this study is

435   a common proxy for measuring uptake of treatment in wildlife species given its

436   properties to stably mark the hair follicle and shaft with a fluorescent signal[17,46,47]. Based

437   on assumptions about population size and demographic distribution of bats in this trial,

438   an estimated gel uptake by 88.0% (CI: 76.9% - 94.0%) of the colony was measured

439   after initial treatment of 20.5% of the colony. This corresponds to gel uptake in 3.29 bats

440   (CI: 2.75 – 3.58) per initial bat treated which improves upon the transfer reported in

441   Bakker et al. (2019) of 1.8-2.1[17]. This difference is likely related to the increase in

442   volume of gel applied per bat from 0.45 mL to 1 mL and the higher proportion of

443   remaining non-treated bats available for gel uptake. Given the near 100% uptake

444   observed in adult females and juveniles in our study, gel transfer rates may be even

445   higher given the finite number of untreated bats within the colony.

446

447   Importantly, adult male bats are disproportionately implicated in the dispersal of VBR[48].

448   While our field trial suggests that adult male bats may be less likely to consume

449   orotopically applied vaccines vehicles, we still demonstrate predicted uptake by 48.4%

450   (CI: 30.4%-69.6%) of non-treated adult male bats. It has also previously been

451   documented that males may be less effective than females at spreading vampiricide

452   within a colony[32]. However, this result differs from Bakker et al. (2019) who found a

453   nonsignificant difference in the proportion of males that showed uptake of RB compared

20

454   to females[17]. This previous finding could be associated with the high proportion of

455   treatment (up to 50%) in the colonies reported, variation in the social dynamics within

456   the colonies between the studies, or potential regional or seasonal influences. Vampire

457   bat colonies are differentiated as either maternity colonies with resident and non-

458   resident males or primarily-male bachelor roosts[49,50]. Social grooming interactions and

459   thus topical vehicle uptake may vary between different sites with different structures and

460   sex and age ratios[51]. Notably, 2.3-9 times the proportion of juveniles (31.5%) were

461   captured in the roost in the present study compared to the three roosts studied in

462   Bakker et al. (2019) (3.5%, 8.7% and 13.6%) during the first diurnal capture[17].

463

464   While RFID detection remained stable between captures 1 and 2 in this study, the

465   number detected declined by around 50% a week later. In addition to the multiple

466   captures at this site, disturbance was caused by the euthanasia of 20 of the bats from

467   our colony carried out by authorities of the Mexican Rabies Campaign as equine rabies

468   cases were reported near the roost site. As previously reported[13,17], vampire bats are

469   prone to roost abandonment when disturbed, relocating to nearby manmade and natural

470   roosts. For example, five months before the initiation of this biomarker study, we visited

471   a large colony of over 100 bats within an abandoned facility 10 km away as a potential

472   study site. However, before captures could be initiated, the colony relocated with only a

473   few bats remaining at the site.

474

475   Epidemiological models indicate that vaccination significantly reduces duration and

476   frequency of rabies outbreaks compared to culling even at low rates of vaccination[17].

21

477   The estimated gel consumption rates by nearly 100% of adult females, and male and

478   female juveniles found in this study will likely aid in preventing rabies virus introduction

479   to naive colonies. As a lower proportion of adult males consumed the gel, targeting

480   males as juveniles may result in higher rates of vaccination; however, this will require

481   investigation of the development and duration of the immune response in vaccinated

482   juvenile bats. Further field trials are needed to understand gel uptake dynamics within

483   multiple bat colonies to maximize vaccine uptake by adult males. Additional models may

484   also investigate the effect of differing rates of vaccination uptake between males and

485   females. Given that vampire bat rabies has been known to spread among colonies in a

486   slow wavelike fashion[1,52,53], strategies of ring vaccination around areas of predicted

487   spread may be effective at containing expansion of VBR[17].

488

489   CMC gel may also work well as a topical vehicle for vaccine delivery to other bat

490   species. In the United States, an estimated 40,000 individuals receive rabies

491   prophylaxis each year after exposure to various bat species[10]. Furthermore, 36 of 40

492   (90%) fatal rabies cases acquired in the United States between 2000 and 2022 were of

493   bat origin[11]. CMC could additionally be used to deliver a white nose syndrome (WNS)

494   vaccine[54] or to scale up deployment of a herpesvirus-vectored rabies vaccine[55]. As

495   WNS-affected bats often inhabit colder regions in the United States and Canada, it is

496   notable that CMC additionally maintains its physical properties at colder temperatures.

497   However, vampire bats participate in allogrooming at higher rates than other bat

498   species[56], and field studies would be needed to determine gel transfer within colonies of

499   other targeted bat species.

500

501 The identification of vampire bats within 50 km of the southern border of the United

502 States[57] and the expectation that their geographic range will continue to expand

503 emphasizes the need for superior control methods[7]. While topical application of

504 vaccines presents an appealing alternative to culling practices that may exacerbate

505 rabies transmission, further studies are needed to understand the dimorphism of uptake

506 rates in adult male and female bats observed in this study. Likewise, additional studies

507 are needed to determine the impact of vaccination on vampire bat colony sizes,

508 although it is proposed to include contraceptives as a method for population control

509 while minimizing unintended ecological impacts of anticoagulant poisons[58].

510

511 **Conclusion**

512 This study details the characterization of a carboxymethyl cellulose (CMC) gel vehicle

513 for orotopical rabies vaccine delivery to vampire bats. The new CMC formulation

514 maintains optimal physical properties across a broad range of temperatures improving

515 on a past topical vehicle. CMC gel is also able to sufficiently preserve our recombinant

516 viral-vector rabies vaccine candidate. A field study in Jalisco, Mexico demonstrates the

517 capacity for high rates of uptake of the CMC-based delivery formulation within a wild

518 vampire bat colony at a low level of application. In summary, CMC gel provides an

519 orotopical vehicle for vaccine delivery to bat species that socially groom and may be

520 tested to treat other diseases and species beyond rabies in vampire bats.

521

522 **Data Availability**

23

523   The data and code that support the findings of this study are openly available in github

524   at https://github.com/zamanianlab/Bat_vaccine_gel-ms.

525

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545 Any use of trade, firm, or product names is for descriptive purposes only and does not

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547

548 **Author Contributions**

549 Conceptualization, C.K., E.M.C.C., D.P.W., D.G.S., J.E.O., M.Z., T.E.O.; data curation,

550 C.K., T.M.G., M.Z.; formal analysis, C.K., T.M.G., funding acquisition, C.K., D.P.W.,

551 D.G.S., J.E.O., M.Z., T.E.O.; investigation C.K., E.M.C.C., T.M.G., M.M.R.M., D.L., L.P.,

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554 M.M.R.M, J.E.O., M.Z., T.E.O.; software, C.K., T.M.G.; supervision, E.M.C.C., M.M.R.M.,

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556 writing – original draft preparation, C.K.; writing – review & editing, C.K., E.M.C.C.,

557 T.M.G., M.M.R.M., D.L., L.P., D.P.W., D.G.S., J.E.O., M.Z., T.E.O.

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707

708

709   **Figure and Table Legends**

710   **Figure 1. Rheological properties of carboxymethyl cellulose (CMC) gel and**

711   **glycerin jelly (GJ). A)** The viscosity of 10%, 12.5% and 15% solutions of

712   carboxymethyl cellulose (CMC) at 40 °C and **B)** the viscosity of 12.5 % CMC and GJ at

713   0 °C, 20 °C, and 40 °C were measured by a steady rate sweep test. Panel **C)** displays

714   the viscosity of CMC and GJ depicted over shear rates associated with spreading (20 –

715   200 s^{-1}). **D)** Viscoelastic properties of CMC and GJ at 40 °C were measured by a

716   dynamic strain sweep test. G' represents the elastic modulus – energy stored during

717   deformation, while G" represents the loss modulus – energy dissipated during

718   deformation. Error bars represent standard error of three experimental replicates.

719

720   **Figure 2. Viral stability of a recombinant raccoonpox vaccine expressing a mosaic**

721   **lyssavirus glycoprotein (RCN-MoG) stored within carboxymethyl cellulose (CMC)**

722   **and glycerin jelly (GJ) formulations at 4 °C, 23 °C, and 40 °C.** Log fold change in

723   vaccine titer measured over **A)** seven days and **B)** three months of storage by plaque

724   assay in Vero CCL-81 cells at each time point after three days of infection. Error bars

725   depict the standard error of three experimental replicates. **C)** Weekly decay rates of

32

726 RCN-MoG titer within CMC and GJ at each storage condition were determined by a

727 linear regression model. Statistical comparisons were made using emtrends (ns = no

728 significance; * = p < 0.05; *** = p < 0.001). **D)** MoG antigen expression of cell lysates

729 infected with RCN-MoG after storage in CMC at 4 °C for one and three months was

730 detected by western blot. Cells were infected with 100 μL of dissolved gel and protein

731 was collected 18 hours post-infection.

732

733 **Figure 3. Study design and demographics of captured vampire bats (*Desmodus**

734 *rotundus*) during three capture sessions in Jalisco, Mexico. A)** In Mexico, the field

735 study location is marked with the yellow star, while regions with the northernmost

736 sightings of vampire bats are labeled with red circles (map retrieved from Google Earth

737 v 10.80.0.1 on May 15, 2025). **B)** The vampire bat colony inhabited the photographed

738 abandoned ranch-house. Photograph by C.K. on May 28, 2024. **C)** In the ranch-house,

739 a radio frequency identification (RFID) system was installed (overhead schematic) to

740 detect movement of microchipped bats. **D)** Three captures were performed for gel

741 application with rhodamine B biomarker during capture 1 and hair collection during

742 captures 2 and 3. **E)** During each capture, bats were sexed and aged. Lines represent

743 the number of recaptures from the previous capture session, or in the case of capture 1,

744 bats with RFID microchips from previous captures at this site. **F)** Each night, the RFID

745 system detected the movement of microchipped bats into and out of the roost. The y-

746 axis represents the number of unique RFIDs detected each night (x-axis).

747

748 **Figure 4. Estimated gel uptake within a vampire bat (*Desmodus rotundus*) colony**

749 **in Jalisco, Mexico, after approximately 20.5% of the colony was treated topically**

750 **with 1 mL of carboxymethyl cellulose (CMC) gel containing 0.3% rhodamine B**

751 **(RB).** Panels **A** (adult males), **B** (adult females), **C** (juvenile males), and **D** (juvenile

752 females) depict capture and treatment status during capture 1 and collection and

753 positivity of hair samples of bats captured during captures 2 and 3. During capture 1,

754 orange squares represent topically treated bats, while open circles were captured, but

755 not treated. The presence of orange fluorescence was used to distinguish between **E)**

756 positive and **F)** negative hair samples. Hair samples were visualized with a Zeiss

757 Imager A2 microscope (Oberkochen, Baden-Württemberg, Germany) and a Zeiss

758 424931 Microscope Fluorescence Filter Cube (excitation 546 nm / emission 590 nm).

759 **G)** A Bayesian model was used to calculate estimates and confidence intervals of

760 estimated coverage within each subpopulation of bats. Statistical significance indicates

761 difference in coverage of non-treated bats and was determined by brms hypothesis

762 testing.

763

764 **Table 1. Age and sex distribution of unique captured vampire bats (*Desmodus***

765 ***rotundus*) during three capture sessions in Jalisco, Mexico.** Age was determined

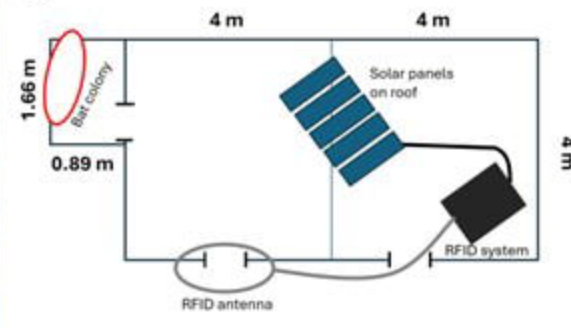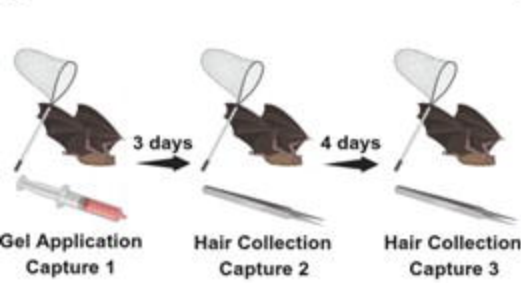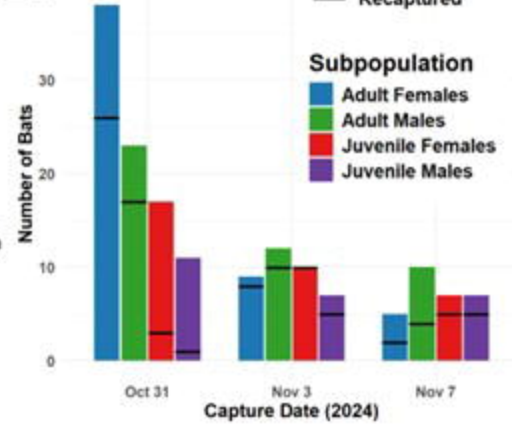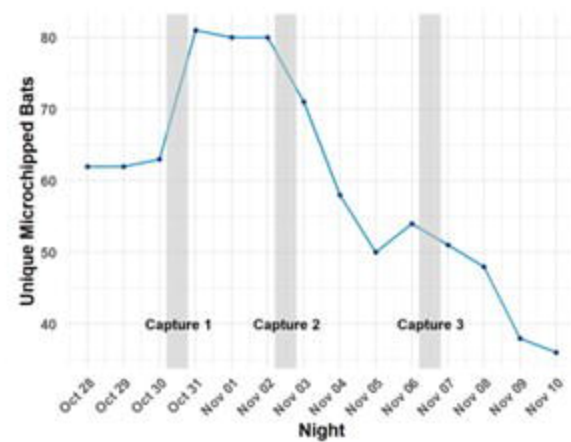766 by fusion of the phalangeal epipyhyses. Subadults were grouped with juveniles.
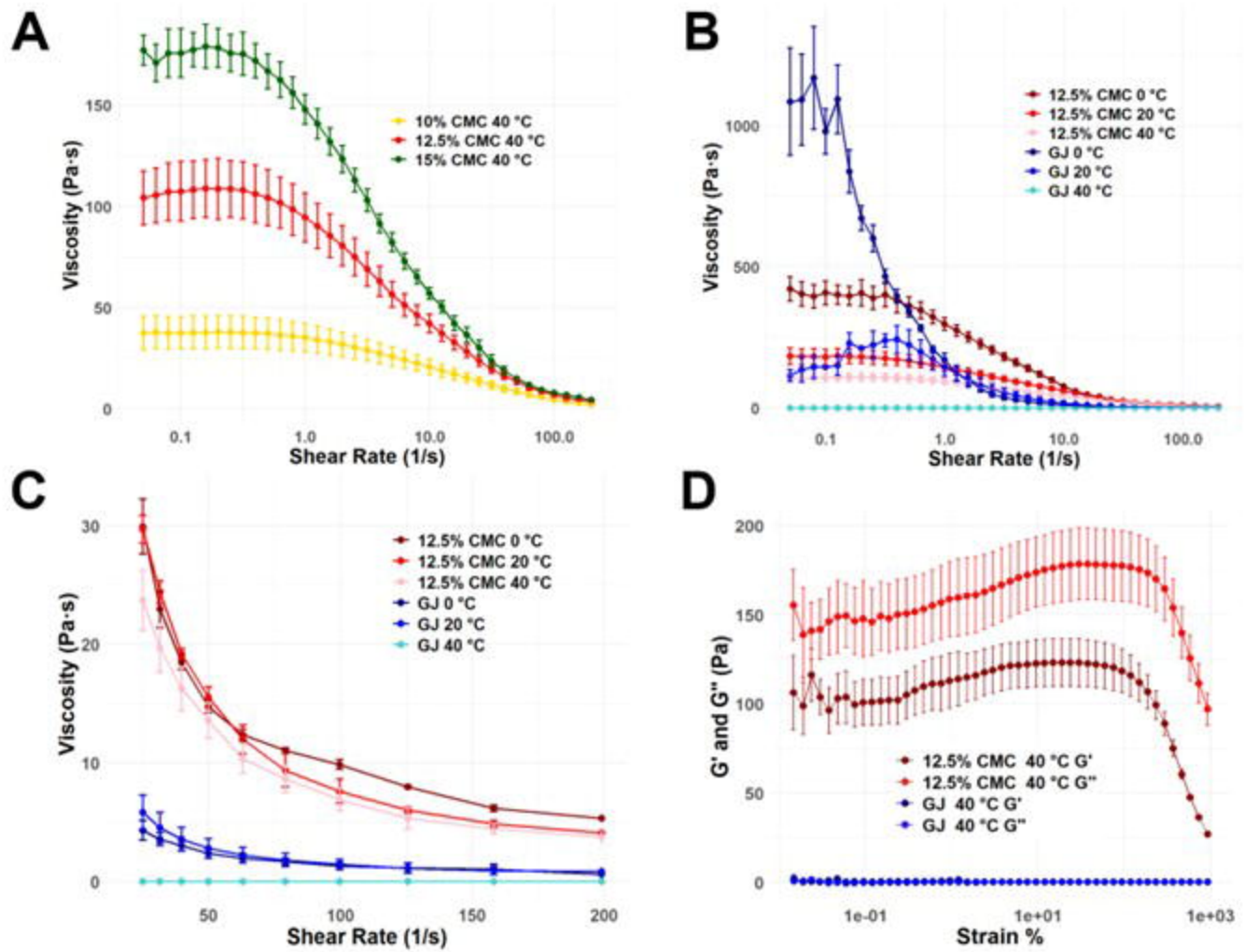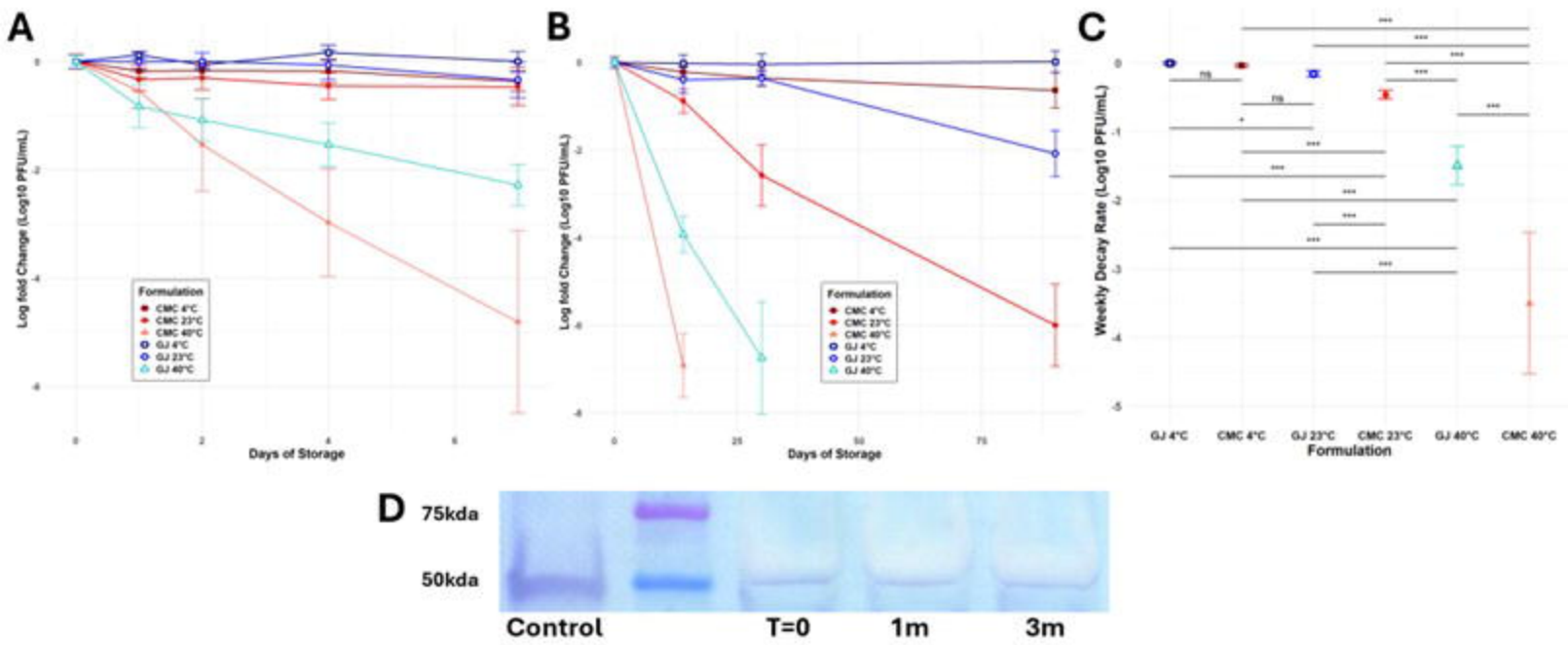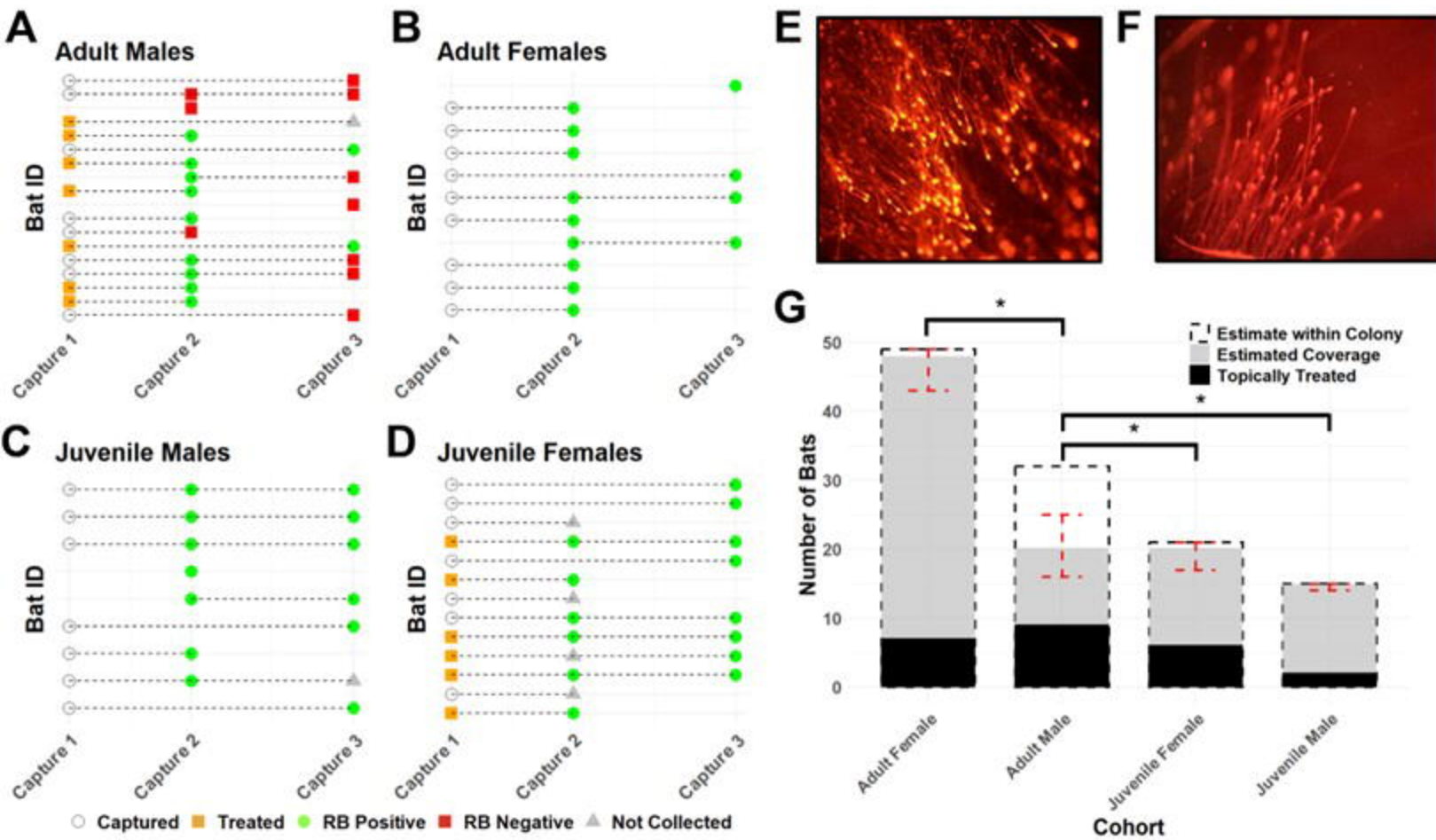
Cohort	Unique Bats Captured	Percentage of Captures
Adult Female	40	42.11%
Adult Male	26	27.37%
Juvenile Female	17	17.89%
Juvenile Male	12	12.63%
Total	95	100%