University of Nevada, Reno

Upper Respiratory Microbes in North American Tortoises (Genus *Gopherus*)

A dissertation submitted in partial fulfillment of the
requirements for the degree of the Doctor of Philosophy in
Ecology, Evolution, and Conservation Biology

by

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May, 2017

THE GRADUATE SCHOOL

We recommend that the dissertation
prepared under our supervision by

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Entitled

Upper Respiratory Microbes in North American Tortoises (Genus Gopherus)

be accepted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Infectious disease can play a vital role in species conservation, as some diseases can cause massive population die-offs. In North American tortoises, population declines have been attributed to an upper respiratory tract disease, but the importance of this disease within and among tortoise hosts is controversial. In this dissertation, I use samples from the upper respiratory tract of wild tortoises representing multiple species in the genus *Gopherus* to answer questions regarding pathogen prevalence and co-infection, pathogen genetic diversity, and the ecology of pathogens within the upper respiratory tracts of tortoises. Using genetic methods including polymerase chain reaction (PCR), quantitative PCR, Sanger sequencing, and 454-pyrosequencing, the data presented herein supports hypotheses that pathogens in this disease system interact with each other and with other microbes in the respiratory tract differently in different tortoise host species.

In this system, both transmission and disease progression are found to be extremely slow in these long-lived hosts. Though pathogens were widespread among the sampled tortoises, few individuals were found with clinical signs of disease. Few individuals capable of transmitting disease (requiring nasal mucus and pathogen), along with few opportunities for pathogen transmission (requiring long periods of direct contact), indicate that it is unlikely for tortoises to fully clear themselves of these microbes without risk of the pathogen's extinction. If this is the case, then it is likely that more tortoises than we can detect have these microbes in their upper respiratory tracts. With this in mind, and considering the abundance of visibly healthy tortoises over ill tortoises, my collaborators and I suspect that pathogens in this system likely form

commensal relationships with their hosts much of the time, until a stressor alters the system and leads to a parasitic interaction.

The results of this dissertation indicate that this disease is context dependent, depending on the host and likely other microbes in the community. Our understanding of the ecology of this disease system would greatly benefit from experimental inoculation and long-term resampling studies.

ACKNOWLEDGEMENTS

This project would not have been initiated without Fran Sandmeier, and I wouldn't have wound up in this lab or at UNR if it wasn't for her. Thanks for your chronic fatique/mono delirium that provided this project with its very own special version of enthusiasm from the very beginning. Field sampling would not have been possible without many volunteers, friends, and collaborators giving their time to search the desert (and Texas, Alabama, and Florida) for those moving rocks we call tortoises. Thank you to Fran Sandmeier, Cristina Jones, Brett Addis, Jenny Todd, Tim Ichien, Lauren Schumacher, Martyn Drabik-Hamshare, Ann McLuckie, Sarah Snyder, Michael Tuma, Jeff Goessling, Molly Bechtel, Claire Crow, Daren Riedle, Adam Emerick, Brian Folt, and the many Arizona volunteers for their help in the field. Additional thanks to the Guyer and Hermann labs at Auburn University, the McCoy/Mushinsky lab at the University of South Florida, Brian Sullivan at Arizona State University, and USGS in Henderson for all of the field and logistic support. Thanks to Mike Teglas and Jen Hsueh for allowing me to use the lab all of these years, and for the guidance and support required to maintain my sanity while doing so. Additional thanks to Nate Nieto, Ryan Gov, and Stephanie Fuetsch for assistance in the lab and Kris Kruse and Craig Osborne at the Nevada Genomics Center for their help over the years with sequencing and troubleshooting. I would especially like to thank the NSF Graduate Research Fellowship Program, the U.S. Fish and Wildlife Service, the Community Foundation, and the UNR Graduate Student Association for financially supporting me and this research.

I would like to thank my committee members, Dick Tracy, Fran Sandmeier, Mike Teglas, Matt Forister, and Scott Mensing, for providing support over the years and

making it not too difficult to get all six of us in a room at the same time. I also thank Sarah Snyder, Josh Jahner, and Michelle Gordon, for providing valuable feedback on earlier drafts of my manuscripts.

I especially thank my advisor, Dick Tracy, for his neverending encouragement, advice, and support throughout my graduate career. Thank you for letting me forge my own path. Thanks to the Tracy lab members over the years, for the camaraderie and neverending lab meetings, and for being part of this weird family Dick has created. A world of thanks as well to my Reno family, who have driven me to succeed and kept me entertained over the years; there are too many of you to list in full here. Cynthia Scholl, Kevin Burls, and Sarah Snyder, my years here would have been much lonelier without you. Thanks for keeping me going. Josh, Carrie, and Michelle, thanks for the late-night karaoke. I have so much gratitude to the EECB program, both students and faculty, and the community we have formed and maintained here. Thank you for being amazing.

To my family, thank you for your support and for schlepping to Reno for the last six years. And a very special thanks to the hundreds of tortoises I've met over the years. You certainly kept things interesting.

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INTRODUCTION

The tortoise genus *Gopherus*, a group of land turtles most closely related to the Asian genus *Manouria* (Le et al. 2006), includes six species inhabiting North America. Of those species, four have partial or entire distributions in the U.S.: the gopher tortoise (*G. polyphemus*) lives in the southeastern U.S.; Texas tortoises (*G. berlandieri*) are found from southern Texas into Mexico; Mojave desert tortoises (*G. agassizii*) are found in the Mojave and Colorado Deserts of the U.S. southwest; and the recently described Sonoran desert (Morafka's) tortoise (*G. morafkai*; Murphy et al. 2011) inhabits the Sonoran Desert of Arizona into Mexico. Previous research on the phylogeny of these species splits them into two groups, one including the desert tortoises and Texas tortoise, with the gopher and bolson (*G. flavomarginatus*) tortoises splitting off as sister taxa to each other (Berry et al. 2002, Reynoso and Montellano-Ballesteros 2004, Le et al. 2006).

In 1990, the Mojave desert tortoise was listed as threatened under the Endangered Species Act of 1973 (U.S. Fish and Wildlife Service 1994), and an upper respiratory tract disease (URTD) has been implicated in population declines of the species (Jacobson et al. 1991). Since the early 1990s, URTD has also been implicated as a cause of population declines in the gopher tortoise (Seigel et al. 2003, Enge et al. 2006), though further investigation indicates lower impacts of this disease on individual mortality and population declines than previously thought (Sandmeier et al. 2009, Florida Fish and Wildlife Conservation Commission 2012). This disease has also been detected in wild Sonoran desert tortoises (Dickinson et al. 2005), Texas tortoises (Judd and Rose 2000, Guthrie et al. 2013) and captive bolson tortoises (Truett and Phillips 2009).

Clinical signs of URTD include nasal exudate, palpebral edema, and wheezing, with severe cases resulting in lethargy and potentially mortality (Brown et al. 1994). Multiple pathogens are associated with URTD in North American tortoises, including *Mycoplasma agassizii*, *M. testudineum*, *Pasteurella testudinis*, testudinid herpesvirus 2, and an iridovirus (Snipes et al. 1980, Snipes and Biberstein 1982, Brown et al. 1994, 1999b, 2004, Westhouse et al. 1996, Jacobson et al. 2012). Importantly, each pathogen does not necessarily cause all of the clinical signs associated with URTD. For example, *M. agassizii* has fulfilled Koch's postulates, though palpebral edema was not a disease result from this pathogen (Brown et al. 1994).

Mycoplasmal URTD was first detected in the western Mojave Desert in California after a population crash in the late 1980s (Berry 1990). This population crash, posited to be due to disease, spurred a flood of research regarding how URTD is spread, the extent to which the disease threatens tortoises, and detecting disease in tortoises via antibody responses (Jacobson et al. 1991, Schumacher et al. 1997, Brown et al. 1999a, Diemer Berish et al. 2000, Brown et al. 2002, Dickinson et al. 2005, Diemer Berish et al. 2010, Sandmeier et al. 2013). However, until now, there has not been an organized study on pathogen presence and characteristics of mycoplasma populations across the Mojave Desert.

Historically, the presence of URTD in tortoise populations has been deduced through several different assays that assessed for different aspects of disease: (a) presence of clinical signs (generally indicates disease, but without identifying the cause of the disease), (b) *M. agassizii*-specific antibody detection using both enzyme-linked immunosorbent assay (ELISA) and Western blot (an indicator of a physiological

response to a particular pathogen; e.g., *Mycoplasma*), and (c) mucus cultures, which directly assesses for the pathogen (Schumacher et al. 1993, Brown et al. 2002, Hunter et al. 2008). More recently, a less-expensive assay using quantitative PCR assay was developed to detect pathogens directly rather than inferring pathogen presence from the presence of physiologically produced antibodies (Braun et al. 2014). There are still many gaps in our understanding of this disease, and not much is known about the bacterium, *M. agassizii*, itself – its virulence, its strain diversity, or its ecology.

An important aspect of understanding a disease system is understanding pathogen transmission. In the case of *M. agassizii*, extended periods of direct contact between individuals is required for pathogen transmission (Aiello et al. 2016), followed by extremely slow disease progression (Maloney 2011). Among wild tortoises, this direct contact is likely associated with burrow sharing (Bulova 1994, Radzio et al. 2016), which could be defined by social interactions (Wendland et al. 2010). Importantly, there is no evidence for vertical transmission of *M. agassizii* (Schumacher et al. 1999), and the limited conditions in which this mycoplasma can grow means it is unlikely to survive outside of the host in the environment (Orlowski et al. 2015).

Once a pathogen has colonized its host, its success is affected by interactions with co-infecting pathogens, the microbial community in the host, and the host's immune system. Pathogens within a host can interact both directly and indirectly through the host's immune response to result in positive, negative, and neutral interactions. Furthermore, these interactions are affected by the pathogens' location within the host, recency of infection, and how chronic the infection is (Pedersen and Fenton 2007, Telfer et al. 2010).

The complexity of interactions within a host is compounded by the host's immune strength, which can be shaped by host genotype and genotype-by-environment interactions. Immune genes determine the possible extent of an immune response, while environmental factors affect the performance of immune physiological traits. Food availability, affected by timing and amount of annual rainfall, along with harshness of winter, are environmental factors that could impact body condition and health. McCoy and colleagues (2011) found that amount of rainfall influenced body condition in Mojave desert tortoises, but not in gopher tortoises. In the Mojave Desert, local tortoise populations have been reported to have increased prevalence of antibodies to *M. agassizii* (indicating an assumed increase in disease) with an increase in number of days below freezing (Sandmeier et al. 2013), though this factor could be confounded by genotypic differences in tortoises inhabiting different environments across the desert southwest.

URTD has been observed in populations across the Mojave Desert at varying frequencies through antibody detection (Sandmeier et al. 2013), and its importance at the population level continues to be controversial (Sandmeier et al. 2009). It is, thus, expected that pathogens are prevalent in tortoises across the Mojave Desert, but the presence within local populations may not necessarily coincide with prevalence of immune response. The likely range-wide prevalence of *M. agassizii,* and the varying prevalences of URTD in the gopher tortoise (McCoy et al. 2007) and Mojave desert tortoise (Sandmeier et al. 2009, 2013) populations indicate a broad range of disease dynamics. In a study by Brown et al. (2001), multiple strains of *M. agassizii* were found in Mojave desert tortoises, though differences in virulence corresponding with strain variation has not yet been determined. In the pathogen *Pasteurella testudinis*, many

strains have also been detected by ribotyping in Mojave desert tortoises, with seemingly variable virulence corresponding with ribotypes (Snipes et al. 1995).

In the last two decades, URTD has been extensively studied in Mojave desert tortoises and gopher tortoises, with no research focusing on the ecology of this disease across *Gopherus* species. The first larger study searching for *M. agassizii* and mycoplasmal URTD in Texas tortoises was only recently published (Guthrie et al. 2013), and found both the pathogen and antibodies to *M. agassizii* in tortoises in southern Texas. Regardless of decades of research focused on tortoise URTD, little is understood about the disease ecology and dynamics of URTD. The work presented in this dissertation answers questions on the ecology of URTD-associated pathogens through research at multiple spatial and ecological scales.

This dissertation aims to address a few of the many questions involving URTD host-pathogen dynamics in tortoises, and will aid in our understanding of infectious disease ecology in long-lived ectotherms. To supplement studies on tortoise ecoimmunology associated with URTD, I use nasal flush samples to investigate microbial patterns, incorporating a range-wide study focused on Mojave desert tortoises and comparative studies discussing differences in the ecology of this disease among four tortoise host species.

CHAPTER ONE OVERVIEW

Mojave desert tortoises inhabit a distribution in the Mojave and Colorado Deserts of the U.S. southwest. Through genetics, individuals of this species have been divided into three main genotypes (Hagerty and Tracy 2010), and prevalence of antibodies to the

M. agassizii pathogen differs among the genotypes (Sandmeier et al. 2013). Chapter One analyzes prevalence and genetic diversity of *M. agassizii* in hundreds of nasal flush samples from wild-caught Mojave desert tortoises. Analyses in this chapter address the following questions:

1) To add to recent diagnostic measures for URTD (Sandmeier et al. 2017), how does conventional PCR and Sanger sequencing compare with quantitative polymerase chain reaction (qPCR) methods?
2) How prevalent is *M. agassizii* in local populations of Mojave desert tortoises, and how does prevalence differ among genotypes and local populations within genotypes?
3) Can sequencing of three genetic markers of *M. agassizii* help us to differentiate strains and their relative virulence in Mojave desert tortoises?

CHAPTER TWO OVERVIEW

The main goal of Chapter Two is to detect whether there are differences in the ecology of two species of *Mycoplasma* pathogens in different host species. Multiple pathogens are associated with URTD in *Gopherus* tortoises, and recently, a probe-based qPCR assay was developed to detect some of these pathogens (Braun et al. 2014). We use this assay to detect *M. agassizii* and *M. testudineum* in samples from four *Gopherus* tortoise host species, in addition to conventional PCR and DNA sequencing to detect genetic variation in *M. agassizii* to answer:

1) Do different tortoise host species have genetically different types of *M. agassizii*?

2) What is the prevalence of each *Mycoplasma* pathogen, as well as prevalence of their co-infection, in each tortoise species, and do these frequencies vary among host species?
3) Are clinical signs of URTD in the form of current or recent nasal discharge predicted by infection or co-infection of these *Mycoplasma* spp.?

CHAPTER THREE OVERVIEW

In disease ecology, pathogens interact with each other and their host to affect the manifestation of disease. However, pathogens also interact within the microbial community within the host. Microbial interactions within a host span the range of positive, negative, and neutral interactions also found in communities of macro-organisms. Little is known about the nasal microbiome within tortoise hosts. In the context of the tortoise upper respiratory tract, studies have assessed quantity and diversity of microbes present within the confines of culture-based methods (Dickinson et al. 2001, Ordorica et al. 2008). The influx of next-generation DNA sequencing techniques allows for more thorough and time-effective means of quantifying microbiomes.

In Chapter Three, my collaborators and I analyze microbiome data from 454-pyrosequencing, a technique that has allowed us to detect nearly 20,000 bacterial operational taxonomic units in nasal flush samples representing four *Gopherus* tortoise species. Data from this assay are used to answer:

1) What bacterial taxa are present in the noses of these tortoise hosts?
2) Are upper respiratory bacterial communities similar within and among tortoise host species?

3) Does the presence of URTD-associated bacteria affect community composition present in these samples?
4) Are certain bacterial taxa predictably found in each tortoise species?
5) How does the presence of nasal discharge affect bacterial diversity and community composition in the upper respiratory tract?

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CHAPTER 1: PREVALENCE AND DIVERSITY OF THE UPPER RESPIRATORY PATHOGEN *MYCOPLASMA AGASSIZII* IN MOJAVE DESERT TORTOISES (*GOPHERUS AGASSIZII*)

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in press, *Herpetologica* June 2017

ABSTRACT

Upper respiratory tract disease (URTD), caused by *Mycoplasma agassizii*, has been deemed a threat to populations of Mojave Desert Tortoises, *Gopherus agassizii*. Previous work on URTD has focused on serology and visual health examinations to determine the extent of this disease in some natural tortoise populations. Here, we present the first range-wide study of the presence of the pathogen, *M. agassizii*, in Mojave Desert Tortoises. We detected *M. agassizii* in tortoise populations throughout the Mojave Desert, with notable differences in prevalence of *M. agassizii* among sampling sites within tortoise genotypes, and sampling years. Analyses of three genetic markers in the *M. agassizii* genome indicated very low nucleotide diversity and no relevant spatial structuring of *Mycoplasma* haplotypes. We use published lines of evidence to discuss the roles of rare transmission events and long-term mycoplasmal persistence in individual hosts on tortoise URTD dynamics.

Key words: Chelonian; Disease; Host; *Mycoplasma*; Polymerase chain reaction; Quantitative PCR; Strain; Turtle; URTD

INTRODUCTION

DISEASES can have important effects on wildlife populations. When a pathogen is invasive and infects naïve host species and populations, such as the spreading of the fungus that causes chytridiomycosis (*Batrachochytrium dendrobatidis*), it can cause severe population declines in its amphibian hosts (Skerratt et al. 2007). Some host species can act as carriers, not directly affected by disease, but able to spread the pathogen to additional hosts (Eskew et al. 2015). Even within a single host species, a disease could

affect some individuals, while others are carriers, such as in the typhoid disease system in humans (reviewed in Merrell and Falkow 2004).

An upper respiratory tract disease (URTD) caused by a species of *Mycoplasma* was first detected in Mojave Desert Tortoises, *Gopherus agassizii* (Cooper 1861), in California after a population crash in the late 1980s (Jacobson et al. 1991; reviewed in Sandmeier et al. 2009). This population crash was posited to be caused by URTD, and it spurred an abundance of research regarding the pathology (including mortality and morbidity) induced by the putative causative pathogen (Jacobson et al. 1991; Brown et al. 1999). The population crash, as well as other population declines, led to the listing of Mojave Desert Tortoises as threatened under the Endangered Species Act in 1990 (U.S. Fish and Wildlife Service 1994). Brown et al. (1994) verified that the pathogen causing URTD was the newly named *Mycoplasma agassizii* (Brown et al. 1994, 2001). In tortoises, *M. agassizii* forms a close association with the nasal epithelium (Jacobson et al. 1991), and URTD can cause clinical signs of nasal exudate, palpebral edema, and wheezing, with severe cases resulting in lethargy and potentially mortality (Brown et al. 1994). Subsequent research continued to investigate the extent to which the disease threatens tortoise populations (Schumacher et al. 1997; Dickinson et al. 2005; Sandmeier et al. 2013) and resulted in the development of serological tools (ELISA and Western blot) that can be used to detect *Mycoplasma* antibodies in tortoises (Schumacher et al. 1993; Brown et al. 2002; Hunter et al. 2008). Recently, a probe-based quantitative polymerase chain reaction for *M. agassizii* was developed and is used to detect the presence of the pathogen itself, to supplement the use of serological tools (Braun et al. 2014).

The ecological importance of URTD for tortoise populations continues to be controversial, because published reports have noted that this disease correlates with a high risk of morbidity, mortality, and population crashes (reviewed in Sandmeier et al. 2009). It is currently thought that *M. agassizii* is present in tortoises throughout the Mojave Desert and prevalence, measured by serology, differs among geographic areas (Sandmeier et al. 2013). Additional data indicate that multiple strains of *M. agassizii* are present in Mojave Desert Tortoises (Brown et al. 2001), but the extent to which different strains are found in tortoise populations remains unknown. Until now, no study has comprehensively investigated the presence of *M. agassizii* in Mojave Desert Tortoises across their geographic range, from southern California to southwest Utah in the Mojave and Colorado Deserts. Furthermore, genetic variation within and among *Mycoplasma* populations has not been assessed.

Here, we use samples of microbes obtained from nasal passages of Desert Tortoises to address the following questions: (1) What is the relative efficacy of conventional PCR and DNA sequencing compared with qPCR in detecting *M. agassizii*? (2) What is the prevalence of *M. agassizii* across the geographic distribution of Mojave Desert Tortoises? (3) Can different strains of *M. agassizii* be detected in our samples, and are certain strains more associated with clinical signs of disease in the tortoises than other strains? (4) Is prevalence of *M. agassizii* or disease correlated with tortoise genotype? We predict that *M. agassizii* is present in tortoise populations across the Mojave Desert, in accordance with previously measured seroprevalence (Sandmeier et al. 2013). We also predict that multiple strains of *M. agassizii* will be found, and that the presence of some strains in tortoises will be more closely associated with clinical signs of URTD than

others.

The present study adds PCR and DNA sequencing to the diagnostic methods recently compared for their efficacy (Sandmeier et al. 2017). Additionally, we use the diagnostic methods presented here to answer broader questions regarding ecological patterns among the component parts of this disease system.

MATERIALS AND METHODS

Field Sites and Sample Collection

Samples of upper respiratory bacteria were taken from wild-caught Mojave Desert Tortoises in the southwestern United States during the active season (April–October) between 2010 and 2012. Each tortoise was sampled only once. Our goal was to sample from sites relatively evenly spaced across the tortoise distribution (Fig. 1), and sites were generally delineated by mountain ranges, which confine tortoises to valley floors. Sites were selected to represent three previously determined tortoise genotypes: California (CA), Las Vegas (LV), and Northeast Mojave (NEM; Table 1; Hagerty and Tracy 2010). At most sites, tortoises are sparsely distributed (with home ranges ranging from 1–89 ha; Berish and Medica 2014); to increase the probability that our sampling was representative for each population, we found most tortoises along haphazardly selected 10 km transects. Approximately 1 tortoise encountered per 10 km transect can be expected based upon U.S. Fish and Wildlife surveys (available at https://psw.databasin.org/galleries/af8e55a0197a4c95a3120b278075a2b1), and our goal was to sample 20 tortoises per site. While tortoises can move long distances, they are not likely to move among local populations, particularly not far enough to occupy a new

range that falls within a different genotype group. Thus, we used the assumed genotypes relevant for each local population as determined by Hagerty and Tracy (2010), and we did not verify genotype group per tortoise. Genotype was used to detect general patterns across the tortoise distribution. We additionally sought to mitigate biases in sampling among years by sampling sites during multiple years, when possible (Table 1).

Visible presence of clinical signs of URTD, including nasal mucus or signs that the tortoise had recently exuded nasal discharge (occluded or asymmetrical nares, or eroded scales around nares), was evaluated for each tortoise. Tortoises with any of these signs of URTD were considered positive for clinical signs. Palpebral edema or any other ocular syndrome was not included in the list of relevant clinical signs of disease as they have not been included in the disease results from Koch's postulates regarding *M. agassizii* and URTD (Brown et al. 1994). To determine whether *Mycoplasma* DNA was present in the upper respiratory tract, a nasal lavage sample was collected by flushing 3 mL of sterile saline solution (0.9% NaCl) through the tortoise nares (Brown et al. 2002). Nasal lavage samples were immediately preserved in RNAlater Stabilization Solution (Ambion Inc., Austin, TX) at a ratio of 1:5 preservative to sample volume. Preserved samples were placed on ice in the field and frozen within 12 hr of collection. In the laboratory, DNA from 500 µL of preserved nasal lavage samples was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) protocol for gram-negative bacteria.

Mycoplasma Detection

qPCR.—Presence of *M. agassizii* was detected by quantitative polymerase chain reaction (qPCR) using the protocol described in Braun et al. (2014). This protocol can

detect 50 copies of the 16S ribosomal RNA (rRNA) gene in *M. agassizii* with a Cq value of 38.5, maximum intra-assay variation of 0.97, and a detection limit of a Cq of 40 (Braun et al. 2014). In qPCR, Cq value is a calculation of the number of cycles necessary for amplification of the desired region above a given threshold. Thus, a lower Cq corresponds to more of the desired template DNA in the sample. All qPCRs were conducted in triplicate with TaqMan Environmental Master Mix 2.0 using a Step One Plus Real-Time PCR System with 40 thermal cycles and Step One Software v2.3 (Applied Biosystems, Foster City, CA). Following the recommendation of Braun et al. (2014), samples were considered to include the DNA of *M. agassizii* (qPCR-positive), if two or three of the triplicates had a Cq value below 40.

Positive results were categorized as being either strongly positive (with a mean Cq < 38) or weakly positive (with 38 < mean Cq < 40). Although this qPCR assay can be used to detect the presence of *M. agassizii* in a sample up to a Cq value of 40, we divided qPCR results into three data sets in order to detect differences in patterns associated with strong versus weak infections: Data Set 1 (DS1) = both strongly positive and weakly positive samples were categorized as positive for *M. agassizii*, all other samples were categorized as negative; Data Set 2 (DS2) = only strongly positive samples were categorized as positive for *M. agassizii*, all other samples were categorized as negative; and Data Set 3 (DS3) = results from weakly positive samples were excluded from analyses, with only strongly positive samples categorized as positive and only negative samples categorized as negative.

PCR.—Samples were screened for three *M. agassizii* genetic markers using PCR: 16S rRNA, 16-23S intergenic spacer region (IGS), and ribosomal polymerase β subunit

(rpoB; see Table S1 in the Supplemental Materials available online). PCRs were conducted with Qiagen Multiplex PCR mix (Qiagen Inc.) with a 15-min hot start at 95°C and a final extension at 72°C. Nested and pseudo-nested PCR products of appropriate length were extracted from agarose gel using Qiagen QIAquick Gel Extraction Kit. Gel-extracted products were sequenced at the Nevada Genomics Center on an ABI3730 DNA Analyzer (Applied Biosystems). Sequences were verified as *M. agassizii* using NCBI's BLAST search (available at http://blast.ncbi.nlm.nih.gov). Samples were considered positive for *M. agassizii* if at least one PCR amplified the appropriate marker.

qPCR and PCR Comparison.—Correspondence of *M. agassizii* results from qPCR and PCR assays per sample was assessed by calculating unweighted kappa statistics using the kappa2 function in the irr package (Gamer et al. 2010) in the programming language R (v3.2.1; R Core Team 2015). Cohen's kappa (κ) ranges from –1.00 to 1.00, where values < 0.00 = poor agreement, 0.00–0.20 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–1.00 = almost perfect agreement (Landis and Koch 1977). PCR results were compared with the three qPCR data sets described above.

Mycoplasmal Genetic Diversity and Geographic Structure

DNA sequences were aligned in ClustalX v2.1 (Larkin et al. 2007) and trimmed in MEGA v6.06 (Tamura et al. 2013). Following Clement et al. (2000), TCS haplotype network analyses were conducted in PopART (Leigh and Bryant 2015). Haplotype networks included sequences from GenBank (available at http://www.ncbi.nlm.nih.gov) for *M. agassizii* (Accession numbers U09786.1, NR_114450.1, AY780802.1, AY780801.1, EU925153.1) to determine similarity between mycoplasmas in our samples

and known strains, such as PS6 (Brown et al. 2001). Spatial analyses of molecular variance (SAMOVAs) were conducted separately for each marker in SAMOVA v1.0 (Dupanloup et al. 2002) to detect spatial patterns of haplotypes. Sampling localities were input as the mean latitude and longitude coordinates of all tortoises sampled per site. SAMOVA was used to produce *F*-statistics by grouping sampling sites based upon their spatial coordinates and associated haplotypes. We ran SAMOVA with groups K = 2 to 16 (when appropriate) and 100 simulated annealing processes (Dupanloup et al. 2002). The output having the highest F_{ct} value (i.e., the number of groups that maximized among-group variance relative to the total variance) defined the best number of groups. Sequences shorter than 75% of the average length for each genetic marker were excluded from haplotype analyses (excludes three samples for IGS and four samples for rpoB).

Range-wide Prevalence

Transmission of *M. agassizii* in adults is likely associated with social behaviors, and little is known about transmission or disease progression in juveniles. Thus, we chose to exclude possible confounding effects from juveniles by excluding four samples from tortoises smaller than 100 mm midline carapace length (MCL) from the range-wide analyses. One sample from the large-scale translocation site in Clark County, Nevada, was also excluded from further analyses because of ambiguities surrounding the historical origin of the tortoise. Analyses regarding genotypes included all remaining samples, while those regarding site-level patterns in prevalence exclude sites with fewer than ten samples assayed.

Presence of *Mycoplasma* in nasal lavage samples (via qPCR) and clinical signs of URTD were analyzed separately using generalized linear mixed effects models (GLMM)

using the glmer function in the lme4 and lmertest packages in R (Bates et al. 2015; Kuznetsova et al. 2016). Predictor variables included tortoise genotype, sampling year, sex, and MCL, with a random variable of site nested within genotype. Analyses of clinical signs of URTD also included *Mycoplasma* presence or Cq as a predictor variable. We used Tukey's post-hoc tests to conduct pairwise comparisons to compare factors within the genotype, year, and sex variables by implementing the glht program in the R package multcomp (Hothorn et al. 2008). We analyzed Cq values using a linear mixed-effects model with the lmer program in the lme4 package using the same predictor and random variables as for the analysis of *Mycoplasma* presence and Tukey's post-hoc tests as above. To detect whether some sites differ in *Mycoplasma* presence from the rest of the genotype (Table 1), we analyzed *Mycoplasma* as predicted by site for each genotype with logistic regressions using the glm function. We compared average Cq values with *Mycoplasma* prevalence by site using linear regressions with the lm function in R to determine if higher average *Mycoplasma* loads tended to correspond with higher prevalence of *Mycoplasma* among sampling sites.

RESULTS

Sample Collection and *Mycoplasma* Detection

Nasal lavage samples were collected from 402 wild Mojave Desert Tortoises from 2010 to 2012. At least 10 tortoises were sampled from each of 21 sampling sites, and fewer samples were collected from six additional sites (Table 1, Fig. 1; and see Table S2 in Supplemental Materials available online). Out of the 402 samples, PCR detected *M. agassizii* in 64 samples, while 198 samples were qPCR-positive with Cq < 40.

Detection of *M. agassizii* via PCR produced similar results with slight agreement to two qPCR data sets (DS2, $n = 402$, $\kappa = 0.159$, $P < 0.001$; DS3, $n = 315$, $\kappa = 0.138$, $P < 0.01$; Table S3) in which the weakly positive samples were considered to be negative or excluded. The κ statistic between PCR and Data Set 1 was not significant (DS1, $n = 402$, $\kappa = 0.045$, $P = 0.23$).

Mycoplasmal Genetic Diversity and Geographic Structure

The PCR reactions for 16S rRNA, IGS, and rpoB detected *M. agassizii* in 49, 51, and 26 samples, respectively. Nucleotide diversity of aligned, trimmed sequences was two to three times higher in IGS (47 samples, 352 bp, $\pi = 0.0016$) than rpoB (23 samples, 548 bp, $\pi = 0.0005$) and 16S (47 samples, 453 bp, $\pi = 0.0007$). TCS network analyses for each genetic marker detected one principal haplotype, with additional haplotypes differentiated by one to three single-nucleotide polymorphisms, represented by a single sample (Fig. 2). A second haplotype was detected in five samples in the IGS marker (Fig. 2b). Despite this shared secondary haplotype, SAMOVAs did not detect any meaningful spatial distribution of haplotypes for any of the three genetic markers, with or without the inclusion of indels in the analyses (Table S5; Fig. S1). Because each genetic marker resulted in one common haplotype, we could not determine whether uncommon haplotypes were more or less associated with clinical signs of disease.

Range-wide Prevalence

Tortoises with MCL ≥ 100 mm were sampled from California ($n = 156$), Las Vegas ($n = 106$), and Northeast Mojave ($n = 135$) genotypes (Table 1). The following results were calculated using Data Set 1 to include all positive samples. In the California

genotype, 57% of tortoises tested positive by qPCR for *M. agassizii* (Cq < 40), and Chuckwalla Bench had lower prevalence than other California sites (25%, $t = –2.15$, $P = 0.03$; Fig. 3). Fifty-two percent of tortoises from the Las Vegas genotype had a detectable amount of *M. agassizii*. Of the sites within this genotype, Pahrump (25%, $t = –3.141$, $P = 0.002$) and South Las Vegas (45%, $t = –2.103$, $P = 0.04$) samples had significantly lower levels of prevalence compared to other sites in the Las Vegas genotype, and prevalence in the Northwest Vegas site was marginally different (50%, $t = –1.803$, $P = 0.07$). In the Northeast Mojave (36% prevalence), Gold Butte, Mormon Mesa/Halfway, Red Cliffs, and Zion all stood out with significantly lower prevalence ($P < 0.05$).

The GLMM did not detect effects of genotype, sex (female, $n = 168$; male, $n = 179$; unknown sex, $n = 47$), or MCL on presence of *M. agassizii* (in all cases, $P > 0.1$). There was an effect of year on *Mycoplasma* presence, with 2011 having higher prevalence than 2010 ($z = 2.354$, $P = 0.05$), and marginally higher prevalence than 2012 ($z = –2.173$, $P = 0.08$).

Clinical signs of URTD ($n = 91$) were marginally correlated with presence of *M. agassizii* in nasal lavages ($z = 1.879$, $P = 0.06$), and were only predicted by sampling year, with 2010 having a lower occurrence than both other sampling years (2011, $z = 2.502$, $P = 0.03$; 2012, $z = 2.860$, $P = 0.01$). When *Mycoplasma* presence was replaced with Cq value in the GLMM, Cq predicted presence of clinical signs ($z = −1.948$, $P = 0.05$), with similar effects of sampling year. Thus, animals with more *Mycoplasma* per sample generally had an increased likelihood of exhibiting clinical signs of URTD.

By site, there was a trend for the prevalence of *M. agassizii* to be negatively correlated with higher Cq values (inferring decreased average *Mycoplasma* load; $F_{1,18} =$

3.77, $R^2 = 0.17$, $P = 0.07$; Fig. 4). Importantly, the Cq variable does not represent a linear change in starting quantity of target DNA, but rather the number of PCR cycles necessary to reach a threshold. Thus, a decrease in Cq by 3.5 is associated with an approximately ten-fold increase in target DNA. Cq values from our sample DNA ranged from 28.98 in a sample from Ord Rodman to 39.93 in a sample from Gold Butte, representing a 1000-fold increase in mycoplasmal DNA from the strongest to weakest infection load detected.

DISCUSSION

Methods Comparison

From the kappa statistics, we found that PCR and qPCR provided similar diagnostic value on the scale of the individual host. The best agreement between these techniques occurred when weakly positive samples were considered to be negative, indicating that qPCR might be more sensitive than PCR. Further supporting this notion was the observation that most of the disagreement between the two techniques resulted from positive qPCR results corresponding with negative PCR results (20–40%, depending on the data set), whereas we detected up to 14.7% negative qPCR results corresponding with positive PCR results (Table S3). In addition to lower detection sensitivity, PCR and DNA sequencing is expensive and time-consuming compared to qPCR. Thus, as a diagnostic technique to detect this pathogen in nasal lavage samples from tortoises, we recommend qPCR over PCR, interpreting Cq values under 40 as positive for *M. agassizii*, as suggested in Braun et al. (2014). Although our previous work detected higher intra-assay variability at Cq values of 38 and above (Sandmeier et al. 2017), the high chemical specificity of a probe-based qPCR indicates that Type II errors

increase more than Type I errors at low DNA concentrations. Thus, even Data Set 1 could underestimate true prevalence of the microbe (Sandmeier et al. 2017), especially considering the imprecision of the nasal lavage sampling process.

Mycoplasmal Diversity

We sequenced three genetic markers of *M. agassizii* from Mojave Desert Tortoises and found very little genetic variation. The type strain of this pathogen (PS6) was cultured from a Mojave Desert Tortoise and had similar rpoB and IGS sequences to the mycoplasmas collected in our study, but its 16S rRNA sequence differed slightly from the main haplotype that we detected (Fig. 2). This type strain (PS6) is considered to be especially pathogenic (Brown et al. 1994, 2001), and the fact that it differs from the rest in 16S rRNA could indicate that the pathogen has evolved since its first discovery, or that we did not sample tortoises in areas where the type strain originally occurred (e.g., Desert Tortoise Natural Area, Kern County, CA). Importantly, this pathogenic strain was not detected in our survey.

The three genetic markers that we sequenced are in conserved regions of the genome or linked to conserved regions. In a previous report, multiple strains that differed in physical size and cell viability after storage were found in the host species (Brown et al. 2001). Whereas we did not find evidence of distinct, shared strains, the true determinant of genetic differentiation requires genome-level analyses of isolates from field samples. A better understanding of the *M. agassizii* genome could allow for identification of virulence factors, such as adhesins, which might be an important indicator of virulence at a population level of the host. If there are virulence factors associated with disease, it would be important to determine which local host populations

seem most at risk based on the *Mycoplasma* strains present. With this kind of information, managers could better address the implications of tortoise translocation programs and the potential effects of habitat-altering stressors on disease outbreaks at a local population scale.

Range-wide Patterns

Mycoplasmal URTD in Mojave Desert Tortoises is categorized by slow transmission and slow disease progression (Maloney 2011; Aiello et al. 2016). Previous work by Jacobson et al. (1995) found that tortoises appearing to be healthy could in fact be infected, and it is possible that an infection is not detectable by field techniques that preserve the tortoise's life. Although histology is the best determination of URTD (Jacobson et al. 1995), it is not a practical method to determine population-level disease and infection patterns in a threatened species. Whereas we detected samples that were qPCR-negative for *M. agassizii*, many more of our cultured field samples contained *M. agassizii*, even though its presence was not detected by pre-culture qPCR (C.L. Weitzman, personal observation). Thus, it is important to note that many more individuals likely harbor this pathogen than can be detected.

Although there is a downward trend in population sizes over most of the Mojave Desert Tortoise distribution (U.S. Fish and Wildlife Service 2015), since the crashes of 1989–1990 (Corn 1994; Peterson 1994), we know of only one crash in the mid 1990s, seemingly caused by drought (Longshore et al. 2003; reviewed in Sandmeier et al. 2009). *Mycoplasma agassizii* is widespread across its host's distribution, but to the best of our knowledge, no die-offs have been linked to disease in the last 25 yr, despite locations with high pathogen prevalence detected in our study (e.g., 84% in Fenner Valley). High

prevalence paired with low morbidity and mortality rates supports the hypothesis that this pathogen maintains colonies in the tortoise host, becoming infective at higher loads that could be triggered by environmental stressors or interactions within the upper respiratory microbiome.

We found that the presence of *M. agassizii* was associated with clinical signs of disease in individual tortoises, supporting previous findings of a similar correlation at the population level (Sandmeier et al. 2017). This correlation was tighter when only strongly positive samples were included in the pool of those positive for *M. agassizii* (Data Sets 2 and 3), indicating that a higher pathogen load might be more likely to cause URTD. Low pathogen loads might be attributable to recent colonization; alternatively, pathogen load could oscillate between high and low levels, with similar cycles in morbidity.

Currently, it is unknown how *M. agassizii* persists in hosts in a subclinical state. While *M. agassizii* is an extracellular pathogen that binds to the epithelium of its host (Jacobson et al. 1991), some predominantly extracellular mycoplasmas can also survive and reproduce within host cells, including *M. pneumoniae, M. penetrans*, *M. genitalium*, *M. gallisepticum*, and *M. fermentans* (Dallo and Baseman 2000; Rosengarten et al. 2000; Waites and Talkington 2004). It seems that this pattern of life-history traits might be common among *Mycoplasma* species. Hosts of *M. pneumoniae* might spread the pathogen without experiencing morbidity, and the presence of a carrier state might explain the cyclical epidemic trends in *M. pneumoniae* that occur every 3–5 yr (reviewed in Waites and Talkington 2004). Possibly, *M. agassizii* also has an intracellular stage or hides elsewhere in the respiratory tract of the host, where sampling cannot reach. Alternatively, the microbe could exist in such small population numbers that they are not

easily represented in lavage samples. The presence of infection cycles in this host-pathogen system could explain the differences that have been observed in *M. agassizii* prevalence among years and sites. Sandmeier et al. (2013) found higher serological prevalence in the Northeast Mojave tortoise genotype than in the California genotype, but our sampling for *Mycoplasma* by qPCR years later did not reveal a similar pattern. The large time delay between serological and pathogen assays could have allowed for cycling of disease dynamics, explaining why patterns from the serological survey did not correspond with similar patterns in the present pathogen survey. Additionally, if serology and pathogen presence are not correlated on a local population scale (sampling locality) at a single time point (see Sandmeier et al. 2017), then cyclical patterns in URTD-associated variables might also explain these discrepancies. Alternatively, tortoise immune responses to similar loads of *M. agassizii* could vary among populations, possibly caused by differences in thermal environment or nutrition (Sandmeier et al. 2013; Drake et al. 2016).

Most disease models focus on parameters of pathogen transmission. We propose that this tortoise/*Mycoplasma* system might be unique in the interplay between persistence in a long-lived host and rare transmission events. There are currently no available data on the recovery of tortoises from URTD, as the progression of this disease is extremely slow. If an individual host cannot clear itself of the pathogen, then even a low transmission rate could allow *M. agassizii* to accumulate across entire populations of Desert Tortoises, as this host species exhibits a long life-span and low turnover of adults (2%; U.S. Fish and Wildlife Service 1994). If that is the case, then predictive models need to incorporate the long-term persistence in individuals as well as in populations.

Lastly, it is important to note that pathogens other than *M. agassizii* are also associated with respiratory disease in *Gopherus* tortoises. *Mycoplasma testudineum* is another causative agent of URTD in Desert Tortoises (Jacobson and Berry 2012). *Pasteurella testudinis* and a herpes virus are also associated with tortoise respiratory disease (Snipes et al. 1980; Snipes and Biberstein 1982; Jacobson et al. 2012). Mycoplasmas can be present in co-infections in tortoises and other vertebrate species (Salinas et al. 2011; Ley et al. 2012), yet we know neither the extent to which co-infections of URTD-associated pathogens occur in *Gopherus* tortoises, nor the importance of co-infections in the development from sub-clinical to clinical disease. Although *M. agassizii* can cause URTD (Brown et al. 1994), it is possible that in wild tortoise populations, an interaction among pathogens is required before clinical signs of disease are detected.

Acknowledgments. We thank S. Snyder, M. Gordon, J. Jahner, and two anonymous reviewers for comments on previous versions of this manuscript. Our work was partially supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1447692. Funding was also provided by the U.S. Fish and Wildlife Service (1320-114-23DZ) and the Desert Legacy Fund from the Community Foundation. Work was conducted under the following permits: US Fish and Wildlife Service 10(a)1(A) Recovery Permit Nos. TE076710-7 and TE076710-8; California Department of Fish and Game Scientific Collecting Permit SC-007374; Nevada Department of Wildlife Scientific Collecting Permits S-33080 and S-35517; Utah Division of Wildlife Resources Permit 5COLL8886; National Parks Service Permits MOJA-2011-SCI-0022,

MOJA-2012-SCI-0009, ZION-2012-SCI-0006, and JOTR-2011-SCI-0021; and University of Nevada, Reno Institutional Animal Care and Use Committee Protocols 00465 and 00555. We thank M. Tuma for field samples, M. Teglas and N. Nieto for laboratory assistance and advice, and multiple field and laboratory technicians.

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TABLE 1. Sampling localities by tortoise genotype and their associated sampling year(s), sample sizes, prevalence of *Mycoplasma agassizii*, and average Cq value. Sample sizes in parentheses are quantities used in population-level analyses if different from *n* to exclude four tortoises < 100 mm midline carapace length and one tortoise from the large-scale translocation site for which we do not know its pre-translocation source site (and thus its genotype). Data on qPCR prevalence for Data Sets 2 and 3 are provided in Table S2.

Tortoise genotype	Year(s) sampled	Site	*n*	Percent positive (PCR)	Percent positive (qPCR, Data Set 1)	Average Cq
California	2011, 2012	Chemehuevi	16 (15)	6.7	60.0	37.55
	2011, 2012	Chuckwalla Bench	20 (19)	31.6	26.3	36.65
	2011, 2012	Fenner Valley	19	21.1	84.2	37.84
	2011	Fort Irwin Study Site	10	10.0	90.0	36.3
	2010	Fremont-Kramer	21	0.0	33.3	37.6
	2011, 2012	Kelso Wash	16	25.0	31.3	38.13
	2010	Laughlin	1	100.0	0.0	
	2011, 2012	Ord Rodman	19	26.3	73.7	36.03
	2011, 2012	Pinto Mountains / Joshua Tree	15	0.0	80.0	36.76
	2010, 2011	Piute Valley	21	4.8	57.1	36.1
Las Vegas	2010	Eldorado Valley	15	0.0	80.0	38.09
	2011, 2012	Ivanpah	16	37.5	56.3	37.54
	2010, 2012	Northwest Vegas	21 (20)	10.0	50.0	37.73
	2010, 2012	Pahrump	16	25.0	25.0	39.31
	2011	River Mountain Valley	13	23.1	69.2	35.9
	2011, 2012	Shadow Valley	5	20.0	40.0	38.74
	2010, 2011	South Las Vegas	21 (20)	35.0	45.0	37.06
	2010	Yucca Mountain	1	0.0	0.0	
	2010	Large-Scale Translocation Site	1 (0)	0.0	100.0	34.06
Northeast Mojave	2010	Bitter Springs	2	0.0	100.0	36.06
	2010, 2011	Coyote Springs	16	56.3	68.8	36.6
	2010	Gold Butte / Pakoon	47	6.4	29.8	37.46
	2010	Mormon Mesa / Halfway	17	17.6	0.0	
	2011, 2012	North Coyote Springs	21	9.5	61.9	37.31
	2011	Paranaghut Valley	1	0.0	0.0	
	2012	Red Cliffs	21	23.8	28.6	36.59
	2012	Zion	10	0.0	30.0	38.76

FIGURE 1. Sampling sites of *Gopherus agassizii* from 2010–2012. Colors denote tortoise genotype (following Hagerty and Tracy 2010): California = blue, Las Vegas = green, Northeast Mojave = orange. Map made with USGS imagery from nationalmap.gov.

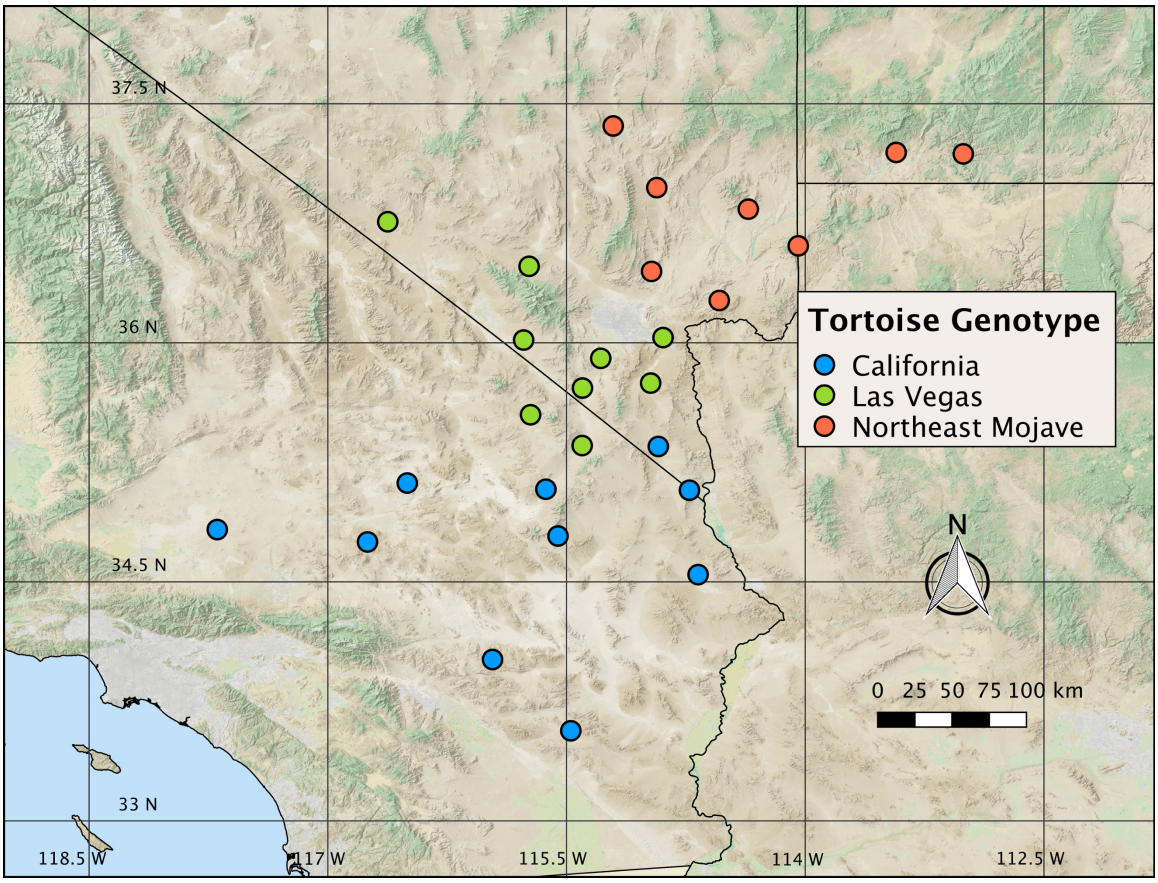


FIGURE 2. TCS haplotype networks for three genetic markers of *Mycoplasma agassizii*. Circle sizes are based on number of individuals in the haplotype. Colors denote tortoise genotype (following Hagerty and Tracy 2010): California = blue, Las Vegas = green, Northeast Mojave = orange, GenBank sequences = black. (a) 16S rRNA; (b) 16-23S intergenic spacer: of the GenBank sequences, PS6 shares the common haplotype and Karina09 is separated by one single-nucleotide polymorphism from the secondary haplotype; (c) ribosomal polymerase beta subunit.

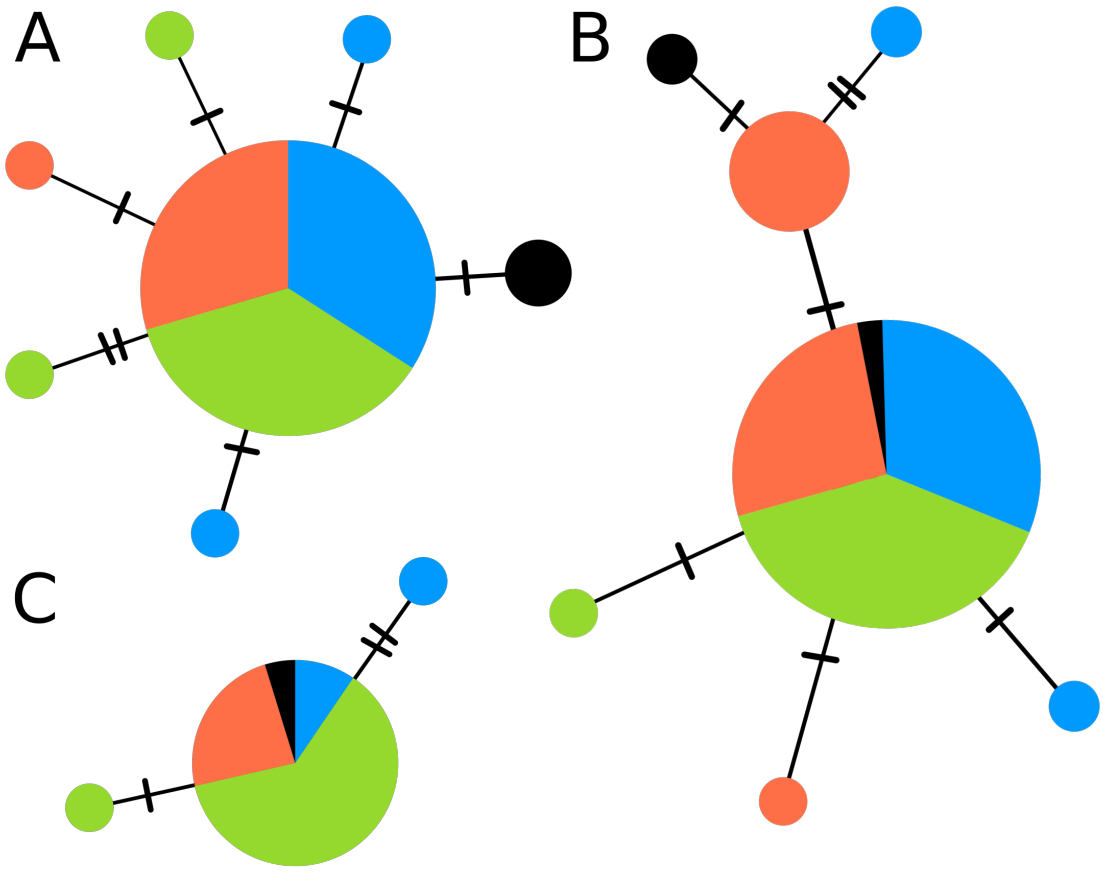


FIGURE 3. Prevalence of *Mycoplasma agassizii* at 21 sites in the Mojave Desert where at least 10 tortoises were sampled. Map made with USGS imagery from http://nationalmap.gov.

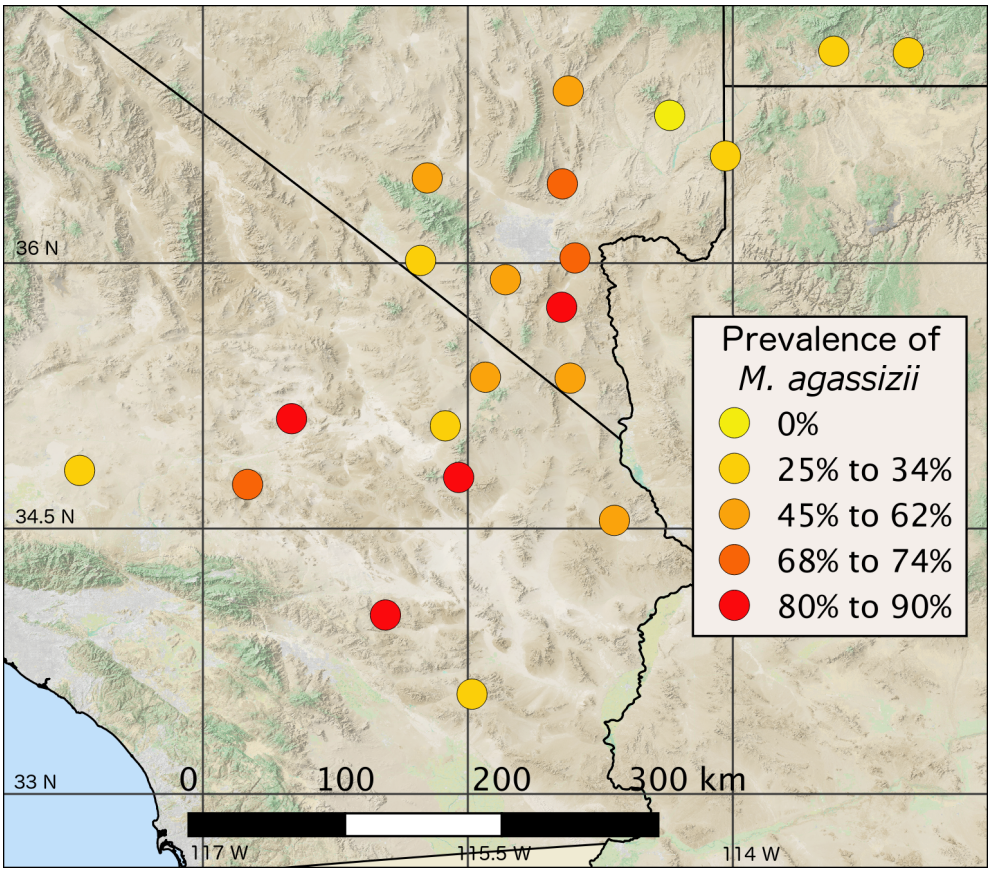
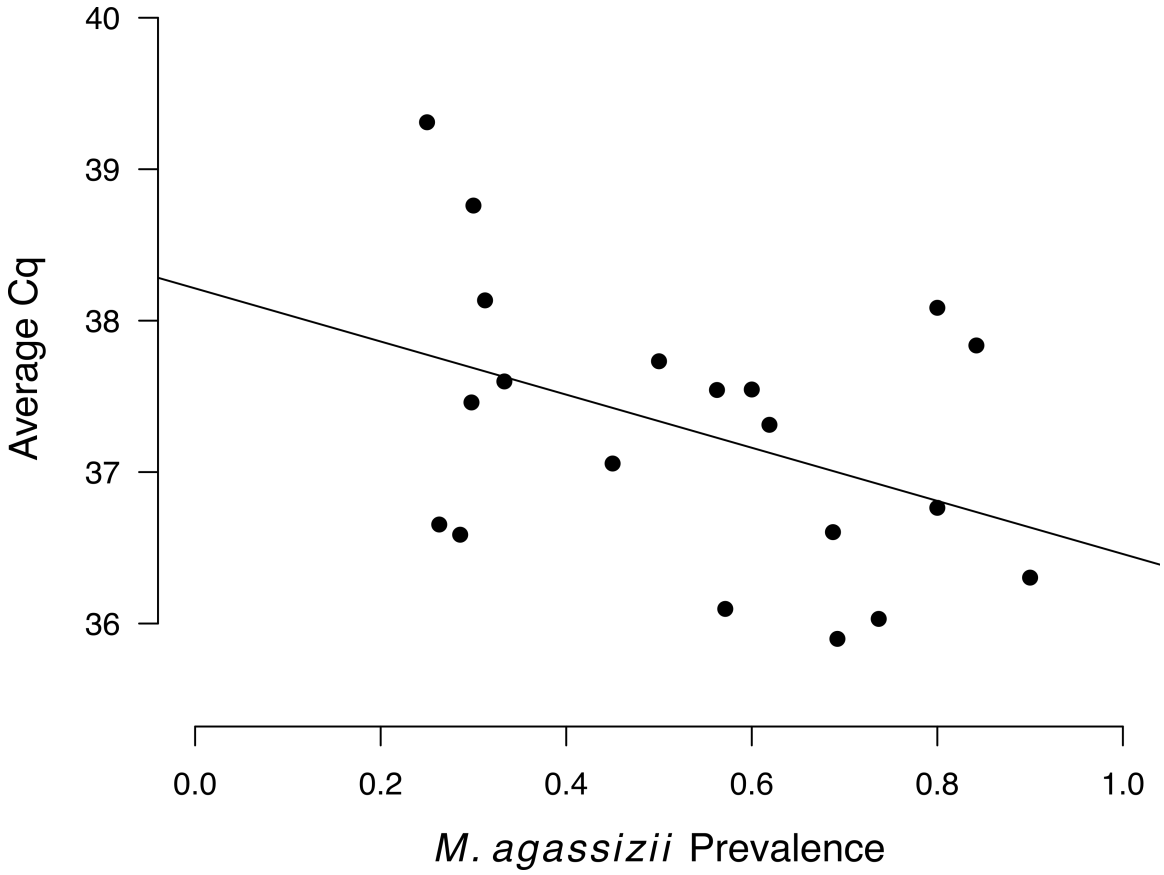


FIGURE 4. Average Cq value as a function of qPCR prevalence of *Mycoplasma agassizii* by tortoise sampling site (Data Set 1, $R^2 = 0.17$, $P = 0.07$).



APPENDIX

Table S1. PCR protocols for three genetic markers of *Mycoplasma agassizii*. All PCRs were conducted with Qiagen Master Mix, a 15-minute hot-start at 95°C, 95°C denaturation period, 72°C extension period, 35 cycles, and a final extension for 5 minutes at 72°C (7 minutes for rpoB).

Locus	PCR	Primer Length	Primer Sequence	Denaturation Time (min.)	Annealing Temp (°C)	Annealing Time (min.)	Extension Time (min.)	Primer Reference
16S	PCR	22	FOR-agagtttgatcctggctcagga	0:45	59	1:00	2:00	(Robertson et al. 1993; Brown et al. 2001) (van Kuppeveld et al. 1992; Brown et al. 2001)
		27	REV-tgcaccatctgtcactctgttaacctc					
	Nested PCR	24	FOR-agcggcaaatgggtgagtaacacg	0:45	58.5	1:00	2:00	
		20	REV-ccccacgctttcgtccctca					
IGS	PCR	20	FOR-gtaatgcccaaagtcggttt	1:00	59.5	1:00	1:30	
		20	REV-gattcagacagggtctcacg					
	Nested PCR	20	FOR-ttcggaagcgactgcctaag	1:00	60.5	1:00	1:30	
		20	REV-gcagtcctacaaccccacaa					
rpoB	PCR and pseudo-nested PCR	20	FOR-gggatgggtggacttaaccg	1:00	60	1:00	2:00	
		20	REV-cgttgtcccccgttttgaga					

Table S2. Sampling localities by genotype and their associated sample sizes, median distances between each tortoise and its nearest neighbor, levels of prevalence of *M. agassizii*, and average Cq value. Sample sizes in parentheses are quantities used in population-level analyses.

Tortoise Genotype	Site	n	Percent Positive (PCR)	Percent Positive (qPCR, Data Set 1)	Percent Positive (qPCR, Data Set 2)	Percent Positive (qPCR, Data Set 3)	Average Cq
California	Chemehuevi	16 (15)	6.7	60.0	40.0	50.0	37.55
	Chuckwalla Bench	20 (19)	31.6	26.3	15.8	17.6	36.65
	Fenner Valley	19	21.1	84.2	47.4	75.0	37.84
	Fort Irwin Study Site	10	10.0	90.0	80.0	88.9	36.30
	Fremont-Kramer	21	0.0	33.3	14.3	17.6	37.60
	Kelso Wash	16	25.0	31.3	18.8	21.4	38.13
	Laughlin	1	100.0	0.0	0.0	0.0	
	Ord Rodman	19	26.3	73.7	57.9	68.8	36.03
	Pinto Mountains/Joshua Tree	15	0.0	80.0	53.3	72.7	36.76
	Piute Valley	21	4.8	57.1	42.9	50.0	36.10
Las Vegas	Eldorado Valley	15	0.0	80.0	33.3	62.5	38.09
	Ivanpah	16	37.5	56.3	31.3	41.7	37.54
	Northwest Vegas	21 (20)	10.0	50.0	5.0	9.1	37.73
	Pahrump	16	25.0	25.0	0.0	0.0	39.31
	River Mountain Valley	13	23.1	69.2	53.8	63.6	35.90
	Shadow Valley	5	20.0	40.0	0.0	0.0	38.74
	South Las Vegas	21 (20)	35.0	45.0	25.0	31.3	37.06
	Yucca Mountain/Beatty	1	0.0	0.0	0.0	0.0	
	Large-Scale Translocation Site	1 (0)	0.0	100.0	100.0	100.0	34.06
Northeast Mojave	Bitter Springs	2	0.0	100.0	100.0	100.0	36.06
	Coyote Springs	16	56.3	68.8	50.0	61.5	36.60
	Gold Butte/Pakoon	47	6.4	29.8	10.6	13.2	37.46
	Mormon Mesa/Halfway	17	17.6	0.0	0.0	0.0	
	North Coyote Springs	21	9.5	61.9	23.8	38.5	37.31
	Paranaghut Valley	1	0.0	0.0	0.0	0.0	
	Red Cliffs	21	23.8	28.6	19.0	21.1	36.59
	Zion	10	0.0	30.0	10.0	12.5	38.76

Table S3. Sample-level comparison of PCR and qPCR results. Data Set 1 – all Cq values categorised as positive, all other samples are negative; Data Set 2 – only strongly positive samples (Cq < 38) are categorised as positive for *M. agassizii*, all other samples are categorised as negative; Data Set 3 – results from weakly positive samples (Cq > 38) are excluded from analysis, with only strongly positive samples (Cq < 38) categorised as positive and only samples with no Cq value categorised as negative. Cohen's kappa (κ) and p-values from calculated unweighted kappa statistics.

		qPCR Data Set 1		qPCR Data Set 2		qPCR Data Set 3	
		$\kappa = 0.0453$, $P = 0.23$		$\kappa = 0.159$, $P < 0.001$		$\kappa = 0.138$, $P < 0.01$	
		+	−	+	−	+	−
PCR	+	38	30	30	38	30	30
	-	160	174	81	253	81	174

Table S4. Sites represented in SAMOVAs for each genetic marker.

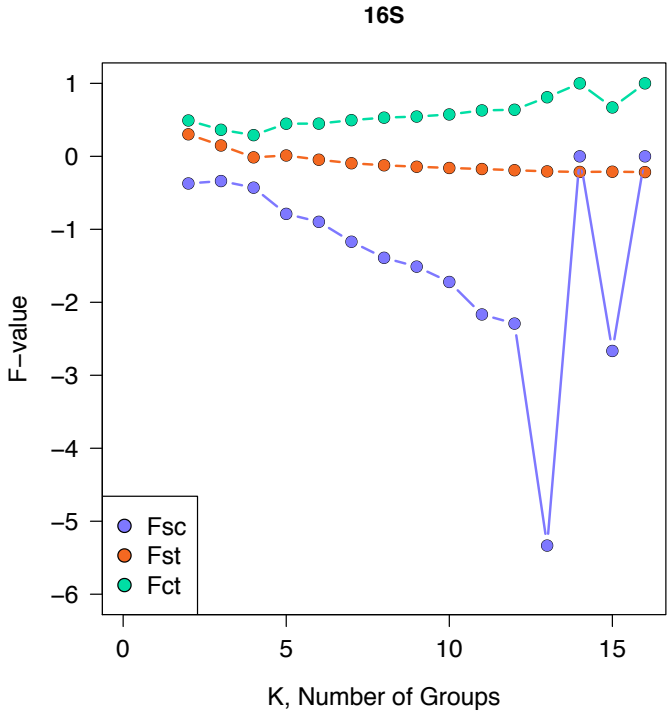
Site	16S rRNA	IGS	rpoB
Chuckwalla Bench	yes	yes	yes
Coyote Springs	yes	yes	yes
Fenner Valley	yes	yes	no
FISS	yes	no	no
Gold Butte	yes	yes	no
Ivanpah	yes	yes	yes
Kelso Wash	yes	yes	no
Laughlin	yes	yes	no
Mormon Mesa	yes	yes	no
North Coyote Springs	yes	yes	yes
NW Vegas	yes	yes	yes
Ord Rodman	yes	yes	yes
Pahrump	yes	yes	yes
Piute	no	yes	no
Red Cliffs	yes	yes	no
River Mtn. Valley	yes	yes	yes
S. Las Vegas	yes	yes	yes
Shadow Valley	yes	yes	yes

Table S5. Optimal number of groups of sampling sites from SAMOVAs, and site groupings associated. Sites not listed are each assigned individual groups.

SAMOVA	Optimal K	Group 1	Group 2	Group 3
16S	14	Laughlin Red Cliffs	FISS Pahrump	NW Vegas North Coyote Springs
16S with indels	15	Laughlin Red Cliffs	FISS NW Vegas	
IGS	2	[Piute is alone, remaining are grouped together]		
IGS with indels	16	Laughlin Piute		
rpoB	8	NW Vegas Shadow Valley North Coyote Springs		
rpoB with indels	8	Chuckwalla Bench NW Vegas North Coyote Springs		

Figure S1. *F*-values from SAMOVA 1.0 with trimmed haplotypes with and without indels. Colors indicate *F*-statistics: F_{sc} = purple, F_{st} = orange, F_{ct} = green. a/b) 16S without and with indels; c/d) 16-23S intergenic spacer without and with indels; e/f) rpoB without and with indels.

a



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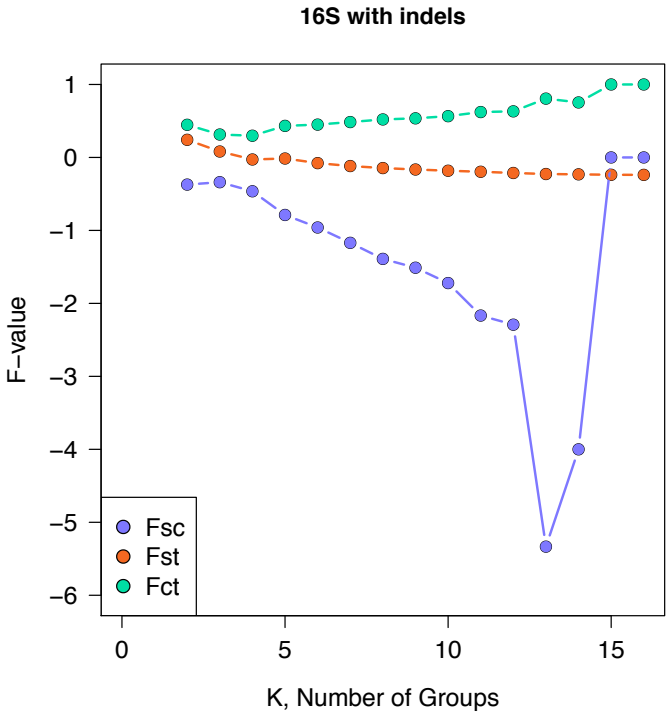
16S with indels
F−value
K, Number of Groups
Fsc
Fst
Fct
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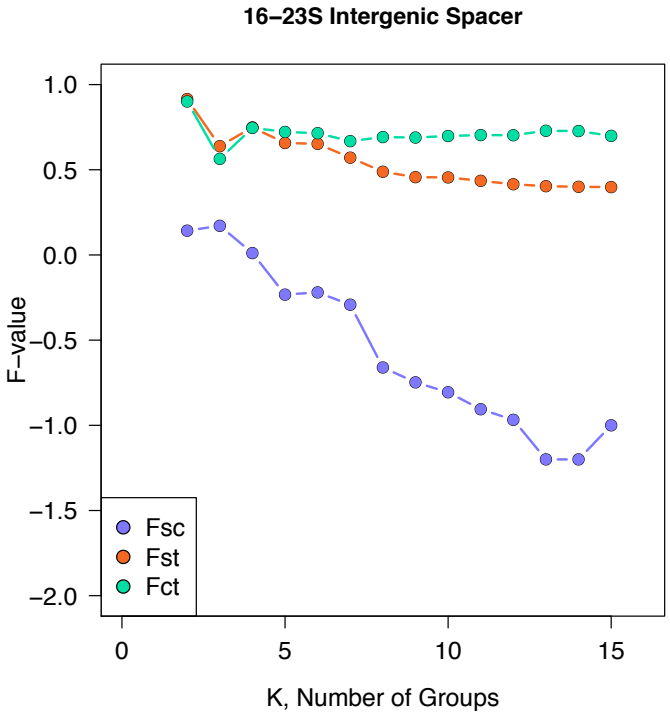
−2
−3
−4
−5
−6
0
5
10
15

c


16−23S Intergenic Spacer
F−value
K, Number of Groups
Fsc
Fst
Fct
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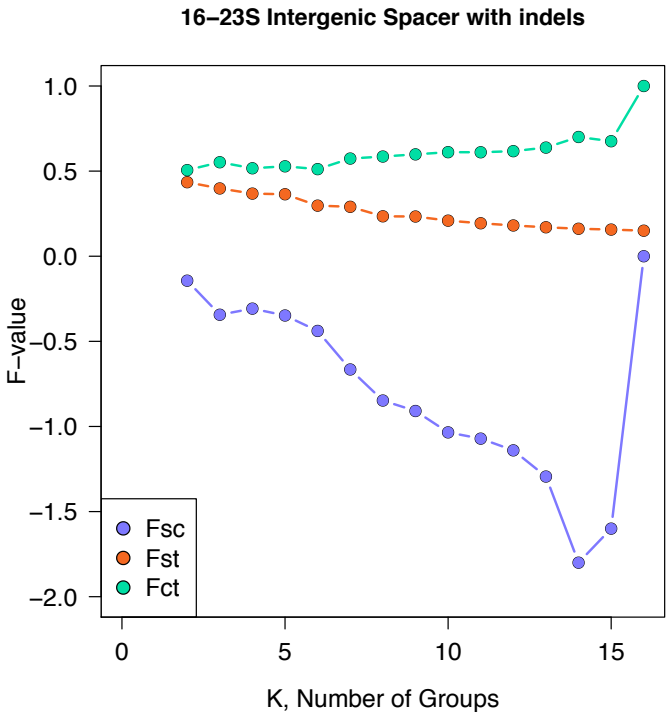
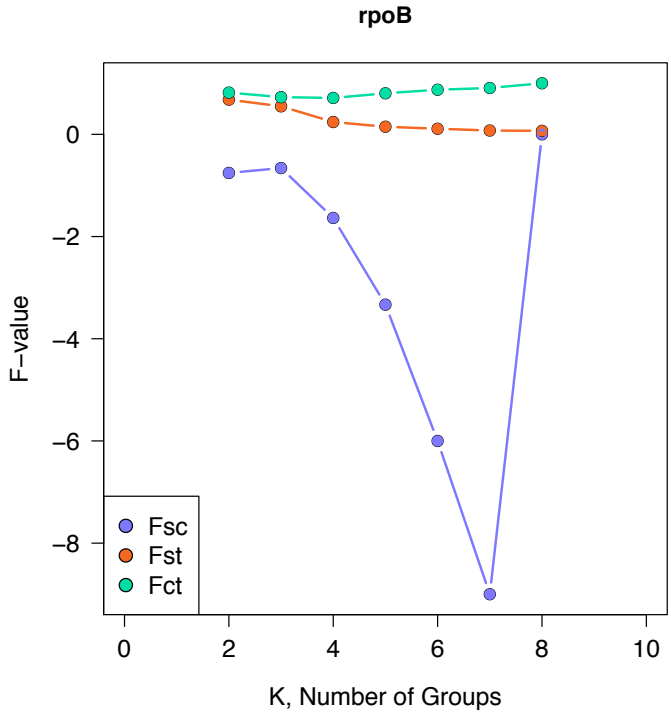
0.5
0.0
−0.5
−1.0
−1.5
−2.0
0
5
10
15

d


16–23S Intergenic Spacer with indels
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
F−value
Fsc
Fst
Fct
0
5
10
15
K, Number of Groups

e


rpoB
0
−2
−4
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0
2
4
6
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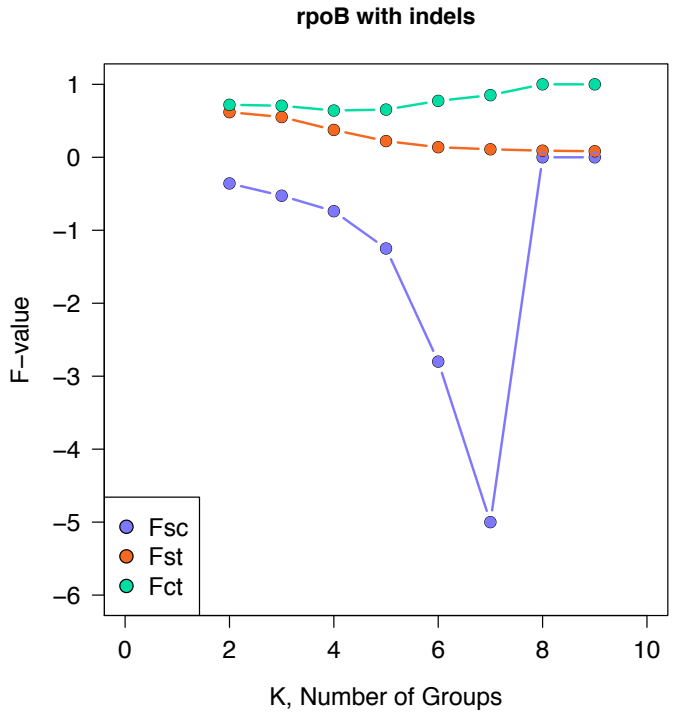
10
K, Number of Groups

f



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CHAPTER 2: CO-INFECTION DOES NOT PREDICT DISEASE IN *GOPHERUS* TORTOISES

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ABSTRACT

In disease ecology, the host immune system interacts with the environmental conditions of the host and properties of the pathogen(s) to affect the impact of disease on the host. Within the host, pathogens also may interact to facilitate or inhibit each other's growth. In addition, pathogens interact with different hosts in different manners, allowing some individuals to be carriers of pathogens that can cause great harm to others. We investigated co-infection patterns of two *Mycoplasma* pathogens (*M. agassizii* and *M. testudineum*) and the association of infection with clinical signs of disease in four congeneric tortoise host species (*Gopherus*) in the United States as a means to detect differences in infection risk and disease dynamics in these species. Mojave desert tortoises had greater prevalence of *M. agassizii* than both Texas tortoises and gopher tortoises, while there were no differences in *M. testudineum* prevalence among the host species. Patterns of sole infection and co-infection were statistically similar among host species. In some, but not all host species, the presence of each pathogen influenced the infection intensity of the other pathogen species. Hence, these two pathogens of *Mycoplasma* interact differently within different hosts. Neither infection, nor co-infection, were associated with clinical signs of disease. To detect strain variation, which may influence disease outcome if strains have variable pathogenicity, we sequenced three genetic markers of *M. agassizii* and detected no meaningful differentiation of haplotypes among host species. Experimental inoculation studies, as well as recurrent resampling of wild individuals, could help to decipher the underlying mechanisms of disease dynamics in this system.

Key words: co-infection, disease ecology, *Gopherus*, *Mycoplasma*, parasite, quantitative PCR, Testudinidae, turtle, URTD

INTRODUCTION

The manifestation of disease depends on interactions between the host and pathogen, in relation to the environment of the host. Pathogens can directly or indirectly affect other pathogens when co-infecting a host, and these microbial interactions can, in themselves, positively or negatively affect the survival of each pathogen within the host (e.g., Telfer et al. 2010). Mutualistic, commensalistic, or neutral interactions of pathogens within a host can result in additive or synergistic rates of clinical signs of disease. Alternatively, co-infection of pathogens that interact with each other negatively could result in disease with lower impact than what is expected additively.

Species in the tortoise genus *Gopherus* range from the Mojave Desert in the western United States to the longleaf pine forests of the southeastern U.S., with different tortoise species inhabiting distinct vegetative ecosystems and climates. Four *Gopherus* spp. are found in the United States: Mojave desert tortoises, *Gopherus agassizii*; Sonoran desert tortoises, *G. morafkai*; Texas tortoises, *G. berlandieri*; and gopher tortoises, *G. polyphemus*, which are broadly distributed in the southeastern U.S. Additionally, multiple pathogens are known to be associated with URTD in *Gopherus*, including *Mycoplasma agassizii*, *M. testudineum*, *Pasteurella testudinis*, an iridovirus, and Testudinid herpesvirus 2 (Snipes et al. 1980, Snipes and Biberstein 1982, Brown et al. 1994, 2004, Westhouse et al. 1996, Jacobson et al. 2012). Thus, there is potential for different biological interactions among hosts and pathogens in the different ecosystems inhabited

by different host species. Previous studies have detected upper respiratory tract disease (URTD), and pathogens known to cause this disease, in each of the four tortoise species found in the U.S. (Jacobson et al. 1991, Judd and Rose 2000, Diemer Berish et al. 2000, Jones 2008, Guthrie et al. 2013). Nevertheless, the majority of research on URTD has focused on *M. agassizii*.

Population declines in Mojave and gopher tortoises have been hypothesized to be caused by URTD, although the actual impacts of this disease to individuals and populations remains controversial (McCoy et al. 2007, Sandmeier et al. 2009). Understanding disease dynamics in this system is especially important as both the Mojave desert tortoise (U.S. Fish and Wildlife Service 1994) and the gopher tortoise in the western portion of its range (U.S. Fish and Wildlife Service 1990) are listed as threatened under the Endangered Species Act of 1973.

Here, we report on the prevalence of two pathogens in the tortoise-URTD system (*M. agassizii* and *M. testudineum*) in the four tortoise species within the U.S. to establish whether the occurrence or co-infection of these pathogens affects the manifestation of clinical signs of disease differently among hosts. We use nasal flushes to sample nasal microbes and visual observations of clinical signs of disease to answer: 1) Are there genetically different bacterial types of the pathogen *M. agassizii* among tortoise species? 2) At what frequencies are the pathogens *M. agassizii* and *M. testudineum*, both individually and in co-infection, present in local tortoise populations among tortoise species? 3) Does the presence or co-infection of these pathogens predict the presence of clinical signs of disease?

METHODS

Sampling and DNA Extraction

We used a 3 mL saline (0.9% NaCl) nasal lavage (Brown et al. 2002; Weitzman et al. in press) to sample upper respiratory *Mycoplasma* from four species of wild-caught *Gopherus* tortoises. Nasal lavage samples were immediately preserved in RNAlater stabilization solution (Ambion Inc., Austin, Texas, USA) at a ratio of 1 part sample to 5 parts preservative volume. Samples were placed on ice in the field and frozen within 12 hours. Each tortoise was sampled once, providing a snapshot of disease and pathogen patterns. All tortoises were captured by hand, except for gopher tortoises, which generally were trapped either with a pitfall trap or other live trap. While handling each tortoise, we recorded midline carapace length (MCL) and clinical signs of URTD. Clinical signs were scored based on severity: 0 = no signs; 1 = wheezing breath and/or damaged scales around nares; 2 = occluded nares; 3 = serous discharge from nares; 4 = purulent discharge from nares; 5 = nasal exudate and deeply damaged nares; 6 = nasal exudate and poor body condition (Sandmeier et al. 2017). Data on clinical signs were not available for 13 sampled Texas tortoises. We used secondary sex characteristics (Goin and Goff 1941, Woodbury and Hardy 1948) to determine the sex of sampled tortoises in all adults. Juveniles were classified as animals without clear secondary sex characteristics and were, on average, 40–60% the average adult size.

Using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California, USA) protocol for gram-negative bacteria, we extracted DNA from 500 μL of preserved lavage sample. We conducted polymerase chain reaction (PCR) for three genetic markers of *M. agassizii* (16S ribosomal RNA (rRNA), 16-23S intergenic spacer

region (IGS), and ribosomal polymerase beta subunit (rpoB); Weitzman et al. in press) to detect genetic variation of this pathogen among tortoise species. PCR products of appropriate length were extracted from agarose gel using the Qiagen QIAquick Gel Extraction Kit and sequenced at the Nevada Genomics Center with an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA). Sequences were identified as *Mycoplasma* using NCBI's BLAST search (http://blast.ncbi.nlm.nih.gov).

Differentiation of *Mycoplasma agassizii* Among *Gopherus* spp.

A TCS haplotype network (Clement et al. 2000) was made for each of the three genetic markers from amplified sequences using PopART (Leigh and Bryant 2015). Sequences were aligned in ClustalX 2.1 (Larkin et al. 2007) and trimmed in MEGA6 (Tamura et al. 2013). Because sequences were of varying length, haplotype networks only included sequences at least 75% of the average length for each genetic marker. *M. agassizii* sequences from GenBank (http://www.ncbi.nlm.nih.gov) were included in haplotype networks for all markers (type strain PS6 (Brown et al. 2001) from a *Gopherus agassizii* individual: Accession numbers U09786.1, AY780802.1, EU925153.1). Additional laboratory sequences from the ATCC strain 723 cultured from a *Gopherus polyphemus* were included in the haplotype networks for 16S rRNA and IGS.

Infection and Co-Infection Analyses

We used a multiplex quantitative PCR (qPCR) protocol for *M. agassizii* and *M. testudineum* to detect both pathogens (Braun et al. 2014). qPCRs were repeated in triplicate for each sample with thresholds determined by serial dilution curves. Samples that amplified in at least two reactions with a Cq value under 40 were considered positive for either pathogen of interest (Braun et al. 2014).

We used generalized linear models to detect differences in prevalence of *M. agassizii* and *M. testudineum* among tortoise species. Tukey's post-hoc tests evaluated pairwise differences between tortoise host species. Analyses were run in the programming language R using the multcomp package for post-hoc analyses (version 3.2.1; Hothorn et al. 2008, R Development Core Team 2015).

Samples were assigned the following infection codes based on qPCR results: 0 = neither *M. agassizii* nor *M. testudineum* detected, 1 = positive for *M. agassizii* only, 2 = positive for *M. testudineum* only, or 3 = positive for both *Mycoplasma* spp.

We used the multinom function in the nnet package (Venables and Ripley 2002) to conduct multinomial logistic regressions of sex and species or site to predict infection codes and Tukey's post-hoc tests to evaluate differences among groups in the lsmeans package (Lenth 2016). We corrected for the error rate due to false discovery in multinomial logistic regression post-hoc tests using the Benjamini Hochberg method, with a false discovery rate of 0.05, allowing for a highest significant p-value of 0.0183 (Benjamini and Hochberg 1995, Benjamini and Yekutieli 2001).

To help determine under what circumstances hosts contract disease, we used generalized linear models to detect whether host sex, site, and infection code significantly predict the presence of clinical signs for each host species. We used linear models to detect a correlation of infection intensity between the two *Mycoplasma* spp. (using Cq value as a proxy).

RESULTS

We sampled 256 tortoises between 2010 and 2015, including three geographic sampling sites for each species (Table 1, Figure 1). We used PCR with Sanger sequencing to detect genetic diversity of three markers in the *M. agassizii* genome, and we detected *M. agassizii* in 27 samples using this method (16S rRNA: n = 9; 16-23S intergenic spacer: n = 19; rpoB: n = 9). We produced TCS haplotype networks from these data (Figure 2), which did not suggest clear differentiation of *M. agassizii* populations among tortoise host species. In two *Mycoplasma* genetic markers (16S rRNA and rpoB), this microbe is only represented from two of the four tortoise hosts sampled.

Using a probe-based qPCR assay (Braun et al. 2014), we detected *M. agassizii* in 92 samples and *M. testudineum* in 65 samples, ranging in prevalence across the tortoise host species (Figure 3). In generalized linear models, host species differed in prevalence of *M. agassizii*: desert tortoise species had higher prevalence than Texas tortoises ($P < 0.001$ each), and Mojave desert tortoises had higher prevalence than gopher tortoises ($z = -4.768$, $P < 0.001$). Prevalence of *M. testudineum* did not differ by host species ($P > 0.4$ each; Figure 3). In Texas tortoises, every individual with detectable *M. agassizii* was also co-infected with *M. testudineum* (Figure 3). Infection load of each pathogen was relatively low (average Cq of 37.5±1.52 (s.d.) for *M. agassizii*, 37.2±1.80 for *M. testudineum*), and Cq values ranged from 30.9 to 39.9, representing an approximately 200–500 fold increase (depending on the pathogen) from the lowest to the highest load detected. These high Cq values indicate a generally low infection load of either pathogen. Of the samples positive for *Mycoplasma*, only 6 (*M. testudineum*) or 7 (*M. agassizii*) samples had a Cq value under 35.

For the following analyses, tortoise samples were categorized into four infection groups from qPCR results: 0 = neither *Mycoplasma* detected; 1 = *M. agassizii* detected; 2 = *M. testudineum* detected; 3 = both *Mycoplasma* spp. detected. Presence of these infection groups were analyzed using multinomial logistic regressions. In pairwise comparisons, all host species significantly differed in patterns of these infection groups ($P < 0.015$ each).

Mojave desert tortoise infection

A similar amount of Mojave desert tortoise samples were either uninfected or infected with just *M. agassizii*, and fewer samples had a single infection of *M. testudineum* or co-infection of both species of *Mycoplasma* (Table 1). Tortoises from Eldorado Valley had lower prevalence of *M. testudineum* in sole infection versus co-infection. More individuals in Fenner Valley had a sole infection of *M. agassizii* than *M. testudineum* or co-infection of both mycoplasmas. In Red Cliffs tortoises, lack of infection was more frequent than sole infection with *M. testudineum*.

Infection types were similarly represented in juvenile Mojave desert tortoises (n = 5), while in both adult males and females, *M. agassizii* infection rate was greater than *M. testudineum* infection (Table 2).

Sonoran desert tortoise infection

Regarding infection in Sonoran desert tortoises, more individuals were uninfected by either *Mycoplasma* than infected with one or both pathogens. In Cave Buttes, prevalence of either pathogen in sole infection was lower than absence of infection (Table 1). Infection prevalence of any type was significantly lower than frequency of no infection in Silverbell tortoises. In Sugarloaf tortoises, there was a difference in

prevalence of sole infection between the two *Mycoplasma* spp., with higher prevalence of *M. agassizii*.

Juvenile Sonoran desert tortoises (n = 2) did not have detectable infection. In females, more individuals were uninfected than infected with just *M. testudineum*, while frequency of the remaining infection groups were statistically similar. In males, absence of infection was more frequent than all other infection groups (Table 2).

Texas tortoise infection

In Texas tortoises, no individuals had *M. agassizii* as a sole infection, and absence of any infection was more common than presence of *M. testudineum* or co-infection (Table 1). At Chaparral WMA, no samples were positive for *M. agassizii*, and thus no individuals had co-infection of both *Mycoplasma* spp., but presence and absence of *M. testudineum* occurred at similar rates. Tortoises from East Rio Grande had *M. testudineum* infection less frequently than no infection, and at West Rio Grande, absence of infection was more frequent than either *M. testudineum* infection or co-infection (Table 1).

A similar number of juvenile Texas tortoises (n = 13) had co-infection, *M. testudineum* infection, and no infection (Table 2). In adults, however, absence of either pathogen was more frequent than co-infection, and females were also more likely to have no infection than *M. testudineum* infection alone.

Gopher tortoise infection

Pairwise comparisons in gopher tortoises discovered significantly more individuals with an absence of infection than any other infection status (Table 1). At Perdido and Rayonier sites, the rate of no infection was greater than any other infection

group. In Perdido tortoises, prevalence of *M. agassizii* in sole infection was higher than prevalence of *M. testudineum*. At the University of South Florida site, absence of infection was more frequent than sole infection with either *Mycoplasma*, but not more frequent than co-infection (Table 1).

In female gopher tortoises, infection rate of any type was significantly lower than no infection. In males, infection rate of *M. testudineum* was lower than absence of infection, and sole infection of either *Mycoplasma* occurred at lower rates than absence of infection in juveniles (Table 2).

Clinical Signs of Disease

From generalized linear models, none of the infection groups significantly predicted the presence of clinical signs of URTD for any host species, and most of the tortoises with clinical signs of disease did not have detectable amounts of either *M. agassizii* or *M. testudineum* in their upper respiratory tracts ($P > 0.1$; Figure 4). In gopher tortoises, we found that individuals from the University of South Florida site had significantly lower rates of clinical signs of URTD than Perdido tortoises (USF 3%, Perdido 28%, Tukey's post-hoc, $z = –2.408$, $P = 0.041$).

Although we found clinical signs of disease in the form of nasal discharge or tissue damage from recent discharge in each of the four tortoise species, none of the Texas or Sonoran desert tortoises sampled had nasal mucus present at the time of sampling. Rather, clinical signs of disease in those species manifested as eroded scales around the nares or occluded nares (severity score 1–2). Serous nasal mucus (severity score 3) was found in gopher (n = 3) and Mojave desert (n = 6) tortoises at the time of sampling. One additional gopher tortoise had severely damaged nares and purulent nasal

mucus (severity score 5), and we did not detect either species of *Mycoplasma* in her lavage sample.

Inter-Pathogen Associations

A greater *M. agassizii* load (lower Cq value) was correlated with the presence of *M. testudineum* in tortoise noses ($F_{(1,90)} = 5.49$, $P = 0.02$). This relationship was likely driven by its significance in the Mojave desert tortoise ($F_{(1,37)} = 6.428$, $P = 0.016$; Figure 5a). Because all Texas tortoises with detectable *M. agassizii* also had *M. testudineum*, we could not compare differences in *M. agassizii* load between presence and absence of *M. testudineum*.

Within all host species combined, the presence of *M. agassizii* in nasal lavage samples was marginally associated with less *M. testudineum* ($F_{(1,63)} = 3.859$, $P = 0.054$), though in gopher tortoises, more *M. testudineum* was correlated with the presence of *M. agassizii* ($F_{(1,18)} = 4.447$, $P = 0.049$; Figure 5b).

Finally, an increase in amount of either bacterium correlated with an increase in amount of the other species ($F_{(1,39)} = 7.39$, $P = 0.0097$; Figure 5c). However, no host species followed this pattern without it being driven by a single, strongly-infected sample.

DISCUSSION

We detected both *Mycoplasma agassizii* and *M. testudineum* in all four tortoise species inhabiting the United States. These pathogens have been reported to cause upper respiratory tract disease (URTD) in tortoise hosts, and some have hypothesized that URTD has caused population declines in Mojave desert tortoises and gopher tortoises

(Brown et al. 1994, 1999, 2004, Seigel et al. 2003, Sandmeier et al. 2009, Jacobson et al. 2014). We aimed to decipher the evolutionary history of this pathogen and detect the bacterial strain diversity within and among host populations with sequences of three genetic markers of *M. agassizii*. Multiple strains of *M. agassizii* have been found in Mojave desert tortoises (Brown et al 2001), but no efforts have since been made to determine the variation of *Mycoplasma* diversity within and among hosts. If some strains are more associated with disease than others, then relevant host populations could be appropriately managed. However, we did not detect any meaningful differentiation of this pathogen among its tortoise hosts. Because our current understanding of the *M. agassizii* genome lies in its conserved regions, we suggest that genomic data of these bacteria would allow us to determine bacterial strain diversity within and among host populations, and potentially to discover the relative virulence of strains. However, variable virulence can also occur with minimal genetic diversity (Razin et al. 1998). Even clonal mycoplasmas can present high diversity of surface antigens through phenotypic plasticity (Razin et al. 1998). Infection of mycoplasmas by viral phages can also impact the bacteria's pathogenicity (Razin et al. 1998). Furthermore, diversity of tandem repeat sections of the genome can allow for increased chromosomal rearrangement and phase variation in the cell surface antigens of *Mycoplasma* (Razin et al. 1998).

Thus far, the focus of research regarding URTD has been on the pathogen *M. agassizii*, even though URTD in tortoises is also associated with *M. testudineum*, *Pasteurella testudinis*, an iridovirus, and Testudinid herpesvirus 2 (Snipes et al. 1980, Snipes and Biberstein 1982, Westhouse et al. 1996, Brown et al. 2004, Jacobson et al. 2012). We assayed tortoise samples for both *M. agassizii* and *M. testudineum*, and our

results did not detect an infection or co-infection status that best associated with the presence of clinical signs of disease (Figure 4). In disease systems, there is an incubation period between colonization of the nasal passages and the onset of clinical signs (and the ability to transmit pathogens). Frequently, there is an assumption that the presence of clinical signs of disease, such as nasal discharge, is associated with shedding of bacteria, enabling disease spread; more often than not, we were unable to detect either *Mycoplasma* in samples collected from tortoises with clinical signs of disease (Figure 4). Half (5/10) of the tortoises exhibiting nasal discharge at the time of sampling did not have detectable amounts of either pathogen in their lavage samples. Thus, while these pathogens or their interactions with yet additional pathogens may be the cause of disease, the presence of nasal discharge may be a necessary, but clearly not sufficient, variable in the spread of *Mycoplasma*.

Little is known about the prevalence or progression of infections in juvenile *Gopherus* tortoises. Recent research by Aiello et al. (2016) found that, on average, long periods of direct nose-to-nose contact are required for *M. agassizii* to be transmitted between adult Mojave desert tortoises. It seems that burrow-sharing at night is the most likely means of exposure, and reproductive Mojave desert tortoises are the most likely individuals to interact with conspecifics via burrow sharing or using multiple different burrows (Sah et al. 2015). Previous research has also reported disease in sub-adults, even though its spread is associated with social interactions attributed to adults (Schumacher et al. 1997, Wendland et al. 2010). We included pre-reproductive tortoises in the present study and found infection by both mycoplasmal pathogens in young individuals of each host species except Sonoran desert tortoises. Though vertical transmission cannot be

ruled out, research has shown that vertical transmission is unlikely for *M. agassizii* (Schumacher et al. 1999) and has not been studied for *M. testudineum*. *Gopherus* can reach sexual maturity in their teens (Germano 1994), and thus they have many years to interact with others before the onset of social interactions associated with mating. Juvenile desert tortoises do occasionally share burrows with adult tortoises (Bulova 1994), as do juvenile gopher tortoises with other juveniles (Radzio et al. 2016), and the extremely low likelihood of *Mycoplasma* surviving in burrow soil (Orlowski et al. 2015) suggests that direct contact is required for pathogen transmission. A recent study found that aggressive behaviors lead to contact between juveniles (Radzio et al. 2016). Additional research examining pre-reproductive behaviors of tortoises that allow them to become infected would greatly help in our understanding of transmission of pathogens associated with URTD. Importantly, we suspect that these *Mycoplasma* species, as in other systems, may have life-long, chronic, cyclical interactions with their tortoise hosts (Weitzman et al. in press). If this is the case, the rate of infection in young tortoises may be an important factor in the prevalence and dynamics of these pathogens among local host populations.

Our analyses suggest that Mojave desert tortoises are more likely than other tortoise species to have detectable amounts of *M. agassizii* in their upper respiratory tracts. Local populations of Mojave tortoises are declining range-wide, and understanding threats by pathogens is important for management initiatives. When tortoises are translocated as part of management schemes, knowing the pathogen prevalence in the source and destination populations could be critically important in predicting survival of local populations and ensuring translocation success. Although this disease has been

hypothesized to cause population declines via host mortality, it seems more likely that under normal conditions, infected tortoises will be minimally harmed, with a chronic latent infection (Sandmeier et al. 2009). However, if disease severity increases as a consequence of environmental stressors, then factors such as habitat alterations and a changing climate could compound to influence the disease trajectory in hosts, as is the case in other systems (Acevedo-Whitehouse and Duffus 2009). *Gopherus* tortoises can live for up to 80 years, and individuals reproduce throughout adulthood, with low adult mortality rates (U.S. Fish and Wildlife Service 1994). Some local populations are likely more at risk due to disease than others (Tuma et al. 2016). For the long-term management of Mojave desert tortoises, focus should be placed on minimizing stressors to decrease the risk of exacerbating the severity of URTD, as stronger infection loads have been linked to higher risk of transmission (Aiello et al. 2016). URTD can also lower the reproductive success of adult females, and high prevalence of URTD in reproductive females might significantly reduce recruitment, contributing to tortoise population declines (Rostal et al. 1996).

Interestingly, while we detected mild clinical signs of disease in Sonoran and Texas tortoises, none of the tortoises examined exhibited mucus discharge. This suggests that mycoplasmal pathogens might interact differently with different host species. Variance in disease dynamics among host populations occurs in other host-pathogen systems, such as with the fungus *Batrachochytrium dendrobatidis* (Bd) in frog species. Among frog species, some experience very high morbidity and mortality rates due to chytridiomycosis, the disease caused by Bd, while others are carriers, unaffected by the pathogen, yet they are able to spread it (Eskew et al. 2015). Species that experience

morbidity due to chytridiomycosis do so along a spectrum of harm (Kilpatrick et al. 2010). The four tortoise species in our study experience different ecological interactions in different ecosystems, so we should not assume that pathogens affect these host species similarly. In the Texas tortoise, *M. agassizii* was only detected in co-infection with *M. testudineum*. Future research should determine whether or not this pattern persists within larger samples and at different sites within the Texas tortoise distribution. None of the Texas tortoises sampled (n = 56) had nasal discharge present at the time of sampling, even though URTD has been detected in this species, particularly in captive individuals (Judd and Rose 2000). As most research on this disease has focused on gopher and Mojave desert tortoises, further studies should determine how pathogens that cause URTD in other tortoise hosts affect species for which this disease system is less well understood.

The interactions between pathogens within a host could be facilitative, competitive, antagonistic, or neutral. Telfer and colleagues (2010) investigated the range of interactions among four pathogens infecting locations throughout a vole host species and found a wide range of positive and negative interactions. The type and strength of interactions were not necessarily reciprocal and depended on recency and whether an infection was acute or chronic. In our data, while the presence of each pathogen affected the infection intensity of the other (Figure 5), this pattern was not consistent across host species. These results indicate that the two pathogens might facilitate each other in a context-dependent manner, and that these pathogens interact differently with their hosts and each other under differing conditions. These hypotheses that should be further investigated.

Here, we provide a snapshot view of *Mycoplasma* prevalence in four North American tortoise species. While we have data on co-infection rates between *Mycoplasma* spp., we still lack prevalence data on other associated pathogens in the system. From preliminary pyrosequencing data, we have found *Pasteurella testudinis* in 50–92% of samples tested from the same four tortoise species (Weitzman et al. in prep). A probe-based qPCR approach to assay for *P. testudinis* would enable us to add a piece to the puzzle to best determine how pathogens interact to cause URTD.

In our study system, two *Mycoplasma* pathogens are present in differing co-infection patterns depending on the host species. Our data suggest a low amount of genetic variation in *M. agassizii* among four allopatric host species that span the two coasts of the United States. Furthermore, a majority of the tortoises sampled with clinical signs of URTD did not have detectable amounts of either *Mycoplasma* in their nasal lavage samples. The two species with lower pathogen prevalence, gopher tortoises and Texas tortoises, had greater prevalence of clinical signs than the other species sampled (Figures 3, 4). These results suggest that long-term data, assaying for additional pathogens, and monitoring environmental stressors might be necessary to understand more fully the role of each pathogen type in the cause and severity of disease. Recurrent resampling of individuals and assaying for all known pathogens would provide a more thorough view of pathogen dynamics over time, and an artificial experiment study on co-infections would disentangle how these pathogens interact within their hosts.

Acknowledgements. We thank Cristina Jones, Darren Riedle, Brian Sullivan, Jeffrey Goessling, Sharon Hermann, Craig Guyer, Adam Emerick, Henry Mushinsky, and Earl

McCoy for help with logistics and sampling, as well as multiple volunteers and field technicians. This material is based on work supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1447692. Funding was also provided by the US Fish and Wildlife Service (1320-114-23DZ); the Bureau of Land Management, United States federal grant number L11C20384; the University of Nevada, Reno Graduate Research Association Awards Program through the Research Grant Program; and the Desert Legacy Fund from the Community Foundation. Work was conducted under the following permits: US Fish and Wildlife Service 10(a)1(A) Recovery Permit Nos. TE076710-7 and TE076710-8; California Department of Fish and Game Scientific Collecting Permit SC-007374; Nevada Department of Wildlife Scientific Collecting Permits S-33080; Utah Division of Wildlife Resources Permit 5COLL8886; Arizona Game and Fish Department permits SP793245, SP609678, and SP654202; Texas Parks and Wildlife Scientific Research Permit SPR-0413-047; National Parks Service Permits MOJA-2011-SCI-0022 and MOJA-2012-SCI-0009; National Wildlife Refuge Permits LRGV-04-10-13-CJP and LA-13-19; and University of Nevada, Reno Institutional Animal Care and Use Committee Protocols 00465 and 00555.

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TABLE 1. Sample size in each *Gopherus* tortoise species and geographic sampling site, with relative proportion of each infection/co-infection result. *Mycoplasma* spp. infection are values outside of co-infection. Letters next to proportions denote significant differences within species or site. Results are from Tukey's post-hoc tests of multinomial logistic regressions at a significant probability level of 0.0183.

Species, Site	n	No Infection	*M. agassizii*	*M. testudineum*	Co-infection
G. agassizii	57	0.28 a	0.44 a	0.04 b	0.25 a
Eldorado	17	0.12 ab	0.18 ab	0.06 a	0.65 b
Fenner	19	0.16 ab	0.74 a	0.05 b	0.05 b
Red Cliffs	21	0.52 a	0.38 ab	0.00 b	0.10 ab
G. morafkai	51	0.53 a	0.25 b	0.02 b	0.20 b
Cave Buttes	8	0.50 a	0.13 b	0.13 b	0.25 ab
Silverbell	18	1.00 a	0.00 b	0.00 b	0.00 b
Sugarloaf	25	0.20 ab	0.48 a	0.00 b	0.32 ab
G. berlandieri	56	0.68 a	0.00	0.23 b	0.09 b
Chaparral WMA	30	0.63 a	0.00	0.37 a	0.00 b
East Rio Grande	13	0.62 a	0.00	0.08 a	0.31 a
West Rio Grande	13	0.85 a	0.00	0.08 b	0.08 b
G. polyphemus	92	0.64 a	0.14 b	0.09 b	0.13 b
Perdido	29	0.69 a	0.24 b	0.00 b	0.07 b
Rayonier	32	0.69 a	0.03 b	0.19 b	0.09 b
U. South Florida	31	0.55 a	0.16 b	0.06 b	0.23 ab

TABLE 2. Sample sizes for each sex per *Gopherus* tortoise species, with proportions of each infection result. *Mycoplasma* spp. infection are values outside of co-infection. Letters denote significant differences within each row at a significant probability level of 0.0184. Results are from Tukey's post-hoc tests of multinomial logistic regressions.

Species, Sex	n	No Infection		*M. agassizii*		*M. testudineum*		Co-infection	
G. agassizii									
Female	25	0.24	ab	0.40	a	0.04	b	0.32	ab
Male	27	0.30	ab	0.48	a	0.04	b	0.19	ab
Juvenile	5	0.40	a	0.40	a	0.00	a	0.20	a
G. morafkai									
Female	26	0.38	a	0.35	ab	0.04	b	0.23	ab
Male	23	0.65	a	0.17	b	0.00	b	0.17	b
Juvenile	2	1.00	a	0.00	b	0.00	b	0.00	b
G. berlandieri									
Female	30	0.70	a	0.00		0.23	b	0.07	b
Male	14	0.64	a	0.00		0.29	ab	0.07	b
Juvenile	12	0.67	a	0.00		0.17	a	0.17	a
G. polyphemus									
Female	46	0.63	a	0.17	b	0.07	b	0.13	b
Male	31	0.58	a	0.16	ab	0.13	b	0.13	ab
Juvenile	15	0.80	a	0.00	b	0.07	b	0.13	ab

FIGURE 1. Sites sampled representing three geographic locations for each of four tortoise species: Red = *Gopherus agassizii*, Mojave desert tortoises; Orange = *G. morafkai*, Sonoran desert tortoises; Green = *G. berlandieri*, Texas tortoises; Blue = *G. polyphemus*, gopher tortoises. FV = Fenner Valley; ED = Eldorado Valley; RC = Red Cliffs; CB = Cave Buttes; SL = Sugarloaf; SB = Silverbell; CH = Chaparral Wildlife Management Area; WE = West Rio Grande; EA = East Rio Grande; PD = Perdido River; RY = Rayonier; USF = University of South Florida.

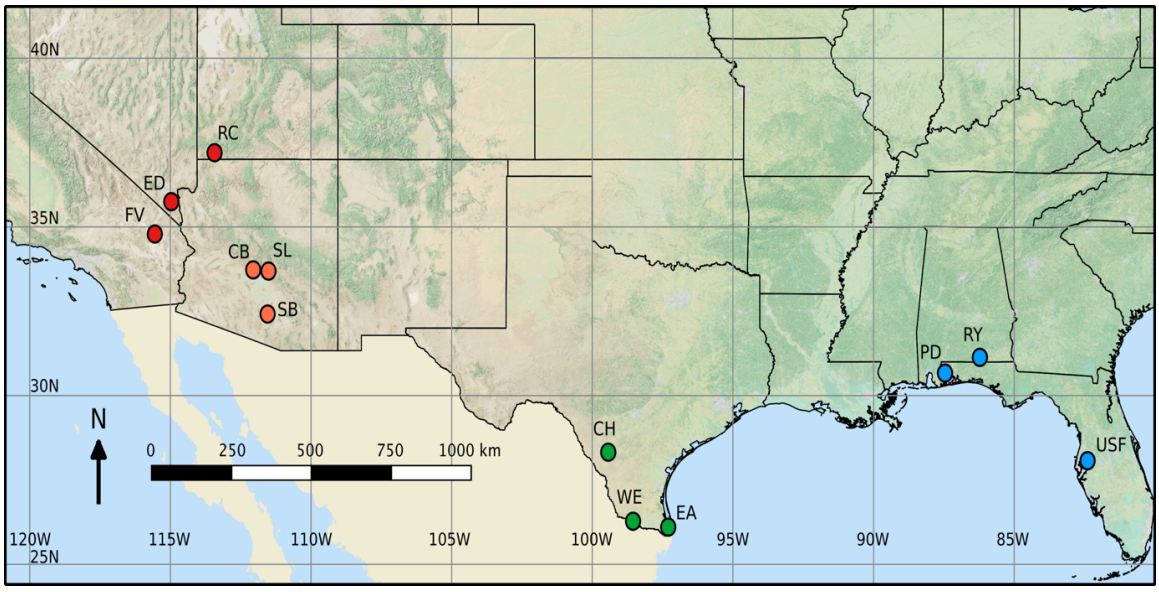


FIGURE 2. TCS haplotype networks of *Mycoplasma agassizii*. Colors based on host species or culture sequence: Red = *Gopherus agassizii*, Orange = *G. morafkai*, Green = *G. berlandieri*, Blue = *G. polyphemus*, Black = Culture sequences (from GenBank or in-lab sequencing). Each square represents a single sequence. a) 16S ribosomal RNA with trimmed sequences 468 bp in length. GenBank sequence for type-strain PS6 split from the main haplotype, while the in-lab sequence for ATCC strain 700617 from a gopher tortoise grouped with the main haplotype. b) 16-23S intergenic spacer region, sequences 476 bp in length. GenBank sequence for PS6 grouped with the core haplotype, while the in-lab sequence for ATCC strain 700617 separated from the core haplotype by two single nucleotide polymorphisms. c) Ribosomal polymerase beta subunit sequences of 568 bp in length.

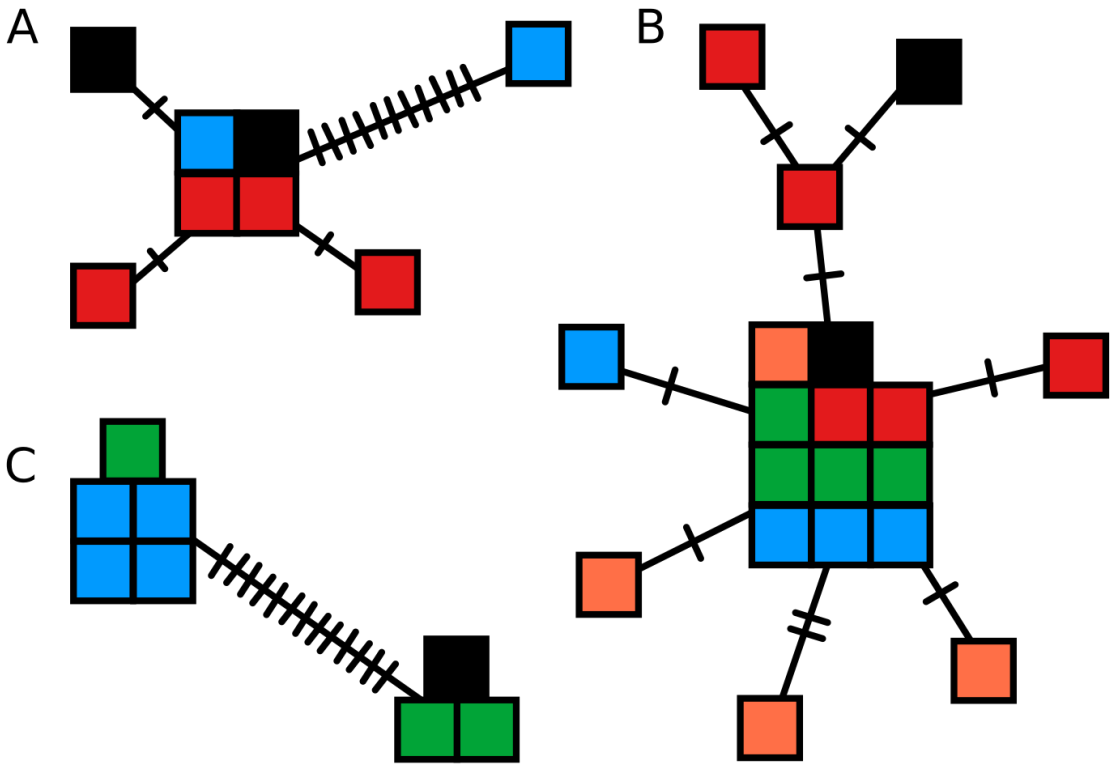


FIGURE 3. Barplot of prevalence of *Mycoplasma agassizii* (light grey), *M. testudineum* (stripes), and co-infection of the two *Mycoplasma* spp. (dark grey) by host species. Letters above *M. agassizii* bars denote significant differences in prevalence of that pathogen between the host species from Tukey's post hoc tests on a generalized linear model at a significance of 0.05.

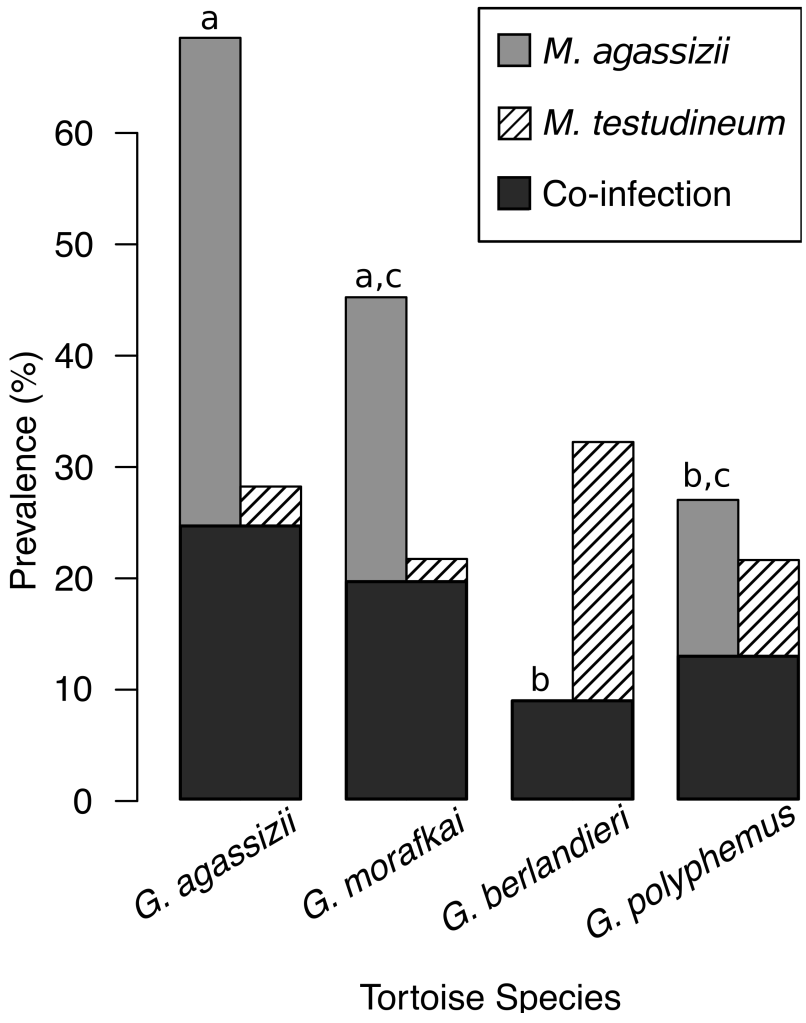


FIGURE 4. Stacked bar plot of infection groups split by presence of clinical signs of disease in each *Gopherus* tortoise host species. Colors denote infection group, with lighter shades indicating absence of clinical signs of disease: Purple = no infection; Blue = *M. agassizii* sole infection; Green = *M. testudineum* sole infection; Red = co-infection of both *Mycoplasma*. In *G. berlandieri*, 13 tortoises were not evaluated for clinical signs, and are thus excluded from the total.

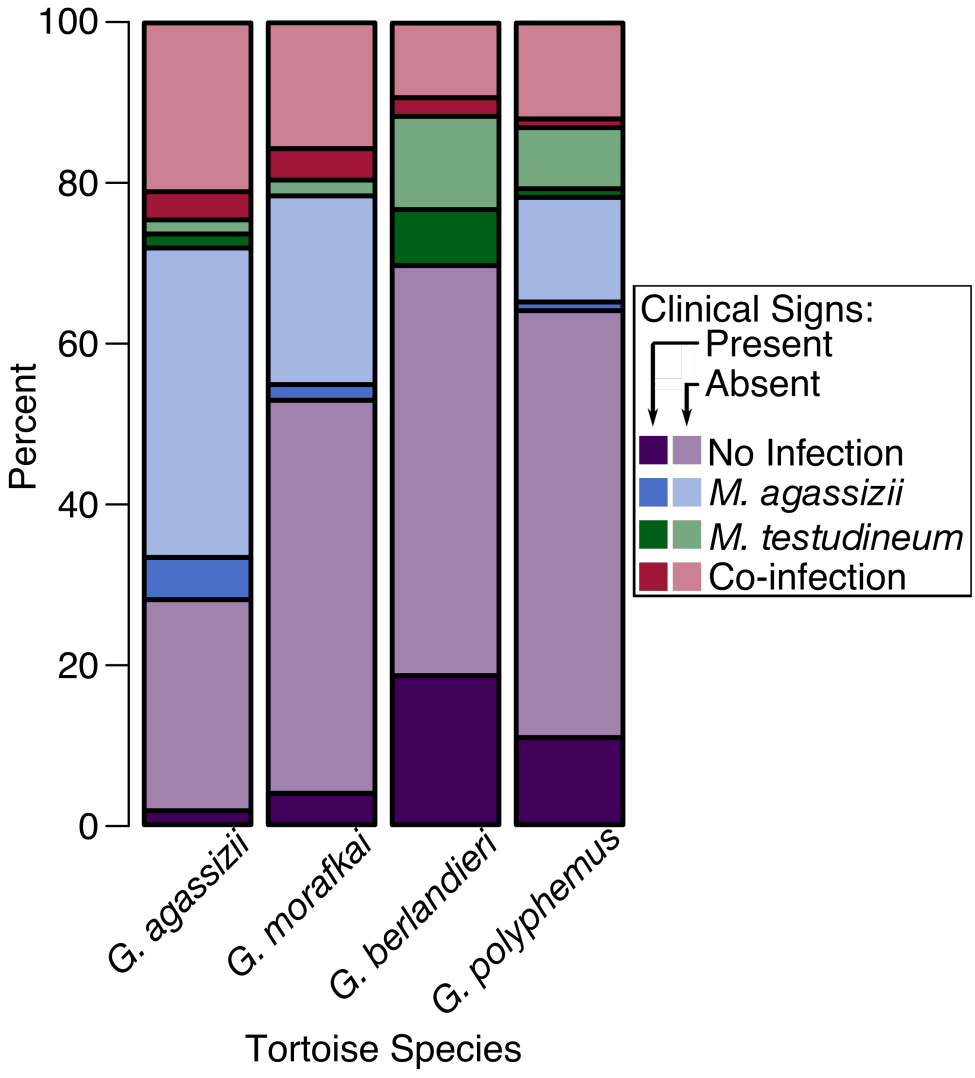
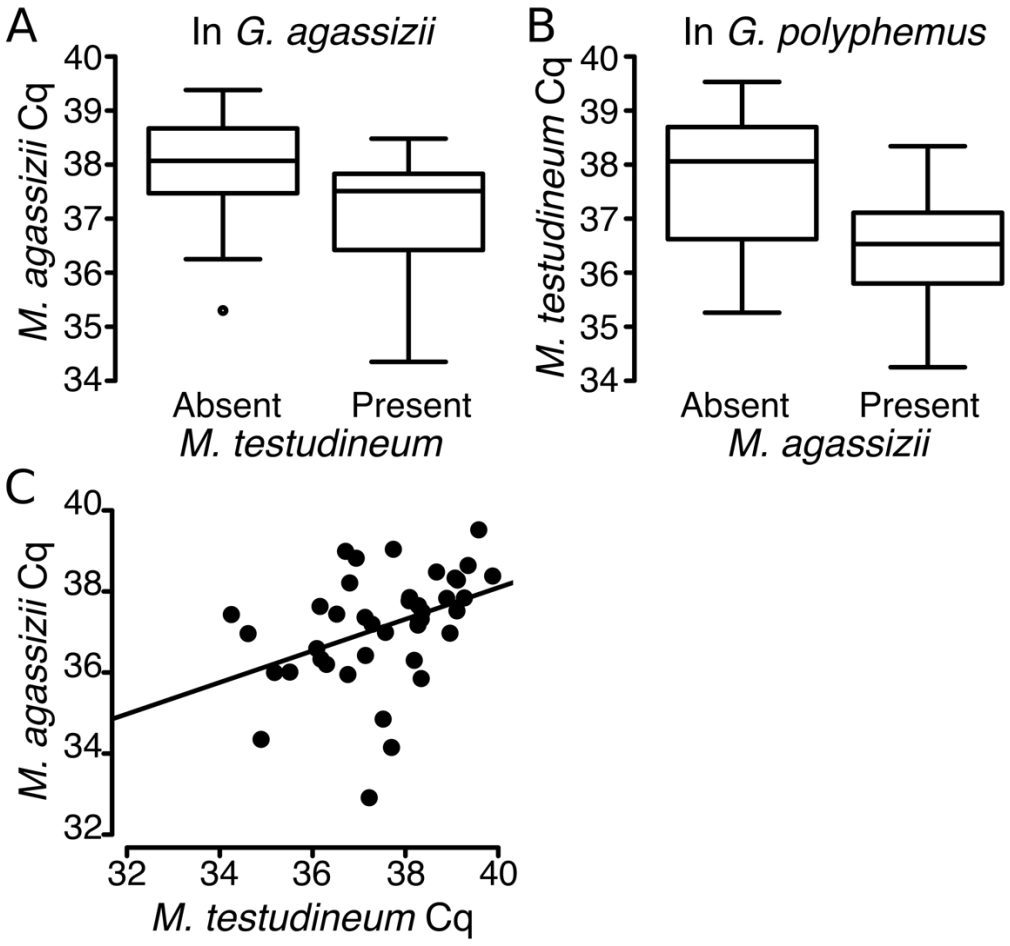


FIGURE 5. Presence and infection intensity (Cq as a proxy) of each *Mycoplasma* species based on the other. a) *Mycoplasma agassizii* with and without *M. testudineum* in *Gopherus agassizii* (Mojave desert tortoises; $F_{(1,37)} = 6.428$, $P = 0.016$); b) *M. testudineum* in samples with and without *M. agassizii* in *G. polyphemus* (gopher tortoises; $F_{(1,18)} = 4.447$, $P = 0.049$); c) Correlation of Cq values between the two *Mycoplasma* spp. assayed ($R^2 = 0.159$, $F_{(1,39)} = 7.39$, $P = 0.0097$).



CHAPTER 3: ALTERED MICROBIAL COMMUNITIES WITH THE PRESENCE OF PATHOGENS AND DISEASE IN TORTOISES

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ABSTRACT

Astoundingly rich and diverse bacterial communities are found on every surface of macro-organisms, and they play important roles in maintaining normal physiological functions in their hosts. While the study of microbiomes has expanded with the influx of data enabled by next-generation DNA sequencing technologies, microbiome research in reptiles is lagging behind other organisms. We sequenced the nasal microbiome in four North American tortoise species, and we found differing community compositions among tortoise species and geographic sampling sites, with higher richness in Texas and Sonoran desert tortoises. Using the microbiome data, we investigated the prevalence and OTU diversity of the potential pathogen *Pasteurella testudinis* within and among tortoise species, and found it to be common, abundant, and highly diverse. However, the presence of this bacterium was not associated with a change in bacterial community composition. We also found that the presence of nasal discharge from tortoises at the time of sampling was associated with a decline in nasal microbiome species richness and a change in microbiome composition, which we posit is due to the harsh epithelial environment associated with upper respiratory disease. Repeated sampling across seasons, and at different points of pathogen colonization, should contribute to our understanding of the causes and consequences of different bacterial communities in the nasal passages of these long-lived hosts.

Key words: bacteria, *Gopherus*, microbiome, pyrosequencing

INTRODUCTION

The influx of next-generation DNA sequencing technology has allowed the study of community ecology to delve more effectively beyond the focus of macro-organisms into the world of the microscopic. As in communities of macro-organisms, members of microbial communities participate in positive, negative, and neutral interactions with other community members. Furthermore, microbial taxa interact with the larger organisms in or on which they reside. Many microbiome studies have been discovery-based, focusing on the identification of microbial species and their location on a host. Gut microbes, and their roles in evolutionary transitions between carnivory and herbivory, have been especially well represented in the literature. A recent review of non-mammalian microbiome studies found that most reptilian studies fail to address microbiomes other than those related to the gut (Colston and Jackson 2016).

Microbiomes may be particularly important for understanding infectious diseases. In disease ecology, characteristics of the host, its environment, pathogens, and their interactive effects are synthesized to discuss the manifestation of disease. Recently, Hanson and Weinstock (2016) proposed to add the microbiome as a fourth node to the disease ecology triangle, emphasizing the role of the microbial community in determining the way a pathogen harms its host. Epidemiological studies, particularly those involving human diseases (Hanson and Weinstock 2016), but also some wildlife diseases, consider the role of microbiomes increasingly more frequently. One such disease garnering recent attention is chytridiomycosis, a pandemic affecting amphibian hosts caused by fungal pathogens. The skin of amphibians harbors microbial communities that can inhibit pathogenic fungi (Harris et al. 2006, Lauer et al. 2007). Thus, the existence and

antimicrobial abilities of a host's microbiome could greatly impact whether an invading pathogen succeeds in the host. Conversely, when fungal pathogens are able to invade amphibian skin, the composition of the skin microbial communities may be altered (Jani and Briggs 2014).

While the microbiome of amphibian skin has been at the forefront of emerging disease research, the microbiomes associated with other herpetofaunal diseases have been largely neglected thus far. One disease that could benefit from more microbial community research is an upper respiratory tract disease (URTD), associated with bacterial and viral pathogens, which is found in North American tortoise species. The tortoise genus *Gopherus* includes six species, four of which are found in the southern portion of the U.S. mostly in allopatric habitats: Mojave desert tortoise (*G. agassizii*) in the southwest (California, Nevada, Utah, Arizona); Sonoran desert tortoise (*G. morafkai*) in the southwest (Arizona into Mexico); Texas tortoise (*G. berlandieri*) in Texas and into Mexico; and gopher tortoise (*G. polyphemus*) in the southeast (Florida, Georgia, South Carolina, Alabama, Mississippi, and Louisiana). In these tortoises, multiple pathogens are associated with URTD, including *Mycoplasma agassizii*, *M. testudineum*, *Pasteurella testudinis*, an iridovirus, and testudinid herpesvirus 2 (Snipes et al. 1980, Snipes and Biberstein 1982, Brown et al. 1994, Westhouse et al. 1996, Brown et al. 2004, Jacobson et al. 2012). This respiratory disease causes nasal exudate and lesions on the nasal epithelium, with extreme cases resulting in lethargy or even death (Brown et al. 1994). It is likely that pathogen proliferation in tortoise hosts occurs after an environmental stressor, affecting the host's immune system or causing a shift in microbial community interactions (Acevedo-Whitehouse and Duffus 2009).

The study of URTD has focused on detecting relevant pathogens, visually detecting signs of disease, and using serological tools to determine exposure to pathogens (Schumacher et al. 1997, Brown et al. 2001, Hunter et al. 2008, Jones 2008, Sandmeier et al. 2013, Guthrie et al. 2013). In Mojave desert tortoises (*Gopherus agassizii*), few studies have addressed the upper respiratory tract microbial community, and those that have used labor-intensive culturing methods. Dickinson et al. (2001) detected 19 taxa in nasal and cloacal samples from desert tortoises, while Ordorica et al. (2008), found 260 bacterial isolates in the nasal passages of desert tortoises. In the present study, we use next-generation DNA sequencing technology to determine not only the members of tortoise upper respiratory microbiomes, but also to search for patterns of microbial community structure. We compared upper respiratory tract microbial communities in four congeneric *Gopherus* tortoise species to test the following hypotheses: (1) Microbes associated with URTD are found in microbial communities with different compositions from those without pathogens; and (2) The presence of nasal mucus correlates with differing microbial communities from those in tortoises without nasal mucus. We also use the microbiome data to evaluate the population diversity of one potential pathogen of interest, *Pasteurella testudinis*.

METHODS

DNA Collection and Microbiome Sequencing

We sampled upper respiratory bacteria from all four species of *Gopherus* found in the U.S. using a 3 mL nasal lavage (0.9% NaCl; Brown et al. 2002, Weitzman et al. in press). Samples represent three sites for each of the four *Gopherus* species, with two

additional sites for Mojave desert tortoises to address patterns regarding the presence of nasal mucus (Table 1). Nasal lavage samples were immediately preserved in RNAlater Stabilization Solution (Ambion Inc., Austin, Texas, U.S.) at a ratio of 1:5 preservative to sample volume, placed on ice in the field, and frozen within 12 hours of sampling. We extracted the DNA from lavage samples with the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, U.S.). Amplification of the variable regions V1-V3 of the bacterial 16S ribosomal RNA gene with the 28F/519R primer set and subsequent 454-pyrosequencing were conducted at the Research and Testing Laboratory (Lubbock, TX, U.S.).

Using methods described in Braun et al. (2014), we conducted qPCRs to determine the presence or absence of the URTD-causing pathogens *Mycoplasma agassizii* and *M. testudineum* in our samples, which have been analyzed elsewhere (Weitzman et al. in prep, F. C. Sandmeier unpublished data).

The following analyses were conducted using the QIIME bioinformatics pipeline (Caporaso et al. 2010), with default parameters, unless otherwise stated. Where applicable, we used unweighted Unifrac distance matrices (Lozupone and Knight 2005).

After quality filtering and demultiplexing, we clustered operational taxonomic units (OTUs) using an open-reference OTU-picking process, which clusters sequences based on 97% similarity and identifies taxonomy using the GreenGenes database (DeSantis et al. 2006, Edgar 2010, McDonald et al. 2012). We subsetted the resultant OTU table separately to compare patterns among the four host species and among Mojave desert tortoises (*Gopherus agassizii*) with and without the presence of nasal mucus.

Bacterial Communities Among Host Species

We compared patterns of upper respiratory bacterial communities among tortoise species using data from three sampling sites per species, excluding data on Mojave desert tortoises from South Las Vegas and Red Cliffs (Table 1). OTUs were filtered to exclude those represented by only one sequence, and to exclude those identified as mitochondrial, as well as Chloroplast, indicating plant origin. The resulting dataset included 19,163 OTUs from 24 to 11950 sequences per individual sample (2784 ± 2234 s.d.).

Because OTU richness was correlated with the number of sequences per sample (Figure 1a), we compared the residuals of this correlation among species and sites using ANOVA and Tukey's post-hoc HSD tests in the programming language R (R Development Core Team 2015). To detect differences in bacterial community composition among species and sites and based on the presence of *Mycoplasma* spp., we rarefied data to 1000 sequences per sample and conducted analyses of similarity (ANOSIMs) with 999 permutations.

Using the pyrosequencing results, we wanted to determine whether *Pasteurella testudinis*, a possible pathogen in *Gopherus* tortoises (Snipes and Biberstein 1982) was present in each sample. Gregerson et al. (2009) published a study separating the genus *Chelonobacter* from that of *Pasteurella*, identifying *Chelonobacter* sp. as a pathogen of European tortoises, sister taxon to *Pasteurella testudinis* present in North American tortoise species. Although the reference database used in the present study, GreenGenes (DeSantis et al. 2006, McDonald et al. 2012), has data on both genera, OTUs in our analyses were identified as being within the *Chelonobacter* genus. Another method of taxonomic identification, using USEARCH with the NCBI database, identifies these

sequences as *P. testudinis*. Because the identification method used in this study did not identify any additional sequences as *P. testudinis,* and because only a small DNA sequence was used to determine taxonomic identity, we analyze and discuss *Chelonobacter* with the assumption that it is, in fact, the *P. testudinis* bacterium previously found in North American tortoises.

To compare communities with and without this microbe, the data were trimmed to exclude *Chelonobacter* OTUs and we conducted principal coordinates analysis and ANOSIMs on rarefied 500 sequences per sample. Furthermore, we used pairwise ANOSIMs to compare the OTUs identified as being in the genus *Chelonobacter* by producing an OTU table containing only sequences from this genus.

The core microbiome is the group of bacterial types present in most or all (depending on cut-off percentage) of the samples in a group. We computed the core microbiome OTU composition for each tortoise species and each site separately. Lastly, we applied supervised learning to determine the predictability of bacterial communities among tortoise hosts by using an algorithm to predict host species identity based on its bacterial community (Knights et al. 2011). Supervised learning was run after excluding OTUs represented by fewer than 20 sequences and rarefying to 1000 sequences per sample.

Disease Signs in Mojave Desert Tortoises

Because zero Texas and Sonoran desert tortoises had nasal discharge at the time of sampling, and few gopher tortoises had discharge, we sequenced the microbiomes from tortoises with and without visible mucus in Mojave desert tortoises sampled in Fenner Valley, South Las Vegas, and Red Cliffs (Table 1). Mojave tortoise samples were

filtered to exclude singletons and OTUs that were likely derived from plant tissue in the tortoise nares and mitochondria, as above. Data from Coyote Springs and Eldorado were excluded in these analyses, as no individuals from those sites had nasal discharge at the time of sampling.

To determine whether the presence or absence of nasal discharge was associated with differing levels of OTU richness, we compared residuals from a linear model between OTU richness and the total number of sequences using an ANOVA in program R. Beta diversity, comparing diversity between samples, was evaluated with ANOSIM and principal coordinates analysis on unweighted Unifrac distances. Diversity of *Chelonobacter* populations was also compared between samples from tortoises with and without nasal mucus using ANOSIM.

RESULTS

Pyrosequencing resulted in 24-11950 sequences in any one sample, totaling 19,644 bacterial OTUs represented by >1 sequence over all samples.

Comparisons of Bacterial Communities Among Tortoise Hosts

Samples from three sites per tortoise species contained an average of 2784 sequences. In each tortoise species, bacteria in the phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes collectively amounted to 84% to 94% of the rarefied sequences (Figure 2a). Ten bacterial classes represented at least 5% of sequences for any one sampling location (Figure 2b). The class Gammaproteobacteria accounted for >25% of OTUs in all host species except Texas tortoises.

The number of observed OTUs was correlated with the number of sequences per

sample (Figure 1a; $F_{(1,133)} = 31.57$, $P < 0.0001$), and residuals of that regression were significantly different among host species, with Texas and Sonoran desert tortoises supporting higher OTU richness than gopher and Mojave desert tortoises (pairwise Tukey's HSD, $P < 0.015$ each; Figure 1b). Within tortoise species, sites had similar OTU richness, one exception being that Sonoran desert tortoises from Sugarloaf had higher richness in comparison to those from Silverbell (pairwise Tukey's HSD, $P = 0.0006$; Figure 1c).

When data were rarefied to 1000 sequences per sample, 29 samples were excluded (Mojave desert tortoise, $n = 13$; Sonoran desert tortoise, $n = 8$; Texas tortoise, $n = 6$; gopher tortoise, $n = 2$). Pairwise ANOSIMs detected significantly different communities among all tortoise host species ($P = 0.001$ each; Figure 3a). Sites sampled within tortoise species also hosted significantly different microbiota (ANOSIMs, $P \leq 0.002$ each). The presence of *Mycoplasma agassizii* was correlated with different bacterial communities in Mojave and Sonoran desert tortoises (*G. agassizii*, $R = 0.2433$, $P = 0.014$; *G. morafkai*, $R = 0.102$, $P = 0.046$), but this was not the case in Texas or gopher tortoises (ANOSIMs, $P \geq 0.9$ each). Presence or absence of *M. testudineum* was not associated with differing microbiomes in any tortoise host species (ANOSIMs, $P > 0.06$ each).

To compare bacterial communities with and without *Pasteurella testudinis*, we made the assumption that OTUs identified as genus *Chelonobacter* represented *P. testudinis* (see methods; Gregerson et al. 2009). We excluded *Chelonobacter* sequences to compare bacterial communities with and without this microbe for each host species (Figure 3b). Bacterial communities within host species did not differ between samples

with and without *Chelonobacter* (ANOSIMs, $P > 0.07$ each in Mojave, Sonoran, and Texas tortoises). Only three gopher tortoise samples were negative for this possible pathogen, and while they seem to separate in principal coordinates space, more samples without *Chelonobacter* are needed to verify this pattern.

A total of 268 OTUs were identified as *Chelonobacter* across all species of hosts. *Chelonobacter* comprised up to 98% of the bacterial sequences in a sample (gopher tortoise from Rayonier; including 31 OTUs) with up to 84 different *Chelonobacter* OTUs detected per sample (Sonoran desert tortoise from Sugarloaf). *Chelonobacter* populations were only significantly different between Texas and Sonoran desert tortoises (ANOSIM, $R = 0.1592$, $P = 0.001$; Figure 3c).

The purpose of a core microbiome analysis is to determine which OTUs are present in many or most individuals of a group, possibly allowing us to detect potentially ecologically important taxa. The presence of a core microbiome varied widely by host species. In Mojave desert tortoise samples, only one OTU was present in 50% of the samples (a *Pseudomonas*), while 50% of Texas tortoise samples shared 41 OTUs, one of which (*Dietzia*) was found in 90% of the samples. Core microbiomes (50% cut-off) for gopher and Sonoran desert tortoises each included 30 OTUs. The number of OTUs found in 75% of samples from each site also varied, from one in Coyote Springs samples (Mojave desert tortoise) to 26 in East Rio Grande samples (Texas tortoise; Figure 4). Four *Chelonobacter* OTUs were common in samples from gopher tortoises (all three sites) and Sonoran desert tortoises from Sugarloaf. Core microbiota (75% cut-off) from Sugarloaf, Perdido, and University of South Florida shared three *Chelonobacter* OTUs, while Rayonier samples frequently had two of those common *Chelonobacter* OTUs plus

an additional type not common in samples from any other site. One *Chelonobacter* OTU was found in all samples from the University of South Florida reserve (gopher tortoise).

We ran a supervised learning algorithm to determine the likelihood of identifying host species based on bacterial community association. Supervised learning, excluding rare OTUs and run with 1000 sequences per sample, resulted in perfect or near perfect assignment for gopher tortoise and Texas tortoise communities (<5% error rate), though the error rates for assigning the bacterial communities in the two desert tortoises to the correct hosts were higher (13% Mojave desert tortoises; 25% Sonoran desert tortoises).

Interaction with Clinical Signs of Disease

In Mojave desert tortoises, the presence of exuded nasal mucus at the time of sampling was associated with a decrease in OTU richness in lavage samples (Figure 5a; $F = 16.26$, $P = 0.0006$). Samples from tortoises with mucus also had significantly different bacterial community composition than those from tortoises without mucus ($R = 0.376$, $P = 0.002$; Figure 5b). This pattern was observed whether all sequences were included in analyses or samples were rarefied to 500 sequences. Therefore, we present the full analysis because the smaller subset of data discards four of the nine samples from mucus-positive tortoises. We analyzed *Chelonobacter* populations between tortoises with and without secretions of nasal mucus and found no difference between the two groups ($R = -0.0039$, $P = 0.387$).

DISCUSSION

Tortoise Upper Respiratory Microbiome

Using a next-generation DNA sequencing platform, we have determined the

microbial diversity, community composition, and core microbiome in the nares of four North American tortoise species in the context of an upper respiratory tract disease and its associated bacterial pathogens. Our four tortoise species, and sites sampled within those species, had significantly different bacterial communities (Figure 1,3). Accordingly, we did not find strong core microbiomes for each tortoise species, particularly for the two species of desert tortoise, and site-level core microbiomes varied in size across the sampling locations (Figure 4).

We used a Unifrac distance metric to assess beta diversity in our samples (Lozupone and Knight 2005). This metric uses phylogenetic distance to characterize community differences. The spread of principal coordinates values from Unifrac distances (Figure 3) suggests a high diversity of bacterial types capable of surviving in these varied tortoise host environments. Further research should address the degree to which microbial community composition or diversity is influenced by both abiotic and biotic environmental conditions for the host, such as rainfall, and transmission among tortoises. Moreover, we do not know if the patterns that we found are stable through time or space, and we know little about how nasal microflora change across the seasons (sampling in this study was not restricted to the same season), with no studies addressing changes over the course of a host's lifetime. Using culturing techniques, Ordorica et al. (2008) found changes in total bacteria count between seasons, with higher counts in the summer versus autumn. They also found that tortoises with clinical signs of URTD had larger and more diverse upper respiratory communities, opposite to what we found in the present study (Figure 5a).

Mojave desert tortoises live in a harsh, arid, and highly seasonal environment,

while gopher tortoises inhabit forests of the southeastern United States, experiencing frequent rainfall events and less seasonality. Differences found among Sonoran tortoise bacterial communities (Figure 3) could be a result of sampling time, as tortoises were sampled after the beginning of the monsoon season, though they also inhabit a dry environment. The lack of a strong core microbiome in the two desert tortoise species could mean that those hosts act more like distinct islands, with little opportunity for movement of upper respiratory-associated bacteria among individual hosts. Thus, in desert tortoise species, microbiomes may function as isolated communities rather than as large contiguous metapopulations of associated bacteria.

Interestingly, the tortoise species most studied with regard to social structure and social interactions, the gopher tortoise (Guyer et al. 2014), had a more widespread range of bacterial community composition, visualized in principal coordinates space, than Mojave desert tortoises or Texas tortoises. However, gopher tortoises did have a stronger core microbiome than Mojave desert tortoises. We currently do not know if these differences are a function of social structure or host physiology, or if they are affected by the environment of the host or an artifact from sample sizes of three sites per tortoise species. Burrow sharing and other social interactions could increase the similarity in the upper respiratory microbiome, as such activities may aid in the direct transmission of upper respiratory pathogens (Bulova 1994, Wendland et al. 2010, Aiello et al. 2016).

In our samples, care was not taken to avoid bacteria that would be associated with the face, particularly the scales around the nares, as these samples were collected for other studies regarding disease ecology in tortoises. Thus, some taxa found in the present study could be associated with the skin, and we do not know if those bacteria could be

maintained in the tortoise respiratory tract. Although there have been no published reports on skin-associated bacteria in reptiles (Colston and Jackson 2016), we predict that because reptile skin is not a mucus membrane, the skin microbiome would be more similar to the environment than that found in amphibians. In sympatric amphibian species, cutaneous bacterial community composition is tightly linked to species identity (McKenzie et al. 2012, Kueneman et al. 2014). If our upper respiratory tract data were strongly influenced by skin and environmental bacteria, then the additional bacteria should increase the presence of a core microbiome, a result not supported in our data. Nevertheless, we acknowledge that a portion of our sequencing results likely includes transient taxa or bacteria found only in close proximity to the upper respiratory tract, but not necessarily maintained in it.

Similar to the skin microbiome in many other taxa (Avena et al. 2016), the majority of OTUs from the upper respiratory tracts of tortoises were in the bacterial phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. These phyla, plus Fusobacteria, are also commonly found in human upper respiratory tracts (de Steenhuijsen Piters et al. 2015), with Bacteroidetes and Firmicutes dominating human lung bacteria (Dickson et al. 2016). Actinobacteria, however, comprised a greater percentage of Sonoran desert tortoise (19%) and Texas tortoise (27%) bacteria than that found on the skin of other non-human animals.

In North American tortoises, microbiome research involving next-generation sequencing technology has also been conducted on the fecal gut microbiome in gopher tortoises in Florida. Yuan et al. (2015) discovered over 1000 core OTUs found in at least 90% of the samples. The large discrepancy between the presence of a strong core

microbiome in the gut versus the lack of strong core microbiome found in our nasal samples is likely due to different roles played by the microbiome in different regions of the body, as well as different methods used to describe the host microbiome. Tortoises are herbivorous hind-gut fermenters, requiring a gut microbiome with large responsibility in the process of food digestion. The nasal flora, alternatively, likely play a role in disease avoidance (Wilson and Hamilos 2014). Furthermore, data collected by Yuan et al. (2015) used Illumina MiSeq data, a sequencing platform that results in a higher sequence count than the pyrosequencing conducted here. MiSeq technology allows for an increase in sequencing speed, resulting in a higher sequence count and lower cost. The role of the microbiome in respiratory mucosal immunity, a field still nascent in human immunology, influences whether or not we should expect to find a looser relationship between a tortoise and its nasal microbiome than with its gut microbiome.

On epithelial tissue, microbes are important for mucosal homeostasis (McDermott and Huffnagle 2014), in addition to their role in stimulating the immune system, resulting in greater immunocompetence in organisms with a healthy microbiome than those with an altered or absent microbial community (Brown and Clarke 2016). Microbes in the digestive tract also play an important role in digestion, a task not required by the respiratory tract. Importantly, microbes in any one mucosal location do not have effects limiting to that location, and gut microbes can aid in fighting off respiratory pathogens (Brown and Clarke 2016). As most research focuses on the importance of gut microbes, the importance of respiratory bacteria, particularly outside of the context of human disease, is still widely unknown.

Associations with Pathogens and Disease

Studies of URTD in North American tortoises have focused on *Mycoplasma* pathogens, with little research on the presence or effects of *Pasteurella testudinis* in the tortoise host. When *P. testudinis* was first described, multiple strains were found among different locations in the tortoise host, and this pathogen was described as being usually commensal with healthy tortoise hosts, though capable of causing disease (Snipes and Biberstein 1982). Furthermore, within *P. testudinis* isolated from the upper respiratory tracts of Mojave desert tortoises, there is some differentiation between types more or less associated with URTD (Snipes et al. 1995). In our analyses, we assume that what was identified as *Chelonobacter* represented *P. testudinis* (see methods; Gregerson et al. 2009), though further research should better distinguish between these two genera.

The amount of diversity in our samples in the genus *Chelonobacter* was extremely large, though a previous study also found high ribotype diversity of this pathogen in tortoises (Snipes et al. 1995). Three *Chelonobacter* OTUs were common and shared among gopher tortoises and Sonoran tortoises from Sugarloaf. *Chelonobacter* populations were similar among most host species studied, indicating that this bacterium and its genotypic diversity are widespread in North American tortoises. Mojave desert tortoises with and without nasal mucus did not have differing *Chelonobacter* populations, suggesting that if this pathogen is associated with disease, then a variable beyond bacterial strain is likely the cause of disease progression. Furthermore, the presence of *Chelonobacter* in the microbiome did not impact the remaining bacterial community composition.

Only in the two desert tortoise species was the presence or absence of

Mycoplasma agassizii associated with differing bacterial communities. This result indicates that different strains could be present in different tortoise host species, the pathogen interacts differently with the different hosts, affecting the bacterial communities in differing ways, or some other aspect of the ecology of this mycoplasma in its bacterial community differs among hosts. Where *M. agassizii* is associated with a shift in microbiome community, it is possible that tortoise hosts are colonized with *Mycoplasma* without disease-causing impacts until the community is pushed away from equilibrium by a stressor, with the shift in microbiome allowing *Mycoplasma* to switch from a commensal organism to one that causes URTD.

Our data on Mojave desert tortoises additionally found that nasal exudate was associated with a decrease in bacterial diversity (Figure 5a) and a change in the bacterial community composition (Figure 5b). If nasal exudate is a sign of upper respiratory tract disease, then it is also associated with lesions, inflammation, and sloughing off of mucosal and bacterial cells from the nasal epithelium. This inflammatory response caused by the immune system does seem to influence the microbiota, possibly by forming an environment inhospitable to the maintenance of a healthy nasal microbiome. Pathogens of the *Mycoplasma* genus are considered to be "ideal parasites" in that the damage to the host is associated with immune defenses and not from direct effects of the pathogens themselves (Razin et al. 1998). Nevertheless, immune responses to mycoplasma are frequently intermittent and ineffective at fully removing the pathogen, resulting in chronic infections (Razin et al. 1998). The decrease in richness with the presence of nasal discharge could cause a decrease in pathogen over the short term, but this shift in microbial community might make animals more susceptible to re-infection.

CONCLUSIONS

Tortoise upper respiratory tracts harbor thousands of bacterial types, forming diverse community assemblages. The presence of pathogens and disease may alter the natural microbiome in tortoise hosts, depending on both the pathogen and host in question. Importantly, clinical signs of disease, associated with inflammation and sloughing off of cells, could dramatically alter the upper respiratory microbiome, and long-term effects of microbial shifts is yet unknown. The tortoise upper respiratory microbiome is diverse and likely important for normal host physiology; the role of tortoise interactions, wet and dry seasons, and ontogenetic changes in the maintenance and stability of the microbiome is yet to be addressed.

Acknowledgements. We thank Sarah Snyder, Cristina Jones, Darren Riedle, Brian Sullivan, Jeffrey Goessling, Sharon Hermann, Craig Guyer, Adam Emerick, Henry Mushinsky, and Earl McCoy for help with logistics and sampling, as well as multiple volunteers and field technicians. This material is based on work supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-1447692. Funding was also provided by the US Fish and Wildlife Service (1320-114-23DZ); the Bureau of Land Management, United States federal grant number L11C20384; the University of Nevada, Reno Graduate Research Association Awards Program through the Research Grant Program; and the Desert Legacy Fund from the Community Foundation. Work was conducted under the following permits: US Fish and Wildlife Service 10(a)1(A) Recovery Permit Nos. TE076710-7 and TE076710-8; California Department of Fish and Game Scientific Collecting Permit SC-007374;

Nevada Department of Wildlife Scientific Collecting Permits S-33080; Utah Division of Wildlife Resources Permit 5COLL8886; Arizona Game and Fish Department permits SP793245, SP609678, and SP654202; Texas Parks and Wildlife Scientific Research Permit SPR-0413-047; National Parks Service Permits MOJA-2011-SCI-0022 and MOJA-2012-SCI-0009; National Wildlife Refuge Permits LRGV-04-10-13-CJP and LA-13-19; and University of Nevada, Reno Institutional Animal Care and Use Committee Protocols 00465 and 00555.

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TABLE 1. Sites sampled representing four North American *Gopherus* tortoise species, with sample size, average (± s.d.) number of sequences per sample, number of samples with *Mycoplasma agassizii* and *M. testudineum* (adjusted sample size for missing data), number of individuals with nasal mucus at the time of sampling, and number of samples with *Pasteurella testudinis* (*Chelonobacter*).

	n	Sequences	*M. ag.*	*M. test.*	Mucus	*P. test.*
G. agassizii						
Eldorado	11	2168 ± 1970	10	7	0	4
Fenner Valley	12	1336 ± 1554	9	1	3	7
Coyote Springs	9	1524 ± 856	1 (8)	0 (8)	0	5
Red Cliffs	5	828 ± 656	3	0	3	3
South Las Vegas	6	1349 ± 1051	4	0	3	4
G. morafkai						
Cave Buttes	7	2993 ± 2533	3	3	0	2
Silverbell	11	2666 ± 2808	0	0	0	7
Sugarloaf	13	4631 ± 3558	11	4	0	11
G. berlandieri						
Chaparral WMA	13	3034 ± 2486	0	4	0	9
East Rio Grande	12	2032 ± 1547	4	4	0	5
West Rio Grande	11	1627 ± 903	1	2	0	7
G. polyphemus						
Perdido	13	4352 ± 1468	5	0	2	12
Rayonier	11	3341 ± 1296	3	3	0	9
University of South Florida	12	3058 ± 1776	4	4	1	12

FIGURE 1. OTU richness in North American *Gopherus* tortoises. (a) Correlation between OTU richness and the number of sequences in a sample ($F_{(1,133)} = 31.57$, $P < 0.0001$). (b) Boxplot of residuals of the correlation presented in (a), separated by tortoise host species. (c) Boxplot of residuals of OTU richness in three sampling locations of Sonoran desert tortoises (*G. morafkai*). Letters above the boxplots denote significantly different groups. Boxplots indicate the median, interquartile range, reasonable range of the data, and outliers.

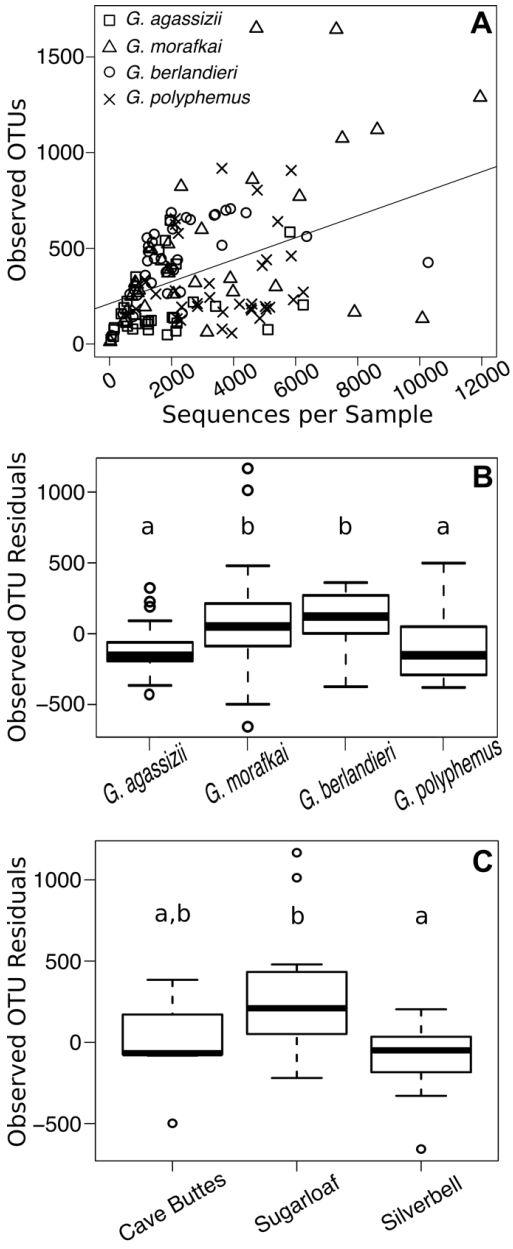


FIGURE 2. Proportion of bacterial taxa in rarefied samples. (a) Bacterial phyla represented in the four *Gopherus* tortoise host species. (b) Classes representing at least 5% of the sequences in any one site, with the remaining classes grouped as "Other Classes".

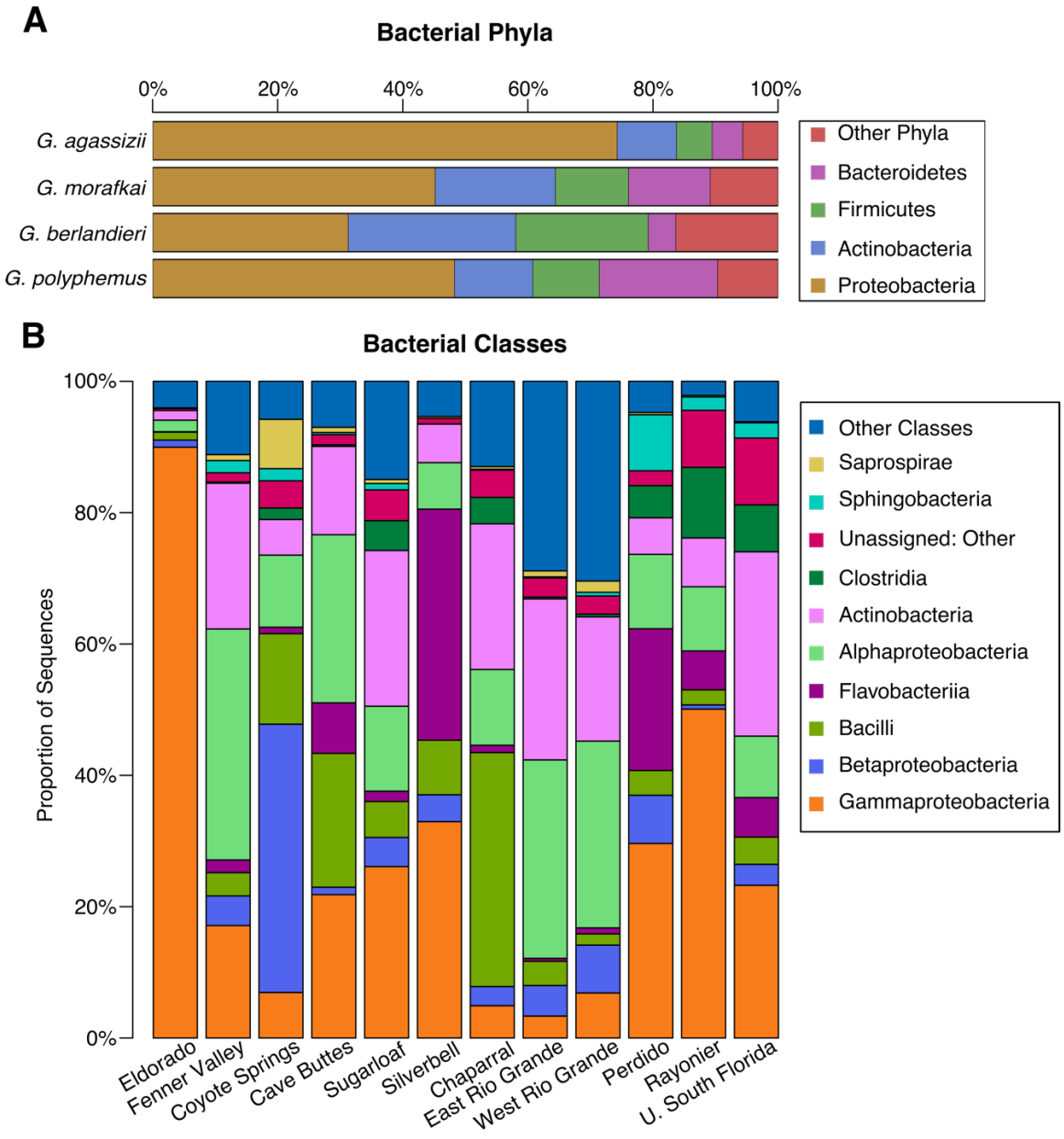


FIGURE 3. Principal coordinates analysis of nasal bacterial communities in four North American *Gopherus* tortoise host species from unweighted Unifrac distance matrices. (a) Communities of 1000 sequences per sample. (b) Data excluding sequences identified as *Chelonobacter*, with data rarefied to 500 sequences per sample. Closed circles = *Chelonobacter* was present in the sample; Open boxes = *Chelonobacter* not detected in the sample. (c) Diversity of *Chelonobacter* populations in tortoise host species. (a-c) Color-coded by host species: Red = *G. agassizii*; Orange = *G. morafkai*; Green = *G. berlandieri*; Blue = *G. polyphemus*.

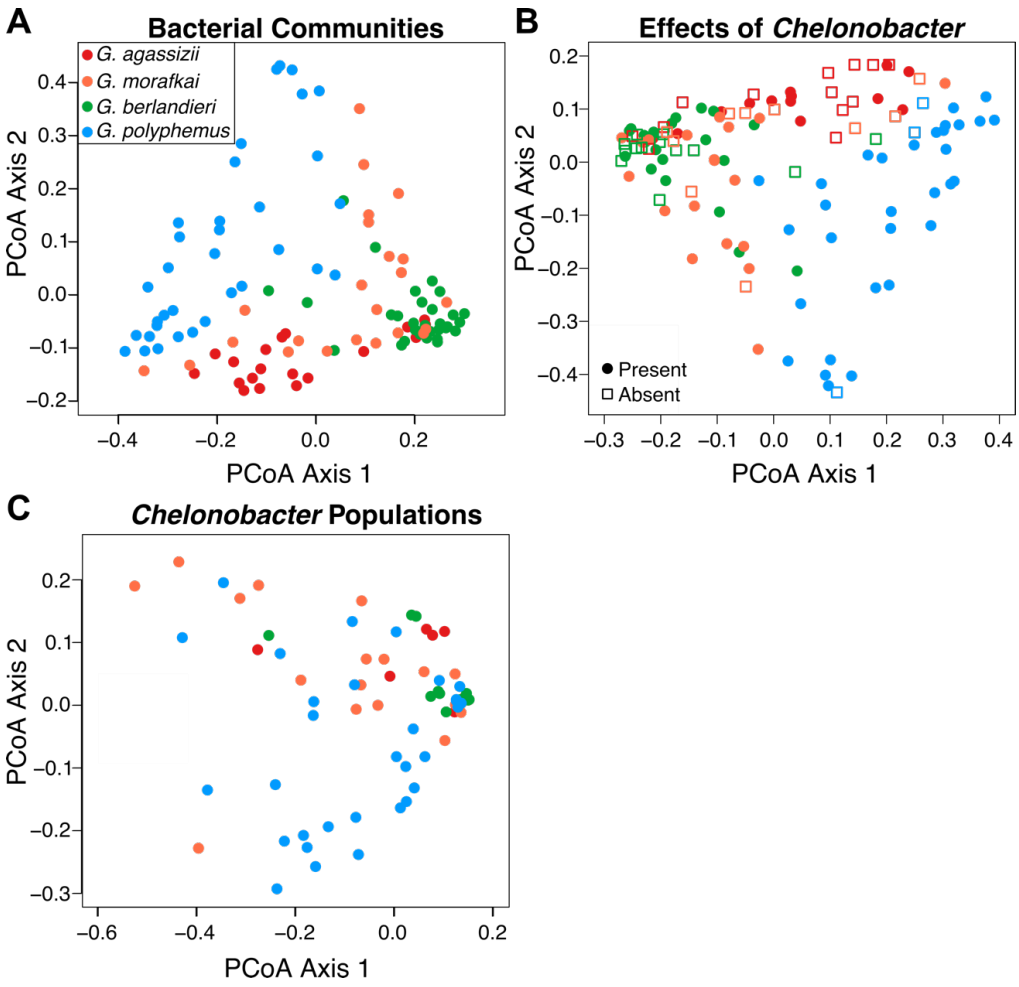


FIGURE 4. Core microbiome, designated as OTUs present in 75% of samples per sampling location, for four North American *Gopherus* tortoise species. Core OTUs color-coded by bacterial family.

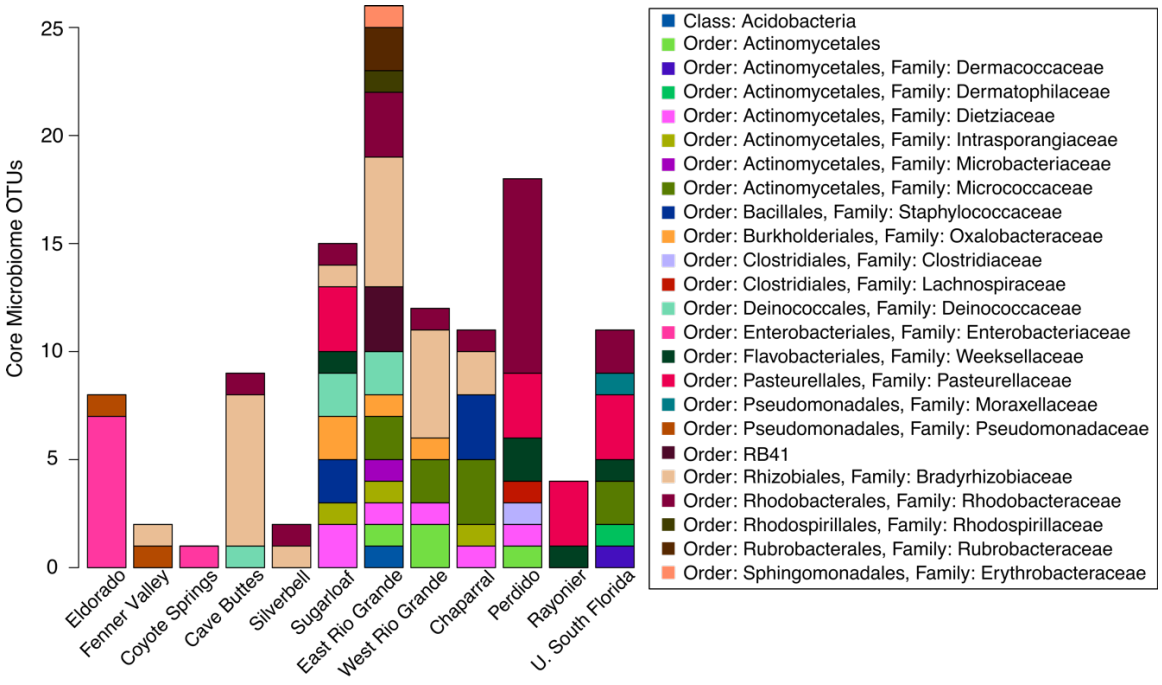
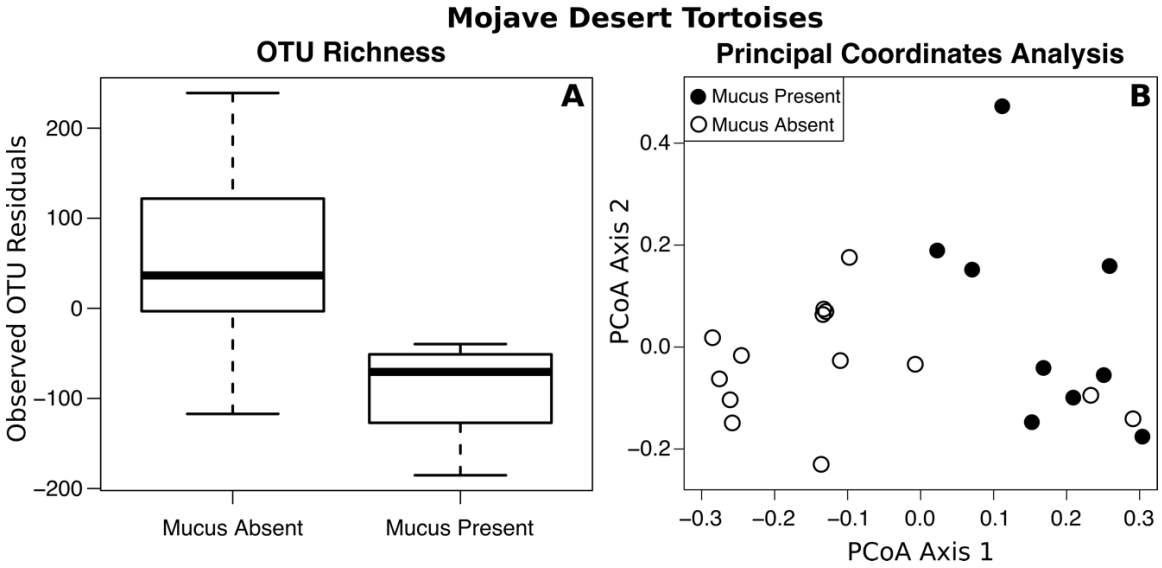


FIGURE 5. Effects of nasal mucus on upper respiratory bacteria in Mojave desert tortoises (*Gopherus agassizii*). (a) Boxplots of residuals of OTU richness (vs. number of sequences) with and without nasal discharge of mucus ($F = 16.26$, $P = 0.0006$). Boxplots indicate the median, interquartile range, and reasonable range of the data. (b) Principal coordinates analysis, with unweighted Unifrac distance matrix, in tortoises with (closed circles) and without (open circles) nasal discharge of mucus.



CONCLUSIONS

The research presented in this dissertation addresses a number of questions regarding the ecology of host-pathogen interactions, in addition to interactions with other members of the microbial community, in the context of tortoise upper respiratory tract disease. From thorough sampling of the nasal flora of individuals in four species of tortoises, two mycoplasmal pathogens were found in four host species, but prevalence of these pathogens differed among local populations. Co-infection of two mycoplasmas was also found across the tortoise species, but in statistically different patterns among host species. Furthermore, pathogens interact within the microbial community differently among hosts, as presence of *Mycoplasma agassizii* was only found to influence community composition in the two species of desert tortoise. Microbiome data also had very high diversity of *Pasteurella testudinis*, a third pathogen in this system. Lastly, the presence of clinical signs of disease was not predicted by infection or co-infection patterns, though mucus as a clinical sign of URTD was associated with declines in microbiome richness and a shift in bacterial community composition.

The intersection of pathogens, other microbes, hosts, and their environment forms a complicated web of disease ecology. The results in this dissertation introduce many new questions yet to be addressed. Sequencing of three genetic markers of *M. agassizii* did not detect meaningful differentiation, and thus we have yet to address questions of co-evolution between hosts and pathogens or variation in virulence. While it is possible that the lack of genetic variation found here represents genomic variation present in the wild, it is more likely that deeper sequencing of the mycoplasma genome is necessary to

best address evolutionary and disease-related questions in this pathogen. Regarding co-infection dynamics, we know nearly nothing about how pathogens interact with each other in this system, and how those interactions differ among host species. Experimental inoculation studies and long-term monitoring of wild individuals would answer whether and how the order of infection by multiple pathogen types influences manifestation of disease, and how those infections and co-infections perturb a healthy upper respiratory microbiome. In Texas tortoises, *M. agassizii* was only found in co-infection with *M. testudineum*, and this difference in pattern from other host species, if not a statistical product of small sample size, suggests additional differences in disease ecology among North American tortoise hosts. Importantly, research should also focus on how human settlements and encroachment affect pathogen transmission and disease manifestation.

Most research in this field has focused on Mojave desert tortoises and gopher tortoises, largely in the context of concern due to population declines. While tortoises are not model species in the conventional sense, population perturbations from habitat degradation or decline and climate change could send these long-lived species down a slippery slope toward extinction.