COYOTES EXHIBIT IMMUNE AND GENETIC EVIDENCE OF RICKETTSIAL INFECTIONS IN ARIZONA

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ABSTRACT

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ERIC N. GREEN

Rocky Mountain Spotted Fever (RMSF), caused by the bacterium *Rickettsia rickettsii*, was recognized as endemic in Arizona after a 2004 outbreak,[1] and has been a public health concern since that time. The brown dog tick (*Rhipicephalus sanguineus*) is the primary vector in the state. Since it selects domesticated dogs as the primary host, free-roaming dogs in peridomestic areas have been named as the primary risk factor for human cases of RMSF. However, the sudden emergence and long-distance transmission of the pathogen have not been adequately explained, and one possible mechanism could include a wildlife component. Since coyotes are prevalent in Arizona,[2] wide-ranging,[3] and genetically-similar to dogs,[4] we predicted that brown dog ticks might parasitize coyotes and that coyotes would therefore have evidence of pathogen exposure. We tested coyote sera for antibodies to *R. rickettsii*. Eight out of 94 (8.5%) samples were seropositive at 1:256 or higher titers. Subsequent qPCR analyses of coyote skin showed evidence for Spotted Fever Group *Rickettsia* in 4 out of 138 (2.9%) samples, but not for *R. rickettsii*. Antibodies are cross-reactive for many rickettsiae, so the positive results in serology could indicate coyotes' exposure to diverse, nontypical rickettsial species with various levels of pathogenicity. This cross-reactivity may play a role in the maintenance of rickettsial bacteria in Arizona. Although the specific mechanisms remain unknown, it is possible that *Rh. sanguineus*

are responsible for transferring rickettsial pathogens across canine host species. Alternatively, a different pathogen-vector complex could be operating on coyotes than on dogs.

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PREFACE

This thesis was prepared in the journal format, with Chapter 2 being formatted for publication in *The American Journal of Tropical Medicine and Hygiene*. I have moved the references to the end of the thesis document, since the other chapters share some of the same references. All other chapters are formatted in accordance with the requirements of Northern Arizona University policies. Some redundancy exists due to the journal formatting style.

CHAPTER 1

INTRODUCTION

Rocky Mountain spotted fever (RMSF) is a tick-borne infection of the Americas caused by *Rickettsia rickettsii*, a gram-negative, obligate intracellular bacterial pathogen.[5,6] Acute infections present with flu-like symptoms such as fever, headache, nausea, vomiting, and muscle aches, along with a characteristic petechial rash in about half of the cases.[7,8] With prompt diagnosis and treatment, the case fatality rate can range from <0.5-5%.[7,9–11] Untreated, the case fatality rate is usually estimated at 10-25%[1,9,11,12] but has been reported as high as 29% in Brazil,[11] 38% in Mexico,[7] and an unusual 100% in an outbreak in Panama.[7] These higher rates may be due to misdiagnoses and delayed or inappropriate treatment.[11] Cases that are not treated within 5-7 days of onset of symptoms can lead to severe and widespread vascular injury, resulting in irreparable organ damage, organ failure, or long-term disability in patients that survive.[7,8,13]

Human cases of RMSF have been reported in Arizona since 1912, but until 2004, no more than two cases were reported per year.[14] An investigation by the Centers for Disease Control and Prevention (CDC) concluded that RMSF had become endemic in Arizona, and that it was vectored by the brown dog tick (*Rhipicephalus sanguineus* sensu lato), a previously-unidentified vector of *R. rickettsii* in the United States.[1,15]

The ecology of the brown dog tick makes it especially efficient at transmitting *R. rickettsii* in Arizona. First, the brown dog tick is better-adapted to Arizona's arid climate than other North American tick species.[1,16] Second, it selects domesticated dogs as its primary host.[12,17,18] This host-specificity, combined with the tick's nidicolous life history (a tendency to

share a nest with another animal) and large numbers of free-roaming dogs in affected communities present ample opportunities for ticks to move among dog hosts, heavily infest homes, and parasitize humans as incidental hosts.[12,18]

During the early RMSF outbreaks in the state (2004-2012), human cases occurred almost exclusively on six Native American reservations.[19] The reservations with non-contiguous borders were separated by 21-270 km. While free-roaming dogs are considered to be the primary risk factor for human cases of RMSF within Arizona's communities,[12] these distances seemed too great for dog-mediated dispersal from one community to another, although humans could facilitate long-distance dog movements.

The cause of the sudden emergence and long-distance transmission of the disease have not been empirically tested, but one possibility is that wildlife could contribute to maintaining and dispersing the pathogen in nature. The coyote (*Canis latrans*) is one species that could potentially fill this role due to its genetic similarity to dogs,[4] its ubiquitous presence across the state,[2] and its ability to readily urbanize when resources such as food and movement corridors are available.[20–23] If brown dog ticks parasitize coyotes, or if coyotes have been exposed to *R. rickettsii*, then further investigation is warranted.

I obtained coyote blood and skin for serological and genetic analyses. I then screened the blood sera for antibodies to spotted fever group (SFG) rickettsiae and used the blood cells and skin for genetic analyses. These two approaches are complimentary. A successful test for antibodies is an indirect way to show which individuals have 1) been exposed to the pathogen and 2) developed a strong adaptive immune response to the infection. It can take up to four weeks to develop a strong antibody response, but the signal can be detectable for several months in a live host.[24] In contrast, the genetic approach relies on polymerase chain reaction (PCR) to

amplify a target gene directly from pathogen DNA. This is only successful when tissues are taken from the site of an active infection, in which sufficient copies of bacterial DNA are present. Donor coyotes were also inspected for ticks, but none were found.

All tissues were collected during routine predator control operations, under an Interagency Service Agreement between Northern Arizona University and the Arizona Game and Fish Department. As such, this study was exempt from having an Institutional Animal Care and Use Committee (IACUC) protocol. IACUC approved the transfer of tissues between agencies via a standard Tissue Transfer Form. Tissues were considered Biosafety Level 2, so my work was conducted under the purview of the Institutional Biosafety Committee (IBC) (protocol number BARB-0115).

CHAPTER 2

COYOTES EXHIBIT IMMUNE AND GENETIC EVIDENCE OF RICKETTSIAL INFECTIONS IN ARIZONA

ABSTRACT

Rocky Mountain Spotted Fever (RMSF), caused by the bacterium *Rickettsia rickettsii*, was recognized as endemic in Arizona after a 2004 outbreak,[1] and has been a public health concern since that time. The brown dog tick (*Rhipicephalus sanguineus*) is the primary vector in the state. Since it selects domesticated dogs as the primary host, free-roaming dogs in peridomestic areas have been named as the primary risk factor for human cases for RMSF. However, the sudden emergence and long-distance transmission of the pathogen have not been adequately explained, and one possible mechanism could include a wildlife component. Since coyotes are prevalent in Arizona, wide-ranging, and genetically-similar to dogs,[4] we predicted that brown dog ticks might parasitize coyotes and that coyotes would therefore have evidence of pathogen exposure. We tested coyote sera for antibodies to *R. rickettsii*. Eight out of 94 (8.5%) samples were seropositive at 1:256 or higher titers. Subsequent qPCR analyses of coyote skin showed evidence for Spotted Fever Group *Rickettsia* in 4 out of 138 (2.9%) samples, but not for *R. rickettsii*. Antibodies are cross-reactive for many rickettsiae, so the positive results in serology could indicate coyotes' exposure to diverse, nontypical rickettsial species with various levels of pathogenicity. These new data suggest that coyotes may play a role in the maintenance of rickettsial bacteria in Arizona. Although the specific mechanisms remain unknown, it is possible that *Rh. sanguineus* are responsible for transferring rickettsial pathogens across canine host

species. Alternatively, a different pathogen-vector complex could be operating on coyotes than on dogs.

INTRODUCTION

Rocky Mountain spotted fever (RMSF) is an infectious tick-borne disease of the Americas[6] that affects humans. With prompt diagnosis and treatment, the case fatality rate can range from <0.5-5%.[7,9–11] Untreated, the case fatality rate is usually estimated at 10-25%[1,9,11,12] but has been reported as high 38% in Mexico,[7] 55% in Brazil,[11,25] and an unusual 100% in an outbreak in Panama.[7] These higher rates may be due to misdiagnoses, delayed or inappropriate treatment.[11] The disease is caused by *Rickettsia rickettsii*, a gram-negative, obligate intracellular bacterial pathogen that primarily infects endothelial tissue in the small and medium blood vessels.[5,8] Acute infections present with flu-like symptoms such as fever, headache, nausea, vomiting, and muscle aches, along with a characteristic petechial rash in about half of the cases.[7,8] However, cases that are not treated within 5-7 days of onset of symptoms can lead to severe and widespread vascular injury, resulting in irreparable organ damage, organ failure, or long-term disability in patients that survive.[7,8,13] Doxycycline is the recommended treatment for patients of all ages and should be administered immediately when any rickettsial infection is suspected.[8]

Regardless of pathogenicity, members of this genus are commonly placed into one of four phylogenetically-distinct antigen groups: spotted fever group (SFG), typhus group (TG), transitional group (*R. canadensis*), and ancestral group (*R. bellii*).[7,26] We placed our emphasis on SFG *Rickettsia* since the scientific literature lack reported cases of the other groups in Arizona.

In addition to *R. rickettsii*, several other species within SFG *Rickettsia* cause RMSF-like diseases that are collectively reported as spotted fever group rickettsioses (SFGR).[5,7] The Centers for Disease Control and Prevention report SFGR as a single disease, since antigenic and genetic characters between the bacterial species are difficult to differentiate for clinical diagnoses.[7,8] However, RMSF is the most severe rickettsial disease in the world.[7,11]

Human cases of RMSF have been reported in Arizona since at least 1912, but until 2004, no more than two cases were reported per year.[14] Human RMSF cases in Arizona then began to rise, with 313 cases reported from 2004 to 2014.[19] Arizona Department of Health Services personnel indicated that 389 cases, including 23 mortalities, occurred from 2003-2017 (Figure 1; Yaglom, H., personal communication). From 2003-2014, Arizona experienced a case fatality rate of 7-10% because the novelty of the disease led to delays in proper diagnosis and treatment.[8,13] Human infections have occurred throughout the state, but most cases were among Native Americans in tribal communities.[13] This is attributed to high densities of free-roaming dogs (*Canis lupus familiaris*) that are not regularly treated for ticks, and the peridomestic presence of the primary tick vector in these communities, the brown dog tick (*Rhipicephalus sanguineus* sensu lato).[12]

Spotted Fever Group rickettsiae are transmitted by all life stages of several species of hard-bodied ticks (Acari: Ixodidae),[5,8] including *Rh. sanguineus*. Specifically, the American dog tick (*Dermacentor variabilis*) and the Rocky Mountain wood tick (*D. andersoni*) are commonly associated with RMSF.[5] In Arizona, these tick species are rare due to the dry climate.[15] Aridity, however, is tolerated by the brown dog tick, and may explain its abundance in Arizona.[1,16]

The brown dog tick is a three-host tick with worldwide distribution, but its taxonomic status as a single species is disputed, partially due to the loss of its type specimen.[17] This may

account for some of the variation in reports of host-specificity. Captive and free-roaming dogs are often considered the primary host for all life stages of the brown dog tick.[17,18] Previous research cites a variety of mammalian and avian hosts, including humans, but also suggests that these are incidental hosts during explosive tick population growth.[18] Historically, *Rh. sanguineus* is believed to have parasitized burrowing carnivores such as foxes and mustelids, and then co-evolved with the domestication of dogs.[17]

This makes dogs likely candidates for dispersing *R. rickettsii*-infected ticks near humans. Indeed, dogs are widely considered to be sentinels for RMSF,[1,27,28] and areas in which > 20% of dogs are seropositive are considered high-risk for human infections.[29] Free-roaming dogs were host to *R. rickettsii*-infected ticks in two neighboring Native American communities in Eastern Arizona during an outbreak of RMSF (2002 to 2004), and titers ≥ 1:32 for *R. rickettsii* were detected in 70% and 54% of free-roaming dogs in those two communities, respectively.[15] Although sentinel surveillance of dogs is useful in modeling the risk of human infections within communities, it reveals little about the origin and long-distance transmission of a pathogen between communities.

Both the sudden emergence of RMSF in Arizona and the pathogen's transmission between geographically-separated communities might have occurred because of the relocation of infected or tick-infested domestic dogs.[15,30] This hypothesis has not been empirically tested, but at least one documented human case of RMSF was linked to a pet dog transporting infected ticks from Mexico to California.[31] However, it is also possible that spillover events involving wildlife-dispersal of the tick vector are involved.[15] Since dogs tend to stay near human habitations, it is possible that another canid – the coyote (*Canis latrans*) – acts as a long-distance dispersal mechanism of brown dog ticks and SFG *Rickettsia*. Antibodies for SFG *Rickettsia* have been

found in coyotes in Texas, Oklahoma and Nebraska,[32,33] but this has not been documented in Arizona.

Evidence of parasitism by brown dog ticks on coyotes is limited. Two nymphal *Rh. sanguineus* were found on a single coyote in Oklahoma, yet domestic dogs in the area frequently had infestations of hundreds of this species per host.[34] This is consistent with the widely-held notion that brown dog ticks have host-specificity for dogs,[12,17] but perhaps coyotes are incidental hosts when their ranges overlap with infested areas.

Other researchers have suggested a similar dynamic regarding domestic infestations. For example, humans become incidental hosts for brown dog ticks when infestations within homes are extreme or when dogs are removed.[18] Experimental evidence has shown that brown dog ticks also attached more readily to humans when exposed to high temperatures,[35] such as those encountered in Arizona. We find it plausible that such a decrease in host-specificity could also occur with coyotes, being genetically similar to dogs.[4]

In the present study, we investigated whether coyotes act as hosts for either brown dog ticks or *R. rickettsii* in Arizona. If brown dog ticks parasitize coyotes, then there is a risk for coyotes to acquire *R. rickettsii* or other tickborne pathogens from them. In addition, urbanized coyotes could disperse infected ticks into peridomestic areas or acquire infected ticks from a peridomestic infestation and transport them to novel urban or wild environments. If coyotes are being exposed to *R. rickettsii*, then the nature of rickettsial pathology must be investigated to determine whether coyotes can amplify or dilute the pathogen's prevalence in nature. Furthermore, exposure to *R. rickettsii* might make urbanized coyotes useful in sentinel surveillance for RMSF.

MATERIALS AND METHODS

Specimen Collection

The Arizona Game and Fish Department (AGFD) conducts predator-reduction operations each spring, and we used this opportunity in 2016 and 2017 to search coyote carcasses for ticks and to collect blood and skin specimens for serological and genetic analyses. We searched carcasses thoroughly but did not find any ticks (of any species) either year. Cardiac blood and ear biopsies were collected post-mortem from coyotes in Apache, Cochise, and Graham counties in 2016; and from Cochise, Coconino, Graham, Mohave, Navajo, and Yavapai counties in 2017. We placed blood specimens into Corvac Integrated Serum Separator Tubes (Medtronic, Minneapolis, MN, USA) containing a coagulation factor and acrylic gel barrier and stored all blood and other tissues at 4 °C in the field. In the lab, serum was separated from cells via centrifugation (15 min at 2500 RPM) and aliquoted into labeled microcentrifuge tubes. We used disposable syringes with 20-gauge needles to puncture the gel barrier, infused the clot with phosphate-buffered saline and air, and collected the cells in 1.5 ml microcentrifuge tubes for subsequent DNA extraction. We froze all tissue specimens at -20 °C until assays and/or DNA extraction could be performed.

To examine whether coyotes are exposed to *R. rickettsii* in Arizona, we performed an indirect immunofluorescence antibody assay (IFA) on coyote serum samples, followed by genetic screening of DNA from whole blood and skin samples using quantitative polymerase chain reaction (qPCR).

Rickettsia rickettsii IFA

First, we screened serum for *R. rickettsii* antibodies using commercial RMSF IFA substrate slides, per the manufacturer's technical instructions (VMRD, Inc., Pullman, WA,

USA). For the secondary antibody in this assay, we used an affinity purified, polyclonal, anti-canine Immunoglobulin G (IgG) of rabbit origin. The secondary antibody is specific for canine IgG and is conjugated to a fluorescein isothiocyanate (FITC) fluorescent label. To ensure that all reagents were working properly, we included positive and negative controls on each slide. All serum samples were initially screened after diluting 1:64 with serum diluting buffer. For any specimens that were positive at 1:64 – the manufacturer's recommended diagnostic cutoff titer – we performed serial two-fold dilutions to determine the endpoint titer.

To minimize observer bias, the same individual viewed and evaluated all slides. We examined slides on a ZEISS Axio Scope.A1 fluorescence microscope at 400x magnification, using ZEISS filter set 10, designed for Alexa Fluor 488 dye. This filter set has an excitation band pass of 450-490 nm and an emitting band pass of 515-565 nm, using a 510 nm dichromic mirror beam splitter (Carl Zeiss Microscopy, LLC, USA). Red blood cells were fixed to each well of the slides as a visual focal reference for negative samples. Positive samples exhibited fluorescence at a level visible to the human eye. The amount of fluorescence in each well varied based on both the strength of the antibody response and the dilution factor. Each trial (sample-dilution combination) was manually-scored using qualitative categories (negative or positive across a dilution series) because we could not obtain quantitative fluorescence data. Although this scoring method is subjective, a discernable pattern of gradually-decreasing fluorescence was reliably observed in all samples that were positive at dilutions 1:64 through endpoint dilution. Trials were scored as negative when no fluorescence was present or when fluorescence was deemed anomalous (e.g., one or two spots in a localized section of the well).

Genetic Screening

We then attempted to confirm any positive IFA results and screen all IFA-negative samples using a hybridization probe-based qPCR from DNA extracted from blood cells and ear biopsies. We extracted DNA from both skin and blood cells using DNeasy kits, per the manufacturer's instructions (QIAGEN, Inc., Valencia, CA, USA), with minor modifications, including extending the initial incubation period from 10 minutes to overnight, and eluting the DNA with 50 µl of deionized water. Skin samples from 2017 were extracted using a slightly different protocol: on these we used glass beads and a Geno/Grinder® (Thomas Scientific, Swedesboro, NJ, USA) prior to the overnight incubation.

We followed a stepwise approach to screen total DNA from both sample types, using a previously-designed real-time TaqMan® assay with primers and probes that target the 23S rRNA gene to detect the *Rickettsia* genus, and the hypothetical protein A1G_04230 gene to detect *R. rickettsii*.[36] The qPCR mixture for each reaction consisted of 3.6 µl of template DNA, 3 µl of deionized water, 1.2 µl each of forward and reverse primers (5 µM), 1.0 µl of probe (5 µM), and 10 µl of master mix (SsoAdvanced Universal Probes Supermix, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The final reaction mixture resulted in 20 µl reactions, each containing final concentrations of 300 nM primers and 250 nM probes. We included at least two negative controls on each plate and followed the two-step thermal cycling protocol recommended by the manufacturer. All reactions were run using a CFX96 Touch real-time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples that had a quantification threshold (C_T) value < 40 and expansion plots that demonstrated a logarithmic curve were considered positive. Positive samples were re-run twice to obtain triplicate trials.

Statistical Analyses

To characterize the distribution of positive titers, we ran initial analyses using the manufacturer's diagnostic cutoff titer of 1:64. However, this is a clinical recommendation based on the comparison of titers from acute (at presentation of symptoms) and convalescent (2-4 weeks later) serum samples, in which a primary infection is confirmed by a four-fold increase in titer. We could not do this since we collected all samples post-mortem. As previously mentioned, we also lacked the equipment to quantify objectively the fluorescence of each assay. Both limitations could have resulted in more false positives, so we chose a conservative cutoff titer of 1:256 based on the natural decline in positives by titer (Figure 2).

In the interest of being conservative, we ran statistical analyses based on both cutoff titers. First, we calculated summary statistics for apparent prevalence, range of positive titers, geometric mean titer (GMT), and a geometric standard deviation factor (GSD). GMT and GSD were calculated using reciprocal titers. Next, we compared the titer distributions between years using a two-sided Fisher's exact test. We also compared positive and negative samples against sampling year, age, and sex of the donor animal; for these we used chi-square contingency table tests for the 1:64 cutoff, and Fisher's exact test for the 1:256 cutoff due to some categories in the latter contingency tables having fewer than five observations. Finally, for the adjusted titer only, we used Fisher's exact test to analyze the titer distribution based on year, age, and sex. All data analyses were conducted in R Version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria), within RStudio Version 1.0.136 (RStudio, Inc., Boston, MA, USA).

RESULTS

Rickettsia rickettsii IFA

Predator-reduction operations in 2016 and 2017 resulted in 125 blood specimens. After centrifugation, we had 94 usable serum samples (53 in 2016, 41 in 2017). The remaining samples were not used for IFA assays because serum did not separate from the cellular components of the blood. However, we still collected cellular material from these samples as described above, to increase the sample size for subsequent genetic testing.

At the manufacturer's recommended cutoff titer of 1:64, 14 (26.4%, CI: 15.7-40.6) of the 53 samples from 2016 were seropositive, with a GMT of 115.93 and GSD of 2.35; and 18 (43.9%, CI: 28.8-60.1) of the 41 samples from 2017 were seropositive, with a GMT of 161.27 and GSD of 3.05. Overall, 32 (34.0%, CI: 24.8-44.6) of the 94 samples were seropositive (titer range: 1:64-1:4096; GMT: 139.59; GSD: 2.74). Chi-square tests showed no difference by sampling year ($p = 0.12$), sex ($p = 0.83$), or age ($p = 1.00$). Fisher's exact test also showed non-significance of titer distributions between sampling years ($p = 0.27$).

At our adjusted cutoff titer of 1:256, eight (8.5%, CI: 4.01-16.56%) of the 94 samples were seropositive (titer range: 1:256-1:4096), with a GMT of 608.87 and GSD of 2.43. Fisher's exact test showed no significant difference in positive samples by year ($p = 0.29$), sex ($p = 1.00$), or age ($p = 0.34$). Titer distribution was also not significantly different by year ($p = 0.29$), sex ($p = 0.26$), or age ($p = 1.00$).

Small per-county sample sizes and variation in sampled counties between years inhibited spatial statistics. However, we found positive samples at 1:64 in all sampled counties except for Mojave and Navajo. At 1:256 or higher, we found positive samples in Apache (2016), Coconino (2017), Cochise (both years), and Graham (2016) counties (Table 1).

Genetic Screening

Using the same samples described above, we had 125 blood samples (53 in 2016; 72 in 2017) that yielded cellular components, and 138 skin samples (66 in 2016; 72 in 2017). Initial qPCR assays resulted in seven samples that amplified logarithmically in the Pan-*Rickettsia* assay, all with high C_T values (range: 34-38). Zero samples were positive for *R. rickettsii*. The repeated qPCR run of the Pan-*Rickettsia* positive samples resulted in three duplicate plus one triplicate positive sample. These four were all sourced from skin; the other three were from blood. We did not find any matched positive skin and blood samples from the same animal. This is consistent with the migration of RMSF bacteria from blood into skin during the infection (W. Nicholson, personal communication). However, since the three blood-sourced DNA samples had high C_T values and we were unable to duplicate positive results, we interpreted them as negative. These blood samples were from animals on which we were unable to perform serology. However, we did perform serology on three of the four animals from which skin was qPCR-positive. The antibody titers for those three animals were 1:64 (1) and 1:128 (2). This provides evidence that some of the low titer samples were likely from positive animals, even though they fell below our 1:256 cutoff.

Out of the four DNA samples that we officially interpreted as qPCR-positive, one was collected in Cochise County, about 60 km south of an RMSF-affected community. The other three were collected in a cluster less than 10 km from each other, on the Coconino-Navajo county line, located roughly equidistantly between two affected communities, which lay 41.6 km north and 49.5 km south of the cluster, respectively.

DISCUSSION

Rocky Mountain spotted fever is an important public health concern in Arizona, and significant knowledge gaps regarding the pathogen-vector-host relationships remain. Although the dog-*Rh. sanguineus*-*R. rickettsii* relationship is of primary importance in the state, other rickettsial pathogens, tick vectors, and/or vertebrate hosts may also factor into human cases of rickettsial diseases. For example, a small proportion of annual SFG rickettsiosis cases in Arizona are attributed to *R. parkeri*, which presents with similar symptoms to RMSF,[7] is commonly associated with the gulf coast tick (*Amblyomma maculatum*), and was recently linked to *A. triste* in Arizona.[7,37] Additionally, *A. maculatum* Koch group ticks were recently found in Cochise County and were infected with *R. parkeri*.[38] Since *A. maculatum* group ticks do not appear to have a host preference,[39] non-dog vertebrate hosts seem likely in the wild.

Even among brown dog ticks, several biological and ecological factors require further investigation. They are known to have distinct, non-hybridizing, temperate and tropical lineages in the Americas,[40,41] and both lineages occur in Arizona.[42] According to these data, the temperate lineage occurred in the mountainous, northern region of the state, while the tropical lineage occurred in the low-lying, southern region. Moreover, the vector competency of each lineage may vary by pathogen.[40] Solis (2017) demonstrated a higher prevalence of rickettsial DNA in the tropical lineage, but whether this effect is due to the lineage or the differences in regional climate is not clear. It is also possible that the two lineages exhibit different degrees of host-specificity. Indeed, tropical *Rh. sanguineus* in Brazil have been recorded infesting dogs, rabbits, cats, rodents, pigeons, wild canids, and humans,[18] and a wild race in Egypt has also been shown to parasitize a variety of hosts.[17]

In our study, we present the first evidence in Arizona that coyotes are exposed to rickettsial bacteria, highlighting the need to investigate the pathogen cycle further. While cross-reactivity can occur in serology,[43] and the Pan-*Rickettsia* primer set is sensitive to all species in the genus,[36] typhus group and other non-SFG rickettsiae have not been found in Arizona. Therefore, we are confident that our data provide evidence of SFG *Rickettsia* in coyotes. In addition, the lack of a significant difference in seroprevalence and titer between years and sexes may indicate that SFG rickettsiae are endemic among Arizona's coyote population.[44] However, our data only cover the spring seasons of two consecutive years. Confirmation of endemism would require widespread sampling in multiple seasons and over several years.

Antibody-based detection is a powerful approach for detecting the presence of a pathogen in wildlife.[43] However, immunofluorescence has been criticized for its lack of specificity in some cases.[45] For instance, a review of tickborne bacterial diseases claimed that certain rickettsial antigens are cross-reactive with all species in the genus and some in related genera.[46] However, IFA has previously been described as the "gold standard" for diagnosing rickettsial infections,[43] and a later review of guidelines for diagnosing rickettsioses found that for the *R. rickettsii* IFA, the use of a cutoff titer of 1:64 resulted in a sensitivity and specificity of 84.6% and 100%, respectively in clinically-relevant samples.[47] Literature also support our use of IgG instead of IgM to reduce the chances of cross-reaction false positives.[45] In addition, a comparative analysis of serological tests has shown that increasing the cut-off titer for positive diagnoses improves specificity.[26] However, the manufacturer of our assay has not conducted a study of its sensitivity and specificity.

It is worth noting that we used anti-canine IgG that was designed for testing dogs rather than coyotes. Any differences in the immunology of the two species could alter the specificity

and sensitivity of the test, which would affect the number of false positives and/or false negatives detected. Unfortunately, a known prevalence value for *R. rickettsii* antibodies in coyotes is not available, so we were not able to calculate the sensitivity and specificity of our test. However, since specificity increases with higher titers, our adjusted cutoff titer of 1:256 should have increased the specificity and decreased the sensitivity of our assay. As such, we might have inadvertently discarded some true positives in an attempt to eliminate false positive samples, but we consider the majority of our adjusted seropositive results as true positives for SFG *Rickettsia* rather than *R. rickettsii*. This is in acknowledgement of the potential for cross-reactivity and the knowledge that in addition to *R. rickettsii*,[15,38] numerous *Rickettsia* species including *R. massaliae*[48] and *R. parkeri*[37,38] have been detected in the state.

Another factor that might have affected the accuracy of our serological results was that all sera exhibited gross hemolysis, perhaps due to unavoidable delays in centrifuging blood samples in the field. It is possible that non-specific binding of intracellular components to the slide substrate could have masked or amplified the reaction and led to an increased number of false negatives or false positives, respectively.

The range of seropositive titers we found is comparable to those found in experimental *R. rickettsii* infections of dogs, which presented with clinical illness and sometimes death.[49] In a 2014 study, Levin et al. experimentally infected dogs with *R. rickettsii* and monitored IgG antibody titers daily. Detectable titers began 7-10 days post-infection (DPI), rose to a maximum of 1:2048, and began falling 28-33 DPI.[49] If the relationship between titer and infection in dogs is also true in coyotes, then the highest titers should indicate more recent exposure. Given the genetic similarity between the two species, we would expect to see similar pathology in both, but to our knowledge this has not yet been investigated.

Currently, the relationship between the infection chronology and antibody titer for coyotes is not known. In the three qPCR-positive samples we were able to match with serology from the same animals, the titers were relatively low (range: 1:64-1:128). This is in keeping with the relationship between early-stage primary acute infections and antibody titer in dogs, as described above. Although these titers were below our adjusted cutoff, they are at levels we would expect from a qPCR-positive sample if rickettsial pathophysiology is similar in coyotes as in the dogs in Levin et al. (2014).

We were surprised not to find any ticks on any sampled coyotes. Although female brown dog ticks feed for only 2-9 days (depending on life stage), males may remain attached to the host for several weeks.[17] Thus, we expected to find ticks on coyotes during specimen collection but found none. The reason for this is unclear, but there are several possibilities. The first is seasonality. Specimens were collected in March and April, when brown dog tick activity is just beginning to recover from a winter lull; they are most active in autumn.[17] Second, a tick's life stage is a major factor in its detectability due to size. For example, a larval brown dog tick (length: 0.54 mm; width: 0.39 mm)[18] is easily mistaken for a speck of dust among the hairs of a furry mammal. Next, as discussed previously, brown dog ticks are presumed to be host-specific for domesticated dogs. If this dogma holds true, perhaps coyotes in Arizona are developing antibodies to rickettsiae via another mechanism or another vector. The nidicolous nature of brown dog ticks led us to wonder whether coyotes in our study could have developed antibodies through the ingestion of infected burrowing mammals such as rodents and lagomorphs, or the incidental ingestion of infected ticks on these prey species. This possibility is minimally-studied, but domesticated dogs have been experimentally infected with *R. rickettsii* by feeding them infected rodents.[50,51] Note that in that experiement, Magalhães and Moreira (1935) used the

Portuguese name "Typho Exanthematico", which translates to exanthematic typhus, a typhus group rickettsial disease. However, later research clarified that the disease was actually Brazilian Spotted Fever, another name for RMSF.[52] Finally, it is possible that any ticks present on coyotes dropped off post-mortem and before we reached the carcasses. Members of the Ixodidae family of ticks secrete a cement-like cone of material around the bite site that produces a weak seal within minutes and a strong anchor in about 24 hours. Very little is known about tick detachment rate, but *D. andersoni* can detach in 1-2 minutes, and limited research shows that a few microliters of saliva is enough to dissolve a cement cone, possibly with the help of a protease.[53]

Thus far, we have not described a specific pathogen-vector-host relationship involving coyotes, ticks, and rickettsial pathogens. This is a complex problem since Arizona is home to several tick species that may parasitize coyotes and vector multiple rickettsial species with varying degrees of pathogenicity. *Amblyomma triste* ticks are present in Arizona and are known to transmit *R. parkeri*.[37] In the eastern United States, there are multiple accounts of coyotes being parasitized by Gulf Coast ticks (*A. maculatum*), an important *R. parkeri* vector.[33,54] This species has been reported in Arizona as well, but due to the recent discovery of *A. triste* being misidentified as *A. maculatum* in Arizona,[55] it is unclear whether some of these accounts might actually be *A. triste*. Indeed, an *Amblyomma* species with similar morphological characteristics to both *A. maculatum* and *A. triste* was recently found in Cochise County. In the sampled ticks of this species, 24% were infected with *R. parkeri*, 1.6% with "*Candidatus* Rickettsia andeanae", and 0.5% with *R. rhipicephali*.[38] In addition, the American dog tick (*D. variabilis*) is rare but extant in Arizona, can carry *R. rickettsii*, and is known to parasitize coyotes.[1,54] Finally, if brown dog ticks parasitize coyotes, they could transmit other rickettsial bacteria, such as *R. massaliae* or *R. rhipicephali*, the latter of which is presumed to be non-pathogenic to humans.[48]

The immunological cross-reactivity between rickettsial species further complicates our understanding of this system. For instance, a 1992 study found that inoculating guinea pigs with the non-pathogenic *R. rhipicephali* produced protective immunity against *R. rickettsii*.[56] Such an effect might attenuate the prevalence of sympatric rickettsial species in the wild. For example, if coyotes turn out to be reservoir competent for *R. rickettsii*, a coyote with antibodies for *R. rhipicephali* might more-easily be able to fight off a primary *R. rickettsii* infection, thereby preventing its spread to subsequent vectors. If such an effect exists, coyotes could serve to reduce the prevalence of rickettsial pathogens in nature.

We do not have sufficient evidence to classify coyotes as bridge hosts for *R. rickettsii* or other SFG *Rickettsia* bacteria. However, if the prevalence of rickettsial antibodies remains constant in the coyote population, blood samples might prove useful for sentinel surveillance during routine predator-reduction operations. Clearly, there is a need for additional research on the role of wildlife in the maintenance of *Rickettsia* spp. in Arizona and surrounding areas, including comprehensive studies of pathogen-vector-host dynamics, cross-reactive adaptive immune responses, and the potential for wildlife-mediated pathogen transmission into peridomestic habitats.

CHAPTER 3

CONCLUSION

I demonstrate for the first time in Arizona that coyote sera react to *R. rickettsii* in IFA, which I interpret as coyotes having been exposed to SFG *Rickettsia* bacteria. I also provide the first evidence in Arizona of rickettsial DNA in coyote tissues, indicating that some of the sampled animals had acute infections at the time of sampling. However, the bacterial species present could not be identified.

The nature of coyotes' exposure to SFG rickettsiae (e.g. bacterial species, tick host, etc.) is still unknown, as pathogen-vector-host relationships can be complex. We were unable to find a thorough investigation into which tick species commonly parasitize coyotes in Arizona. This relationship would affect the list of pathogens to which coyotes are exposed. In turn, coyotes' physiological responses to those pathogens would determine whether they are reservoir, dead-end, or other types of hosts. In addition, the feeding behavior of each tick species, combined with the level of coyote urbanization, could influence the possibility of coyotes dispersing ticks into peridomestic areas.

In the meantime, I suggest a preventative approach to wildlife management. Although coyotes have not been demonstrated to transport infected ticks into peridomestic environments, taking measures to reduce this possibility may also reduce the incidence of coyote-human and coyote-dog interactions. Strategies such as eliminating outdoor water catchments around homes, feeding pets indoors, and making garbage inaccessible to wildlife may reduce the occurrence of coyotes being attracted to homes. Free-roaming domestic cats may be an important prey item for coyote in urban areas,[20] and free-roaming dogs might be perceived as threats to territory.[23,57,58]

Keeping pets confined to yards or homes may prevent coyotes in suburban areas from being attracted to the central areas of cities. In addition, if urbanized coyotes do pose a risk of transporting infected ticks to peridomestic areas, then it is important for pet owners to remain vigilant about treating their pets and yards for ticks.

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APPENDIX 1

FIGURES

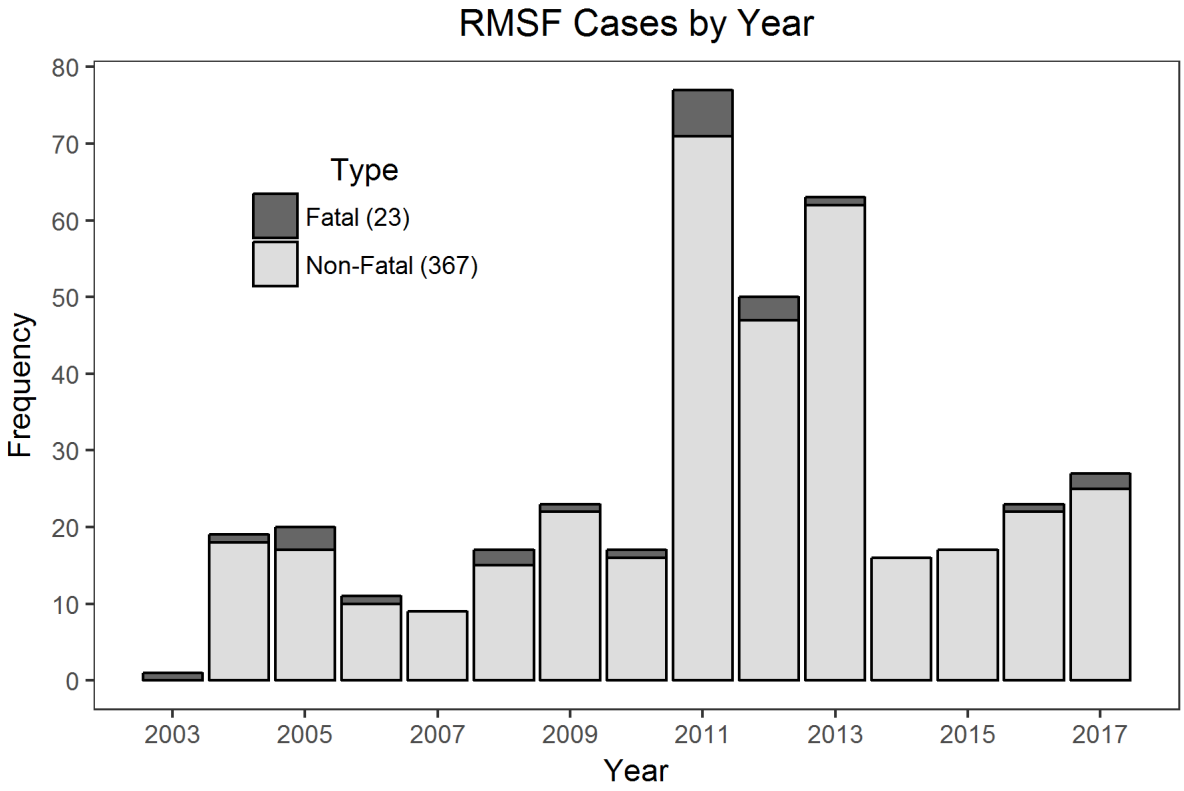

Figure 1 - RMSF Cases in Arizona 2003-2017. Light gray indicates non-fatal cases. Dark gray indicates fatal cases. Data sources: AZDHS (http://www.azdhs.gov/); Hayley Yaglom, AZDHS (personal communication).

FIGURES

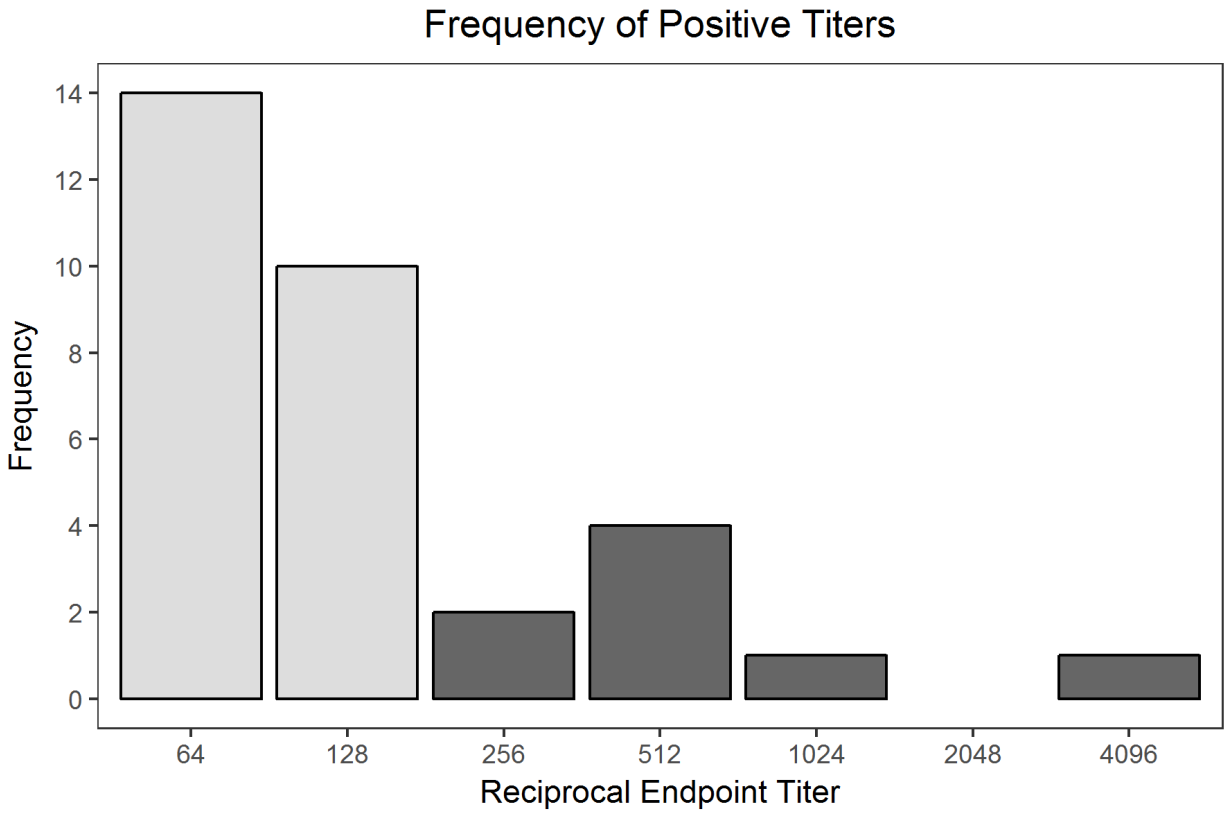

Figure 2 – Frequency of reciprocal endpoint titers in IFA-positive samples. Light gray indicates samples that were positive at the manufacturer's recommended diagnostic cutoff titer of 1:64. Dark gray indicates samples that were positive at our adjusted cutoff titer of 1:256.

APPENDIX 2

TABLES

Table 1 - Endpoint titers by year and county. Shaded columns were interpreted as seronegative, being below the adjusted cutoff titer of 1:256. Hashed cells indicate no usable sera were collected in the given year-county combination.

	2016					2017						
County	n	1:64	1:128	1:256	1:512	n	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Apache	10	2			1	-	-	-	-	-	-	-	-
Coconino	-	-	-	-	-	18	2	2	2				1
Cochise	26	3	2		1	4	1	1		1	1		
Graham	17	3	1		1	5	2	2					
Mohave	-	-	-	-	-	2							
Navajo	-	-	-	-	-	4							
Yavapai	-	-	-	-	-	8	1	2					