DISSERTATION

EVOLUTIONARY AND ECOLOGICAL PROCESSES IN MICROPARASITE COMMUNITIES OF BATS

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ABSTRACT

EVOLUTIONARY AND ECOLOGICAL PROCESSES IN MICROPARASITE COMMUNITIES OF BATS

The majority of parasites infecting humans come from animals, so it is necessary to study how parasites are maintained in nature to understand which human populations are at risk of spillover. Parasites are also highly diverse in their own right, with their own fascinating ecology, so studying parasite communities will give us a full perspective of Earth's biodiversity. Research has shown that bats are significant hosts of parasites globally, including important pathogens of humans. The unique evolution of flight in bats has influenced their ability to disperse parasites, and may have modified their immune systems to be more tolerant of infections compared to other mammals. Thus, studying bat parasite communities could deepen our knowledge of the evolutionary history of mammalian parasites and the importance of flight in the maintenance of parasite community diversity in bats.

In this dissertation, I focus on the evolutionary history and ecological forces affecting diversity in blood-borne microparasite communities of bats. There is a particular focus in this dissertation on *Bartonella* bacteria, a common parasite in mammals. To determine the importance of bats in the historical diversification of *Bartonella* bacteria, I performed the most comprehensive phylogenetic analysis of the genus to date, including data from 121 strains cultured from bats globally. I discovered that *Bartonella* bacteria began infecting mammals 62 million years ago and likely originated from bats. In a review of other bat parasites, including eukaryotic trypanosomes and haemosporidian parasites, I find that bats have had a similarly deep influence on the evolution of these taxa, and their historical spread across continents and to other mammalian hosts.

To examine the importance of dispersal on parasite community diversity at smaller ecological scales, I focused on *Bartonella* communities in African fruit bats. I investigated differences in the

Bartonella communities in fruit bat populations across a West African island chain. In addition, I examined the population genetics of bat flies, the presumed vectors of *Bartonella* in bats, and bat fly symbionts to compare with the genetic population structure of the bat hosts. *Bartonella* communities differed across islands and showed a pattern of isolation by geographic distance, indicating that dispersal of parasite species is constrained by bat movement patterns. Population structure was reduced in bat flies and symbionts compared to that of the bat hosts, suggesting that bat movements between islands are going undetected from population genetics of the hosts alone.

Finally, I investigated *Bartonella* community dynamics in a captive colony of fruit bats in Ghana over a sampling period of three years. In this study, the population density of bat flies declined over time and was then restored via reintroduction of flies from a wild source population, causing predictable changes in *Bartonella* prevalence within the bat colony. These results provide the first experimental confirmation of bat flies as vectors of *Bartonella* in bats. In addition, changes in *Bartonella* diversity within the colony that occurred in the absence of bat flies might be attributed to ecological drift and selection through interspecies competition mediated by the host immune system. These projects highlight the ecological and evolutionary processes affecting microparasite communities of bats, providing useful information for understanding how parasite biodiversity is created and maintained in natural populations.

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DEDICATION

This dissertation is dedicated in loving memory of my father, Scott McKee. My academic journey started at home, with the curiosity you always fostered.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGEMENTS iv
DEDICATION vi
LIST OF TABLES x
LIST OF FIGURES xi

Chapter 1 Introduction 1

Chapter 2 The influence of bats on the evolution of mammalian parasites: a generalizable 'bat seeding' hypothesis? 5
2.1 Overview 5
2.2 Introduction 5
2.3 *Trypanosoma* 9
2.4 Haemosporida 17
2.5 *Bartonella* 24
2.6 Other parasite taxa 27
2.7 Conclusions and future directions 28

Chapter 3 Comprehensive time tree analysis identifies bats as key to the radiation of mammal-associated *Bartonella* bacteria 34
3.1 Overview 34
3.2 Introduction 34
3.3 Methods 37
3.3.1 Molecular data collection 37
3.3.2 Phylogenetic analysis 39
3.3.3 Tip-association tests 41
3.3.4 Host clade definitions and divergence dates 41
3.3.5 Assessing the influence of hosts on *Bartonella* diversification and geographic spread 42
3.4 Results 43
3.4.1 Revised topology of *Bartonella* phylogeny 43
3.4.2 *Bartonella* divergence dates and clock rates 45
3.4.3 Diversification of bartonellae with hosts 46
3.4.4 Influence of host groups on *Bartonella* evolution 48
3.5 Discussion 52

Chapter 4 Vector population and bacterial community structure suggest bat dispersal across an island chain 61
4.1 Overview 61
4.2 Introduction 61
4.3 Methods 66

4.3.1 Specimen collection . . . 66
4.3.2 Laboratory methods . . . 67
4.3.3 Phylogenetic analysis . . . 69
4.3.4 Statistical analysis . . . 69
4.4 Results . . . 70
4.4.1 Collection and identification of bat flies . . . 70
4.4.2 Patterns of *Bartonella* prevalence and diversity . . . 71
4.4.3 Detection and identification of bat fly symbionts . . . 74
4.5 Discussion . . . 75

Chapter 5 Manipulating vector transmission reveals local processes in bacterial communities of bats . . . 80
5.1 Overview . . . 80
5.2 Introduction . . . 80
5.3 Methods . . . 83
5.3.1 Study system . . . 83
5.3.2 Bacterial detection and gene sequencing . . . 85
5.3.3 Data recording and statistical analyses . . . 86
5.4 Results . . . 87
5.4.1 Phylogenetic analysis of detected bacteria . . . 87
5.4.2 *Bartonella* infection prevalence and effects of bat fly reintroduction . . . 87
5.4.3 Patterns of *Bartonella* diversity . . . 89
5.4.4 Shift in *Bartonella* species abundance . . . 90
5.4.5 Interactions between *Bartonella* species . . . 93
5.5 Discussion . . . 94

Chapter 6 Concluding remarks . . . 99

References . . . 104

Appendix I . . . 148
I.1 Sequencing of bat-associated *Bartonella* strains and molecular data validation . . . 148
I.2 Testing alternative models in BEAST . . . 150
I.3 *Bartonella* lineages associated with arthropods . . . 153
I.4 Additional figures and tables . . . 156

Appendix II . . . 165
II.1 Additional figures and tables . . . 165

Appendix III . . . 171
III.1 Supplementary methods . . . 171
III.1.1 Sampling protocol . . . 171
III.1.2 DNA extraction . . . 171
III.1.3 Bacterial detection and sequencing . . . 172
III.1.4 Phylogenetic analysis of bacterial sequences . . . 173

III.1.5 Regression analyses . . . 174
III.1.6 Likelihood ratio tests . . . 175
III.1.7 Assumptions . . . 176
III.2 Supplementary results . . . 176
III.2.1 Phylogenetic analysis of detected *Bartonella* and *Rickettsia* species . . . 176
III.2.2 Demographic patterns of *Bartonella* prevalence during experiment . . . 181
III.2.3 Effects of bat fly reintroduction on treatment versus control bats . . . 182
III.2.4 Differences in *Bartonella* prevalence and diversity between bats and bat flies . . . 183
III.2.5 Shift in *Bartonella* community diversity using relative counts . . . 184
III.2.6 Individual infection histories and duration of infections . . . 184
III.3 Additional figures and tables . . . 186

LIST OF TABLES

2.1 *Trypanosoma* host and vector associations . . . 31
2.2 Haemosporida host and vector associations . . . 32
2.3 *Bartonella* host and vector associations . . . 33

3.1 Clock rates . . . 47
3.2 Tip-association test results . . . 47
3.3 Stochastic character mapping resampling results . . . 50
3.4 Node properties of state transition networks . . . 52

4.1 Sampling sites . . . 67
4.2 *Bartonella* infection prevalence . . . 71

I.1 Features of sequenced loci . . . 159
I.2 Pairwise homoplasy index test results . . . 159
I.3 Substitution saturation test results . . . 160
I.4 Robustness of divergence dates . . . 161
I.5 *Bartonella* clades and host associations . . . 162
I.6 *Bartonella* clade descriptions . . . 162
I.7 Host clade divergence dates . . . 163
I.8 BEAST priors . . . 163
I.9 Transition rates . . . 164

II.1 PCR primers . . . 165
II.2 PCR thermocycler protocols . . . 165
II.3 Counts of bacterial symbionts . . . 168
II.4 Counts of *Bartonella* species . . . 169
II.5 *E. helvum* age distribution . . . 169
II.6 Genetic and physical distances . . . 169
II.7 Bacterial symbiont detection results . . . 170

III.1 Captive colony cohorts . . . 172
III.2 PCR primers . . . 173
III.3 PCR thermocycler protocols . . . 173
III.4 Segmented regression results . . . 190
III.5 LR test results, *Bartonella* relative abundance . . . 191
III.6 LR test results, comparison of bats and bat fly populations . . . 196
III.7 LR test results, *Bartonella* relative counts . . . 197

LIST OF FIGURES

1.1 Dissertation conceptual figure . . . 3

2.1 *Trypanosoma* composite cladogram . . . 13
2.2 Haemosporida composite cladogram . . . 19
2.3 *Bartonella* cladogram . . . 27

3.1 *Bartonella* time tree . . . 45
3.2 Divergence date comparison . . . 49
3.3 Order and ecozone transitions . . . 51

4.1 Gulf of Guinea island study conceptual figure . . . 63
4.2 Map of study area . . . 64
4.3 Genotypes of bat flies and bacterial symbionts . . . 72
4.4 Patterns of *Bartonella* infection . . . 73
4.5 Correlation between *Bartonella* dissimilarity and distance . . . 75

5.1 Captive colony experiment conceptual figure . . . 82
5.2 *Bartonella* infection prevalence and load . . . 88
5.3 *Bartonella* infection diversity . . . 91
5.4 *Bartonella* relative abundance . . . 92
5.5 Duration of *Bartonella* infections . . . 93
5.6 *Bartonella* coinfection frequencies . . . 94

I.1 GC content . . . 149
I.2 *Bartonella* network phylogeny . . . 150
I.3 Substitution saturation, uncorrected versus adjusted distances . . . 151
I.4 Substitution saturation, adjusted distances versus transitions/transversions . . . 152
I.5 16S rRNA molecular clock . . . 156
I.6 Lineages over time . . . 157
I.7 Comparison of Bayesian and maximum likelihood trees . . . 157
I.8 *Bartonella* time tree, uncollapsed . . . 158

II.1 *Bartonella* concatenated *ftsZ* and *gltA* tree . . . 166
II.2 Genetic and physical distance correlations . . . 167

III.1 *Bartonella gltA* tree . . . 177
III.2 *Bartonella ftsZ* tree . . . 178
III.3 *Bartonella* ITS tree . . . 179
III.4 *Bartonella* concatenated *ftsZ* and *gltA* tree . . . 180
III.5 *Rickettsia gltA* tree . . . 181
III.6 Change in *Bartonella* prevalence during experiment . . . 182
III.7 Segmented regression results, prevalence and infection load . . . 186
III.8 *Bartonella* markers positive . . . 187

III.9 *Bartonella* positive markers and coinfection 188
III.10*Bartonella* inverse Simpson index 189
III.11Segmented regression results, colony-level *Bartonella* diversity 192
III.12Segmented regression results, individual-level *Bartonella* diversity 193
III.13*Bartonella* relative counts 194
III.14Correlations between *Bartonella* relative abundance and counts 195

Chapter 1

Introduction

Humans do not exist in isolation from the diseases of animals. In fact, most infectious pathogens of humans originate from wild or domestic animals. Reviews have estimated that 60% of recently emerging human pathogens originated from animals (Jones et al., 2008; Woolhouse and Gowtage-Sequeria, 2005). Research into the origins of important human pathogens such as malaria, HIV, smallpox, and others have shown that they were acquired from animals and later became established within human populations (Babkin and Babkina, 2015; Esposito et al., 2006; Loy et al., 2017; Sharp and Hahn, 2011). Ultimately, all infectious agents of humans can be fit into a spectrum of specificity, ranging from accidental infections from animals (spillover) to fully exclusive infections of humans that are evolutionarily derived from their zoonotic predecessors (Lloyd-Smith et al., 2009; Wolfe et al., 2007). Considering this framework, it is necessary to assess the diversity of pathogens that exist in animals and the ecological processes that maintain infections in nature in order to predict where and how spillover occurs.

Beyond the immediate consequences of human disease, parasites and pathogens are fascinating to study in their own right. Parasites are enormously diverse in terms of number of species and distinct traits that mediate their relationships with their hosts. Potentially half of all species are parasites, with many still awaiting discovery (Morand, 2015; Poulin, 2014). Host-parasite interactions also require multiple scientific disciplines to fully characterize, including chemistry, molecular biology, population biology, evolution, and ecology. Consquently, we only have a shallow understanding of the life history and host-parasite interactions of most parasites.

From the perspective of ecology, parasites are interesting to study because they can serve as microcosms through which to evaluate broader ecological theories. In addition, parasites not only interact with their hosts, but potentially other co-infecting parasites within the same host. Thus, parasites ought to be studied in a community ecology context to understand how parasite diversity is generated and maintained, and how within-host interactions might introduce nonlinearity into infection dynamics (Johnson et al., 2015; Pedersen and Fenton, 2007). However, this is not yet a

common approach for studying parasites, but could lead to important insights about how parasites persist in natural systems (Seabloom et al., 2015). Additionally, by studying the community ecology of parasites, we might discover general processes that govern biological diversity in nature (Sutherland et al., 2013).

Community ecology theory posits that four fundamental processes determine diversity in communities (Vellend, 2010): speciation, ecological selection, ecological drift, and selection. However, the nested nature of parasitism produces multiple scales at which to study the effects of these four processes on parasite community diversity (Johnson et al., 2015; Seabloom et al., 2015): within individual hosts, among hosts in a population, and among host species in a community (Figure 1.1). The process of dispersal, or parasite movement, influences parasite community diversity across all scales. Within host populations and communities, parasite dispersal takes the form of transmission between individual hosts or spillover between co-occurring host species (Figure 1.1, inset). Increasing the scale to the study of multiple host populations or communities in a geographical region, parasite dispersal occurs due to the movement of infected host individuals between populations or colonization of parasite hosts between regional communities (Figure 1.1). This theoretical framework is powerful because it allows us to discern at which scale we are studying parasite communities, and pose questions about how ecological processes are acting at that scale (Levin, 1992).

In this dissertation, I will focus on the ecological processes affecting parasite communities in bats. Bats are an interesting group to study because their parasite communities are highly diverse and they are capable of long-distance dispersal through flight. Furthermore, bats host many important human viral pathogens: SARS and MERS coronaviruses (Lau et al., 2005; Li et al., 2005; Memish et al., 2013), Ebola and Marburg filoviruses (Goldstein et al., 2018; Leroy et al., 2005; Negredo et al., 2011; Towner et al., 2009; Yang et al., 2019), paramyxoviruses (Chua et al., 2002; Drexler et al., 2012; Halpin et al., 2000), and rhabdoviruses, including rabies (Hayman et al., 2016; Kuzmin et al., 2006; Rupprecht et al., 2011). Bats are the only mammals capable of powered flight, dramatically increasing their dispersal potential relative to other terrestrial mammals. Flight has

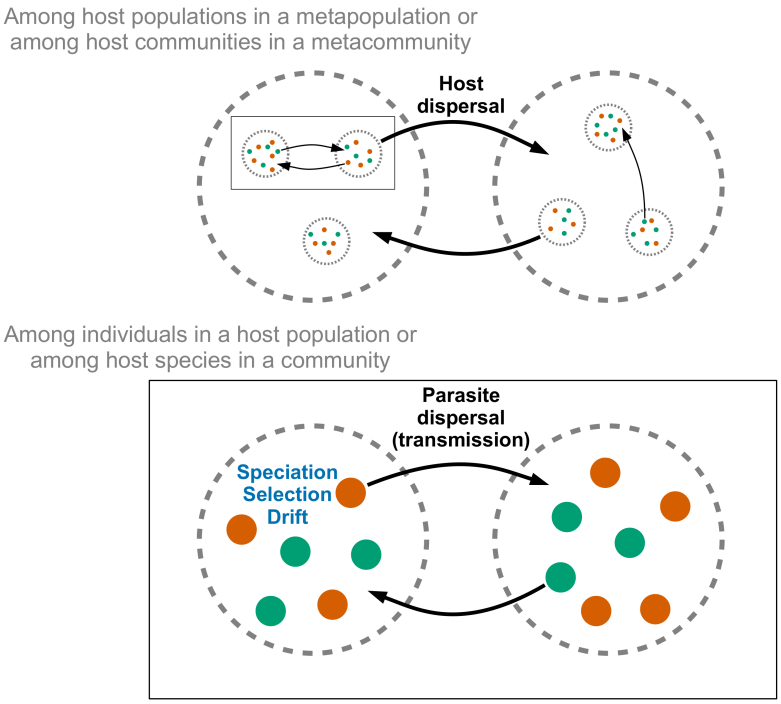

Figure 1.1: Multiple scales of parasite community ecology. Parasite communities (colored dots) can be studied at the level of within individual hosts or host species (inset), and among host populations or communities (top). Within host populations or communities (inset), parasites disperse between host individuals or host species via transmission. Other ecological processes, including speciation, selection, and drift, act on within-host communities to affect diversity. At the level of separate host populations or regional communities (metapopulations and metacommunities), dispersal is through movement of infected host individuals or species. At this level, within-host processes are occurring simultaneously (box in top panel).

allowed bats to colonize all continents except Antarctica and a few isolated islands. Additionally, the evolution of flight may have reshaped bats' immune responses to make them highly tolerant of infections (Hayman, 2016, 2019; Zhang et al., 2013). Bats' long lifespans, gregarious behavior, and habituation to urban environments also contribute to their ability to carry parasites, and potentially spread them to humans (Foley et al., 2018; Peel et al., 2017; Plowright et al., 2011). However, much of the literature on bat parasites has focused on viruses, so less is known about bacterial or endoparasitic eukaryotes of bats. My focus in this dissertation on bacterial communities, particularly of the genus *Bartonella*, will help to close this knowledge gap.

This dissertation will focus on the evolutionary origins of bat-associated parasites and the ecological processes affecting parasite communities in bat populations. Chapters 2 and 3 assess the

evidence that bats are key hosts in the diversification of the parasite taxa *Trypanosoma*, Haemosporida, and *Bartonella*, their geographic distribution, and spread to other animals. From an ecological perspective, these two chapters touch on parasite speciation and host dispersal among regional communities of animals (Figure 1.1), through patterns of diversification and host switching that have occurred over millions of years. Chapter 4 investigates the influence of fruit bat movement patterns on *Bartonella* communities and the population genetics of bat fly vectors and symbionts in a chain of isolated islands in West Africa. This chapter touches on speciation, drift, and host dispersal between island populations (Figure 1.1). Finally, Chapter 5 involves a study of long-term *Bartonella* community dynamics in a captive colony of bats in Ghana. In the absence of transmission via bat flies, changes in *Bartonella* communities were attributed to within-host selection via the host immune system and inter-species competition, and loss of rare species due to drift (Figure 1.1, inset). These chapters indicate that studying ecological processes affecting communities of parasites is similar to free-living organisms, although with influence from additional scales of organization. The emphasis on bat dispersal across these chapters demonstrates its importance in maintaining parasite community diversity across scales.

Chapter 2

The influence of bats on the evolution of mammalian parasites: a generalizable 'bat seeding' hypothesis?

2.1 Overview

Bats are regarded as important reservoir hosts for viruses; however there is less understanding of their role as hosts for vector-borne eukaryotic and bacterial infections. In this review, I synthesize the literature on the role that bats play in the evolutionary history of vector-borne parasites of mammals, including trypanosomes, haemosporidians, *Bartonella* bacteria, and other taxa. Common trends across these parasites suggest that bats have been highly influential in the diversification of these organisms and their transitions to other mammalian orders.

2.2 Introduction

A broad goal in parasitology and disease ecology is to understand the life history and evolutionary origins of parasites. Many parasites have undergone a convoluted series of host switches throughout their history, so identifying the ancestral hosts and the ecological drivers that lead to host switching has become a central theme in these disciplines. Furthermore, most infectious diseases of humans are either recently acquired zoonoses or ancestrally derived from infections of animals (Jones et al., 2008; Loy et al., 2017; Sharp and Hahn, 2011). Understanding how parasites switch hosts and how generalist parasites evolve from often specialist predecessors can give context to spillover events into atypical hosts, including humans and domesticated animals, and lead to predictions about which zoonoses may become established in atypical hosts (Lloyd-Smith et al., 2009).

Among the many reservoir hosts of human zoonotic infections, bats have been identified as important reservoirs for zoonotic viruses (Calisher et al., 2006; Hayman, 2016; Luis et al., 2013). High profile human viral infections associated with bats include coronaviruses (Lau et al., 2005; Li et al., 2005; Memish et al., 2013), filoviruses (Goldstein et al., 2018; Leroy et al., 2005; Negredo et al., 2011; Towner et al., 2009; Yang et al., 2019), paramyxoviruses (Chua et al., 2002; Halpin

et al., 2000), and lyssaviruses (Hayman et al., 2016; Kuzmin et al., 2006; Rupprecht et al., 2011). Other viruses associated with bats include hepadnaviruses (Drexler et al., 2013; Rasche et al., 2016), hepaciviruses and pegiviruses (Quan et al., 2013), bornaviruses (Cui and Wang, 2015), hepatoviruses (Drexler et al., 2015), and hantaviruses (Guo et al., 2013; Witkowski et al., 2016), although their zoonotic potential is unknown. Some of these viruses have wide, potentially global, geographic distributions (Anthony et al., 2017; Han et al., 2016b; Hayman, 2016; Hayman et al., 2016; Witkowski et al., 2016); long evolutionary histories with bats (Halpin et al., 2007; Plyusnin and Sironen, 2014; Taylor et al., 2010; Wertheim et al., 2013); and show frequent patterns of host switching from bats into other mammalian orders (Badrane and Tordo, 2001; Drexler et al., 2012, 2015; Rasche et al., 2016; Wilkinson et al., 2014). While our understanding of bats as viral reservoirs is nacent, even less is known about their role as hosts of vector-borne eukaryotic and bacterial agents. However, what we do know about bats and their interactions with viruses seems to be somewhat non-specific to viruses. Here, I review our current understanding of bats as viral reservoirs and extend these ideas to vector-borne eukaryotic and bacterial pathogens.

Bats have a combination of traits that is unique among mammals and may contribute to their reservoir potential (Calisher et al., 2006). Bats are an evolutionarily ancient lineage of mammals that began diversifying around 58–66 million years ago (mya) (Meredith et al., 2011; Shi and Rabosky, 2015; Teeling et al., 2005). Bats (Chiroptera) are the second most diverse order of mammals next to rodents, constituting 20% of all mammal species (Teeling et al., 2005). The age and diversity of bats provided the time and available niche space opportunity for many parasites to colonize and evolve within bats. Bats are exceptionally long-lived for their body size (Austad and Fischer, 1991; Foley et al., 2018; Healy et al., 2014; Munshi-South and Wilkinson, 2010), and frequently use torpor and hibernation (Geiser and Stawski, 2011). Some bats species form large, dense aggregations of millions of individuals during parts of the year (Hristov et al., 2010; Peel et al., 2017). Bats also show a range of social organizational behaviors (Kerth, 2008), including the temporary fragmentation of social groupings, i.e., fission-fusion structures (Kerth and Konig, 1999; Peel et al., 2017; Willis and Brigham, 2004), that may link networks of populations through

individual movements over time and across landscapes. Such traits of bats particularly have been shown to enhance various metrics of viral infections within and among species and contribute to the broad spatial distribution of bat-associated infections (Blackwood et al., 2013; George et al., 2011).

Chiropteran immune responses to infections also appear to differ in important ways from other mammals. Although there are some exceptions, bats generally show limited pathology to intracellular pathogens (Calisher et al., 2006; Hayman, 2016; Schountz, 2014). While bats share much of the same innate and adaptive immune machinery with other mammals (Baker et al., 2013b), there are documented losses of important immune genes (Ahn et al., 2016; Zhang et al., 2013; Zhou et al., 2016) or expansion of others (Bratsch et al., 2011), modifications of loci involved in pathogen recognition (Abduriyim et al., 2019; Escalera-Zamudio et al., 2015; Ng et al., 2016; Pavlovich et al., 2018), and changes in gene expression related to inflammatory responses (Ahn et al., 2019; Xie et al., 2018). These unusual aspects of bat immunology may allow bats to tolerate infection by viruses and other intracellular parasites without the excessive inflammatory responses that cause pathology (Hayman, 2019; Schountz et al., 2017). This attenuated inflammatory response may have evolved to prevent DNA damage by reactive oxygen species produced from the high metabolic demands of powered flight (Brook and Dobson, 2015; Munshi-South and Wilkinson, 2010; Zhang et al., 2013), a hypothesis that could explain the exceptional longevity and low rates of cancer in bats (Foley et al., 2018; Wang et al., 2011). Thus, the singular evolution of flight in bats may have produced pleiotropic effects on other traits that contribute to the uniqueness of bats as infectious reservoirs (Wang et al., 2011).

The importance of flight as a key innovation in bat evolution and their role as parasite hosts is reflected in the geographic distribution of Chiroptera. Flight enables extant bats to disperse over large distances, sometimes hundreds of kilometers, during regular foraging or seasonal migrations (Fahr et al., 2015; Popa-Lisseanu and Voigt, 2009; Richter and Cumming, 2008) or accidental dispersal or translocation events (Constantine, 2003; Jimenez and Hazevoet, 2010). This trait has allowed bats to repeatedly colonize landmasses and islands throughout their evolutionary history

(Almeida et al., 2014, 2016; Bonaccorso and McGuire, 2013; Eick et al., 2005; Lim, 2009; O'Brien et al., 2009; Russell et al., 2015; Stribna et al., 2019; Teeling et al., 2005). The result is that bats are one of the most widely distributed orders of mammals, occupying all continents except Antarctica and some isolated oceanic islands. The dispersal of bats globally would thus be expected to expand the geographic range of their parasites, which is illustrated by the global distribution of some bat-associated viruses (Hayman, 2016) and the presence of other bat viruses on isolated islands (Hall et al., 2014; Peel et al., 2012). As the only mammals capable of flight, this trait alone potentially singles out bats as unique vectors of parasite dispersal compared to other mammals.

However, much of the research and theory developed on the role of bats as unique infectious reservoirs has focused primarily on viruses (Hayman et al., 2013a). Bats host a number of other microparasite taxa, including bacteria, protozoa, fungi, and helminths (Beltz, 2018; Klimpel and Mehlhorn, 2014; Mühldorfer, 2013) that may share similarities with viruses in terms of host-parasite interactions and their coevolutionary history with bats. Whether bats are 'special' reservoirs of these other parasite taxa is still an area of much needed research that must surmount some existing challenges to studying the evolution of these organisms. Compared to directly transmitted viruses, these taxa can have more complicated life histories, including free-living (i.e., environmental) stages or transmission to additional vertebrate hosts or invertebrate vectors. Such traits may lead to less predictable patterns of host-parasite coevolution. This is especially true for vector-borne parasites, since vectors can vary in their own host specificity which may counteract the adaptation of the parasite to any one vertebrate host. Thus, specialist and generalist parasite lineages may exist even in the same taxon, depending on the specificity of the vectors for each lineage. Secondly, some of the earliest descriptions of bat parasites relied completely on morphological descriptions (Dionisi, 1898; Gardner et al., 1987; Goedbloed et al., 1964). These data can be challenging to align with modern methods of detection and phylogenetic analysis that rely on DNA sequences, producing 'ghost' taxa that have never been identified with DNA sequences (Perkins and Schaer, 2016).

Despite these challenges, there are several vector-borne parasites harbored by bats that are particularly well studied and thus good candidates for review: protozoa in the genus *Trypanosoma*, Haemosporida parasites, and *Bartonella* bacteria. In this review, I attempt to draw parallels between these taxa and link any emerging patterns with the literature on bat-associated viruses. One clear pattern is that bats have had a profound influence on the evolution and geographic distribution of these parasites, similar to patterns that are emerging in bat-borne viruses. Drawing on the 'bat seeding' hypothesis proposed by Hamilton et al. (2012b) to explain the broad geographic and host distribution of the clade of trypanosomes that include *T. cruzi* (the etiological agent of Chagas disease), I posit that this same hypothesis might be broadly applicable. Such a hypothesis could unite often siloed realms of research on infectious agents and generate discussion and collaborations that would lead to a broader understanding of the biogeography and evolutionary history of mammalian parasites.

I will review the literature on trypanosomes, haemosporidia, and *Bartonella* in mammals and summarize current knowledge on the life cycle, host and vector specificity, geographic distribution, and evolutionary history of these taxa, emphasizing the unique role that bats play in the diversification and spread of these taxa. This synthesis highlights knowledge gaps that remain in the study of these three groups. Finally, I discuss the broad applicability of the 'bat seeding' hypothesis to other mammalian parasites and suggest directions for future research.

2.3 *Trypanosoma*

Trypanosomes are unicellular flagellate eukaryotes (Euglenozoa: Kinetoplastea: Trypanosomatida: Trypanosomatidae) that parasitize vertebrates. Two *Trypanosoma* species, *T. brucei* and *T. cruzi*, cause significant disease in humans. *T. brucei*, the agent of African trypanosomiasis (sleeping sickness), is endemic to sub-Saharan Africa and is transmitted by tsetse flies (Diptera: Glossinidae). Approximately 65 million people are at risk of infection with *T. brucei*, but ongoing control efforts have successfully reduced the number of actual cases to below 10,000 per year since 2009 (World Health Organization, 2019a). Chagas disease (American trypanosomiasis) is caused by *T. cruzi*, is endemic in Central and South America, and is transmitted by kissing bugs in the

subfamily Triatominae (Hemiptera: Reduviidae). Approximately 109 million people are at risk of infection in Latin America, and between 6–8 million people are estimated to be currently affected by Chagas disease, with 41,200 new cases arising annually in 2006 (Moncayo and Silveira, 2009; Rassi et al., 2010; World Health Organization, 2019b).

Trypanosoma parasites are dixenous, forming asexual developmental stages (extracellular trypomastigotes or intracellular amastigotes) in vertebrate blood and other stages (epimastigotes) in their invertebrate vectors depending on the transmission route. Two different forms of vector-borne transmission are used by trypanosomes to infect hosts: salivarian (inoculative) and stercorarian (contaminative). The salivarian route used by *T. brucei* and allies (*T. congolense*, *T. vivax*) involves the development of epimastigotes in the salivary glands of tsetse flies and formation of infective trypanomastigotes that are injected into the host along with saliva during the insect bite. The stercorarian route used by *T. cruzi*, *T. rangeli*, *T. theileri* and other mammalian trypanosomes involves development of epimastigotes and infective trypanomastigotes in the gut of invertebrate vectors that are transmitted to vertebrate hosts through contamination of bite wounds by feces or ingestion of infected vectors. Some species in the Stercorarian group, including *T. cruzi*, also produce intracellular amastigote stages within host tissues.

Along with the related dixenous genus *Leishmania*, trypanosomes appear to have evolved from monoxenous ancestors harbored by mainly invertebrates, which in turn evolved from free-living euglenids (Hamilton et al., 2004; Lukeš et al., 2014, 2018). *Trypanosoma* is an early branching lineage within the Trypanosomatidae family; however it is still unclear exactly when and how this lineage evolved (Lukeš et al., 2018). While limited fossil evidence of trypanosomes exist, it points to an advanced age for the clade. Trypanosomes identified as *Paleotrypanosoma burmanicus* were observed in the midgut and salivary ducts of the biting midge *Leptoconops nosopheris* preserved in Early Cretaceous Myanmar amber (Poinar Jr., 2008). While extant *Leptoconops* feed on saurupsids and mammals, the age and characteristics of the *L. nosopheris* specimen pointed to an association with reptiles that may have extended back at least 120 million years (Poinar Jr., 2008, 2014). *Trypanosoma antiquus* was described from metatrypanosomes in fecal droplets adjacent to a *Triatoma*

dominicana nymph preserved in Dominican amber (Poinar Jr., 2005). The exact age of Dominican amber is not clear, with dates ranging from 15–45 mya (Poinar Jr., 2005). The metatrypanosomes morphologically resembled that of *T. cruzi*, and mammalian hairs resembling bat hairs were also found next to the fecal droplets, suggesting that the ancestral vertebrate host of the triatomine and the trypanosome was a bat. Such a finding is supported by the extant association of *T. cruzi* and allies with bats and triatomines, as will be discussed below.

Molecular dating approaches likewise indicate an ancient origin of *Trypanosoma*, although estimates vary widely depending on calibration methods. Considering the exclusive distribution of *T. brucei* in Africa and *T. cruzi* in the Americas, Stevens et al. (1999) estimated that these two lineages diverged during the breakup of the continents in the mid-Cretaceous 100 mya. Haag et al. (1998) used the estimated evolutionary rate of 0.85% per 100 million years for metazoan ribosomal RNA genes (Escalante and Ayala, 1995) to date the separation of two non-salivarian trypanosome groups about 150 mya, and the separation of salivarian trypanosomes from the other trypanosomes about 300 mya. Other methods of dating the evolution of *Trypanosoma* have been based on host associations, particularly the separation of rodent, bird, and fish clades, and the separation of *Trypanosoma* from the monoxenous trypanosomatid parasites *Crithidia* (Lake et al., 1988), producing an average estimate of 335 mya for the divergence of deep *Trypanosoma* clades (Haag et al., 1998). While the exact age of trypanosomes may be challenging to capture with any one approach, it is probable that these parasites have had long relationships with their hosts and vectors.

One of the main challenges to understanding the evolution of trypanosomes is the broad host distribution of the genus. Phylogenetic analysis using molecular data and broad taxon sampling has been able to resolve deep splits between *Trypanosoma* clades (Figure 2.1): a clade infecting aquatic and amphibious vertebrates (amphibians, testudines, fish, platypus) and likely vectored by leeches and another clade containing the remaining *Trypanosoma* lineages infecting terrestrial vertebrates (Hamilton et al., 2007). Such a topology could indicate that *Trypanosoma* derived from free-living aquatic trypanomastids that subsequently adopted a parasitic lifestyle in aquatic

vertebrates and invertebrates, with a later transition to terrestrial animals. Within this terrestrial clade there are also deep separations between the salivarian trypanosomes (*T. brucei* clade) and several distinct clades infecting mammals and sauropsids (Hamilton et al., 2007). Despite these broad class-level associations, there is very little host specificity observed in the trypanosomes or their suspected vectors. *T. brucei* and its allies, *T. vivax* and *T. congolense*, infect a broad range of mammals in Africa, following the range of tsetse flies (Table 2.1). *T. evansi*, a species within the *T. brucei* clade, is not transmitted by tsetse flies and has thus expanded out of Africa (Desquesnes et al., 2013), where it is transmitted mechanically by tabanid flies and potentially by vampire bats. The remaining trypanosome lineages are associated with a polyphyletic assemblage of terrestrial vertebrates and invertebrate vectors (Table 2.1), with little to no evidence of cospeciation (Hamilton et al., 2007). Instead, the evolutionary history of trypanosomes appears to be marked by multiple host switching events.

Given these patterns of host switching, the wide geographic distribution of trypanosomes, and a growing number of *Trypanosoma* species associated with bats, researchers began to speculate about the role of bats in the spread of trypanosomes to other animals. As originally proposed by Hamilton et al. (2012b), the 'bat seeding' hypothesis proposes that the common ancestor of trypanosomes in the *T. cruzi* clade was a bat trypanosome that then diversified in other mammals and spread across continents. At this time the *T. cruzi* clade included *T. cruzi* from New World mammals (including bats), *T. cruzi marinkellei* from New World bats, *T. erneyi* from African bats (Lima et al., 2012), *T. dionisii* from Old and New World bats (Hamilton et al., 2012a), *T. conorhini* found globally in rats, two trypanosomes from a civet and a monkey in Africa (Hamilton et al., 2009), *T. vespertilionis* from Old World bats, *T. rangeli* found in New World mammals (including bats), a trypanosome from an African *Rousettus* bat, and *T. noyesi* from an Australian kangaroo (Botero et al., 2016; Noyes et al., 1999). Against these data, the 'bat seeding' hypothesis proposed that trypanosomes in this group switched from bat to terrestrial animal hosts at least five times, including *T. cruzi*.

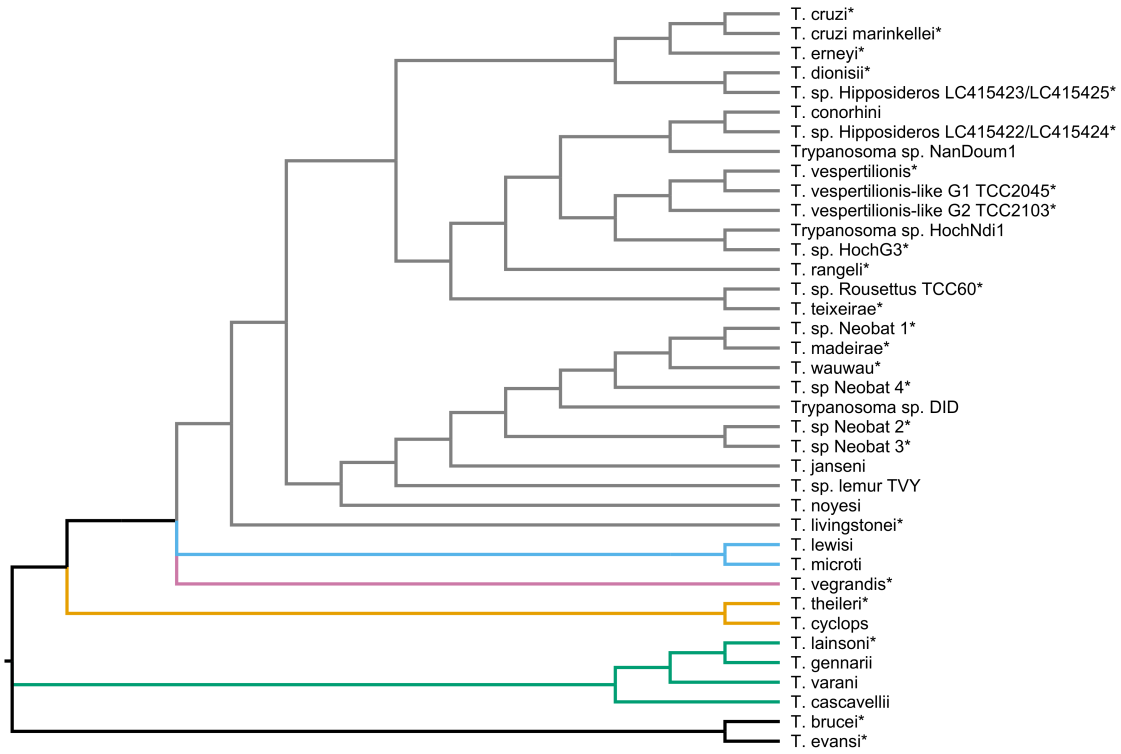

Figure 2.1: Composite cladogram of *Trypanosoma* species of terrestrial animals produced from published topologies (Barros et al., 2019; Botero et al., 2016; da Costa et al., 2016; Espinosa-Álvarez et al., 2018; Fraga et al., 2016; Hamilton et al., 2007; Lima et al., 2013; Qiu et al., 2019; Rodrigues et al., 2019; Thompson et al., 2014; Viola et al., 2009). Branches are colored according to clades identified in the literature: black – *T. brucei* clade, green – *T. varani* clade, orange – *T. theileri* clade, pink – *T. vegrandis*, blue – *T. lewisi* clade, gray – *T. cruzi* clade. Species names with asterisks have been identified in bats.

In the intervening years, this clade has expanded to include at least nine more *Trypanosoma* species associated with bats and at least three more from other mammalian groups (Figure 2.1; Table 2.1), lending further credence to the 'bat seeding' hypothesis (Espinosa-Álvarez et al., 2018). Insectivorous bats sampled in Africa were infected with *T. livingstonei*, currently the most deeply branching lineage in the *T. cruzi* clade (Lima et al., 2013). Several new species (Neotropical bat clades 1–4, *T. wauwau*, *T. madeirae*) have now been described from South American bats related to *T. noyesi* (Barros et al., 2019; Cottontail et al., 2014; Lima et al., 2015b; Rodrigues et al., 2019). A new trypanosome named *T. teixeirae* was found in an Australian flying fox and is phylogenetically related to the trypanosome from the African *Rousettus* bat and the subclade that includes *T. rangeli* (Barbosa et al., 2016). Novel lineages have also been discovered in African and Asian bats. Two trypanosomes cultured from *Hipposideros vittatus* in Zambia are related to

T. dionisii and *T. conorhini* (Figure 2.1) (Qiu et al., 2019). One lineage detected in *Hipposideros pomona* in China is distinct from the *T. dionisii-T. cruzi* subclade and may be related to the *T. rangeli* subclade (Cai et al., 2019). Another lineage from Chinese *H. pomona* and *Rhinolophus pusillus* appears to be related to the Neobats clade that includes *T. wauwau*. Nested within this diversity of bat-associated trypanosomes are several newly discovered *Trypanosoma* species from South American marsupials and Madagascan lemurs (Figure 2.1; Table 2.1) (Larsen et al., 2016; Lopes et al., 2018; Rodrigues et al., 2019). The host and geographic range of the bat-associated *T. dionisii* have also been expanded based on detection of this species in bats from the United States, China, and Japan (Cai et al., 2019; Hodo et al., 2016; Mafie et al., 2018), and in marsupials from Brazil (Rodrigues et al., 2019). A fatal case of Chagas disease in a person from Brazil was also attributed to a mixed infection of *T. cruzi* and *T. dionisii* (Dario et al., 2016). These findings are in keeping with the 'bat seeding' hypothesis and increase the number of inferred host switches from bats to other mammals across the phylogenetic tree.

Recent research has also refined our understanding of the genetic diversity of *T. cruzi* and the origin of this species from a progenitor in bats. Phylogenetic analysis of *T. cruzi* isolates has revealed six discrete typing units of *T. cruzi* (DTUs TcI–VI) infecting humans, bats, and other mammals in South America and a bat-associated DTU called TcBat (Lima et al., 2015a; Marcili et al., 2009; Pinto et al., 2012, 2015). Analysis of multiple sequenced loci indicates that TcBat is most closely related to TcI (Lima et al., 2015a; Marcili et al., 2009; Pinto et al., 2012) and not ancestral to all other DTUs (although see (Guhl et al., 2014)). Instead of a single switch from bats to terrestrial mammals including humans, these data suggest that multiple switching events from bats occurred during the evolution of *T. cruzi*. The timing of these host switches and the diversification of *T. cruzi* is still not clear, but there is consensus that all of the DTUs emerged prior to the arrival of humans in South America (Flores-López and Machado, 2011; Guhl et al., 2014; Lewis et al., 2011; Tomasini and Diosque, 2015). The initial switch from bats to terrestrial mammals several million years ago could have been facilitated by generalist triatomine vectors that parasitize both bats and terrestrial mammals. Once humans arrived in the Americas in the late

Pleistocene, multiple host switches of *T. cruzi* from bats and terrestrial mammals would explain the diversity of lineages presently found in humans. Detection of *T. cruzi*, including the TcBat lineage, in mummified human remains suggests that these transitions may have occurred soon after human colonization of the region and that bats were a reservoir of these infections (Aufderheide et al., 2004; Guhl et al., 2014). A recent report of *Trypanosoma* infection with the TcBat genotype in a Colombian child suggests that this genotype may also be a continuing source of infections in human populations (Ramírez et al., 2014a). In summary, bats have had a clear influence on the diversification and geographic distribution of trypanosomes within the *T. cruzi* clade, serving as the probable ancestral hosts of *T. cruzi*, *T. rangeli*, and *Trypanosoma* species associated with marsupials and other mammalian groups across many continents.

Bats also appear capable of naturally harboring a number of other trypanosomes outside of the *T. cruzi* clade, hinting that bats could have had a broader influence on *Trypanosoma* evolution and dispersal. *T. evansi* and *T. theileri* have been detected in vampire bats (*Desmodus rotundus*), which possibly acquired infection by feeding on domesticated animals or their dipteran vectors (Ayala and Wells, 1974; Herrera et al., 2004; Ramírez et al., 2014b; Rojas, 2005). While these bats might act as a vector of infections between domesticated animals, they might also be a reservoir, maintaining infection in a bat population without other hosts (Hoare, 1965). This latter alternative is supported by the findings of *T. evansi* in not only insectivorous bats, but also primarily frugivorous bat species (Herrera et al., 2004; Silva-Iturriza et al., 2013). Additionally, Cai et al. (2019) have reported detection of trypanosomes in the *T. brucei* clade for the first time in bats from China. Whether a sylvatic cycle involving additional vectors is maintaining infection of trypanosomes from the *T. brucei* clade in bats will need to be confirmed with additional research.

In addition to the *T. brucei* clade, bats in Australia were found to be carrying *T. vegrandis*, a species typically associated with Australian marsupials (Austen et al., 2015; Thompson et al., 2014). The prevalence of this species was very high (81.8%) across the pteropodid and vespertilionid bats sampled, suggesting that bats may be the primary hosts for *T. vegrandis*. Additional research on the host range, vectors, and phylogenetic position of *T. vegrandis* will inform un-

derstanding of the role that bats may play in its transmission cycle. Sequences from a clade of trypanosomes associated with lizards, snakes, rodents, and marsupials including *T. lainsoni*, *T. gennarii*, *T. varani*, and *T. cascavelli* have recently been obtained from phyllostomid bats (including *D. rotundus*) from Brazil (Dario et al., 2017b; Rodrigues et al., 2019). The lizard trypanosome *T. cascavelli* is transmitted by sand flies, so it is possible that the shared microhabitats of bats, rodents, and lizards may facilitate transmission among these animals.

Perhaps most intriguing are recent findings of monoxenous trypanosomatids associated with insects in bats. Sequences closely related to *Blastocrithidia* from triatomine bugs were amplified from insectivorous bats in Texas (Hodo et al., 2016). The trypanosomatid *Crithidia mellificae* (a species associated with bees and wasps) was detected in the omnivorous bat, *Anoura caudifer*, in Brazil (Rangel et al., 2019). The bat may have become infected by ingesting a hymenopteran; the ingestion of pollen contaminated with bee excreta, since the parasite is found in the posterior digestive tract of bees; or an infected bee shed its stinger in the bat, which became infected after licking the sting site. Finally, sequence reads from the non-pathogenic kinetoplastid *Bodo saltans* were found in *D. rotundus* (Dario et al., 2017b). *Bodo saltans* is an incredibly resilient and widespread organism, so the authors of this study did not rule out the potential that this finding was due to environmental contamination of the sample. The viabilility of these insect parasites and other trypanosomes outside of the *T. cruzi* clade will certainly need to be confirmed in bats to firmly rule out the possibility that the detections were only of DNA from dead organisms, were caused by contamination, or were accidental infections. Nevertheless, there is room to speculate that if these infections are authentic, they may be related to the tolerant immune systems of bats. If bats are highly permissive to infection by trypanosomes, then this could help to explain why they might be the ancestral hosts of *T. cruzi* and the broader *T. cruzi* clade. Further surveys of bat trypanosomes are certain to reveal new findings that will expand on the 'bat seeding' hypothesis.

Such surveys might be targeted towards answering some important questions regarding bat trypansome diversity and evolution. One question relates to the geographic distribution of *T. dionisii* and *T. vespertilionis*. *T. dionisii* seems to be geographically widespread, being reported now in

Japan (Mafie et al., 2018) and China (Cai et al., 2019; Wang et al., 2019) in addition to South America and Britain (Concannon et al., 2005; Gardner et al., 1987; Hamilton et al., 2012a). The Old and New World lineages of *T. dionisii* are phylogenetically distinct and the divergence of these lineages has been dated to 3.2–5 mya (Hamilton et al., 2012a). One possibility entertained by the authors was that Palearctic bats (e.g., *Myotis* or *Eptesicus* spp.) carried the parasite into the New World by disperal across Beringia. Broader detection of this species in bats could help to ascertain how and when this species spread across continents. There are similar indications that *T. vespertilionis* is also widespread in Africa, Asia, and North America (Edrissian et al., 1976; Keymer, 1971; Mitchell, 1956; Tromba, 1951; Wood, 1943). However, these reports were mostly limited to morphological descriptions, so it is difficult to ascertain whether all of these are truly this same species and not the morphologically similar *T. dionisii*. *T. vespertilionis* stands among other 'ghost' taxa that must be rediscovered and sequenced, including *T. pteropi* and *T. hipposideri* from Australian bats (Mackerras, 1959), *T. megachiropterorum* from Tonga (Marinkelle, 1979), *T. pessoai* from *D. rotundus* (Deane and Sugay, 1963), *T. leonidasdeanei* (Zeledón and Rosabal, 1969), and other *Megatrypanum* species that may be difficult to culture (Baker, 1973; Gardner and Molyneux, 1988b). Characterization of these species and the many others potentially awaiting discovery in bats could lend further support to the 'bat seeding' hypothesis and refine our understanding of the importance of bats in the evolution and spread of trypanosomes globally.

2.4 Haemosporida

Informally known as 'malaria parasites', haemosporidia are unicellular eukaryotic parasites in the phylum Apicomplexa. The most notorious member of this family is *Plasmodium falciparum*, one of the agents of human malaria. This mosquito-borne infection causes hundreds of millions of cases of malaria in Africa annually and is a subject of intense research to improve treatment and control efforts (World Health Organization, 2019c). However, *P. falciparum* is just one particularly virulent species in this highly diverse order of parasites associated with a diverse range of terrestrial animal hosts and hematophagous dipteran vectors (Table 2.2) (Perkins, 2014). The life cycle of haemosporidians involves multiple developmental stages distributed between the ver-

tebrate host and the vertebrate vector (Borner et al., 2016; O'Donoghue, 2017). Vertebrates are injected with sporozoites by the vector during blood feeding, the sporozoites colonize host tissues or blood to undergo asexual merogony, and merozoites eventually invade red blood cells where male and female gametocytes differentiate. Gametocytes are taken up in the blood meal of the next vector and travel to the vector's gut, undergo sexual reproduction and formation of an oocyst which will produce sporozoites, and the sporozoites will migrate to the salivary glands to complete the cycle. Schizogony in red blood cells is a unique feature of the genus *Plasmodium* that is correlated with some of the more severe symptoms of human malaria and its reliance on mosquito vectors (O'Donoghue, 2017). As discussed later, the polyphyly of *Plasmodium* across the haemosporidian phylogeny suggests that this trait has evolved multiple times (Galen et al., 2018; Lutz et al., 2016).

The phylogeny of Haemosporida is divided into clades that are generally associated with either sauropsids or mammals, and are transmitted by a particular family of dipteran vectors (Figure 2.2; Table 2.2). The mapping of these clades onto existing names of genera is still controversial, as recent developments have found that not all genera are monophyletic (Galen et al., 2018). Currently, there are 15–20 extant genera of haemosporidians and several more that are only described morphologically (Perkins, 2014; Perkins and Schaer, 2016). Ten genera are known from mammals – *Biguetiella*, *Bioccala*, *Dionisia*, *Hepatocystis*, *Johnsprentia*, *Nycteria*, *Plasmodium*, *Polychromophilus*, *Sprattiella*, and *Rayella* – and all except *Hepatocystis*, *Plasmodium*, and *Rayella* are exclusively associated with bats (Perkins and Schaer, 2016). The remaining haemosporidian genera are associated with sauropsids, with the exception of *Mesnilium* in fish (Perkins, 2014). Schizogony in genera besides *Plasmodium* occurs outside of erythrocytes, typically in the liver or other tissues or reticuloendothelial cells (Landau et al., 2012a,b; O'Donoghue, 2017). The multiple clades named *Plasmodium* are not recovered as monophyletic in several recent phylogenetic analyses, but are specific to either mammals or sauropsids (Figure 2.2) (Boundenga et al., 2016; Galen et al., 2018; Lutz et al., 2016; Martinsen et al., 2016; Schaer et al., 2015; Templeton et al., 2016a). *Plasmodium* has been reported from bats, primates, rodents, ungulates, Dermoptera, Philodota, and Macroscelidea (Perkins and Schaer, 2016). *Hepatocystis* is a derived genus placed within the

Plasmodium subgenera associated with primates and rodents (Figure 2.2), and has been reported from primates, rodents, ungulates, and bats (Table 2.2).

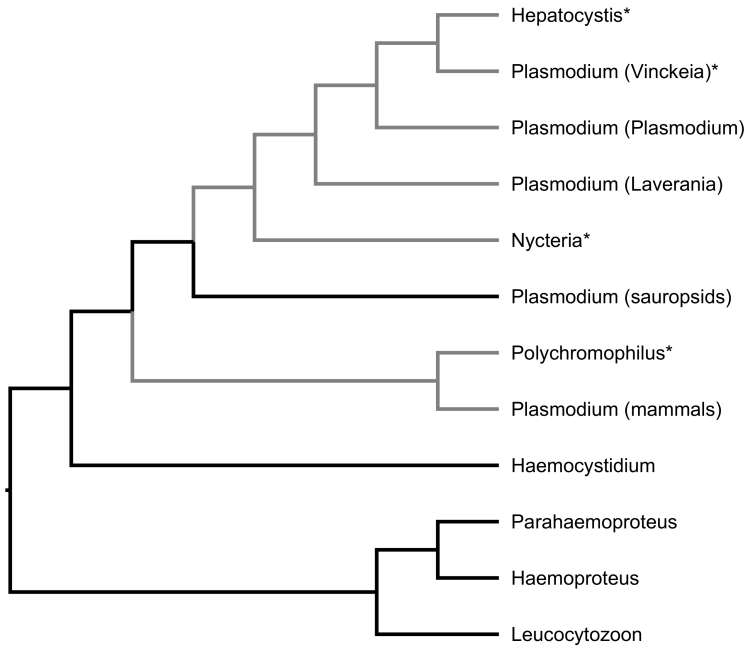

Figure 2.2: Composite cladogram of Haemosporida genera produced from published topologies (Borner et al., 2016; Galen et al., 2018; Lutz et al., 2016; Martinsen et al., 2016; Perkins and Schaer, 2016; Schaer et al., 2015; Templeton et al., 2016a). Branches are colored black for genera associated with sauropsids and gray for mammals. Genera with asterisks have been identified in bats.

Aside from host specificity, haemosporidian clades are also associated with specific families of vectors (Table 2.2) (Martinsen et al., 2008; Pacheco et al., 2018; Perkins, 2014), but the association of vector families across clades of the phylogenetic tree does not indicate a pattern of strict co-diversification. For instance, *Culicoides* midges vector both *Parahaemoproteus* and *Hepatocystis* parasites, and flies in the superfamily Hippoboscoidea vector *Haemoproteus* and *Polychromophilus* (Perkins, 2014). However, there is still much research that needs to be done on the vector associations of Haemosporida, especially among the genera associated with mammals. Vectors have yet to be discovered for bat-associated *Biguetiella*, *Dionisia*, *Johnsprentia*, *Nycteria*, and *Sprattiella*. Additionally, *Culicoides* midges have only been confirmed as vectors for *Hepatocystis* species found in primates (Garnham et al., 1961); whether this can be generalized to other *Hepatocystis* species

in rodents, ungulates, and bats is unclear. Many of these experiments to determine vector potential were done using dissection and microscopy before the advent of sensitive molecular detection methods (Gardner and Molyneux, 1988a; Garnham et al., 1961; Mer and Goldblum, 1947), so it is possible that the vector range of *Hepatocystis* and other bat-associated genera is broader than currently appreciated. For example, an *Anopheles* mosquito was found carrying *Polychromophilus* in Gabon (Makanga et al., 2017), although additional study will be needed to confirm biological competence. Given the broad host and vector associations of haemosporidians, phylogenetic studies can shed light on the host and vector switches that have occurred throughout the evolutionary history of haemosporidians.

The topology of the haemosporidian phylogenetic tree has been a source of some controversy among researchers due to issues involving restricted numbers of molecular loci used for phylogenetic inference, low taxon sampling, choice of appropriate outgroups, and models that account for different rates among lineages (Perkins and Schaer, 2016). Combining the results from recent studies that address these problems (Borner et al., 2016; Galen et al., 2018; Lutz et al., 2016; Martinsen et al., 2016; Schaer et al., 2015; Templeton et al., 2016a), a consensus topology may now be emerging that demonstrates how host and vector associations have evolved (Figure 2.2). Such a topology suggests that Haemosporida first evolved as parasites of sauropsids and then switched to mammals. It is unclear whether this switch occurred once with a subsequent reversion to sauropsids in one clade of Plasmodium or if two switches occurred along the branches leading to Polychromophilus and to *Plasmodium*/*Hepatocystis*. In parallel with these changes, a single switch to mosquito vectors occurred along with the switch to mammals, with at least two additional vector switches to nycteribiid bat flies in *Polychromophilus* and to biting midges in *Hepatocystis*. Knowledge of the vectors for *Biguetiella*, *Dionisia*, *Johnsprentia*, *Nycteria*, and *Sprattiella* may increase this number of switches.

Besides these patterns of host switching among vertebrate groups deep in the evolutionary tree, there is also rampant host switching within haemosporidian genera. Studies that have used different approaches to compare phylogenetic trees of parasites and hosts have found that the coevolutionary

history of haemosporidians and birds has been dominated by host switching, with little evidence of strict cospeciation (Alcala et al., 2017; Santiago-Alarcon et al., 2014). While no such cophylogeny approaches have been used to study the relationships between haemosporidians and mammals, host switching appears to be an equally important pattern in these organisms. Together *Plasmodium* (four distinct clades) and *Hepatocystis* are known to infect bats, rodents, primates, and ungulates (Boundenga et al., 2016, 2018; Perkins, 2014; Perkins and Schaer, 2016; Templeton et al., 2016b). The only host specific clades are *Polychromophilus* and *Nycteria* in bats, and the *Plasmodium* subgenera *Plasmodium* and *Laverania* in primates, however the clades are polyphyletic relative to host orders. Even within bat-associated genera there are equivocal patterns of host specificity. *Hepatocystis* infects pteropodid and hipposiderid bats with no clear specificity for host species or families within a geographic region (Atama et al., 2019; Boundenga et al., 2018; Lutz et al., 2016; Schaer et al., 2013, 2017, 2018, 2019). While *Nycteria* and *Polychromophilus* do show clear evidence of host specificity at the level of bat families, the evidence for species specificity is not obvious, and it is likely that closely related and sympatric bat species can share *Nycteria* and *Polychromophilus* parasites, possibly through shared vectors (Duval et al., 2012; Lutz et al., 2016; Megali et al., 2011; Obame-Nkoghe et al., 2016; Ramasindrazana et al., 2018; Rosskopf et al., 2019; Schaer et al., 2015). Overall, a wealth of studies indicate that haemosporidian host switching is rampant in mammals as well as in birds.

The frequency of host switching, combined with a lack of useful fossil parasites, make calibration of a timed phylogeny a challenging task. The available set of fossils is of dipteran vectors preserved in amber and appear to carry haemosporidian parasites. The ages suggest a long evolutionary history of host-vector-parasite associations: *Paleohaemoproteus* in a biting midge from the Early Cretaceous (Poinar and Telford, 2005), *Plasmodium* in a mosquito from the Paleogene (Poinar, 2005), and *Vetufebrus* in a bat fly from the Miocene (Poinar Jr., 2011). However, inability to relate these parasites to extant relatives erases their usefulness as calibration points (Perkins, 2014). In lieu of fossils, researchers have relied on soft calibration points based on the divergence of haemosporidian lineages associated with particular mammalian clades, particularly primates

(Pacheco et al., 2018; Silva et al., 2011). Pacheco et al. (2018) estimated the origin of haemosporidian parasites to around 70 mya, with most of the main divergence events leading to the extant genera occurring after the Cretaceous-Paleogene boundary 66 mya following the radiation of modern birds. The origin of the *Plasmodium*/*Hepatocystis* clade (not including *Plasmodium* in sauropsids, *Polychromophilus*, or the mammalian *Plasmodium* clade related to *Polychromophilus*) was dated to 33–44 mya by Pacheco et al. (2018) and 20.1–31.8 mya by Hayakawa et al. (2008). The deeper evolutionary origins of Haemosporida are not entirely clear, but it is thought that apicomplexan parasites (haemosporidians, gregarines, coccidia, and piroplasms) ultimately derive from free-living dinoflagellate-like ancestors that transitioned to parasitism (Keeling and Rayner, 2015; O'Donoghue, 2017). Vertebrates likely acquired ancestral coccidians through consumption of infected arthropods, which then reproduced in liver cells and later blood cells, which facilitated the transition to vector-borne transmission via hematophagous insects (considered to be secondary hosts) (Perkins, 2014). These deep evolutionary origins of parasitism in Haemosporida give important context to the transition of haemosporidians from sauropsids to mammals, host switching within mammals, and the geographic distribution of these parasites.

Bats are the hosts that appear especially responsible for host switching of Haemosporida among mammalian groups (Duval et al., 2007; Lutz et al., 2016; Schaer et al., 2013). As already noted above, the majority of Haemosporidian generic diversity in mammals comes from bats (Perkins and Schaer, 2016). The consensus topology in Figure 2.2 indicates that bat-associated *Nycteria* is ancestral to the mammalian *Plasmodium*/*Hepatocystis* clade. Phylogenetic analyses of *Hepatocystis* parasites indicate that the monophyletic clade associated with primates is nested within the broader diversity of bat-associated *Hepatocystis*, suggesting a single host switch from bats to primates (Lutz et al., 2016; Schaer et al., 2018, 2019). The *Plasmodium* species *P. cyclopsi* and *P. voltaicum* from African bats are related to rodent *Plasmodium* in the subgenus *Vinckeia*, the sister clade to *Hepatocystis* (Schaer et al., 2013). The common arboreal lifestyle used by bats, primates, and the rodent hosts of *Vinckeia* (*Grammomys* spp. rats) could have attracted the same generalist vectors, thereby facilitating historical host switching (Schaer et al., 2013). The phylogenetic posi-

tion of *Polychromophilus* relative to the remaining mammalian Haemosporida clades also suggests that bats might be the ancestral hosts of these clades. In this scenario, a presumably sauropsid parasite colonized and subsequently radiatiated in bats, leading to at least four host switches to ungulates, primates, rodents and sauropsids (Figure 2.2; Table 2.2) (Galen et al., 2018). The best available date for this transition is the origin of the *Plasmodium* genus at around 47 mya (Pacheco et al., 2018), a date which is well after the evolutionary origin of bats 58–66 mya. An alternative scenario, based on the close phylogenetic position of *Polychromophilus*, ungulate *Plasmodium*, and pangolin parasites, posits that the ancestral host of mammalian haemosporidia was probably from the Laurasiatheria group, with separate colonizations of bats as hosts (Martinsen et al., 2016; Perkins and Schaer, 2016; Templeton et al., 2016b). However, the more ancient origin of bats compared to ungulates (radiation of Ruminantia 42 mya) and pangolins (radiation of Philodota 25 mya) suggests that the first scenario is more likely (Meredith et al., 2011). Additionally, the permissive immune systems of bats would have been ideal for an initial colonization of mammals by a sauropsid haemosporidian parasite. Given these phylogenetic patterns, the 'bat seeding' hypothesis could equally apply to mammal-associated haemosporidians.

While the role of bats in the diversification of Haemosporida in mammals is generally accepted, the topology of the haemosporidian phylogenetic tree is not fixed. The tree will certainly change with continued global sampling of diverse mammal groups (Perkins and Schaer, 2016). The genera *Biguetiella*, *Dionisia*, *Johnsprentia*, and *Sprattiella* are 'ghost' taxa from bats that have never been sequenced, so additional surveys of bats could revive these taxa and fit them into the existing phylogeny. Some successful examples of this come from the rediscovery of ungulate *Plasmodium* (Martinsen et al., 2016; Templeton et al., 2016b) and of *Polychromophilus* from New World bats (Borner et al., 2016). There is also a need to revisit and confirm some of the fundamental research on haemosporidian vectors using modern molecular techniques. With a broader sample of haemosporidian taxa from mammals and their vectors, combined with multi-locus sequence analysis and appropriate phylogenetic approaches, researchers will be able to answer open questions about the evolution of Haemosporida. These include the number of potential host switches that occurred

between sauropsids and mammals and between bats and other mammalian groups, the true extent of possible malaria vectors, and the possibly different immunological responses of animals to haemosporidian infection.

2.5 *Bartonella*

The genus *Bartonella* (Alphaproteobacteria: Rhizobiales) consists of facultative intracellular bacteria that infect mammals and are transmitted by a range of haematophagous vectors. The most well-known species are *B. bacilliformis*, the agent of Carrion's disease, *B. quintana*, the agent of trench fever, and *B. henselae*, the agent of cat scratch disease. Trench fever was a significant cause of illness in troops during World War I (Anstead, 2016) and bartonellosis caused by these agents remains common in some at-risk populations (Bonilla et al., 2009; Chamberlin et al., 2002; Nelson et al., 2016). Aside from human infections, there are currently at least 40 named species from many mammalian hosts and likely many more novel species that have been characterized but not named. Hosts include rodents, bats, shrews, primates, carnivores, ungulates, marsupials, rabbits, hyraxes, hedgehogs, and tree shrews sampled globally (Table 2.3) (Frank et al., 2018; Kosoy et al., 2018; Marciano et al., 2016; Neves et al., 2018). Vector competence has been confirmed in only a few arthropods, including sand flies, fleas, and lice (Tsai et al., 2011) while replication of bacteria has been demonstrated via artificial feeding of blood inoculated with *Bartonella* to ticks and bed bugs (Cotté et al., 2008; Leulmi et al., 2015). The remaining assemblage of biting flies and mites implicated in the transmission of bartonellae are based mostly on molecular detection (Tsai et al., 2011), although bacteria have been successfully cultured from ked flies and bat flies (Billeter et al., 2012; Dehio et al., 2004; Kosoy et al., 2016).

Transmission from vector to hosts involves either inoculation of bacteria present in vector feces into the skin of the host or directly through a bite during blood feeding (Harms and Dehio, 2012). The bacteria migrate from the dermis into a primary intracellular niche in endothelial cells, from which bacteria periodically invade and replicate inside red blood cells before returning to the primary niche. Hematophagous arthropods take up the bacteria in blood meals, which then replicate in the midgut (and possibly migrate to the salivary glands in ticks (Cotté et al., 2008)). Alternative

routes of infection have also been discussed, potentially through hosts biting or scratching each other or via shedding of viable bacteria in urine or feces, although these routes have not been confirmed (Becker et al., 2018; Dietrich et al., 2017). Given the broad host distribution of bartonellae and the numerous plausible transmission routes, it is likely that spillover between hosts and historical host switching are common in nature.

This is supported by the fact that at least 17 *Bartonella* species are known to infect humans (Breitschwerdt, 2017) and recent phylogenetic analyses that demonstrate transitions of bartonellae between host orders are common (Frank et al., 2018). Nevertheless, *Bartonella* species and clades tend to be host specific to particular mammalian orders (Kosoy et al., 2012; Vayssier-Taussat et al., 2009), with host switches occurring predominantly between related species living in sympatry that are likely to share vectors (Kosoy and Bai, 2019). Such strong biases in host switching patterns can lead to significant signals of coevolution between bats and rodents and their bartonellae, although cospeciation is also an important factor in these host-parasite relationships (Kosoy and Bai, 2019; Lei and Olival, 2014). Given the patterns of host specificity within the genus, bartonellae are likely to have been coevolving with their mammalian hosts for a long time.

Important findings now shed some light on the deep evolutionary history of *Bartonella*. As a member of Rhizobiales, bartonellae are ultimately derived from nitrogen-fixing soil bacteria, some of which form symbiotic relationships with plant roots. Surveys of bees, ants, and other nonparasitic arthropods have revealed a number of clades ancestral to the canonical bartonellae infecting mammals (eubartonellae) (Bisch et al., 2018; Frank et al., 2018; Kešnerová et al., 2016; Neuvonen et al., 2016). *Bartonella tamiae*, a species first discovered in humans in Thailand (Kosoy et al., 2008), appears to be a transitional species in the evolution of bartonellae from symbionts of arthropods to parasites of mammals (Segers et al., 2017). *B. tamiae* and related sequences have been detected from a variety of hematophagous arthropods (Bai et al., 2018; Kabeya et al., 2010), but the only animal tissues that have been positive for this species are bat spleens (Leulmi et al., 2016). Therefore, it is possible that bats were the first mammalian hosts of bartonellae, becoming infecting through fecal contamination or ingestion of an infected hematophagous arthropod.

The importance of bats in the diversification of the *Bartonella* genus has been supported by the high prevalence and many phylogenetically distinct clades of bartonellae discovered in bats in the last decade (Corduneanu et al., 2018; Frank et al., 2018; Stuckey et al., 2017a). Using a comprehensive multi-locus sequence database of *Bartonella* strains from mammalian hosts, including hundreds of strains from bats, I have now shown that bartonellae from bats are dispersed across at least seven distinct clades in the *Bartonella* phylogenetic tree (Figure 2.3; Chapter 3). The phylogenetic positions of clades A, D, and G suggest that bats are the ancestral hosts of the bartonellae in the larger clades that contain them. Ancestral state reconstruction analysis supported bats as both the ancestral hosts of the large clades, but also as the ancestral hosts of all eubartonellae. The authors also used molecular clock analysis to date their phylogeny, producing an estimated divergence date for mammalian bartonellae at 62 mya, a date that falls within the diversification of bats 58–66 mya. The bat *Bartonella* strains in this analysis come from Africa, Central and South America, Europe, and Asia covering 50 bats species from 10/20 extant chiropteran families, suggesting that diversification of bartonellae in bats occurred in concert with the radiation of bats and their colonization of different continents. These results indicate that after an initial acquisition from arthropods, bartonellae radiated along with bats and repeated 'seeding' into rodents, carnivores, ungulates, and other mammals (Figure 2.3). These intra-ordinal host switching may have been facilitated by generalist vectors like ticks (Hornok et al., 2019), through contamination of the environment with bacteria shed in bat feces or urine (Becker et al., 2018; Dietrich et al., 2017), or another unknown transmission route.

Similar to trypanosomes and haemosporidia, it is likely that additional surveys of diverse mammals like rodents, bats, carnivores, ungulates, and other unsampled mammalian taxa will reveal additional *Bartonella* diversity. It will be important to culture and characterize novel strains at multiple genetic loci in order to accurately place these lineages into the *Bartonella* phylogeny (Gutiérrez et al., 2017; Kosoy et al., 2018). Finally, additional surveys and experiments to identify the competent vectors of mammalian *Bartonella* species or hosts of ancestral *Bartonella* symbionts will be needed for a complete picture of the ecology and evolution of these diverse bacteria.

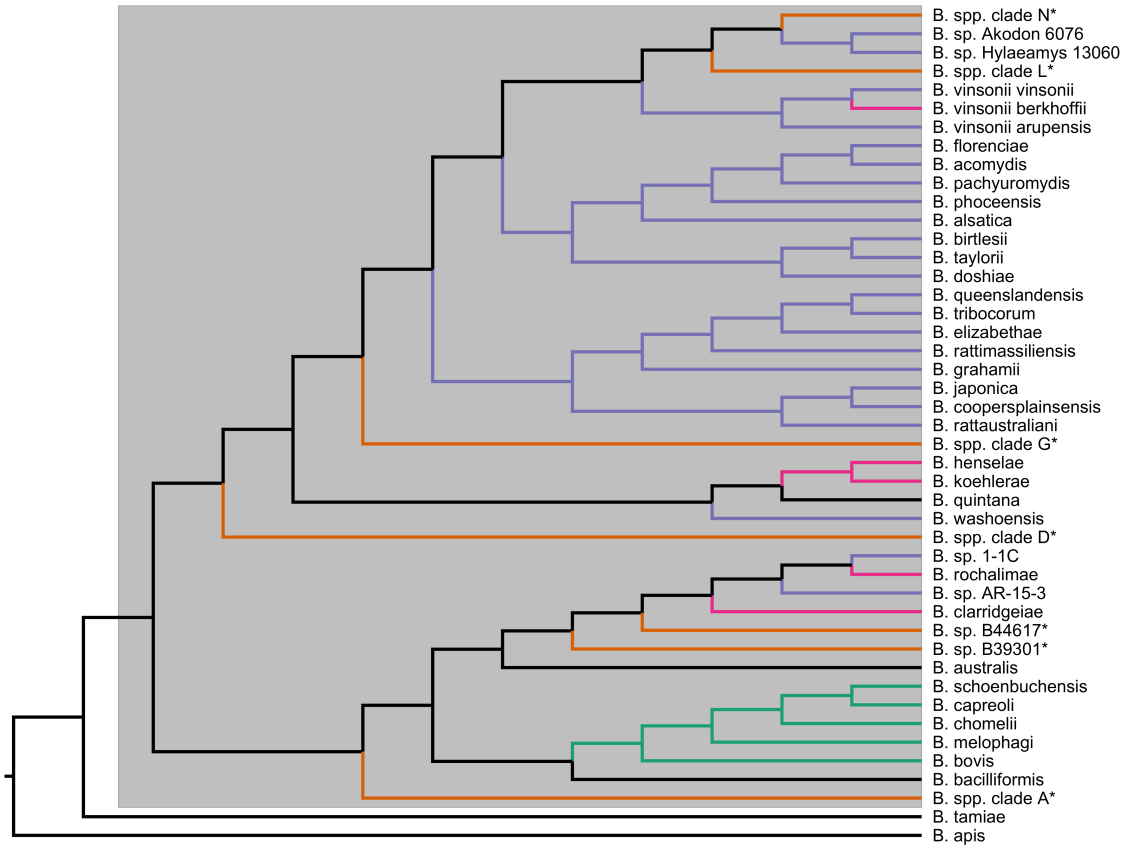

Figure 2.3: Cladogram of *Bartonella* species and clades produced from the topology presented in Chapter 3. Branches are colored according to host associations: green – ungulates; orange – bats; purple – rodents, shrews, and lagomorphs; pink – carnivores. The gray box encloses the current extent of eubartonellae associated with mammals. Clades associated with bats are marked with an asterisk.

2.6 Other parasite taxa

Beyond *Trypanosoma*, Haemosporida, and *Bartonella*, bats are host to other diverse vector-borne parasites including *Neorickettsia*, *Rickettsia*, and filarial nematodes. While we are lacking full understanding of the influence of bats on the diversification of these parasites, recent research has produced some intriguing findings from each of these groups. *Neorickettsia* are bacterial symbionts of digenean trematodes which can be horizontally transmitted to the vertebrate host of the digenean and cause diseases including Sennetsu fever (*N. sennetsu*), salmon dog poisoning (*N. helminthoeca*) and Potomac horse fever (*N. risticii*). Global sampling of bats, bat feces, and bat digeneans have revealed that bats are the definitive hosts of many *Neorickettsia* genotypes related to *N. sennetsu* and *N. risticii* (Cicuttin et al., 2017; Greiman et al., 2017; Hornok et al., 2018).

Diverse strains from across the phylogeny of vector-borne *Rickettsia* bacteria have been detected in bats and bat ectoparasite worldwide (Cicuttin et al., 2017; Dietrich et al., 2016; Hornok et al., 2018, 2019; Izzard et al., 2018; Lv et al., 2018; Moreira-Soto et al., 2017; Sánchez-Montes et al., 2016; Socolovschi et al., 2012; Szubert-Kruszyńska et al., 2019; Tahir et al., 2016). *Litomosa* and *Litomosoides* nematodes infect Old and New World bats. While *Litomosa* are exclusive parasites of bats, *Litomosoides* species are also found in New World rodents and marsupials (Brant and Gardner, 2000). Based on the phylogenetic position of *Litomosa* and bat-associated *Litomosoides* species (Junker et al., 2009; Ramasindrazana et al., 2016), it is possible that bats are the ancestral hosts of these clades of filarial nematodes, with host switching occurring between bats, rodents, and marsupials. These data are intriguing but still sparse, so the explanatory power of the 'bat seeding' hypothesis for understanding the diversity and distribution of these other parasites will require additional investigation.

2.7 Conclusions and future directions

Drawing together the common elements of trypanomes, haemosporidians, and *Bartonella*, it is clear that bats have had a profound influence on the evolution of these taxa. Bat-associated lineages of these parasites are phylogenetically diverse and geographically widespread. Based on the dispersion of bat-associated clades across the phylogenetic trees of these three parasite taxa, bats are potentially the ancestral hosts of particular clades of *Trypanosoma* (*T. cruzi* and allies) and Haemosporida (*Plasmodium*/*Hepatocystis*), and the entirety of mammal-associated *Bartonella*. Similar patterns are emerging in numerous viral taxa (Anthony et al., 2017; Badrane and Tordo, 2001; Drexler et al., 2012, 2013), suggesting that 'bat seeding' may be a generalizable biological phenomenon. The evolution of flight in bats makes them unique among mammals in their ability to travel long distances and transport parasites. Pleiotropic effects potentially related to flight may have also shaped chiropteran immune systems to be tolerant of infections with few signs of disease.

The 'bat seeding' hypothesis is a parsimonious explanation for the diversity and distribution of mammalian parasites, but much work remains to clarify the role of bats. It is highly unlikely that fossil evidence exists or could be successfully identified as the most recent common ancestor of

mammalian trypanosomes, haemosporidians, or bartonellae. The amplification of arthropod parasites *Blastocrithidia* and *Crithidia* (Hodo et al., 2016; Medkour et al., 2019), the transitional *Bartonella* species *B. tamiae* (Bai et al., 2018; Leulmi et al., 2016), and arthropod-associated viruses (Bennett et al., 2019) in bats and bat tissues could indicate that bats are permissive to parasites that have no coevolutionary relationship with bats. While these findings may simply be incidental, they are intriguing and should be investigated further, particularly because they serve as useful proxies for fossils and may reveal more about the evolutionary origins of mammalian parasites. Phylogenetic methods, including ancestral state and phylogeographic reconstructions, using genetic data could provide measures of the statistical likelihood that bats are the ancestral hosts of mammalian parasites. However, such analyses require confidence in a phylogenetic tree as an accurate representation of the evolutionary history of the parasite taxon of interest. There are common issues that can effect phylogenetic analyses of parasites, including reliance on single gene trees, limited taxon sampling, improper choice of outgroup taxa, and inappropriate molecular clock models. A potentially generalizable approach has been presented Chapter 3, using *Bartonella* sequences from multiple loci, broad taxon sampling, proper choice of outgroups for rooting, and relaxed molecular clock models paired with ancestral state reconstruction with resampling to account for biases in taxon sampling from host groups. Application of this approach more broadly would assist in identifying bats as key hosts in the diversification of these parasite taxa.

Most importantly, as mentioned already in this review, additional global surveys of trypanosomes, haemosporidia, *Bartonella* and other vector-borne parasites are needed to fully understand the evolutionary history of these organisms with their vertebrate hosts. Beyond collection of biological materials, advanced molecular techniques could be used to improve detection of parasites in vertebrate blood and tissues, including addition of organic solvents to increase the efficiency of PCR (Farell and Alexandre, 2012; Rodrigues et al., 2019), selective digestion or hybrid capture approaches to isolate parasite DNA from host DNA (Melnikov et al., 2011; Oyola et al., 2013), and next-generation sequencing strategies (metabarcoding, metegenomics, metatranscriptomics) (Dario et al., 2017b; Galen et al., 2019). These techniques may even revive some of the 'ghost'

Trypanosoma and Haemosporida from bats that would allow fitting of these lineages into the phylogenetic tree. With all of the tools available to modern researchers, the future is likely to bring about many insights about the evolution of mammalian parasites and the unique role that bats may have played.

Table 2.1: Host and vector associations of *Trypanosoma* parasites infecting terrestrial animals. Data were summarized from published studies (Barros et al., 2019; Botero et al., 2016; Cai et al., 2019; da Costa et al., 2016; Dario et al., 2017a; Espinosa-Álvarez et al., 2018; Hamilton et al., 2007; Lima et al., 2013; Qiu et al., 2019; Rodrigues et al., 2019; Silva-Iturriza et al., 2013; Thompson et al., 2014; Viola et al., 2009). Generalist taxa infect bats and terrestrial mammals. *T. brucei* can also infect lizards and birds (Hamilton et al., 2007).

Taxon	Vertebrate hosts	Vectors
T. cruzi	generalist	kissing bugs (Triatominae)
T. cruzi marinkellei	bats	kissing bugs (Triatominae)
T. erneyi	bats	unknown
T. dionisii	bats, marsupials	bat bugs (Cimicidae)
T. sp. *Hipposideros* LC415423/LC415425	bats	unknown
T. conorhini	rodents	kissing bugs (Triatominae)
T. sp. *Hipposideros* LC415422/LC415424	bats	unknown
T. sp. NanDoum1	carnivores	unknown
T. vespertilionis	bats	unknown
T. vespertilionis-like G1 TCC2045	bats	unknown
T. vespertilionis-like G2 TCC2103	bats	bat bugs (Cimicidae)
T. sp. HochNdi1	primates	unknown
T. sp. HochG3	bats	unknown
T. rangeli	generalist	kissing bugs (Triatominae)
T. sp. *Rousettus* TCC60	bats	unknown
T. teixeirae	bats	unknown
T. sp. Neobat 1	bats	unknown
T. madeirae	bats	unknown
T. wauwau	bats	unknown
T. sp. Neobat 4	bats	unknown
T. sp. DID	marsupials	unknown
T. sp. Neobat 2	bats	unknown
T. sp. Neobat 3	bats	unknown
T. janseni	marsupials	unknown
T. sp. lemur TVY	primates	unknown
T. noyesi	marsupials, rodents	unknown
T. livingstonei	bats	unknown
T. lewisi	rodents	fleas (Siphonaptera)
T. microti	rodents	fleas (Siphonaptera)
T. vegrandis	marsupials, bats	unknown
T. cyclops	primates	unknown
T. theileri	ungulates, bats	horse flies (Tabanidae)
T. lainsoni	rodents, marsupials, bats	unknown
T. cascavellii	snakes, marsupials	sand flies (Phlebotominae)
T. gennarii	marsupials	unknown
T. varani	snakes, rodents	unknown
T. brucei	generalist	tsetse flies (Glossinidae)
T. evansi	generalist	horse flies (Tabanidae)

Table 2.2: Host and vector associations of Haemosporida parasites infecting terrestrial vertebrates. Data were summarized from published studies (Borner et al., 2016; Boundenga et al., 2016; Galen et al., 2018; Lutz et al., 2016; Martinsen et al., 2016; O'Donoghue, 2017; Pacheco et al., 2018; Perkins, 2014; Schaer et al., 2013). *Anopheles* mosquitoes are also possible vectors of *Polychromophilus* (Makanga et al., 2017). Horse flies are vectors of the subgenus *Simondia* in testudines; the vector of *Haemocystidium* in lizards is unknown.

Taxon	Vertebrate hosts	Vectors
Hepatocystis	bats, primates, rodents, ungulates	biting midges (*Culicoides*)
Plasmodium (*Vinckeia*)	bats, rodents	mosquitoes (*Anopheles*)
Plasmodium (*Plasmodium*)	primates	mosquitoes (*Anopheles*)
Plasmodium (*Laverania*)	primates	mosquitoes (*Anopheles*)
Nycteria	bats	unknown
Plasmodium (sauropsids)	birds, lizards	mosquitoes (Culicinae)
Polychromophilus	bats	bat flies (Nycteribiidae)
Plasmodium (mammals)	ungulates, primates, pangolin	mosquitoes (*Anopheles*)
Haemocystidium	lizards, testudines	horse flies (Tabanidae)
Parahaemoproteus	birds	biting midges (*Culicoides*)
Haemoproteus	birds	louse flies (Hippoboscidae)
Leucocytozoon	birds	black flies (Simuliidae)

Table 2.3: Host and vector associations of *Bartonella* bacteria infecting mammals. Data were summarized from Chapter 3 and published studies (Billeter et al., 2008; Engel et al., 2011; Frank et al., 2018; Hayman et al., 2013b; Kosoy and Bai, 2019; Kosoy, 2010; Tsai et al., 2011; Wagner and Dehio, 2019). Numerous vector groups are suspected for bat-associated bartonellae, including bat flies (Nycteribiidae, Streblidae), mesostigmatid mites (Macronyssidae, Spinturnicidae), fleas (Ischnopsyllidae), ticks (Argasidae, Ixodidae), and bat bugs (Cimicidae). *B. tamiae* has been detected in mammalian ectoparasites and humans, however the reservoir host is currently unknown.

Taxon	Vertebrate hosts	Vectors
B. spp. clade N	bats	many
B. sp. *Akodon* 6076	rodents	unknown
B. sp. *Hylaemys* 13060	rodents	unknown
B. spp. clade L	bats	many
B. vinsonii vinsonii	rodents	fleas
B. vinsonii berkhoffii	carnivores	fleas
B. vinsonii arupensis	rodents, carnivores	fleas
B. florenciae	shrews	unknown
B. acomydis	rodents	unknown
B. pachyuromydis	rodents	unknown
B. phoceensis	rodents	lice, ticks
B. alsatica	rodents	fleas
B. birtlesii	rodents, shrews	fleas
B. taylorii	rodents	fleas
B. doshiae	rodents	fleas, mites, ticks
B. queenslandensis	rodents, shrews	fleas
B. tribocorum	rodents, shrews	fleas, lice, ticks
B. elizabethae	rodents, shrews	fleas, ticks
B. rattimassiliensis	rodents	fleas, lice, ticks
B. grahamii	rodents	fleas, mites
B. japonica	rodents	unknown
B. coopersplainsensis	rodents	unknown
B. rattaustraliani	rodents	unknown
B. spp. clade G	bats	many
B. henselae	carnivores	fleas
B. koehlerae	carnivores	fleas
B. quintana	primates	lice, bed bugs
B. washoensis	rodents	fleas
B. spp. clade D	bats	many
B. sp. 1-1C	rodents	fleas
B. rochalimae	carnivores	fleas
B. sp. AR-15-3	rodents	unknown
B. clarridgiae	carnivores	cat fleas
B. sp. B44617	bats	many
B. sp. B39301	bats	many
B. australis	marsupials	unknown
B. schoenbuchensis	ungulates	hippoboscid flies (*Lipoptena*)
B. capreoli	ungulates	ticks
B. chomelii	ungulates	hippoboscid flies (*Hippobosca*)
B. melophagi	ungulates	hippoboscid flies (*Lipoptena*)
B. bovis	ungulates	ticks, biting flies (*Haematobia*)
B. bacilliformis	primates	sand flies (*Lutzomyia*)
B. spp. clade A	bats	many
B. tamiae	unknown	mites, ticks, bat flies
B. apis	none (bees)	none

Chapter 3

Comprehensive time tree analysis identifies bats as key to the radiation of mammal-associated *Bartonella* bacteria

3.1 Overview

Bats are notorious reservoirs of several zoonotic diseases and may be uniquely tolerant of infection among mammals. Broad sampling has revealed the importance of bats in the diversification and spread of viruses and eukaryotes to other animal hosts. Vector-borne bacteria of the genus *Bartonella* are prevalent and diverse in mammals globally and recent surveys have revealed numerous *Bartonella* lineages in bats. I assembled a sequence database of *Bartonella* strains, consisting of nine genetic loci from 209 previously characterized lineages and 121 new strains from bats, and used these data to perform the most comprehensive phylogenetic analysis of *Bartonella* to date. This analysis included estimation of divergence dates using a novel molecular clock and ancestral reconstruction of host associations and geography. I discovered that *Bartonella* began infecting mammals 62 million years ago near the Cretaceous-Paleogene boundary. Additionally, the radiation of particular *Bartonella* clades correlate strongly to the timing of diversification and biogeography of mammalian hosts. Bats were inferred to be the ancestral hosts of all mammal-associated *Bartonella* and appear to be responsible for the early geographic expansion of the genus. I conclude that bats have had a deep influence on the evolutionary radiation of *Bartonella* bacteria and their spread to other mammalian orders and relate my findings to broad patterns observed in other mammalian parasites.

3.2 Introduction

A central part of the work done by disease ecologists is to understand the host range of infectious agents. However, host ranges must be understood in a coevolutionary context, specifically how agents have adapted to and diversified in hosts over time. Only by considering both ecological and evolutionary context can we understand how agents come to infect and adapt to new hosts. While cophylogeny is a common tool for studying the codiversification of hosts and parasites, few

studies have examined the relative timing of the diversification of parasite lineages in parallel with that of hosts (Garcia-R and Hayman, 2016; Roth et al., 2019; Weinert et al., 2009; Zhu et al., 2015).

The genus *Bartonella* is an excellent study system for disease ecology and evolution because it is common and diverse in many mammalian hosts (Kosoy, 2010). Clades of *Bartonella* species tend to be host-specific (Vayssier-Taussat et al., 2009, 2010), so it could be hypothesized that the genus diversified along with its mammalian hosts millions of years ago. Successful amplification of *Bartonella* DNA from recent fossils also points to a prolonged history of *Bartonella* infection in some hosts, including *B. quintana*, the agent of trench fever in humans, and *B. henselae*, the agent of cat-scratch disease in domestic cats and humans (Fournier et al., 2015). However, it is unlikely that DNA could be successfully amplified from more ancient fossils to test hypotheses about the origin of bartonellae with mammals. Instead a molecular clock approach can be used to estimate the rate at which substitutions accumulate in *Bartonella* DNA and then extrapolate divergence dates of lineages. Bartonellae are primarily transmitted by arthropod vectors, and new research has shown that mammal-associated *Bartonella* parasites evolved from arthropod symbionts (Bisch et al., 2018; Neuvonen et al., 2016; Segers et al., 2017). I therefore estimated a relaxed molecular clock for the 16S ribosomal RNA (rRNA) gene based on sequence divergence data from bacterial symbionts of arthropod hosts separated for millions of years (Kuo and Ochman, 2009; Ochman et al., 1999). Separate clocks for eight additional genetic loci were then estimated relative to the rate for 16S rRNA, here functioning as a strong prior distribution. The inclusion of multiple loci should provide more accurate estimates of divergence dates on a well-supported phylogeny.

I perform this multi-locus analysis using the most comprehensive database of *Bartonella* strains to date, including a greater number of loci than a recent time tree analysis (14)and broader taxon sampling than previous genomic analyses (Engel et al., 2011; Guy et al., 2013; Harms et al., 2017; Neuvonen et al., 2016; Segers et al., 2017; Zhu et al., 2014). Many new *Bartonella* strains have recently been discovered in bats (McKee et al., 2016), so I have included 121 novel strains of bats in this study to amend current delineation of *Bartonella* clades (Engel et al., 2011; Guy et al., 2013; Harms et al., 2017; Wagner and Dehio, 2019; Zhu et al., 2014) and to determine the influence

of bats on the diversification and spread of *Bartonella* bacteria to other mammalian orders. Bats (Chiroptera) are recognized as reservoirs of many infections, rivaling that of other speciose taxa such as rodents (Beltz, 2018; Han et al., 2016a; Luis et al., 2013, 2015; Olival et al., 2017). They show traits that are amenable to parasite transmission, including their global distribution, ability to fly, seasonal migration, dense aggregations and high sociality in some species, long life spans, and the use of torpor and hibernation (Brierley et al., 2016; Brook and Dobson, 2015; Calisher et al., 2006; Luis et al., 2013; O'Shea et al., 2014). There is also evidence that chiropteran immune systems are highly tolerant of infections, especially of viruses (Baker et al., 2013b; Brook and Dobson, 2015; Schountz et al., 2017; Zhang et al., 2013; Zhou et al., 2016). Thus, their role as reservoirs for *Bartonella* bacteria may be unique among mammals.

Bats are also an ancient lineage of mammals (Foley et al., 2016; Shi and Rabosky, 2015), providing ample time for diversification of bacterial parasites and transitions from bats to other mammals. Research has concluded that bats are potentially ancestral hosts that influenced the diversification and spread of lyssaviruses (Badrane and Tordo, 2001; Hayman et al., 2016; Hughes et al., 2005), paramyxoviruses (Drexler et al., 2012), trypanosomes (Espinosa-Álvarez et al., 2018; Hamilton et al., 2012b), and haemosporidia (Galen et al., 2018; Lutz et al., 2016; Perkins and Schaer, 2016; Schaer et al., 2013) among other mammalian orders. Drawing from Hamilton et al. (2012b), who developed the 'bat seeding' hypothesis to explain the geographic and host distribution of *Trypanosoma* lineages related to the agent of Chagas disease, *T. cruzi*, I hypothesize that bats may have also been influential in the ancient diversification and spread of *Bartonella*.

Using the molecular clock approach outlined above, I extrapolate when the genus *Bartonella* diversified and compare the timing of *Bartonella* clade diversification along with their hosts. I hypothesize that mammal-infecting bartonellae evolved with their hosts starting in the late Cretaceous or early Paleogene when many eutherian and metatherian taxa diversified (dos Reis et al., 2012; Foley et al., 2016; Grossnickle et al., 2019; Meredith et al., 2011; Phillips, 2016). I expect to see highly specific lineages within host orders and correlation between diversification dates of hosts and *Bartonella* clades. Using ancestral state reconstruction and network analysis, I discern

which orders of mammals are highly influential in the diversification and spread of *Bartonella* to other host orders and geographic regions. I predict that the speciose orders Chiroptera and Rodentia are important in the historical expansion of the *Bartonella* genus; however bats may have a more profound influence in this process because of their ability to fly and disperse over wide areas. This study provides a more complete understanding of *Bartonella* evolution and biogeography through a suite of phylogenetic methods that could be adapted to understand these processes in other host-specific parasites and symbionts. Such investigations could lead to a deeper evolutionary understanding of symbiosis and parasitism and the influence of key host groups on the diversification and spread of these organisms.

3.3 Methods

3.3.1 Molecular data collection

While studies using whole genomes have attempted to link *Bartonella* species into deeply branching lineages (Engel et al., 2011; Guy et al., 2013; Harms et al., 2017; Zhu et al., 2014), this has been accomplished with a limited number of taxa for which genomes are available. Furthermore, many past studies using multi-locus sequence analysis (MLSA) or whole genomes from *Bartonella* species have not included strains from bats. The first bat-associated *Bartonella* sequences were published in 2005 (Concannon et al., 2005), but successful cultures were not obtained until later studies (Bai et al., 2011b, 2012; Kosoy et al., 2010; Lin et al., 2012). Considering the wide distribution and broad phylogenetic diversity of recently discovered *Bartonella* lineages in bats (Corduneanu et al., 2018; Frank et al., 2018; Stuckey et al., 2017a), I included cultures from bats in this study to characterize new clades within the established taxonomy of the *Bartonella* genus.

I assembled a comprehensive database of *Bartonella* sequences from published genomes on GenBank, previous studies using MLSA, and archived cultures from bats. To obtain a well-supported phylogenetic tree, I targeted nine genetic markers (Table I.1) commonly used for *Bartonella* detection and phylogenetic analysis (Kosoy et al., 2018; La Scola et al., 2003): 16S ribosomal RNA (rRNA), *ftsZ*, *gltA*, *groEL*, *nuoG*, *ribC*, *rpoB*, *ssrA*, and the 16S–23S internal transcribed spacer (ITS). Data from MLSA studies and genomes published as of 2018 were collected from

GenBank via accession numbers or strain numbers from 74 studies, including recent publications that have isolated bartonellae or related bacterial symbionts in arthropods (Kešnerová et al., 2016; Neuvonen et al., 2016). I excluded any strains that were noted in the studies as showing evidence of homologous recombination between *Bartonella* species to prevent issues with incomplete lineage sorting in phylogenetic analysis.

Data gathered from past studies included bat-associated *Bartonella* strains from Asia, Africa, and North America (Bai et al., 2015; Davoust et al., 2016; Lilley et al., 2017; Lin et al., 2012; Stuckey et al., 2017b; Veikkolainen et al., 2014). Additional molecular data collection of *Bartonella* strains from bats included a subset of cultures archived in our laboratory from previous studies in Africa, North and South America, Europe, and Asia (Bai et al., 2011b, 2012; Kosoy et al., 2010; McKee et al., 2017; Olival et al., 2015; Urushadze et al., 2017) that have been partially characterized at some of the genetic loci listed above, as well as new cultures from bats sampled from Nigeria in 2010 and Guatemala in 2010, 2014, and 2015. The data combined from bat-associated *Bartonella* strains cover 50 species from 10/20 extant chiropteran families (Shi and Rabosky, 2015). Details of DNA extraction from bat-associated *Bartonella* cultures and sequencing can be found in Appendix I.1. The final database contained sequence data from 332 taxa: 209 *Bartonella* reference strains from genomes and MLSA studies, 121 bat-associated strains from our laboratory archive, the ant symbiont *Candidatus* Tokpelaia hoelldoblerii, and the outgroup *Brucella abortus*. With the exception of *B. tamiae* and *B. apis*, I did not include any of the other numerous *Bartonella* lineages associated with arthropods (Bisch et al., 2018; Frank et al., 2018) because they are not yet available or have only been sequenced at a few loci (16S rRNA and/or *gltA*), and the main focus of this study was on the eubartonellae clade associated with mammals.

Sequences from each locus were aligned separately with MAFFT v7.187 (Katoh and Standley, 2013). Ends of alignments and poorly aligned sites were trimmed with Gblocks v0.91b (Castresana, 2000), and final alignments were manually checked for ambiguous base pairs and edited. The final alignment lengths and coverage across taxa are listed in Table I.1, with an average of 78% coverage across the nine loci. I concatenated all loci using Phyutility v2.2 (Smith and Dunn,

2008) to produce a full supermatrix of 8345 base pairs (including gap sites) for later analyses. Sequences were validated as phylogenetically informative by confirming the absence of GC content bias, homologous recombination, and substitution saturation (Appendix I.1).

3.3.2 Phylogenetic analysis

The best sequence evolution model was chosen according to the Akaike information criterion (AIC) using jModelTest v2.1.6 (Darriba et al., 2012) via the CyberInfrastructure for Phylogenetic RESearch (CIPRES) Science Gateway portal v3.3 (Miller et al., 2010). The generalized time-reversible model with a proportion of invariant sites and gamma rate variation across sites (GTR+I+G) was chosen for all loci except *ssrA*, which best fit the Tamura-Nei model (TN+I+G) (Table I.1). Nevertheless, I chose to analyze all loci using the GTR+I+G for consistency and to correspond with the maximum likelihood analysis, which used a GTR+I+G model. A maximum likelihood (ML) tree was generated from the concatenated alignment of nine loci using RAxML v8.2.12 (Stamatakis, 2014) on CIPRES with 1000 bootstrap iterations to estimate node support. The ML tree was used to compare topologies with the Bayesian tree and for tip-association tests.

Bayesian phylogenetic analysis was performed using BEAST v1.8.4 (Drummond and Rambaut, 2007; Drummond et al., 2012) on CIPRES. The nine loci were analyzed separately using GTR+I+G sequence evolution models, estimated base frequencies, four gamma rate categories, an uncorrelated relaxed clock with an exponential distribution of clock rates along branches for each locus, and a birth-death speciation model with incomplete sampling (Stadler, 2009). *Brucella abortus* was set as the outgroup in all analyses. Extensive testing using alternative substitution (with or without codon partitioning), clock, and tree models and subsets of genetic data determined that model choice or the exclusion of the ITS locus had little influence on tree topology and estimated divergence dates (Table I.4; Appendix I.2). The prior distributions for substitution rate and speciation model parameters are listed in Table I.8. To determine a prior for the 16S rRNA locus, I analyzed published 16S rRNA sequence divergence and host divergence times for bacterial symbionts of arthropods (Kuo and Ochman, 2009). A linear regression model was fit to the data in R and a lognormal prior was estimated by moment matching to the normal distribution for the

fitted mean and standard error of the slope (Figure I.5). The prior distribution for the exponential clock rate for 16S rRNA was set to this lognormal distribution while prior distributions for the exponential clocks of the remaining eight loci were set to an approximate reference prior for continuous-time Markov chain (CTMC) rates (Ferreira and Suchard, 2008). Thus, the 16S rRNA clock acts as a strong prior and the rates for the other eight loci are estimated relative to the 16S rRNA rate. This approach allows for external validation of *Bartonella* diversification events based on host diversification dates without explicitly using host diversification dates as calibration points for the parasite tree.

In addition to divergence time estimation, I performed ancestral state reconstruction in BEAST. I assigned discrete traits to each tip based on the taxonomic order of the host and the ecozone (or biogeographical realm (Olson et al., 2001; Udvardy, 1975)) that includes the majority of the host's geographic range. The association of some *Bartonella* with arthropods and not mammals, particularly *B. apis*, *B. tamiae*, *B. bacilliformis*, *B. ancashensis*, *Candidatus* B. rondoniensis, and *B. senegalensis*, are justified in Appendix I.3. Ancestral state reconstruction was performed using a symmetrical rate model to reduce the number of state transitions that needed to be inferred. The prior for all state transitions was a gamma distribution with shape and scale parameters set to one, and the prior for the mean rate of order and ecozone transitions were set to the CTMC approximate reference prior (Ferreira and Suchard, 2008).

I ran three chains in BEAST using the model settings above for the final analysis. The chains were run for $2x10^8$ iterations, sampling parameters every $2x10^4$ iterations. I inspected posterior distributions for all model parameters to assess convergence, mixing, and high effective sample sizes (ESS > 200) using Tracer v1.7.1 (Drummond and Rambaut, 2007). I chose the maximum clade credibility (MCC) tree from the posterior tree iterations after burn-in using TreeAnnotator (Drummond and Rambaut, 2007; Drummond et al., 2012) and the tree with the highest MCC score was used for all subsequent analyses. The final tree was visualized and edited in FigTree v1.4.4. Molecular clocks for the nine genetic loci were summarized by the median and highest posterior density (HPD) of their distributions. The divergence date of the most recent common ancestor of

mammal-infecting *Bartonella* (eubartonellae, excluding *B. tamiae* and *B. apis*) was summarized from the MCC tree by the median and HPD.

3.3.3 Tip-association tests

I performed tip-association tests using the Bayesian Tip-association Significance testing (BaTS) program v1 to assess the clustering of traits along tips of the phylogenetic tree (Parker et al., 2008). I performed four sets of simulations using the same assignments of host orders and geographic ecozones used in the ancestral state reconstruction above. The two sets of traits were simulated on 1000 posterior sampled trees from the final BEAST run and on the single ML tree. Clustering of traits was measured by the association index (AI) and parsimony score (PS), producing a distribution for the 1000 Bayesian trees and a single value for the ML tree. Null distributions for these measures were generated using 100 randomizations of traits onto tips of the trees. The significance of clustering was evaluated based on the overlap between observed values or distributions of AI and PS and their null distributions. For both measures, small values indicate a stronger phylogeny-trait association (Parker et al., 2008).

3.3.4 Host clade definitions and divergence dates

I defined particular host-associated clades a posteriori based on high posterior support (>0.9) and the clustering of host orders from the ancestral state reconstruction (Figure 3.1A). Previous analyses of *Bartonella* host associations have shown that host-switching is common (Lei and Olival, 2014), so a calibration approach that assumes strict cospeciation across the tree would not accurately reflect the evolutionary history of these bacteria. However, *Bartonella* lineages are broadly host-specific within orders (Frank et al., 2018) and host-switching is more frequent between closely related hosts (McKee et al., 2019). I defined 15 host-associated *Bartonella* clades (Tables I.5–I.6) at relevant taxonomic scales below the order level to test the hypothesis that *Bartonella* lineages diversified with their hosts while accounting for frequent host-switching that could occur within a host clade. I collated relevant divergence dates for the most recent common ancestor uniting the taxa of interest within each clade from available studies on the TimeTree website

(http://timetree.org/), summarized by the estimated mean, 95% confidence intervals, and range of dates across studies (Kumar et al., 2017). I then correlated these mean host divergence dates with the median divergence date of the associated *Bartonella* clade (Table I.7). A significant linear fit between these dates would support the hypothesis that *Bartonella* diversified within their hosts after colonization.

To validate measurement of the divergence time for mammal-associated *Bartonella* with the ultrametric tree produced in BEAST, I also generated a calibrated timed phylogeny with the ML tree. Using the RelTime relative rate framework (Tamura et al., 2012, 2018) within MEGA v10.0.5 (Kumar et al., 2018) I generated a timed phylogeny using host clade divergence dates from TimeTree (Table I.7). I inputted confidence intervals (or ranges in the case of clade J) for the 15 host clade divergence dates as minimum and maximum divergence dates in RelTime. The program then calculated divergence dates on the tree using a maximum likelihood approach (Mello, 2018; Tamura et al., 2012), producing mean estimates and 95% confidence intervals for clade dates that I could compare with the eubartonellae date estimated in BEAST. This analysis can confirm that the date estimation is robust to different approaches, either using a calibration-based method on an existing tree or a method that relies on relaxed clock priors during tree estimation.

3.3.5 Assessing the influence of hosts on *Bartonella* diversification and geographic spread

To determine the inferred ancestral host order and ecozone of mammal-infecting eubartonellae, I initially inspected the results of the ancestral state reconstruction on the MCC tree. Specifically, I inspected the posterior support for the node and the posterior probability of the host order and ecozone at the node across all posterior trees. However, due to the large number of *Bartonella* lineages associated with Chiroptera in the database (n = 160) relative to those in other diverse orders (Rodentia, 87; Artiodactyla, 32; Carnivora, 21), I tested the influence of this sampling bias on uncertainty about ancestral states using stochastic character mapping of host orders and ecozones onto trees (Bollback, 2006; Huelsenbeck et al., 2003). I wrote a custom R function to resample tips from the phylogenetic tree and perform stochastic character mapping on the pruned tree using the packages `ape` and `phytools` (Paradis et al., 2004, 2016; Revell, 2012, 2013)

assuming an equal-rates model. The function ran 100 mapping simulations on each pruned tree and calculated the probability that Chiroptera and Palearctic were the inferred host order and ecozone at the node uniting eubartonellae. These states were chosen based on initial reconstructions from BEAST indicating them as ancestral. I performed this simulation using three resampling schemes: equalizing the number of tips associated with bats and rodents (n = 87), equalizing tips associated with bats, rodents, and artiodactyls (n = 32), and equalizing tips associated with bats, rodents, artiodactyls, and carnivores (n = 21). Resampling schemes were run with 100 resampling iterations on the MCC tree and 10 resampling steps on 10 randomly sampled posterior trees. I summarized the resulting probability distributions by the mean and interquartile range.

I further assessed the nature of transitions between hosts and ecozones by performing additional stochastic character mapping simulations on posterior trees followed by network analysis of state transitions. Host orders and ecozones were simulated with phytools over 1000 posterior sampled trees with an equal-rates model. The number of state transitions were then summarized over all 1000 simulations by the median and 95% credible intervals, ignoring state transitions with a median of zero. Separate host order and ecozone networks were then built from these median transitions and node-level properties including degree, out-degree, and betweenness centrality were calculated using the R package `igraph` (Csárdi and Nepusz, 2006; Csárdi, 2015).

3.4 Results

3.4.1 Revised topology of *Bartonella* phylogeny

Using molecular data from nine genetic loci sequenced from 331 *Bartonella* strains (Table I.1), I produced a well-supported phylogeny (Figure 3.1; Figure I.8) that confirmed monophyletic clades of *Bartonella* species identified in past studies (Engel et al., 2011; Guy et al., 2013; Harms et al., 2017; Wagner and Dehio, 2019; Zhu et al., 2014). These included a clade containing rodent-associated *B. elizabethae*, *B. grahamii*, *B. tribocorum*, and *B. rattimassiliensis* (clade H); a clade containing cat-associated *B. henselae* and *B. koehlerae* (clade F), *B. quintana*, and *B. washoensis* (clade E); and all three *B. vinsonii* subspecies (clade K). However, in contrast with these studies that used only maximum likelihood analysis of concatenated genes and limited taxon sampling, I

showed that neither *B. bacilliformis* nor *B. australis* are the most deeply branching lineages in the genus. Instead I found that *B. bacilliformis* and its allies *B. ancashensis* (Mullins et al., 2015) and *Candidatus* B. rondoniensis (Laroche et al., 2017), constituting the clade previously named lineage 1, are in fact most closely related to ruminant-associated *Bartonella* species including *B. bovis*, *B. schoenbuchensis*, and others (Tables I.5–I.6); a finding supported by Wagner and Dehio (2019). This ruminant clade was named clade C in my analysis and lineage 2 in other studies (Engel et al., 2011; Guy et al., 2013; Harms et al., 2017; Wagner and Dehio, 2019; Zhu et al., 2014). Lineage 3 including *B. rochalimae*, *B. clarridgeiae*, and allies was found to be distantly related to a clade containing kangaroo-associated *B. australis* (Fournier et al., 2007) and other *Candidatus* strains from marsupials (Kaewmongkol et al., 2011a,b), and two lineages associated with bats, one from Africa (Bai et al., 2015) and one from Europe (Urushadze et al., 2017). These clades (lineages 1–3) were all found to be part of a strongly supported monophyletic clade (posterior probability, PP = 1) that includes the deeply branching sister group clade A associated with neotropical bats (Figure 3.1). The bat-associated and marsupial-associated clades could potentially be elevated to the level of lineages equal to the others. Alternatively, unification of these lineages into a monophyletic clade would suggest a redefinition of lineages into subclades.

Broad taxon sampling also expanded lineage 4, a well-supported clade (PP = 1) that contains all other *Bartonella* species separate from lineages 1–3 and most of the diversity in the genus (Figure 3.1). Specifically, I discovered four new bat-associated clades (D, G, L, N) within this lineage, with clade D as the sister group to all other lineage 4 clades, and clade G as the sister group to a large clade of predominantly rodent-associated *Bartonella* species (enclosed within clade O). Clade L, containing strains from North American and European vespertilionid bats (Lilley et al., 2017; Stuckey et al., 2017b; Urushadze et al., 2017; Veikkolainen et al., 2014) and *Candidatus* B. mayotimonensis (Lin et al., 2010), along with clade N associated with neotropical bats, are contained within a clade that includes the *B. vinsonii* species complex associated primarily with rodents (Bai et al., 2011a; Kosoy, 2010; Kosoy et al., 1997, 2012; Morway et al., 2008; Rubio et al., 2014; Schulte Fischedick et al., 2016) I also recovered a monophyletic clade (PP = 1) that

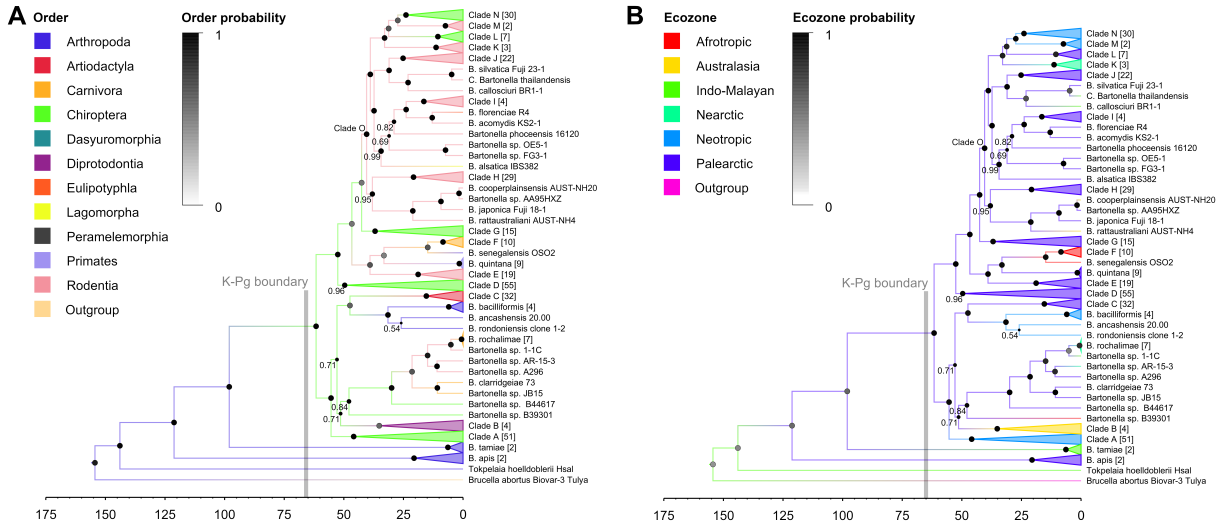

Figure 3.1: Evolution of *Bartonella* lineages, host associations, and geographic origins. Timed maximum clade credibility tree of *Bartonella* lineages. Tips are collapsed into clades of related *Bartonella* species and strains (Tables I.5–I.6); the number of tips in each clade is shown in brackets. Posterior probabilities (PP) for nodes are indicated by the size of circles; ancient nodes had strong support (PP = 1), unless otherwise labeled. Branch lengths are in millions of years. Ancestral state reconstruction of (A) host order and (B) ecozone transitions was performed during the estimation of diversification times. Branches are colored according to their most probable (PP > 0.5) host order or ecozone states, with host or ecozone probability shown by the color of circles at each node. The Cretaceous-Paleogene extinction event is drawn as a gray line at 66 million years ago.

unites rodent-associated *B. birtlesii*, *B. doshiae*, and *B. taylorii*, similar to a previous MLSA study (Buffet et al., 2013). Subdivisions within lineage 4 could be based on radiations within distinct mammalian groups, as I have done (Figure 3.1A; Table I.5–I.6). This revision of the *Bartonella* tree through increased taxon sampling and characterization of bat-associated strains illustrates the diversity in this genus that remains uncharacterized.

3.4.2 *Bartonella* divergence dates and clock rates

Beyond a revised phylogeny, the relaxed clock approach demonstrated that bartonellae are ancient and supports the hypothesis that the genus diversified with mammals (Figure 3.1). I confirm that the genus first evolved as a symbiont of arthropods, represented by the species *B. apis*, *B. tamiae*, and the ant symbiont *Candidatus* Tokpelaia hoelldoblerii, before transitioning to a parasitic lifestyle in mammals. These mammal-infecting eubartonellae (excluding *B. apis* and *B. tamiae*) began diversifying 62 million years ago (mya; 95% HPD: 40–90), near the Cretaceous-Paleogene

boundary 66 mya (Figure 3.1; Figure I.6). Many crown metatherian and eutherian clades began diversifying around this time (dos Reis et al., 2012; Foley et al., 2016; Grossnickle et al., 2019; Meredith et al., 2011; Phillips, 2016), including the diverse placental orders Chiroptera, Artiodactyla, Carnivora, Rodentia, and Primates, suggesting that *Bartonella* diversification is tightly linked with the radiation of mammal lineages during the Paleogene. My estimate for the timing of *Bartonella* emergence in mammals is younger than the date (79 mya) estimated by a previous time tree analysis by Frank et al. (2018), which used only a short fragment of the *gltA* gene for their phylogeny and calibrated their dates using the timed divergence between two *Artibeus* bat species. Estimates of divergence dates using alternative substitution, tree, and clock models placed the origin of mammal-infecting eubartonellae between 57–70 mya (Table I.4; Appendix I.2). The Bayesian tree was structurally similar to a maximum likelihood (ML) tree estimated based on concatenated sequences of all nine loci, with only minor differences in topology for some internal and external branches with low bootstrap support (Figure I.7).

To verify that the molecular clock approach could capture variation across loci using a single strong prior distribution on the 16S rRNA gene, I analyzed clock rates for each of the nine loci. Clock rates predictably varied by gene function (Table 3.1). The 16S locus had a very low median clock rate at 5.2×10^{-10} nucleotide substitutions site^{-1} year^{-1} (95% HPD: $3.4–7.1 \times 10^{-10}$) across branches. As this locus codes for a functional RNA with a conserved 3D structure, this low rate was deemed reasonable and was very close to previous estimates of 16S rRNA divergence of 1–2% per 50 million years in *Buchnera* symbionts of aphids (Moran et al., 1993). Protein-coding loci and the functional transfer-messenger RNA locus *ssrA* had branch rates five to nine times higher than 16S rRNA while ITS had rates 22 times higher than 16S rRNA (Table 3.1).

3.4.3 Diversification of bartonellae with hosts

Following the hypothesis that *Bartonella* lineages evolved along with their mammal hosts, I performed tip-association tests to analyze the clustering of host taxonomic traits and geographic origin along the tips of the tree. Simulations using 1000 posterior sampled trees showed significant clustering of host orders and geographic ecozones across the phylogeny according to association

Table 3.1: Posterior median estimates of clock rates across genetic loci. Numbers in parentheses show the 95% highest posterior density (HPD) interval. UCED, uncorrelated exponential distribution. Median UCED clock rate represents the mean rate for the clock (x10^{-9} substitutions site^{-1} year^{-1}). Median branch clock rate represents the clock rate estimate weighted by branch lengths (x10^{-9} substitutions site^{-1} year^{-1}.)

Locus	Median UCED clock rate	Median branch clock rate
16S	0.47 (0.31–0.63)	0.52 (0.34–0.71)
ITS	9.2 (5.9–13.3)	11.5 (7.5–16.6)
ftsZ	3.1 (2–4.6)	3.8 (2.4–5.4)
gltA	3.8 (2.3–5.5)	3.7 (2.4–5.3)
groEL	2.5 (1.6–3.7)	2.4 (1.5–3.5)
nuoG	3.3 (2–4.8)	3.3 (2.1–4.8)
ribC	4 (2.4–5.6)	3.9 (2.5–5.6)
rpoB	5 (3.1–7.1)	4.8 (3–6.7)
ssrA	2.9 (1.8–4.2)	2.8 (1.7–4.1)

indices (AI) and parsimony scores (PS). Observed distributions for both measures did not overlap their respective null distributions based on random associations of traits to tips (Table 3.2). Simulations using a single ML tree showed similar results. Host orders had smaller values for AI and PS than geographic origin, indicating a stronger phylogeny-trait association with host taxonomy than geographic origin. This phylogeny-trait association with host taxonomy is illustrated in Figure 3.1A through strong support for monophyletic groups associated with host orders.

Table 3.2: Tip-association tests of host trait clustering on trees. Observed credible intervals were drawn from 1000 posterior sampled trees. Null distributions were produced from 100 resampling steps for each sampled tree. ML, maximum likelihood; AI, association index; PS, parsimony score.

	Posterior sampled trees		Single ML tree	
Trait	Order	Ecozone	Order	Ecozone
States	12	7	12	7
Observed AI	1.4 (1.39–1.43)	6.13 (5.87–6.25)	2.2	6.1
Null AI	25.5 (23.1–27.4)	28.1 (25.8–30)	20.9 (19–22.8)	23 (21.2–24.9)
Observed PS	24 (24–24)	61.9 (61–62)	54	102
Null PS	153.5 (147–160)	178.4 (172.5–186.1)	172.2 (148–205)	193.8 (175–216)

I clarified this association with host taxonomy by describing 15 *Bartonella* clades (Tables I.5–I.6) predominantly associated with marsupials (B), ruminants (C), carnivores (F), rodents (E, H,

I, J, K, M, O), and bats (Figure 3.1A). I then compared divergence dates of each *Bartonella* clade with divergence dates of the associated hosts within each clade (Table I.7) collated from TimeTree (Kumar et al., 2017). I found a strong correlation between *Bartonella* and host clade divergence times ($R^2 = 0.72$, $F = 36.4$, $P < 0.0001$); however most (13/15) *Bartonella* clades were younger than their associated host clades; on average, the age of *Bartonella* clades was 76% that of their associated host clades (Figure 3.2, slope of regression is 0.76). Using confidence intervals for host clade divergence dates provided from TimeTree and the RelTime relative rate framework (Tamura et al., 2018), I produced a similar age for mammal-infecting eubartonellae of 66.3 mya (95% CI: 63.5–69.1) on the ML tree compared to the Bayesian relaxed clock approach (Table I.4). This separate analysis validates the inference that *Bartonella* began diversifying with mammals near the Cretaceous-Paleogene boundary.

3.4.4 Influence of host groups on *Bartonella* evolution

Bats appear to be highly influential in the diversification and spread of *Bartonella* geographically and to other host orders. Bat-associated clades are broadly distributed across the tree and form external branches to clades associated with other mammalian orders (Figure 3.1A). This contrasts with clades associated with marsupials, ruminants, carnivores, and rodents, which are less dispersed on the tree and stem from more internal branches. Based on ancestral state analysis using host orders as states, bats were inferred to be the ancestral host of all mammal-infecting eubartonellae with a posterior probability of 0.99. Due to the large number of bat-associated strains in the database (n = 160), this inference of the ancestral host may have been biased towards bats. Yet in all resampling scenarios, the median posterior probability that bats are the ancestral hosts of mammal-infecting eubartonellae exceeded 0.9 (Table 3.3). In further support of this inference, the diversification of mammal-infecting *Bartonella* started almost exactly when bats began their evolutionary radiation around 62 mya (95% CI: 59–64, range: 51.9–74.9) according to the compiled studies from TimeTree (Kumar et al., 2017).

In addition to ancestral host associations, I also wanted to understand more about the ancestral biogeography of *Bartonella* and where these host transitions may have occurred. I performed

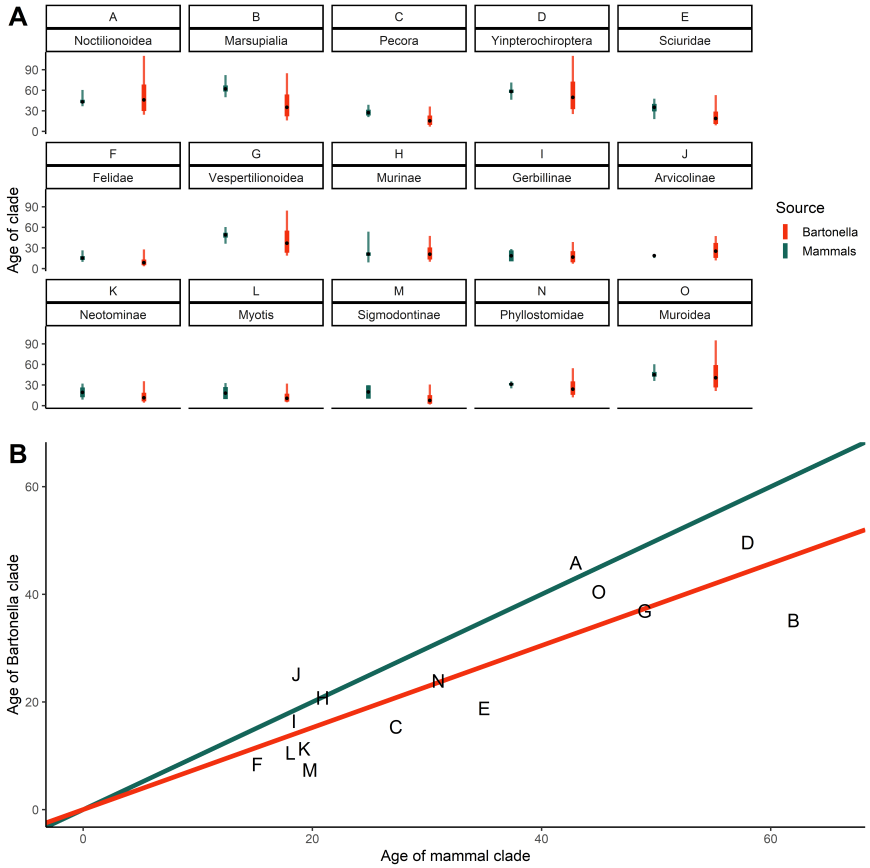

Figure 3.2: Comparison of divergence dates between *Bartonella* clades and associated host mammal clades. (A) Divergence times and intervals for *Bartonella* (in green) and host clades (in red). Clade identifiers correspond to Tables I.5–I.6. Black points show the median estimates and thin bars show divergence date ranges. Thick bars for mammal clades are the 95% confidence intervals estimated from TimeTree and the same bars for *Bartonella* clades are the 95% HPD intervals. (B) Correlation of median divergence dates between host and *Bartonella* clades, with clade identifiers shown as points. The red line indicates the best linear fit through the points and the green line shows the 1:1 line if host and *Bartonella* divergence dates were equal.

ancestral state reconstruction of ecozones based on the current geographical distribution of the host of each *Bartonella* strain. The geographical origin of eubartonellae was inferred to be in the Palearctic (Figure 3.1B) with a posterior probability of 0.99. This fits with the classification of bats within the clade Laurasiatheria and previous reconstructions of chiropteran biogeography, which found that extant bats may have originated in Eurasia (Teeling et al., 2005). However, the inference of the geographic origin of eubartonellae is less certain when host sampling bias was accounted for in the stochastic character mapping analysis. The median posterior probability for a Palearctic origin of eubartonellae ranged from 0.63 to 0.77 across all resampling scenarios (Table

Table 3.3: Posterior probability of host and ecozone states for the mammal-infecting eubartonellae ancestor. For the MCC tree, 100 stochastic character mapping simulations were run on 100 randomly resampled trees and for the posterior sampled trees, 100 stochatic simulations were run on 10 randomly chosen trees with 10 random resampling iterations of tips. The distribution of the posterior probability of the ancestral state over 100 trees is summarized by the median and the interquartile range (in parentheses).

	Sampled tips	Host probability	Ecozone probability
MCC tree	87	0.99 (0.95–1)	0.75 (0.7–0.8)
	32	0.95 (0.91–0.98)	0.63 (0.56–0.69)
	21	0.93 (0.88–0.96)	0.64 (0.56–0.69)
Posterior sampled trees	87	0.99 (0.95–1)	0.77 (0.7–0.82)
	32	0.92 (0.87–0.95)	0.67 (0.55–0.74)
	21	0.93 (0.9–0.97)	0.63 (0.57–0.72)

3.3). Regardless of the exact geographical origin, it is probable that bats have been influential in the ancient geographic spread of *Bartonella* infections (Figure 3.1).

I explored the influence of particular hosts on the spread of *Bartonella* among mammalian orders and across ecozones using stochastic character mapping and network analysis. After mapping the number of host and ecozone transitions across 1000 posterior sampled trees, I built a network consisting of host and ecozones as nodes and the median number of transitions between nodes as edges (Figure 3.3; Table I.9). In general, the ecozone network was more highly connected than the host network (Figure 3.3). Not considering transitions between states and the outgroup (*Brucella abortus*) or transitions between mammalian orders and arthropods, the ecozone network had 22 non-zero median transitions between 6 nodes, resulting in a network density of 73% considering all possible directed transitions. The host order network showed only 10 non-zero transitions between 9 nodes, for a density of 14%. The ecozone network also had higher median counts of transitions than the order network, with up to 12 observed transitions between the Palearctic and Indo-Malayan ecozones (Table I.9). This higher number of connections in the ecozone network corresponds with the results of the tip-association tests (Table 3.2), which showed that clustering of traits was stronger for host taxonomy than geographic origin. That is, the high frequency of transitions between ecozones leads to lower levels of geographical clustering on the tree.

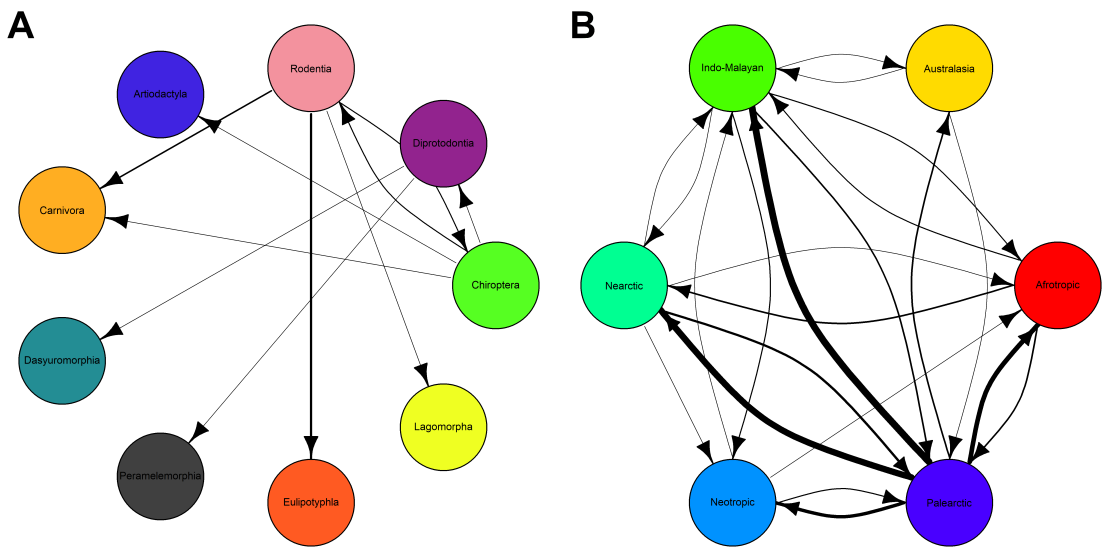

Figure 3.3: Transition network for (A) host orders and (B) ecozones across the *Bartonella* phylogeny. Edges connecting nodes are the median number of transitions between host and ecozone states based on stochastic character mapping on 1000 posterior sampled trees. Edge widths are proportional to the median number of transitions. Edges with a median of zero transitions are not shown. Transitions between the outgroup (*Brucella abortus*) and between mammalian orders and arthropods have been removed for clarity. All transition counts with a median above zero are shown in Table I.9.

Examining the network properties of the nodes, I find that certain host orders are influential in the spread of *Bartonella* among host orders (Table 3.4). Bats and rodents were a source to other mammalian orders (Figure 3.3A), with the highest degree and out-degree of all host orders and high betweenness (Table 3.4). Rodents are a source of transitions to Carnivora, Eulipotyphla, and Lagomorpha, while bats are a source to Diprotodontia and other marsupials, Artiodactyla, and Carnivora. Many of these transitions are strongly supported within the MCC tree with posterior probability greater than 0.9 (Figure 3.1A; Figure I.8A). Notable transitions from Rodentia include those to Carnivora at the ancestor to *B. rochalimae* and to *Bartonella* sp. JM-1 from *Martes melampus* within clade E (including *B. washoensis*); to Eulipotyphla at the ancestor to *B. florenciae*, to *Bartonella* sp. DB5-6 from *Sorex araneus* within clade J (including *B. birtlesii*, *B. doshiae*, and *B. taylorii*), and to *B. tribocorum* and *B. queenslandensis* strains from shrews within clade H; and to Lagomorpha at the ancestor to *B. alsatica*. Well-supported transitions from Chiroptera include to Diprotodontia and other marsupials at the ancestor to clade B (including *B. australis*). Rodents and bats showed an equal number of transitions between each other (Figure 3.3A); however the

sources of these transitions are equivocal with lower posterior probabilities for the ancestral host (Figure 3.1A).

Table 3.4: Node properties of state transition networks. Measures are based on median counts of stochastic character mapping simulations on 1000 posterior sampled trees. The networks exclude transitions between states and the outgroup (*Brucella abortus*) or transitions between mammalian orders and arthropods.

Network	State	Degree	Weighted degree	Out-degree	Weighted out-degree	Betweenness
Order	Artiodactyla	1	1	0	0	0
	Carnivora	2	4	0	0	0
	Chiroptera	5	7	4	5	4
	Dasyuromorphia	1	1	0	0	0
	Diprotodontia	3	3	2	2	4
	Eulipotyphla	1	4	0	0	0
	Lagomorpha	1	1	0	0	0
	Peramelemorphia	1	1	0	0	0
	Rodentia	5	12	4	10	2
Ecozone	Afrotropic	7	20	3	8	0.33
	Australasia	4	6	2	2	0
	Indo-Malayan	10	26	5	9	3.67
	Nearctic	7	22	4	7	0.33
	Neotropic	6	13	3	4	0
	Palearctic	10	53	5	40	3.67

Transitions between ecozones show that the historical movement of *Bartonella* by hosts led to the present global distribution of these bacteria (Figure 3.1B) through bidirectional exchange (Figure 3.3B). Palearctic and Indo-Malayan ecozones showed the highest degree, out-degree, and betweenness. These two regions may have thus played an important role as geographic hubs for *Bartonella* diversification and movement of hosts to other ecozones (Figure 3.1B; Figure I.8B).

3.5 Discussion

Bartonella is a broadly distributed bacterial genus associated with many mammals and arthropod vectors globally. Patterns of host-specificity and phylogenetic diversity in this genus reflect general trends in other zoonotic pathogens, thus *Bartonella* may serve as a model system for understanding the evolution and ecology of animal-borne agents. Specifically, this system could inform theory about how agents adapt to and diversify in hosts over time and the ecological conditions that lead to accidental infections and host-switching. Using a multi-faceted analytical ap-

proach, this study has answered several key questions about the evolution of *Bartonella* bacteria. First, I found that the *Bartonella* genus began diversifying with mammals around the Cretaceous-Paleogene boundary. My novel approach used a strong relaxed clock prior on the 16S rRNA locus based on substitution rates observed in bacterial symbionts of arthropods (Kuo and Ochman, 2009) while accounting for rate variation at eight other genetic loci (Table 3.1) to yield a highly supported phylogenetic tree with presumably more accurate divergence dates (Figure 3.1). Second, I showed that *Bartonella* lineages diversified along with their hosts. Ancestral state reconstruction on the phylogenetic tree showed that *Bartonella* lineages tend to cluster by host taxonomic orders and this clustering was found to be significantly higher than random expectations using tip-association tests (Table 3.2). Additionally, I found a significant correlation between the divergence times of 15 *Bartonella* clades and their associated host clades (Figure 3.2; Table I.7). A separate time tree estimation approach calibrated using these host divergence dates confirmed the dating of eubartonellae diversification (Table I.4). Finally, the ancestral state reconstruction and stochastic character mapping analyses demonstrated that bats have been key to both the origin and spread of *Bartonella* among other mammals, while rodents seemed to be responsible for additional spread. This work elucidates key aspects of the ecology and evolution of *Bartonella* yet there are many new avenues of research to be explored in future studies.

One necessity is to thoroughly catalog the diversity of *Bartonella* lineages. While description of *Bartonella* species was slow through the 20th century, the advent of genetic sequencing has brought about an explosion of *Bartonella* diversity with over 40 named and likely countless unnamed species. My phylogenetic analysis relied on the most comprehensive sequence database to date, including broad taxon sampling of *Bartonella* strains characterized from 10 mammalian orders. These data, along with a relaxed clock approach, have dramatically reshaped the *Bartonella* phylogeny, defining five new clades of bat-associated *Bartonella* strains and reorganizing the relationships of deeply branching clades (Figure 3.1). Attempts to culture and characterize novel *Bartonella* strains from undersampled mammalian orders are needed to further improve taxon sam-

pling. This continued work will undoubtedly reshape the *Bartonella* tree further and may lead to new hypotheses about ancient associations with mammals.

My results also provide context to the biological changes that are associated with the shift of *Bartonella* bacteria from an arthropod symbiont to a mammal parasite. My phylogeny reaffirms work demonstrating this shift (Bisch et al., 2018; Frank et al., 2018; Kešnerová et al., 2016; Neuvonen et al., 2016; Segers et al., 2017) and provides an estimated time for when it occurred, suggesting that an existing bacterial population colonized a new niche in mammals shortly after their emergence as potential hosts. Interestingly, some of the molecular machinery that could have facilitated this colonization was already present in arthropod-associated *Bartonella* and other Rhizobiales (Segers et al., 2017). The majority of virulence factors (69/88) important for host interaction or establishment of intracellular infection are shared across Bartonellaceae, suggesting some latent potential for infecting vertebrates even in arthropod-associated lineages. However, the evolutionary radiation of eubartonellae is associated with a number of other important molecular innovations. Some of these are the acquisition of virulence factors including the *trw* and *virB* type IV secretion systems (T4SS) for translocation of effector proteins into host cells and the expansion of a vertically inherited gene family of hemin-binding proteins for harvesting cofactors such as heme and iron from the environment (Bisch et al., 2018; Segers et al., 2017). The acquisition of T4SS that mediate interactions between host and bacterial cells in independent *Bartonella* lineages has been associated with the evolutionary radiation of eubartonellae in mammals (Engel et al., 2011; Harms et al., 2017; Saenz et al., 2007; Wagner and Dehio, 2019). Additionally, the absence of flagella in *Bartonella* coincides with the acquisition of the *trw* T4SS in *Bartonella* lineage 4, which may have driven the radiation through rapid host adaptation in this lineage (Wagner and Dehio, 2019). Secretion systems have only been detected and characterized in a few *Bartonella* species representing lineages 1–4, so my revision of *Bartonella* lineages highlights a need for future work regarding the machinery (e.g., flagella, T4SS) shared between bat-associated lineages and their relatives.

Given that current mammal-associated bartonellae are vectored by blood-feeding arthropods and ancestral bartonellae were likely arthropod symbionts, it is probable that early adaptation to blood-feeding arthropods facilitated the colonization of the mammalian bloodstream. Hematophagous arthropods frequently harbor endosymbionts to cope with their nutritionally deficient diet (Husnik, 2018), so ancient (and possibly some extant) bartonellae may have had beneficial relationships with arthropod hosts. The switch from symbiont to mammal parasite could then have occurred early in the evolution of mammals. There is evidence that ancestors of extant mammalian ectoparasites implicated as *Bartonella* vectors (Leulmi et al., 2015; McKee et al., 2018; Tsai et al., 2011) were already present by the end of the Cretaceous, including sand flies (Akhoundi et al., 2016), fleas (Zhu et al., 2015), sucking lice (Light et al., 2010), bed bugs (Roth et al., 2019), and hippoboscoid flies (de Moya, 2019). Based on available evidence, the colonization of mammals by *Bartonella* bacteria may have occurred via hematophagous vector, possibly parasitizing early bats. An ancestral relationship with bats is supported by recent detection of *B. tamiae* in bat flies and bat spleens (Bai et al., 2018; Leulmi et al., 2016), suggesting that this species can opportunistically colonize bats from flies even today. The initial transmission may have occurred through contamination of skin with arthropod feces containing bacteria, direct consumption of an infected arthropod, or some other unknown route. Once inside the host, the existing ability of bartonellae to invade host cells may have led to proliferation of bacteria in the blood. The presence of bacteria in blood would have then facilitated the transmission back to hematophagous arthropods, thereby establishing the vector-borne transmission cycle seen in eubartonellae. The bacteria then eventually spread to other mammalian groups and hematophagous vectors, potentially involving shifts in transmission modes catalyzed by molecular innovations (Wagner and Dehio, 2019). As mammals continued to diversify and host-specific vectors evolved, *Bartonella* lineages would become isolated in transmission cycles involving a restricted set of hosts and vectors, resulting in the broadly host-specific patterns we see in extant eubartonellae.

In light of the genetic diversity of eubartonellae and the molecular machinery used for host adaptation, it is worth discussing the seemingly low DNA substitution rates observed in this study.

For the protein-coding genes sequenced in the database, I presume that purifying selection against nonsynonymous substitutions should preserve the function of these genes in hosts and vectors. In fact, low ratios of nonsynonymous to synonymous substitutions indicating purifying selection have been seen in most of these loci sequenced from *Bartonella* strains (Bai et al., 2015; Buffet et al., 2013). A recent experimental study showed that substitution rates ($2.8x10^{-10}$ and $4.5x10^{-10}$ substitutions $site^{-1}$ $generation^{-1}$) in two rodent-associated *Bartonella* strains were lower than rates expected for their respective genome sizes (Gutiérrez et al., 2018b). Horizontal gene transfer (HGT) and homologous recombination may also partly explain the low observed substitution rates. Intraclonal HGT and recombination have been documented in *Bartonella* species (Arvand et al., 2007; Buffet et al., 2013) and could act to repair mutations that accumulate in clonal populations over time. In further support of the low substitution rate in the loci sequenced, I found limited evidence of significant substitution saturation caused by multiple substitutions per site (Appendix I.1). Thus, I believe that the estimated molecular clocks for nine genetic loci accurately reflect their long-term average rate across bartonellae (Table 3.1) and indicate an advanced age for the *Bartonella* genus. Although substitution rates will undoubtedly vary across *Bartonella* lineages, the molecular clock estimates for loci commonly used for *Bartonella* detection and phylogenetic analysis could serve to approximate divergence times in newly characterized *Bartonella* strains that would inform hypotheses about host associations and biogeography.

Regarding biogeographic patterns, I also note that there are several instances of deep separations of host-associated *Bartonella* strains that are most compatible with an ancient origin of the *Bartonella* genus. Inoue et al. (2011) discovered phylogenetically distinct clades of *B. washoensis* infecting ground squirrels in North America and Asia, a result I replicated in my tree within clade E (Figure 3.1; Figure I.8). The squirrels harboring these bacteria are from two separate genera, *Spermophilus* from Eurasia and *Urocitellus* from North America, that diverged 7.8 mya according to studies published on TimeTree (Kumar et al., 2017). Therefore, it is unlikely squirrels from these two genera have been in recent close contact that could lead to *Bartonella* transmission and the divergence observed in the *Bartonella* clades reflects their independent evolution in isolated

hosts. Similar patterns were seen in *Bartonella* clades infecting bats. One involves the separation of two clades within bat-associated clade L (Figure 3.1). One clade within this group (Figure I.8) contains strains from vespertilionid bats in Europe (Urushadze et al., 2017; Veikkolainen et al., 2014) and the other clade contains a strain from North American bats (Lilley et al., 2017) and an agent of human endocarditis, *Candidatus* B. mayotimonensis (Lin et al., 2010). *Myotis* and *Eptesicus* spp. bats in North America diverged from their congeners in Eurasia 16.2 and 15.3 mya respectively according to TimeTree. Within the large clade D harbored by Old World bats (Figure 3.1; Figure I.8) there are two *Bartonella* strains infecting *Hipposideros* spp. bats, *H. larvatus* from Thailand (McKee et al., 2017) and *H. vittatus* from Kenya (Kosoy et al., 2010). While *Hipposideros* species have repeatedly moved between Africa and Asia according to phylogenetic analysis (Foley et al., 2017), these two species have been separate for 34 million years. These divergence times between geographically isolated hosts are reflected in the estimated times for their *Bartonella* divergence times: 5.2 mya for *B. washoensis*-like strains in ground squirrels, 10.5 mya for *Candidatus* B. mayotimonensis-like strains in vespertilionid bats, and 27.6 mya for the two *Hipposideros*-associated strains. These results provide confidence in the molecular clock approach and an ancient diversification of the *Bartonella* genus, but more work is clearly needed to reconstruct historical biogeographical patterns of bartonellae and their hosts.

As mentioned in the text and apparent in Figures 3.1 and 3.3, the evolutionary history of *Bartonella* has involved many host-switching events. Thus, calibrating divergence dates by relying on codivergence between host taxa would poorly reflect this history. Instead I initially avoided a calibration approach in favor of using a relaxed clock prior, then validated estimated divergence dates based on 15 radiation events within particular bat, rodent, ruminant, and marsupial host taxa (Table I.7). The *Bartonella* divergence dates correlate strongly with the host divergence dates, although with a widespread delay in the colonization of *Bartonella* within a clade (Figure 3.2). While it is possible that this delay in *Bartonella* colonization is associated with the divergence date estimation approach and bacteria diverged immediately along with their hosts, it is more likely that the delay reflects some biological reality. According to Manter's rules (Manter, 1955, 1966), parasites evolve

more slowly than their hosts due to the relatively uniform environments they experience within a host. This slow evolution may help to explain rampant *Bartonella* host-switching between related hosts in the tree, since from a parasite's perspective the intracellular environments of phylogenetically similar hosts are unlikely to have significantly changed. Despite these inherent delays, the clustering of *Bartonella* strains with host orders and particular clades within those orders along with the correlation of divergence times strongly suggest a shared evolutionary history between *Bartonella* strains and their hosts, though a more complicated one than simple cospeciation.

Beyond patterns of codiversification, it is clear from this study that *Bartonella* evolution has been shaped by certain hosts, particularly rodents and bats. As the two most speciose groups of mammals, they could be expected to host diverse parasites according to Eichler's rule (Eichler, 1942), which predicts positive covariance between host and parasite diversity. While more studies will need to be done to explicitly test patterns of host and *Bartonella* diversity while accounting for sampling biases, it is clear from the phylogenetic analysis that rodents and bats are important sources for bartonellae switching hosts (Figure 3.3). As abundant taxa within ecosystems, rodents and bats could act as targets for both generalist and specialist ectoparasites. While endemic *Bartonella* infections are likely maintained by transmission by specialist ectoparasite vectors, generalist vectors could target the most abundant species in the community (e.g., rodents or bats) and occasionally infest alternative hosts, resulting in opportunities for accidental *Bartonella* infections in phylogenetically distant hosts over evolutionary time (McKee et al., 2019). Reconstructing some of these ancient host-switching dynamics would require knowledge of ancestral ectoparasite associations and the interactions of hosts and their ectoparasites within communities.

Finally, bats were identified as the most probable host of eubartonellae in mammals even after accounting for sampling bias in the database. The fact that bats can fly would have hypothetically increased their dispersal ability during their early diversification. This is exemplified by numerous long-distance colonization events: from Africa to Madagascar by seven different extant bat families, including the endemic Myzopodidae; from Australia to New Zealand by the family Mystacinidae; and from mainland North America to Hawaii by *Lasiurus cinereus* (Eick et al.,

2005). The dispersal of bats to distant landmasses during the early diversification of mammals could have played a role in the importance of bats as sources of *Bartonella* infection to other mammals. As early colonizers, bats and their associated ectoparasites and bartonellae could have increased in abundance and diversity before the colonization of other mammalian orders. Following the colonization of other mammalian groups, bartonellae could have spilled over from bats into these other potential hosts via generalist vectors or another transmission route. While many of these spillover events likely failed to establish, eventually bartonellae may have successfully colonized and then diversified along with the radiation of the hosts. I also note that bats appear to be highly tolerant of infections, especially intracellular bacteria and viruses (Brook and Dobson, 2015), showing few signs of disease and unique immune responses compared to other mammals (Ahn et al., 2019; Hayman, 2019; Schountz et al., 2017). Such patterns in extant bats may have ancient origins linked with their ability to fly (Zhang et al., 2013), and thus bats may have been ideal hosts for the early colonization of mammals by arthropod-borne bartonellae.

The importance of bats in the evolutionary diversification of mammal parasites has been discussed by other authors working in distinct systems. One of these groups are the *Trypanosoma* parasites that include *T. cruzi*, the agent of Chagas disease. Observing the broad distribution of bat-associated clades in the growing diversity of trypanosomes, Hamilton et al. (2012b) hypothesized that bats may have been highly influential in the geographic spread of the *T. cruzi* clade and host-switching to other mammals. This 'bat seeding' hypothesis has continued to gain support since it was proposed with the discovery of diverse lineages in the *T. cruzi* clade in bats globally (Barbosa et al., 2016; Lima et al., 2012, 2013, 2015b; Qiu et al., 2019). Similar patterns have been noted in malarial parasites (Haemosporida), wherein the transition from sauropsids into mammals likely occurred only once, with bats being a possible bridge to other mammals (Galen et al., 2018; Lutz et al., 2016; Perkins and Schaer, 2016). In light of the results of this study and the patterns in other systems, I contend that the 'bat seeding' hypothesis may apply more widely among mammalian parasites. My approach using comprehensive phylogenetic analysis, estimation of divergence times, and ancestral reconstruction of host associations could be applied to understand

the evolutionary radiation and host-switching patterns of these parasites, and potentially the role that bats have played in their diversification.

Chapter 4

Vector population and bacterial community structure suggest bat dispersal across an island chain

4.1 Overview

Few studies have examined the genetic population structure of vector-borne microparasites, so it is unclear how much these systems can reveal about the movement of their associated hosts. This study examined the complex host-vector-microparasite interactions in a system of bats, ectoparasitic bat flies, vector-borne bacteria (*Bartonella*), and bacterial symbionts of flies (*Enterobacteriales*) across an island chain in the Gulf of Guinea, West Africa. Limited population structure was found in bat flies and *Enterobacteriales* symbionts compared to that of their hosts. Additionally, significant isolation by distance was observed in the dissimilarity of *Bartonella* communities detected in flies from sampled populations of *Eidolon helvum* bats. These patterns indicate that while dispersal of bats between islands may be limited, some nonreproductive movements may lead to the dispersal of ectoparasites and their bacterial symbionts. This study deepens our knowledge of the phylogeography of African fruit bats, their ectoparasites, and associated bacteria. The results presented could inform models of pathogen transmission in these bat populations and increase our theoretical understanding of community ecology in host-parasite systems.

4.2 Introduction

A key question in biology is how populations and communities of organisms are structured across space and time. A uniting theme in the theory of population genetics and community ecology is movement (Vellend, 2010), either gene flow via the movement of individuals between populations or the movement of species between communities. Holding all other processes constant, the frequency of movement produces results ranging from panmixia or community homogeneity to the complete fixation of alleles or species. While organismal movement is challenging to measure directly, researchers can rely on molecular genetic tools to infer the movement of individuals between populations. However, movements that do not lead to reproduction cannot be detected from

such genetic data. A potential solution is to use molecular techniques on the symbionts (mutualists or parasites) of the focal organisms to quantify these nonreproductive movements (Nieberding and Olivieri, 2007).

Successful examples showing that parasites can provide a refined understanding of host movement come from human ecology (Falush et al., 2003; Holmes, 2004) and notable wildlife studies (Biek et al., 2006; Criscione et al., 2006; Nieberding et al., 2004). While these examples have focused on subpopulation structure in individual host and symbiont species, similar patterns might be observable in symbiont communities (Mihaljevic, 2012; Seabloom et al., 2015). In these systems the agents under consideration are not alleles but rather individuals of distinct symbiont species moving between populations within hosts, potentially resulting in varying relative abundance of symbiont species across host populations (Figure 4.1). At either the population or community scale, the ability to detect structure depends on the choice of appropriate molecular markers and the life history of the symbiont (Jarne and Therón, 2001; Nieberding and Olivieri, 2007). Symbionts that rely on vertical transmission, or horizontal transmission without a free-living stage or alternative hosts, would be expected to be ideal proxies for associating population or community structure with host movement since the movement of such symbionts is intimately tied to the behavior of a single host species (Nieberding and Olivieri, 2007; Wirth et al., 2005).

In the case of symbionts with multiple potential hosts, particularly vector-borne microparasites, any structure observed might be challenging to interpret. It has been hypothesized that the population structure of a multi-host parasite should reflect the movement patterns of its most vagile host, since any structure generated by another isolated host will be overwhelmed by frequent dispersal events facilitated by the vagile host (Jarne and Therón, 2001). Yet this expectation might be complicated by the nested levels of dependence in vector-borne microparasite systems, wherein the microparasite is dependent on the vector for transmission between hosts, and the vector, being a parasite itself, is dependent on the host for completion of its own life cycle. Previous studies of host-restricted, ectoparasitic vectors and associated microparasites have shown that vectors can show less population structure than their hosts (van Schaik et al., 2018), and either no genetic struc-

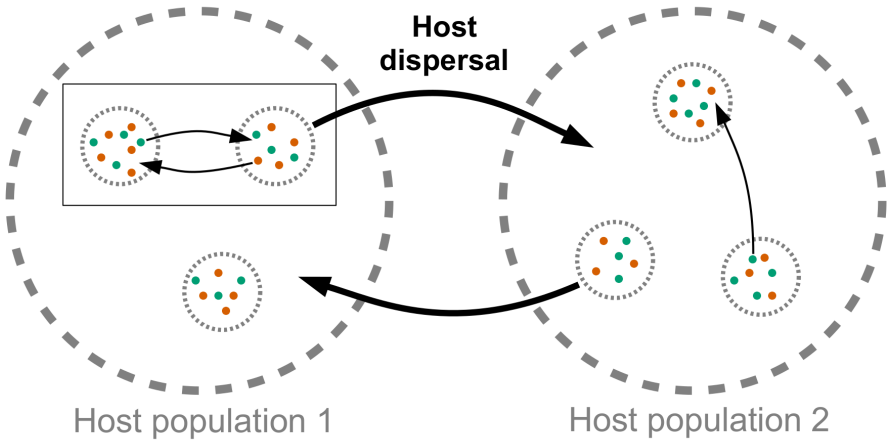

Figure 4.1: Conceptual diagram for parasite community dynamics among host populations. Parasite species (colored dots) exist within hosts (dashed circles). Parasites are transmitted among hosts within a population (inset box). Movement of infected host individuals (or vectors) between host populations may alter the frequency of alleles or species within parasite communities.

ture in the microparasites (Levin and Parker, 2013) or poor correlation between the differentiation in microparasite subpopulations with the structure apparent in their hosts or vectors (Witsenburg et al., 2015). It is possible that the low genetic differentiation in vector-borne microparasites is due to the additive effect of host and vector movements (Witsenburg et al., 2015), facilitating high levels of gene flow in microparasite populations. Additional examinations of population and community structure in hosts, vectors, and microparasites are needed to find general patterns across systems.

The system chosen for this study is especially suitable for this type of investigation because of the contained nature of the focal host populations and the traits of the parasites. This study focuses on two species of fruit bats (Chiroptera: Pteropodidae), *Eidolon helvum* and *Rousettus aegyptiacus*; their ectoparasitic bat flies (Diptera: Nycteribiidae), *Cyclopodia greefi* and *Eucampsipoda africana*; and two taxa of bacteria, the genus *Bartonella* (Alphaproteobacteria: Rhizobiales) and the order *Enterobacteriales* (Gammaproteobacteria). The bat species are distributed across Africa and can be found on several isolated islands in the Gulf of Guinea (Figure 4.2). Studies on both

species have found that island populations are genetically distinct from each other and mainland populations (Juste and Ibanez, 1993; Juste et al., 2000; Peel et al., 2013; Stribna et al., 2019). The two bat fly species are obligate ectoparasites specific to their host species, *C. greefi* with *E. helvum* and *E. africana* with *R. aegyptiacus* (Theodor, 1955, 1957). These hematophagous flies spend almost their entire lives attached to their bat hosts, with gravid females only leaving to deposit a single third-instar larva on the roost substrate (Dick and Patterson, 2006; Dittmar et al., 2015; Marshall, 1970). While both flies are wingless and rely on their hosts for long-distance dispersal, bat flies are agile walkers and could be capable of frequent movements between host individuals within a roost (Dick and Patterson, 2006; Dittmar et al., 2015). Both fly species have been documented across much of their respective hosts' ranges (Billeter et al., 2012; Theodor, 1957), but no studies have evaluated their potential genetic differentiation by geography. Only a few population genetic studies have been performed on nycteribiid bat flies generally (Olival et al., 2013; van Schaik et al., 2015, 2018; Witsenburg et al., 2015).

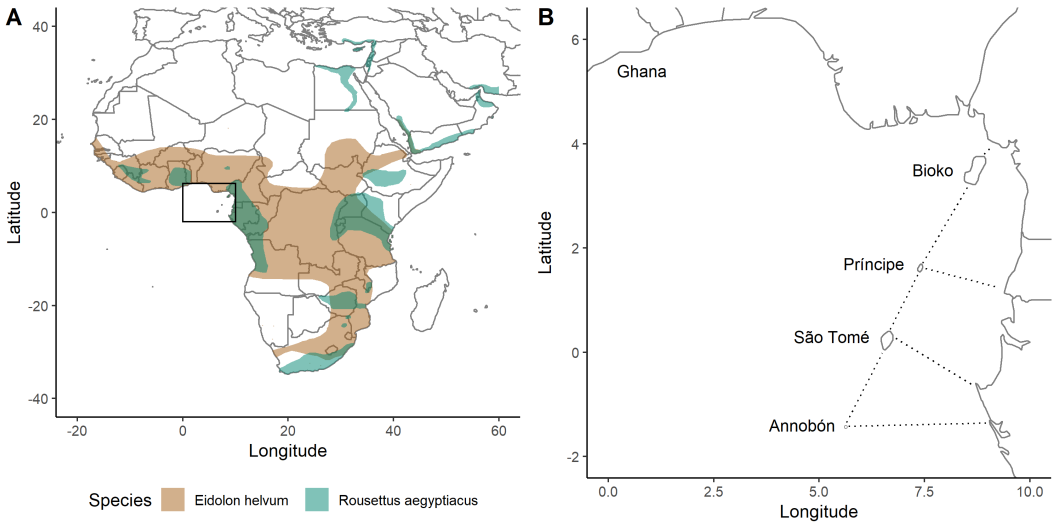

Figure 4.2: Map of study area in West Africa (A), islands in the Gulf of Guinea (B). Axis values are in degrees latitude and longitude. Segments for estimating the shortest distance between islands and the mainland are shown as dotted lines. Bat geographic ranges were retrieved from the IUCN Red List website (https://www.iucnredlist.org/).

The two taxa of bacteria frequently associated with bat flies, *Bartonella* and *Enterobacteriales*, provide an interesting contrast in their relationships with their hosts. Bartonellae are facultative intracellular parasites that produce long-lasting infection in host erythrocytes and are horizontally transmitted among hosts by hematophagous arthropod ectoparasites (Harms and Dehio, 2012). *Bartonella* species have been characterized from both *E. helvum* and *R. aegyptiacus* and similar sequences have been found in *C. greefi* and *E. africana* (Bai et al., 2015, 2018; Billeter et al., 2012; Kamani et al., 2014; Kosoy et al., 2010), suggesting that bat flies are vectors of these bacteria. The diversity of Bartonella species infecting *E. helvum* is especially high, including at least six distinct species (Bai et al., 2015), facilitating not only the potential identification of population structure in separate *Bartonella* species, but also community structure in terms of the relative abundance of species (Figure 4.1). In comparison with *Bartonella* the order *Enterobacteriales* (including the genera *Arsenophonus* and *Aschnera*) are obligate endosymbionts of bat flies and other arthropods (Duron et al., 2008, 2014; Morse et al., 2012a, 2013; Wilkinson et al., 2016). They are thought to be vertically transmitted from mother to offspring via bacteriocytes in the milk glands of nycteribiid and may have mutualistic relationships with flies (Dittmar et al., 2015; Hosokawa et al., 2012). While other studies have reported these symbionts in *E. africana* and other *Eucampsipoda* species (Morse et al., 2013), and in *C. dubia*, a congener of *C. greefi* parasitizing *Eidolon dupreanum* from Madgascar (Wilkinson et al., 2016), these studies have been limited in their geographic extent and have not attempted to identify signals of population structure in these symbionts that may reflect restrictions in the dispersal of bat flies.

Using this complex system involving bacterial symbionts that range from mutualistic to parasitic within their hosts, I tested the effects of geographic restrictions in host bat dispersal on symbiont population or community structure across trophic levels. I hypothesize that the genetic structure of bat flies will reflect that of their specific bat hosts, with distinct genotypes associated with mainland and island populations. Similarly, I predict that obligate *Enterobacteriales* symbionts of bat flies will mirror the phylogenetic separation in their host bat flies. Similar to other vector-borne microparasite systems (Levin and Parker, 2013; Witsenburg et al., 2015), I expect to

see no population structure in the separate *Bartonella* species found in flies. However, it is possible that the relative abundance of *Bartonella* species detected in bat fly species will differ across sampled host populations due to host movement patterns (Figure 4.1). Results from this investigation could indicate the dispersal of bat flies and their symbionts through cryptic, nonreproductive movements of bats that are not captured in their genetic population structure. In addition to evaluating the differentiation of symbiont populations and communities, I assessed patterns in the prevalence of *Bartonella* bacteria across locations, particularly the influence of bat age structure and bat fly density to better understand how these bacteria are maintained in host populations. Knowledge of bat movements across isolated islands and mainland Africa will shed light on their phylogeography and their potential to spread infectious agents. The results of this study also increase our understanding of the ecological processes affecting community diversity in vector-borne parasite systems.

4.3 Methods

4.3.1 Specimen collection

Bat flies were collected during the course of a long-term research program on the demographics and viral transmission dynamics of *E. helvum* across Africa from 2009–2016 (Baker et al., 2014; Peel et al., 2016, 2017). Permits for animal capture were granted by national and local authorities and under ethics approval from the Zoological Society of London Ethics Committee (WLE/0489 and WLE/0467); field protocols followed ASM guidelines (Sikes and Gannon, 2011). Bats of two species, *E. helvum* and *R. aegyptiacus*, were captured using mist nets (6–18 m; 38 mm) as bats departed roost sites at dusk or were returning at dawn; roost sites, sampling dates, and sample sizes are recorded in Table 4.1. Bats were held in individual cloth bags until processing, wherein bat flies were removed from the pelage while under manual restraint. A small minority of flies from Ghana (n = 18) were collected from the clothes of researchers while processing bats or on the ground under roosts (presumably groomed off and returning to the roost). The flies were attributed to *E. helvum* based on the bats being sampled at the time or the predominant species in the roost. Flies were stored in 1.2 ml microcentrifuge tubes, pooled by individual bat, and then stored at 4 or

-20°C until shipment. Flies were initially shipped on dry ice to the Zoological Society of London, then to the Centers for Disease Control and Prevention Division of Vector-Borne Diseases, where flies were stored at -80°C until processing. Distances between islands in the Gulf of Guinea and the mainland (considering Ghana as a representative population) were measured on Google Earth (http://earth.google.com). Age distributions of *E. helvum* populations from sampling locations were taken from Peel et al. (2017). Genetic data from *E. helvum* specifically pairwise distances between populations from mtDNA sequences (*cytb*) and microsatellite loci, were taken from Peel et al. (2013).

Table 4.1: Sampling sites and dates for bat flies from Ghana and Gulf of Guinea islands.

Bat host species	Country/Island	Sampling dates	Region/Site	Latitude	Longitude	Samples
E. helvum	Ghana	2009/03/25-26; 2012/01/17; 2016/03/26-2016/05/05	Accra, 37 Military Hospital	5.5882	-0.1824	151
			Brong Ahafo, Tanoboase	7.6466	-1.8824	7
	Bioko	2010/05/21-26	Malabo, Hess compound	3.7471	8.7701	6
			Malabo, New Spanish embassy	3.7521	8.7723	170
	Príncipe	2010/04/05-12	Micoto	1.6802	7.3895	2
			Novo	1.5897	7.3373	79
	São Tomè	2010/03/19-2010/04/23	Binda	0.2333	6.4833	9
			Canecao	0.3406	6.5629	7
			Cruzeiro	0.2861	6.6781	3
			Monte Cehada/Isla Calici	0.0223	6.5166	30
			Ponta Baleia	0.0430	6.5443	75
			Porto Alegre	0.0289	6.5320	41
	Annobón	2010/05/10-14	Adjo/Mábana	-1.4592	5.6453	131
R. aegyptiacus	Ghana	2012/01/25; 2016/05/07-08	Brong Ahafo, Buoyem Cave	7.6681	-1.9617	45
	Príncipe	2010/04/10-11	Novo	1.5897	7.3373	1
	São Tomé	2010/03/30	Ponta Baleia	0.0430	6.5443	10

4.3.2 Laboratory methods

Bat flies were initially identified to species based on host associations and morphological traits (Theodor, 1955, 1957). Whole bat flies were surface sterilized following published procedures (Billeter et al., 2012) and then homogenized in Navy Eppendorf bead tubes (Next Advance, Averill Park, NY, USA) containing 400 µl of brain heart infusion (CDC, Atlanta, GA, USA) using a Bullet Blender Gold (Next Advance) until no visible appendages remained. Tubes were briefly centrifuged and a 200 µl aliquot of homogenate was taken for DNA extraction. DNA was extracted from homogenates using the KingFisher Flex Purification System and associated MagMAX

Pathogen RNA/DNA Kit (ThermoFisher, Waltham, MA, USA) following manufacturer protocols and then stored at 4°C during the molecular genotyping process.

A subset of flies were genotyped through PCR amplification and sequencing of two mitochondrial DNA (mtDNA) genes, 16S ribosomal RNA (rRNA) and cytochrome b (*cytb*). These markers have previously been used for identification of species and detection of intraspecific diversity in bat flies (Bai et al., 2018; Dittmar et al., 2006; Hosokawa et al., 2012; Olival et al., 2013). *Enterobacteriales* symbionts of bat flies were detected by amplification of the 16S rRNA gene (Duron et al., 2008). *Bartonella* DNA was amplified and sequenced at three markers commonly used for detection and characterization of *Bartonella* species (Gutiérrez et al., 2017; Kosoy et al., 2018; La Scola et al., 2003): 16S–23S ribosomal RNA intergenic spacer region (ITS), citrate synthase gene (*gltA*), and cell division protein gene (*ftsZ*).

All PCR primers and protocols are listed with their associated references in Tables II.1–II.2. Preparation of PCR reagents was performed in a separate room from amplification to prevent cross-contamination. Extraction and negative (nuclease-free water) controls were used in all reactions to detect contamination of reagents; *Bartonella doshiae* was used as a positive control in all reactions for *Bartonella* detection to identify appropriately sized products. Amplification products were visualized by gel electrophoresis using 1.5% agar and GelGreen stain (Biotium, Hayward, CA, USA) and then purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) following manufacturer instructions. Purified products were prepared for sequencing using Big Dye terminator mix (Applied Biosystems, Inc., Foster City, CA, USA) and the same primers as PCR (the second-round primers in the case of nested *ftsZ* and *gltA* protocols) and then sequenced in both directions on an ABI 3130 Genetic Analyzer (Applied Biosystem). Sequence reads were assembled with the SeqMan Pro program in Lasergene v14 (DNASTAR, Madison, WI, USA) and manually checked for ambiguous bases before phylogenetic analysis. Sequences were validated as the correct gene and target organism using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Due to the potential amplification biases of each primer set toward different *Bartonella* species in a sample, the sequences obtained from the three targeted genes were considered as independent measurements of the community of *Bartonella* species in a sample. The presence of coexisting species was confirmed in many samples through observation of multiple peaks in the electropherograms, which were separated into distinct sequences by comparison with previously obtained *Bartonella* sequences from the target bat and bat fly species (Bai et al., 2015, 2018; Billeter et al., 2012; Kosoy et al., 2010). Sequences were identified as belonging to a *Bartonella* species if they shared >95% sequence similarity at any of the three markers with known *Bartonella* species. Counts of *Bartonella* species detected in each bat fly were then summarized for sampling locations.

4.3.3 Phylogenetic analysis

Sequences from each locus were aligned with closely matching references from GenBank using MAFFT v7.187 (Katoh and Standley, 2013) and trimmed to equal length with Gblocks v0.91b (Castresana, 2000). MEGA v7.0.26 was used to select the best sequence evolution model for each locus based on Akaike's information criterion corrected for finite sample sizes (AICc). The best model was GTR+I+G for mitochondrial and bacterial 16S rRNA and TN+I+G for mitochondrial *cytb*. Maximum likelihood trees were generated with these models and 100 bootstrap replicates to estimate support for nodes. Distinct genotypes were delineated by single nucleotide changes and the relative abundance of genotypes was assessed across sampling locations.

4.3.4 Statistical analysis

Confidence intervals for *Bartonella* prevalence were estimated using Wilson score intervals. Differences in *Bartonella* prevalence between bat flies collected from each bat species and location were assessed using two-sided chi-square tests. Relationships between *Bartonella* prevalence, the average number of bat flies per bat, and the proportion of adult and sexually immature bats from each location were tested using Pearson's product-moment correlation. *Bartonella* diversity in bat flies sampled from each location was calculated as species richness, the Shannon number (the exponentiated form of Shannon entropy), and the inverse Simpson index in the R package

`vegan` (Oksanen et al., 2018; R Core Team, 2019). *Bartonella* community dissimilarity was calculated as one minus the Spearman rank correlation among *Bartonella* spp. counts across loci between locations. Isolation by distance patterns were explored between *Bartonella* community dissimilarity, physical distance between islands and the mainland, and genetic distances between bat populations (mtDNA and microsatellites) taken from Peel et al. (2017) using linear regression.

4.4 Results

4.4.1 Collection and identification of bat flies

Bat flies were obtained from *E. helvum* from Ghana, Bioko, Príncipe, São Tomé, and Annobón while flies from *R. aegyptiacus* were obtained only from Ghana, Príncipe, and São Tomé (Table 4.1). A total of 767 flies were initially identified by morphology using available keys and known host distributions (Theodor, 1955, 1957). For a subset of 401 flies, sequences were successfully obtained from one or both of the 16S rRNA or *cytb* loci. All flies from *E. helvum* were identified as *Cyclopodia greefi* Karsch, 1884 while the majority of flies from *R. aegyptiacus* were *Eucampsipoda africana* Theodor, 1955 with the exception of a single *Dipseliopoda biannulata* Oldroyd, 1953 (Table 4.2; Table II.3). All three species are part of the Old World family Nycteribiidae, subfamily Cyclopodiinae (Maa, 1965).

The two mitochondrial loci revealed varying numbers of genotypes across bat fly species (Figure 4.3). Only one 16S rRNA genotype was found in *C. greefi* from all locations (Figure 4.3A,B) while two *cytb* genotypes were found in this species, genotype 1 in all locations and genotype 2 only on Annobón (Figure 4.3C,D). Three individuals from Annobón were confirmed as *cytb* genotype 1 through repeated sequencing. Two 16S rRNA genotypes were found in *E. africana* (Figure 4.3A,B). Genotype 1 was found in Ghana and was identical to sequences from *E. africana* on GenBank (accession numbers MH138033, MH138035, and MH138036) from a previous study in Nigeria (Bai et al., 2018). Genotype 2 was found in specimens from Príncipe and São Tomé. Five *cytb* genotypes were found in *E. africana* (Figure 4.3C,D): genotypes 1–4 were from Ghana and genotype 5 from Príncipe and São Tomé.

Table 4.2: Molecular genotyping and *Bartonella* infection prevalence in bat flies. Samples were considered successfully genotyped if one or both mitochondrial loci were successfully sequenced. Samples were considered positive for *Bartonella* bacteria if one or more genetic markers produced a sequence confirmed as *Bartonella*. Binomial 95% confidence intervals for prevalence were estimated using Wilson score intervals.

Bat host species	Bat fly species	Country/Island	Samples	Genotyped	*Bartonella* positive	Prevalence
E. helvum	*C. greefi*	Ghana	158	50	131	0.83 (0.76–0.88)
		Bioko	176	52	113	0.64 (0.57–0.71)
		Príncipe	81	55	67	0.83 (0.73–0.89)
		São Tomé	165	95	137	0.83 (0.77–0.88)
		Annobón	131	96	121	0.92 (0.87–0.96)
R. aegyptiacus	*E. africana*	Ghana	44	41	19	0.42 (0.29–0.57)
		Príncipe	10	10	4	0.4 (0.17–0.69)
		São Tomé	1	1	1	1 (0.21–1)
	D. biannulata	Ghana	1	1	0	0 (0–0.79)

4.4.2 Patterns of *Bartonella* prevalence and diversity

Bartonella spp. DNA was present in bat flies collected from both *E. helvum* and *R. aegyptiacus* (Table 4.2). On average, *Bartonella* prevalence was higher in flies collected from *E. helvum* (80%) than in flies collected from *R. aegyptiacus* (42%; $\chi^2 = 41$, df = 1, $P < 0.0001$). Prevalence differed across locations for *C. greefi* collected from *E. helvum* ($\chi^2 = 42.2$, df = 4, $P < 0.0001$), and Bioko island had the lowest prevalence with confidence intervals that did not overlap with the other locations (Table 4.2). *Bartonella* prevalence did not differ across locations for *E. africana* collected from *R. aegyptiacus* ($\chi^2 = 1.4$, df = 2, $P = 0.5$). Eight *Bartonella* species were detected in *C. greefi*: E1–E5, Ew, Eh6, and Eh7 (Figure II.1; Figure 4.4A). These species have been detected previously in *C. greefi* and *E. helvum* from other locations and characterized at multiple genetic markers to verify their status as distinct species (Bai et al., 2015; Billeter et al., 2012; Kamani et al., 2014; Kosoy et al., 2010). In contrast, only one species was found in *E. africana* flies from *R. aegyptiacus* (Figure II.1). This species, *B. rousetti*, is the only *Bartonella* species found in *R. aegyptiacus* sampled to date from Kenya and Nigeria (Bai et al., 2018; Kosoy et al., 2010).

Bartonella diversity varied across locations for flies from *E. helvum* but not for flies from *R. aegyptiacus*. Only *B. rousetti* was found in *E. africana* from Ghana, Príncipe, and São Tomé (Table II.4). *Bartonella* spp. E1–E5 and Ew were found in *C. greefi* from Ghana and all islands whereas the rare species Eh6 and Eh7 were detected inconsistently (Figure 4.4A,B). The highest *Bartonella*

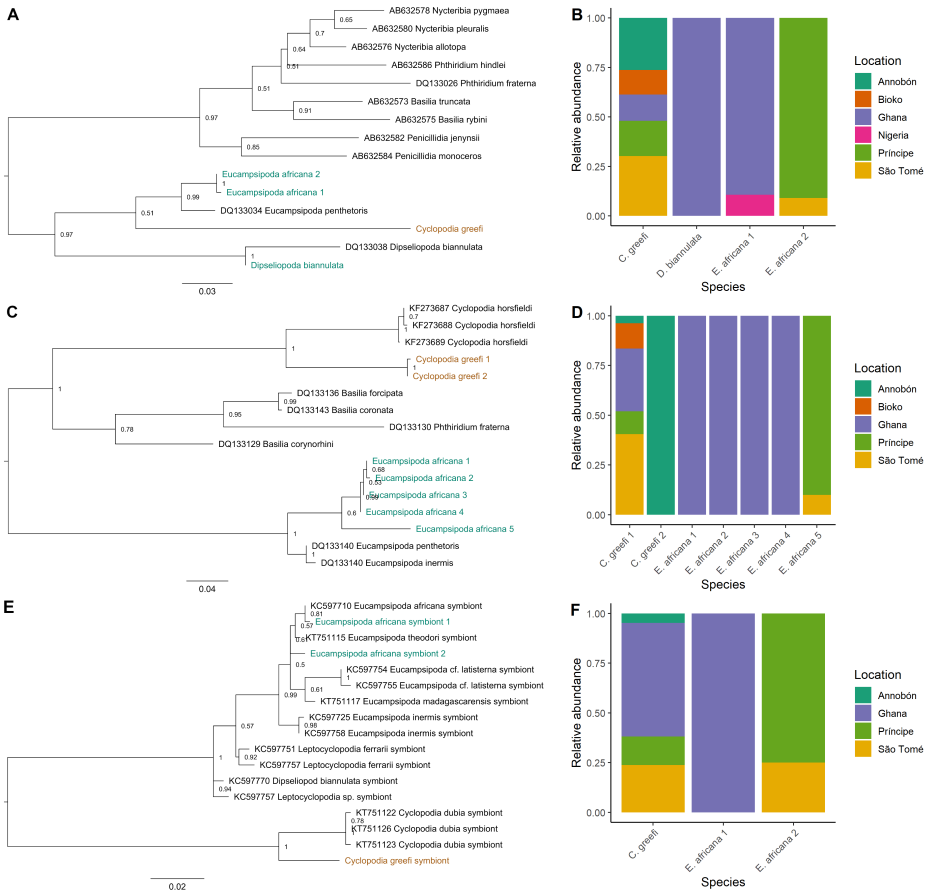

Figure 4.3: Genotyping of bat fly species and bacterial symbionts. Bat fly genotypes were identified by sequencing 411 bp of mitochondrial 16S rRNA (A) and 387 bp of *cytb* (C) while symbionts of flies were identified by sequencing 575 bp of bacterial 16S rRNA (E). Trees were generated by maximum likelihood using the appropriate substitution models (GTR+I+G for 16S rRNA and TN+I+G for *cytb*) and nodal support was estimated from 100 bootstrap iterations. GenBank accession numbers are given next to published reference sequences. Relative abundances of genotypes across locations (B, D, and F) are shown based on the total number of specimens genotyped at each marker.

species richness in *C. greefi* was from Bioko whereas the highest species evenness (Shannon number and inverse Simpson index) was in flies from Príncipe, and São Tomé (Figure 4.4B). No clear evidence of population structure was found in *Bartonella* species at any of the sequenced markers (ITS, *ftsZ*, *gltA*). Genotypes of each species were found broadly across sampling locations, including on isolated islands. A previous study using multi-locus sequence typing to characterize *Bartonella* species E1–E5 and Ew from *E. helvum* from African populations also found identical multi-locus sequence types that were found in geographically distant locations on the continent and from Annobón (Bai et al., 2015).

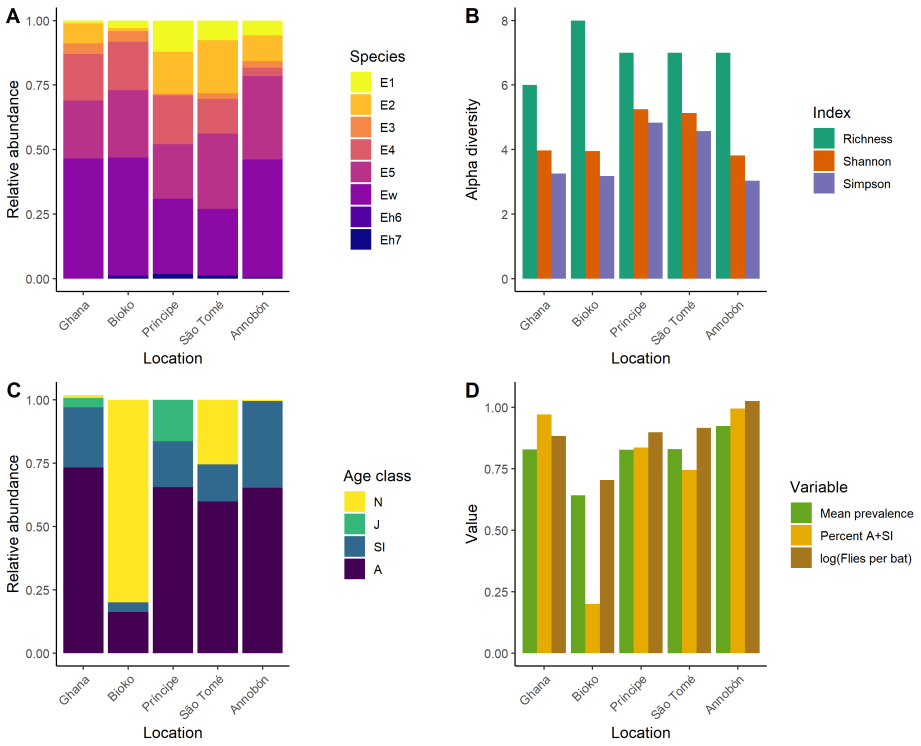

Figure 4.4: Patterns of *Bartonella* infection prevalence and diversity in *C. greefi* bat flies collected from *E. helvum*. (A) Relative abundance of eight *Bartonella* species across sampling locations. (B) *Bartonella* species alpha diversity across locations according to species richness, Shannon number, and inverse Simpson index. (C) Age distribution of *E. helvum* sampled from each location (N – neonate, J – juvenile, SI – sexually immature, A – adult). (D) Population-level correlates of *Bartonella* prevalence across locations.

I found that variation in *Bartonella* prevalence in *C. greefi* populations from different locations can be explained by population-level covariates (Figure 4.4D). According to data published by Peel et al. (2017) for collections in Ghana and the Gulf of Guinea islands that produced the current fly samples from 2007–2010 plus new collections of flies in Ghana from 2016 included in this study, age distributions in *E. helvum* populations varied widely across locations at the time of sampling (Table II.5; Figure 4.4C). In particular, the population sampled on Bioko island consisted almost entirely of neonate bats that were less than two months old. This was due to inadvertent selection of a sampling site near a 'nursery roost' where mothers were caring for pups. The sampling period was during a time when juveniles were being weaned and the bats were caught at night when mothers had apparently gone to feed and left their offspring at the roost (Peel et al., 2017). Coincidentally, *Bartonella* prevalence was also lowest in flies from Bioko (Table 4.2) and a significant

positive correlation was observed between *Bartonella* prevalence and the proportion of adult and sexually immature bats at each location (R = 0.95, t = 5.1, df = 3, P = 0.015). Additionally, the average density of bat flies collected from each population varied from 2 flies per bat from Bioko to 2.8 flies per bat from Annobón (Figure 4.4D). *Bartonella* prevalence was positively correlated with log bat fly density (R = 0.99, t = 13.4, df = 3, P = 0.009) and log bat fly density was positively correlated with the proportion of adult and sexually immature bats (R = 0.9, t = 3.5, df = 3, P = 0.04).

The dissimilarity in *Bartonella* communities in *C. greefi* appear to be related to restrictions in movement of flies between locations. Considering the Ghana population as representative of the African mainland, I assessed the correlation between *Bartonella* community dissimilarity and the physical distances between islands and the mainland (Figure 4.2B; Figure 4.5) and found a positive signal of isolation by distance (R = 0.73, t = 3, df = 8, P = 0.016). Based on data from Peel et al. (2013), similar isolation by distance patterns were observed for *E. helvum* according to $\phi_{ST} = \phi_{ST}/(1-\phi_{ST})$ for *cytb* sequences (R = 0.56, t = 1.9, df = 8, P = 0.096) and $F_{ST} = F_{ST}/(1-F_{ST})$ for microsatellites (R = 0.74, t = 3.1, df = 8, P = 0.014), however no significant correlation was observed between either measure of bat genetic distance and *Bartonella* community dissimilarity (Figure II.2).

4.4.3 Detection and identification of bat fly symbionts

Enterobacteriales symbionts (Gammaproteobacteria) were successfully detected in bat flies from mainland and island populations (Table II.7). The bacteria detected in *C. greefi* was most closely related to symbionts from the congener *C. dubia* collected from *E. dupreanum* from Madagascar (Wilkinson et al., 2016). The phylogenetic group that contains the symbionts from *Cyclopodia* is distinct from other known genera of bat fly symbionts, including *Arsenophonus*, *Arsenophonus*-like organisms, and *Aschnera* (Wilkinson et al., 2016). Only one genotype of the *C. greefi* symbiont was obtained from flies collected from Ghana, Príncipe, São Tomé, and Annobón (Figure 4.3E,F; Table II.3). The bacteria from *E. africana* were most closely related to symbionts detected in *Eucampsipoda*, *Leptocyclopodia*, and *Dipseliopoda* spp. flies from bats in Kenya,

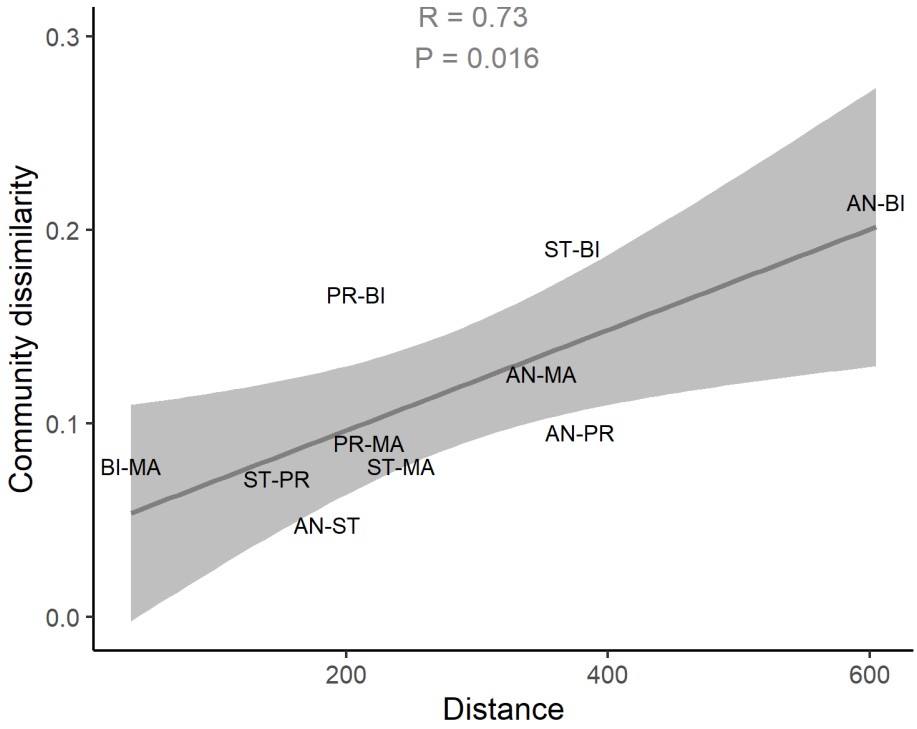

Figure 4.5: Correlation between *Bartonella* community dissimilarity in *C. greefi* and physical distance between locations. R is Pearson's correlation coefficient and P is the p-value for the linear regression. Physical distances match segments in Figure 4.2B, considering Ghana as a representative mainland population. Community dissimilarity was calculated as one minus the Spearman rank correlation between *Bartonella* species counts across locations. Locations are abbreviated AN – Annobón, BI – Bioko, MA – mainland (Ghana), PR – Príncipe, and ST – São Tomé.

China, Philippines, Madagascar, and Comoros; this phylogenetic group is considered part of the genus *Arsenophonus* (Morse et al., 2013; Wilkinson et al., 2016). Two genotypes of *E. africana* symbionts were obtained from the samples (Figure 4.3E,F; Table II.3). Genotype 1 was found in flies collected from Ghana and was most closely related to a symbiont previously detected in *E. africana* from Kenya (Morse et al., 2013). Genotype 2 was found in flies collected from Príncipe and São Tomé and formed a separate branch from symbionts in *E. africana* from the mainland and *E. theodori* from Comoros (Wilkinson et al., 2016).

4.5 Discussion

Host-parasite systems like the one in this study are ubiquitous, but our knowledge of the effects of host movement on parasite populations and communities is still in development. Through joint

analysis of ectoparasitic vectors and bacterial symbionts, this study aimed to infer patterns of host movement beyond those reflected in population genetic analysis of hosts alone. This study contributes to our understanding of the phylogeography of African bats and nycteribiid bat flies, and supports general expectations of limited genetic differentiation in vector-borne microparasites.

Sequencing mitochondrial loci from *C. greefi* and *E. africana* bat flies revealed limited population structure in both species compared with their bat hosts. A unique genotype of *C. greefi* was found only on Annobón island, which corresponds with the presence of a genetically distinct subspecies of *E. helvum* on this island (Juste et al., 2000; Peel et al., 2013). The remaining *C. greefi* specimens from Ghana, Bioko, Príncipe, and São Tomé are a single genotype, failing to capture the genetic differentiation between Príncipe and São Tomé from the mainland and Bioko as seen in *E. helvum* (Peel et al., 2013). Three individuals from Annobón had this widespread genotype, suggesting that they are immigrants from one of these other locations to Annobón. Such distant dispersal events have been reported in *E. helvum*, including one individual recorded from Cape Verde islands 570 km from the African mainland (Jimenez and Hazevoet, 2010) and another recorded traveling 370 km from its roost in Zambia in one night during migration (Richter and Cumming, 2008). The population structure of *E. africana* also partially mirrored that of its host, *R. aegyptiacus*. The single genotype from Príncipe and São Tomé was distinct from the other genotypes found on the mainland. This reflects the distinctiveness of the *R. aegyptiacus* populations from these islands compared to the mainland, but fails to distinguish the island populations from one another (Juste and Ibanez, 1993; Stribna et al., 2019). These results agree with past studies that have shown less structure in bat flies compared to their hosts due to recent or ongoing gene flow (van Schaik et al., 2018; Witsenburg et al., 2015). I conclude that occasional, nonreproductive movements of *E. helvum* and *R. aegyptiacus* between islands or the mainland are resulting in dispersal of their ectoparasitic bat flies.

However, the amount of population structure seen in the flies is sensitive to the choice of genetic marker used for genotyping. In both fly species, mitochondrial *cytb* was able to find more distinct genotypes with greater pairwise distances than 16S rRNA. The 16S rRNA gene may be too

conservative for this type of analysis. Additionally, the low genetic diversity observed in bat flies may be linked to the presence of *Enterobacteriales* symbionts. Previous studies have attributed the lack of population differentiation in mtDNA to selective sweeps caused by reproductive manipulation in those flies not carrying the bacterial symbiont (Hurst and Jiggins, 2005; Lack et al., 2011; Speer et al., 2019). Sequencing of these fly populations at nuclear loci could identify additional population structure in these species and more accurately estimate the amount of gene flow occurring due to bat dispersal. Such data could also clarify the effect that bacterial symbionts have on mitochondrial diversity.

Despite the possible interaction between bacterial symbionts and mtDNA, the population structure of *Enterobacteriales* symbionts reflected the inferred dispersal patterns of their host bat flies. This fits well with expectations that vertically-transmitted parasites are good proxies for inferring movement of their hosts (Nieberding and Olivieri, 2007). The unique phylogenetic group of *Enterobacteriales* symbionts of *C. greefi* was genetically homogeneous across Ghana, Príncipe, São Tomé, and Annobón. The presence of only one genotype may reflect the occasional, indirect dispersal (via bat hosts) of bat flies carrying these bacteria between islands. The *Arsenophonus* symbionts of *E. africana* were split into two genotypes that corresponded to the geographic distribution of the hosts, with one genotype from Príncipe and São Tomé and the other from Ghana. As with genotyping bat flies, bacterial 16S rRNA may be too conserved to successfully identify phylogenetically distinct genotypes of *Enterobacteriales* symbionts, and additional genes should be sequenced. These data would be useful in comparing with the diversity at nuclear loci in bat flies to better detect signatures of selective sweeps in mtDNA due to reproductive manipulation.

The patterns observed in *Bartonella* bacteria reflect their lifestyle as horizontally transmitted, vector-borne parasites. As expected, no population genetic structure was seen in the separate *Bartonella* species from *C. greefi* and *E. africana*. These results are similar to previous studies that have found little correlation between the genetic structure observed in vector-borne microparasites compared to their hosts or vectors (Levin and Parker, 2013; Witsenburg et al., 2015), and lend support to the hypothesis that host and vector movement have additive effects on gene flow in

associated microparasites (Witsenburg et al., 2015). While the markers used for *Bartonella* detection are sufficiently diverse to identify different *Bartonella* species (Kosoy et al., 2018; La Scola et al., 2003), their substitution rates may still be too low to detect microevolutionary patterns. Additional studies using culturing and more extensive methods for genotyping, such as multi-locus sequence typing, amplified fragment-length polymorphisms, or whole genome sequencing, could find additional structure. Nevertheless, by analyzing the relative abundance of the diverse *Bartonella* species found in *C. greefi* from *E. helvum* a significant pattern of isolation by distance was observed, with locations nearer to each other having more similar rank abundances of species, such as Ghana and Bioko or Príncipe and São Tomé. A similar pattern of isolation by distance was seen in *E. helvum* using mtDNA and microsatellites, but there was no correlation between these genetic distances and *Bartonella* community structure. Thus, it is likely that movement of bats (and attached bat flies) is restricted by the distances between islands, and this results in changes in transmission patterns that affect *Bartonella* communities. I encourage future studies to consider analyzing microparasite communites as I have done, since they may help to further clarify patterns of host movements that are uncorrelated with reproduction, but lead to parasite dispersal.

A secondary goal of this study was to find population-level predictors of *Bartonella* prevalence across sampled populations. The *Bartonella* prevalence in both bat fly species was comparable to previous studies using similar molecular detection methods (Table 2). Billeter et al. (2012) reported *Bartonella* prevalence of 57% (26/46), 72% (23/31), and 71% (42/59) in *C. greefi* flies collected from *E. helvum* from Ghana, Annobón, and Bioko, respectively. Bai et al. (2018) reported *Bartonella* prevalence of 42% (21/50) *E. africana* flies from *R. aegyptiacus* from Nigeria. There was no overlap in the species of *Bartonella* found in *C. greefi* and *E. africana* which reflects the specificity of these bacteria to their bat hosts (Bai et al., 2015, 2018; Kosoy et al., 2010). This is reinforced by the ecological separation of the two hosts and bat fly vectors. While these bat species may interact occasionally at feeding sites, they exhibit different roosting behavior, with *E. helvum* roosting predominantly in trees and *R. aegyptiacus* in caves. While *C. greefi* has been occasionally collected from *R. aegyptiacus* and *E. africana* from *E. helvum* (Atama, 2015; Nartey,

2015; Theodor, 1955), these infrequent exchanges of flies do not appear to lead to *Bartonella* transmission from bat flies to an atypical host. Using data from demographic studies of *E. helvum* (Peel et al., 2017), *Bartonella* prevalence was correlated with the age structure of bats and the density of flies in the sampled populations. Specifically, lower prevalence in bat flies was associated with a higher proportion of neonate bats and a lower density of flies, most notably on Annobón. This agrees with results from a captive colony of *E. helvum* in Ghana, wherein neonate bats were found to be initially uninfected with *Bartonella* and become infected if bat flies are present (Chapter 5). *Bartonella* diversity in *C. greefi* did not vary much, with the same common species occurring across locations and only differing in their relative abundance. This is counterintuitive given expectations of island biogeography, which would predict a lower diversity of bacterial communities on the smallest and most isolated islands. This might be explained by chronic or recurrent latent infections, continuous transmission of *Bartonella* in bats within a population, and possible transmission events between populations through occasionally dispersing bats (and bat flies). These factors could sustain populations of *Bartonella* species and prevent the local extinctions that are a fundamental to island biogeography theory.

In summary, the joint analysis of bat parasites and symbionts from African fruit bats has demonstrated these organisms can reveal movement patterns and interactions among bat populations that are not apparent from analysis of host bats alone. Such movements could contribute to the maintenance of other infectious agents in these bats, including viruses (Glennon et al., 2019; Peel et al., 2012). Humans frequently interact with bats in Africa, including through hunting and consumption of bat meat (Kamins et al., 2011; Mickleburgh et al., 2009; Peel et al., 2017), so an understanding of the infection cycles in these pathogens is critical for assessing the risk of spillover into human populations through various exposure routes (Bai et al., 2018; Baker et al., 2012, 2013a; Drexler et al., 2012; Freidl et al., 2015; Hayman et al., 2008, 2012a; Ogawa et al., 2015; Pernet et al., 2014; Suu-Ire et al., 2017). On a broader level, this study increases our knowledge of the complex ecology and population genetics of host-parasite systems that are widespread in nature.

Chapter 5

Manipulating vector transmission reveals local processes in bacterial communities of bats

5.1 Overview

Manipulation of vector populations provides a unique opportunity to test the importance of vectors in infection cycles while also observing changes in pathogen community diversity and species interactions in the absence of pathogen dispersal via transmission. Yet for many vector-borne infections in wildlife, a biological vector has not been experimentally verified and few manipulative studies have been performed. Using a captive colony of fruit bats in Ghana, changes were observed in the community of *Bartonella* bacteria over time after the removal and reintroduction of bat flies. In the absence of transmission, community changes were attributed to ecological drift and potentially selection through interspecies competition mediated by host immunity. This work demonstrates that forces maintaining diversity in communities of free-living macroorganisms act in similar ways in communities of symbiotic microorganisms, both within and among hosts. Simultaneously, this study is the first to validate bat flies as vectors of *Bartonella*.

5.2 Introduction

Knowledge of the processes driving parasite diversity is central to understanding infection dynamics in endemic populations and pathogen emergence in new hosts. In contrast with an historical focus on simple one host-one parasite systems, there is now greater appreciation that parasites exist within communities of other parasites, harbored by hosts that may vary in their responses to parasitism (Johnson et al., 2015). Yet is not clear how well ecological theory developed for free-living organisms applies to communities of microorganisms (Sutherland et al., 2013). This is especially true for parasites and symbionts due to the environmental feedbacks that exist from their dependence on hosts for survival and reproduction (Costello et al., 2012; Miller et al., 2018). Additionally, parasite community dynamics within hosts may occur at differing timescales compared to transmission among hosts. Given these differences, experimental manipulations of natural parasite communities are needed to explore the generality of community theory across organisms.

The metacommunity concept is a useful framework to apply toward analyzing parasites community dynamics within hosts (Leibold et al., 2004; Mihaljevic, 2012). In this framework, hosts are discrete patches harboring potentially interacting parasite species (Figure 5.1). Similar to free-living organisms, four forces might be expected to affect parasite community diversity: speciation, dispersal, ecological drift, and ecological selection (Vellend, 2010). Within a metacommunity, the relative importance of these forces may vary at different scales (Seabloom et al., 2015), i.e., within versus among hosts. Speciation is the only force that generates parasite diversity de novo, but is generally slow and dependent upon dispersal for newly created diversity to penetrate to all scales. Dispersal is the movement of parasite species within a host, among hosts through transmission, or among host populations through host movement. Within metacommunities, parasite species with equal competitive ability may vary stochastically in the production of new parasite individuals or in new infections through transmission. This ecological drift can lead to changes in community composition within hosts (e.g., loss of rare species) or among hosts (e.g., increases in beta diversity), similar to predictions of neutral theory (Hubbell, 2001). Drift happens faster in small communities with few parasite individuals and with little dispersal. Lastly, ecological selection acts within and among hosts. Selection occurs because parasite species vary in replication success within different host individuals or species because of variation in susceptibility or tolerance. Additionally, parasite species may compete within a host, either indirectly through shared resources or common enemies, such as the host immune system, or directly through interference (Pedersen and Fenton, 2007). Species with higher success within a host will dominate and may exclude others, but this can be counterbalanced if fitness is driven by dispersal ability over interspecific competition or there is frequency-dependent selection by the host immune system. These four forces could separately affect parasite community diversity over time. While speciation ultimately creates diversity, the other forces sort parasite species across scales. Thus, a strategy for studying parasite community diversity is to understand the relative importance of these forces both within and among hosts (Seabloom et al., 2015).

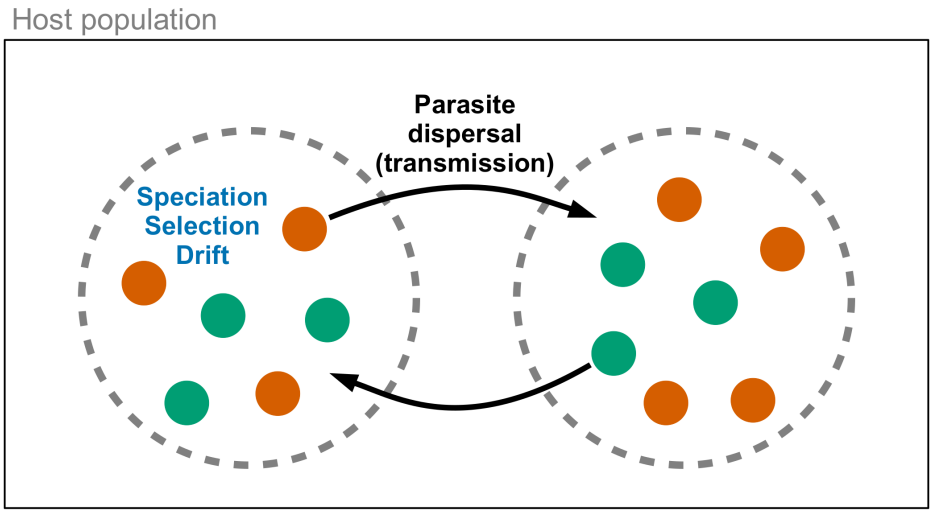

Figure 5.1: Conceptual diagram for parasite community dynamics. Parasite species (colored dots) exist within hosts (dashed circles) and disperse among hosts via transmission. Other ecological forces, including speciation, selection, and drift, act on parasite communities within host individuals. In the absence of among-host transmission, such as when vector populations are removed, the effects of within-host processes can be observed.

Manipulative experiments are one approach to measuring the relative influence of ecological forces acting on communities. By changing the strength of one force, one can observe how others respond and interact across scales. While previous studies have performed parasite community manipulations within and among hosts (see Mihaljevic (2012) and Johnson et al. (2015) for examples), few studies to our knowledge have looked at how manipulating forces that act across scales lead to changes in other forces. Since dispersal is the force that interacts with other processes across within-and among-host scales (Vellend, 2010), it is an appealing target for manipulation.

Vector-borne infections are ideal systems for experimental study because removal of vectors prevents dispersal of parasites between hosts, allowing the analysis of other forces affecting the relative abundance of parasite species. Using a captive colony of straw-colored fruit bats (*Eidolon helvum*) in Ghana, the community dynamics of *Bartonella* bacteria were monitored in bats over three years. During this experiment, the presumed vectors (bat flies) declined in density within the colony, but were then reintroduced. The experiment thus controls parasite dispersal across two scales: the captive colony is closed to immigration (pups enter the colony uninfected) and transmission is manipulated via changes in the bat fly population size. By manipulating parasite

dispersal, the effect of among-host dispersal is minimized and the effects of local, within-host processes (ecological drift and selection) on parasite dynamics and diversity can be observed. I hypothesize that *Bartonella* communities in the colony will respond to changes in among-host dispersal/transmission by bat flies. Specifically, I predict that infection prevalence and diversity will at first decline concurrently with the bat fly population and then increase upon reintroduction of flies, thus providing experimental evidence that bat flies are vectors of *Bartonella* in bats. I hypothesize that limitation of parasite dispersal will result in stochastic losses of rare *Bartonella* species and changes in community beta diversity via ecological drift, and shifts in the rank abundance of *Bartonella* communities due to local selection. Finally, potential interactions among *Bartonella* species will be detectable based on coinfection frequencies, specifically evidence of competition and/or facilitation. This work expands our understanding of *Bartonella* dynamics in natural communities, particularly in bats and their ectoparasites. More broadly, this experiment deepens our understanding of the processes that affect parasite communities, patterns that may be compared with those seen in communities of free-living or mutualistic organisms.

5.3 Methods

5.3.1 Study system

Eidolon helvum (Chiroptera: Pteropodidae) is a long-lived, tree-roosting bat species that can form enormous colonies during the local dry season (Fahr et al., 2015; Hayman et al., 2012b). Bat flies (*Cyclopodia greefi*; Diptera: Nycteribiidae) are obligate blood-feeding ectoparasites that feed continuously on bat hosts. The flies are wingless but can move among hosts within dense roosts. *Bartonella* spp. (Alphaproteobacteria: Rhizobiales) are intracellular bacteria that infect mammals and are transmitted by blood-feeding arthropods (Harms and Dehio, 2012). Six distinct *Bartonella* species have been previously described in *E. helvum* (Bai et al., 2015; Kosoy et al., 2010) and the same species plus additional variants have been detected in *C. greefi* (Billeter et al., 2012; Kamani et al., 2014). Based on these data and other studies (Brook et al., 2015; Morse et al., 2012b; Moskaluk et al., 2018), it has been proposed that bat flies are vectors of *Bartonella* spp. in bats, but no experimental studies have been performed to demonstrate their competenence.

Data for this study come from a captive population of *E. helvum* bats in Accra, Ghana previously detailed by Baker et al. (2014). The captive facility is a fenced hexagonal structure 27.5 m in diameter and 3.5 m high; a metal roof and cladding at the base prevent entry by other animals. The captive population was founded by three cohorts (Table III.1) of mixed age and sex (n = 78) collected from a large seasonal colony in Accra (Hayman et al., 2012b). The cohorts entered the colony in July 2009, November 2009, and January 2010; two additional cohorts were born in captivity in April 2010 (produced by mating between wild bats before entering the colony) and 2011 (produced by mating in captivity). Thirteen of the captive-born neonates were matched to the dam they were attached to at the first sampling point after birth.

Bats were assigned to age classes and sex upon entry to the colony and afterward according to approximate birth date and secondary sexual characteristics detailed previously (Peel et al., 2016). Passive integrated transponder (PIT) tags were implanted in each bat either at entry or shortly after birth to uniquely identify each bat. In addition, adult bats received necklaces with alphanumeric codes. Although 112 total bats entered the colony, 25 bats left the colony either through recorded mortality (n = 12) or presumed mortality after being recorded missing for $\geq$3 sampling points (n = 13). Furthermore, not all bats had complete sample histories throughout the experiment because they escaped into the main area of the enclosure during the cordoning and capture process.

Blood samples were taken from the captive colony every two months in 2009 and 2010 and every four months in 2011 (Table III.1; see Appendix III.1 in for sampling protocol). On 6 March 2010 (denoted M10, day 221), a sample of bat flies (*C. greefi*; n = 28) was removed from the colony for testing and from that point forward the fly population was observed to decline. During this time, it is assumed that little among-host bacterial transmission was occurring. To test the effect of restoring transmission on *Bartonella* community dynamics and to provide evidence that bat flies are vectors, bat flies were experimentally reintroduced to the colony. On 17 January 2012 (J12, day 903), a sample of adult bat flies and nymphs was taken from the original wild source colony, along with paired blood samples from donor bats, and randomly assigned to approximately half the bats in the colony while additional bat flies were collected for testing. Blood samples from captive bats

were subsequently taken at three additional time points after the reintroduction of flies. In total, 910 blood samples were taken from the captive colony over 14 time points from 2009 to 2012 (a period of 961 days), of which 905 samples could be definitively assigned to an individual by PIT tag or necklace ID. An additional 50 blood samples and 18 flies were taken from wild bats on J12 (Figure 5.2).

5.3.2 Bacterial detection and gene sequencing

The focus of this study was on changes in *Bartonella* infection prevalence and the relative abundance of *Bartonella* species in bats, so a molecular detection and sequencing approach capable of distinguishing among potentially coinfecting species was used. Bat blood and fly samples were tested for the presence of *Bartonella* DNA using a multi-locus PCR platform (Bai et al., 2016) targeting fragments of the 16S–23S ribosomal RNA intergenic spacer region (ITS), citrate synthase gene (*gltA*), and cell division protein gene (*ftsZ*). Each of these loci is capable of distinguishing among *Bartonella* species and subspecies (La Scola et al., 2003), but may have amplification biases toward different *Bartonella* species in a sample (Kosoy et al., 2018). Thus, the purpose of this multi-locus approach was to confirm the detection of *Bartonella* DNA and to indicate across loci whether multiple infections were present. Further quantification of *Bartonella* infection load was performed by real-time PCR targeting the transfer-messenger RNA (*ssrA*). Sequences were verified as *Bartonella* spp. using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Samples were only considered positive if a significant match was observed, even if there was a positive real-time PCR result (cycle threshold value (Ct) < 40). *Bartonella* sequences with multiple peaks in the electropherogram were separated into two or more distinct sequences by comparison with previously obtained *Bartonella* sequences from *E. helvum* and *C. greefi* (Bai et al., 2015; Billeter et al., 2012). Due to the frequency of multiple sequences obtained from these loci, conflicting sequences across genes were interpreted as evidence of coinfection rather than homologous recombination, and thus report counts of sequences representing distinct species within a sample as is recommended (Kosoy et al., 2018). All variants of *Bartonella* sequences sharing <95% sequence similarity with previously identified *Bartonella*

species were submitted to GenBank. Additional details on bacterial detection and phylogenetic analysis are provided in Appendix III.1.

5.3.3 Data recording and statistical analyses

Relevant measures of *Bartonella* infection prevalence, infection load, and diversity were recorded or calculated to assess changes that occurred during the experiment, particularly before and after the reintroduction of bat flies to the captive colony. *Bartonella* infection prevalence within the colony, in sampled flies, and from wild bats was reported based on the number of tested bats that were positive at one or more loci (ITS, *gltA*, *ftsZ*, *ssrA*). Wilson scores were used to calculate 95% confidence intervals for single infection and coinfection prevalence. *Bartonella* alpha diversity was measured by *Bartonella* species richness and Shannon number; species richness within each sample based on the number of loci positive was also recorded. *Bartonella* species relative abundances were calculated from the total number of sequences obtained across all loci, including separate sequences obtained from the same locus. A custom bootstrapping procedure with 1000 samples from the observed multinomial distribution of *Bartonella* species relative abundances was used to estimate 95% confidence intervals around measures of alpha diversity. *Bartonella* beta diversity was measured across sampled bats and flies using the binomial index option of the vegdist function in the R package `vegan` (Oksanen et al., 2018; R Core Team, 2019). Infection load was recorded as the number of loci positive and real-time PCR Ct value for each sample. Additionally, for each bat the time until becoming infected after first entering the colony and the duration of infection for the most persistent *Bartonella* species were recorded. These measures help to track whether certain demographic groups are more affected by the reintroduction of flies and to compare with changes in relative abundances of Bartonella species over time, respectively. Change points in *Bartonella* prevalence, infection load, and diversity measures were detected with segmented regression using the R segmented `segmented` (Muggeo, 2017). Chi-square or Fisher's exact tests were performed to compare changes in infection status for bats that did or did not receive bat flies on J12. Multinomial and binomial likelihood ratio (LR) tests adapted from Pepin et al. (2013) were performed to find statistical associations between coinfecting *Bartonella* species and to detect changes in the

relative abundance of *Bartonella* species during the study period. For additional details regarding regression analyses and likelihood ratio tests, see Appendix III.1.

5.4 Results

5.4.1 Phylogenetic analysis of detected bacteria

Bartonella infections in bats and bat flies were identified as six previously characterized species based on ITS, *gltA*, and *ftsZ* sequences: *Bartonella* spp. E1–E5 and Ew (Bai et al., 2015; Kosoy et al., 2010). Two additional genogroups identified by *gltA* sequences, *Bartonella* spp. Eh6 and Eh7 (Figure III.1), were similar to sequences previously obtained from *C. greefi* collected from *E. helvum* in Ghana and two islands in the Gulf of Guinea (Billeter et al., 2012). Phylogenetic analysis of concatenated *ftsZ* and *gltA* sequences distinguished Eh6 and Eh7 from other *Bartonella* species associated with *E. helvum* or other bat species (Figure III.4). See Appendix III.2 for more details on phylogenetic analysis.

5.4.2 *Bartonella* infection prevalence and effects of bat fly reintroduction

As predicted, *Bartonella* prevalence in the captive colony changed with the population density of bat flies. *Bartonella* prevalence in the first three cohorts was high at colony entry, then declined concurrently with the observed decline in the bat fly population (Figure 5.2A). After flies were reintroduced, prevalence increased from 31% at day 903 to 48% on day 961. This change is reflected in the segmented regression analysis (Figure III.7A; Table III.4) with a shift from positive to negative slope at M10 (day 221) and a shift from negative to positive slope at J12 (day 903). The trend in *Bartonella* prevalence in the colony over time was similar if bats were considered positive for *Bartonella* with a threshold of at least one, at least two, at least three, or all genetic markers being positive (Figure III.8).

The effect of bat fly reintroduction affected some age classes of bats more than others. Most sexually immature subadult and sexually mature adult bats initially entered the colony infected (Figure III.6A). All subadult bats were infected at entry and by the end of the study; however there was an increase in the proportion of adult bats (χ^2 = 3.2, df = 1, P = 0.038) infected by the end

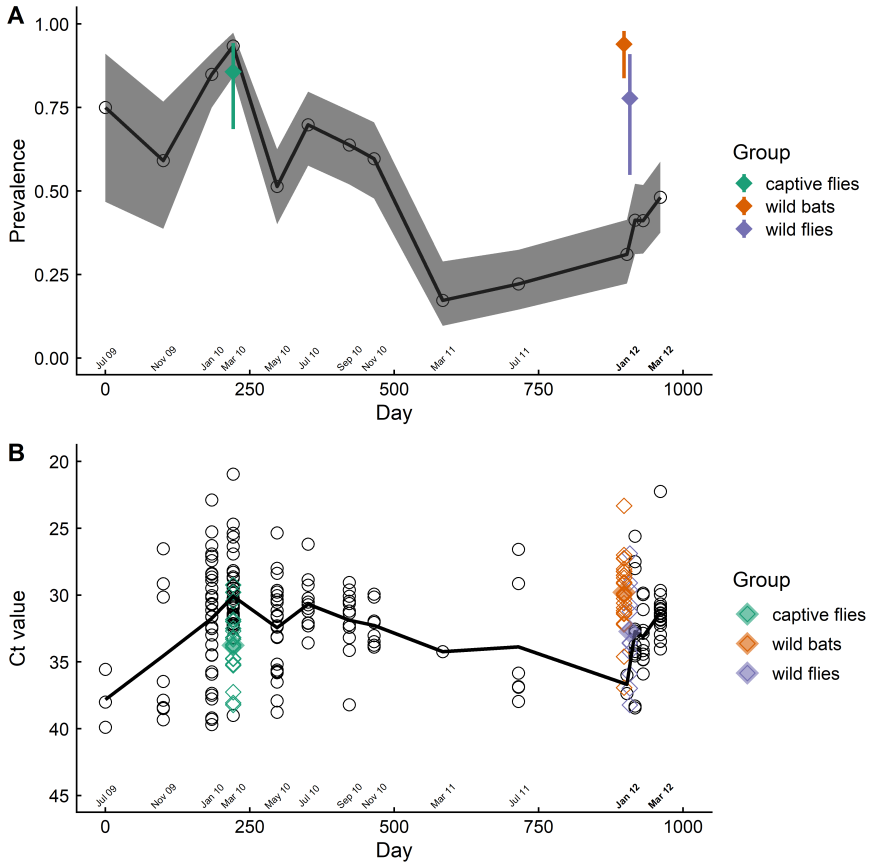

Figure 5.2: *Bartonella* infection prevalence and load in a captive colony of *E. helvum* over time. (A) Bats were considered positive if a *Bartonella* sequence was obtained from one or more genetic markers. Wilson score 95% confidence intervals (dark gray) were drawn around prevalence estimates at each sampling time point. Prevalence and confidence intervals for sampled *C. greefi* flies and wild *E. helvum* are shown as colored points. (B) Only points with RT-PCR Ct values < 40 are shown. Mean Ct values calculated at each time point are drawn as a black line over the data points. Ct values for *C. greefi* flies and wild *E. helvum* are shown as colored points with calculated mean values (filled symbols). Months labeled in bold font on the x-axis show the period after bat flies were reintroduced.

of the study compared to the start. Bats born into the colony in 2010 and 2011 were *Bartonella*-negative at first sampling. By the end of the experiment, 88% of these bats had become infected (Figure S6a), a very significant increase ($\chi^2 = 48.2$, df = 1, $P < 0.001$).

Out of the 53 bats that were negative on J12, 32 bats (60.4%) became positive after flies were reintroduced ($\chi^2 = 43$, df = 1, $P < 0.001$). The effect of flies on prevalence was much more pronounced for bats that were born into the colony in 2010 and 2011 than for adult bats: 16/17 (94.1%) late cohort bats became positive after reintroduction versus 16/36 (44.4%) early cohort bats ($\chi^2 = 9.9$, df = 1, $P < 0.001$). Including bats that were already positive on J12, 48/84 (57.1%)

either became positive or changed *Bartonella* species after fly reintroduction (χ^2 = 64.4, df = 1, P < 0.001). This effect was greater for late cohort bats than for early cohort bats: 22/28 (78.6%) late cohort bats versus 26/30 (46.4%) early cohort bats (χ^2 = 6.6, df = 1, P = 0.005). However, when comparing bats that received flies versus those that did not (i.e., cases versus controls), there were no significant differences between groups in their change in infection status after fly reintroduction (see Appendix III.2 for details). Thus, the effect of bat fly reintroduction was only observable at the population level infection prevalence and within age classes, but not for individual bats.

Bat fly reintroduction had similar effects on measures of infection load in the colony. Infection load in each sample as measured by RT-PCR cycle threshold (Ct) values (Figure 5.2B) and the number of positive genetic markers per sample (Figure III.9A) reached a peak on M10, then declined before sharply increasing after the reintroduction of flies. This trend is reflected in the segmented regression of both measures, with a shift from positive to negative slope at day 221 and a shift from negative to positive slope at day 903 (Figure III.7B,C; Table III.4). Coinfection prevalence also showed a peak at M10 and declined until March 2011 when it began to increase again (Figure III.9B). The shift from positive to negative slope around M10 was not statistically significant, but the shift from negative to positive slope around March 2011 was significant (Figure III.7D; Table III.4). For details on prevalence and load in bat flies and wild bats collected on M10 and J12, see Appendix III.2.

5.4.3 Patterns of *Bartonella* diversity

Similar to infection prevalence and load, *Bartonella* diversity measures changed in response to bat fly population density. *Bartonella* diversity was measured at two scales, at the colony level and at the individual host level. *Bartonella* species richness and evenness (Shannon index) measured colony-level alpha diversity. The number of *Bartonella* species in an individual sample and beta diversity (binomial index) measured individual-level diversity. Diversity measures showed qualitatively similar patterns during the early phase of the experiment (Figure 5.3): an initial increase with the entry of the first three cohorts into the colony reaching a maximum in January 2010 followed by a decline. Diversity measures increased again until the reintroduction of flies on J12 and then

declined slightly (or remained flat in the case of species richness). The pattern in species evenness was nearly identical when the inverse Simpson index was used (Figure III.10). The observed trends were only partially reflected by segmented regression breakpoints. Segmented regression detected only one breakpoint each in the timelines for species richness, species evenness, and the number of *Bartonella* species in an individual sample (Table III.4). A shift from positive to negative slope was detected in January 2010 for species richness (Figure III.11A) whereas a change from negative to positive slope was detected for species evenness and the number of species in an individual sample between November 2010 and March 2011 (Figure III.11B,C; Figure S12A). There were two significant breakpoints detected in the timeline of beta diversity, changing from negative to positive slope in July 2010 and from positive to negative slope in January 2012 (Figure III.12B; Table III.4). For details on diversity measures in bat flies and wild bats collected on M10 and J12, see Appendix III.2.

5.4.4 Shift in *Bartonella* species abundance

Bartonella species observed in the colony varied in their relative abundance, with an apparent shift in the dominant species during the study (Figure 5.4A). While rarer species E1, E2, and Eh7 were not observed at all time points, E1 and E2 were consistently observed over the duration of the study. In contrast, the rarest species Eh7 was not observed after July 2010, even after flies were reintroduced to the colony. Species Eh6 was also uncommonly observed during the study, went unobserved for three time points in 2012, but was observed again in March 2012.

As noted above, beta diversity decreased after January 2010 when the bat fly population was decreasing, reached another maximum in January 2012, and then decreased again after flies were reintroduced (Figure 5.3D). These decreases in beta diversity correspond with periods of expansion by some species within the colony that appear to homogenize beta diversity. During a period from January 2010 to July 2011, Ew became the most abundant species in the colony (Figure 5.4A). Another measure of this species' dominance in the colony is the duration of its infections in individual bats. For each individual bat that was sampled more than once and was recorded as having the same *Bartonella* species for a sequential period, I tabulated which species was present

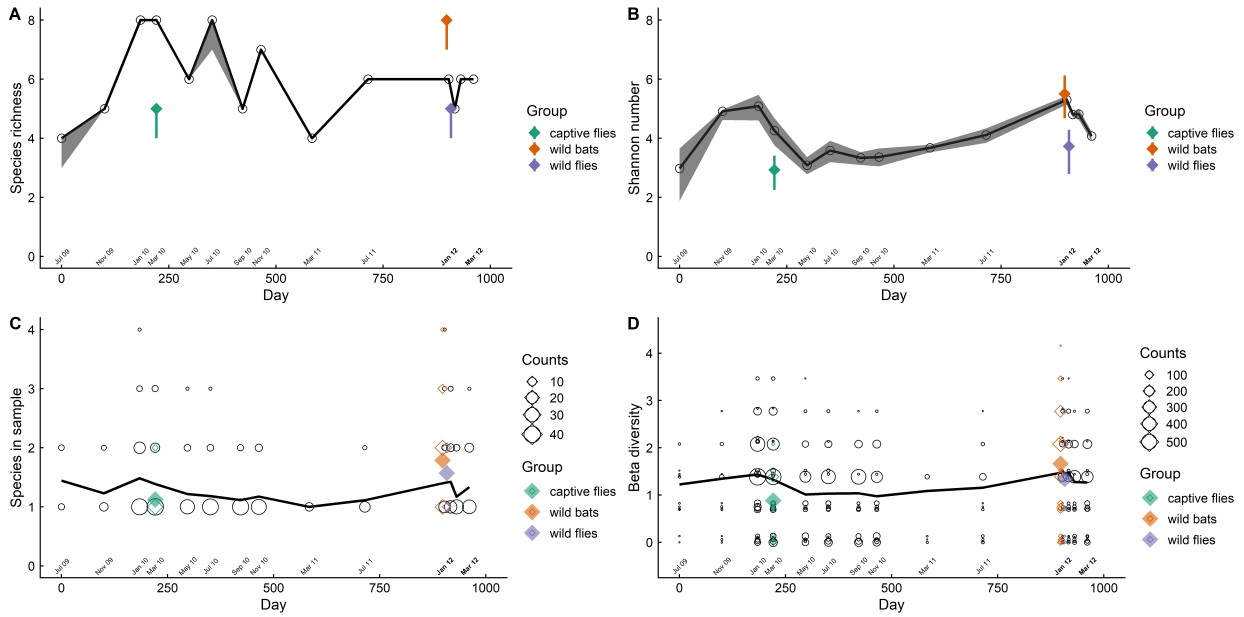

Figure 5.3: *Bartonella* infection diversity measures over time. Species richness (A) and the Shannon index of species evenness (B) are colony-level measures or *Bartonella* alpha diversity, the number of *Bartonella* species in each individual bat and *Bartonella* beta diversity (binomial index) are measures of individual-level diversity. (A–B) Dark gray intervals around richness and evenness are bootstrap 95% confidence intervals from 1000 samples from the observed multinomial distribution of *Bartonella* species relative abundances. Diversity and confidence intervals for sampled *C. greefi* flies and wild *E. helvum* are shown as colored points. (C–D) Points show the number of *Bartonella* species observed (C) and the binomial index of beta diversity (D; compared to all other bats in the colony) for each individual with the width proportional to the number of individuals with that same diversity value. For each measure, lines for the calculated mean trend are shown. Points for sampled bat flies and wild bats are shown as colored points with calculated mean values (filled symbols). Months labeled in bold font on the x-axis show the period after bat flies were reintroduced.

for the most time points (Figure 5.5). Among *Bartonella* species, Ew was the longest lasting infection in the highest number of bats (n = 40). The infection durations for this species ranged from 37 to 610 days with a median of 145 days.

Beginning around March 2011, the relative abundance of Ew began declining and species E1, E2, and E5 increased (Figure 5.4A). Dividing the study into two parts – before flies were introduced (July 2009 to July 2011) and after flies were introduced (J12 and after) – a clear difference in the rank abundance of *Bartonella* species was observed (Figure 5.4B). This shift in abundance after the introduction of flies was significant according to a multinomial LR test ($D = 350.1$, $df = 7$, $P < 0.001$) and individual binomial LR tests for all species (Table III.5). Significant differences were also observed in the relative abundances between bat flies and sampled bat populations on M10 and

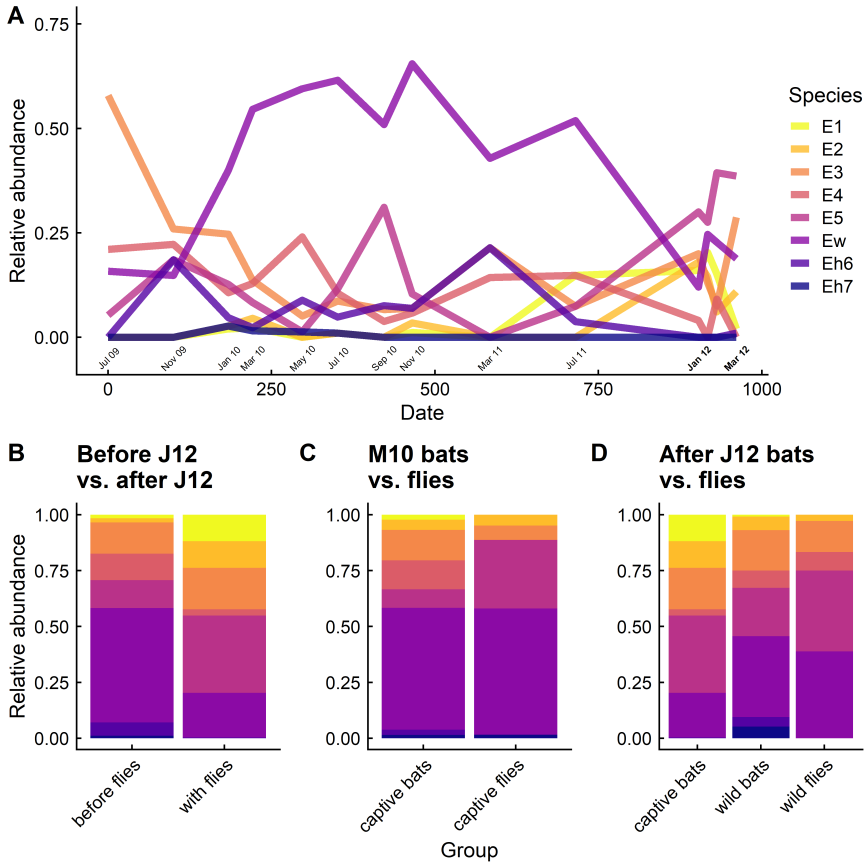

Figure 5.4: Relative abundance of *Bartonella* species in the captive colony over time (A–B) and between sampled bat flies and their respective bat populations (C–D). Relative abundance (A) at each time point was estimated from the total number of counts for each *Bartonella* species based on sequences from ITS, *gltA*, and *ftsZ*. Months labeled in bold font on the x-axis show the period after bat flies were reintroduced. Tests for differences in the relative abundance of species were performed between bats in the captive colony before and after bat flies were reintroduced on 17 January 2012 (B); between bat flies sampled from the colony and the captive bat population in March 2010 (C); and between bat flies and wild bats sampled on 17 January 2012 and the captive colony population after flies were reintroduced (D).

J12 (Figure 5.4C,D; Table III.6). Patterns in the occurrence of species over time and relevant tests of differences in the *Bartonella* community were similar if the relative counts (presence/absence of species across any marker rather than counts across markers) were used instead of relative abundance (Figure III.13; Table III.7). For details on this and tests of differences in the relative abundance of species in bat flies and wild bats, see Appendix III.2.

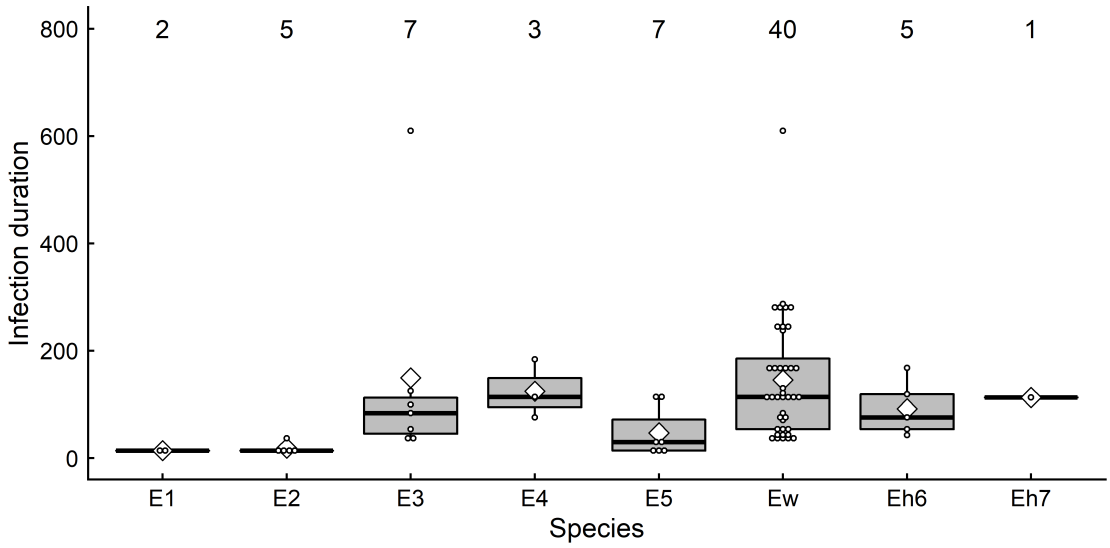

Figure 5.5: Duration of *Bartonella* sp. infections in serially infected individuals. For each *Bartonella* species, the numbers above the bars are counts of individual bats that had the *Bartonella* species as its longest lasting infection (i.e., the *Bartonella* species was present for the most sequential time points). The infection durations in days for all serially infected bats (white circles) are plotted on top of box plots. Stacked data points are aggregated into bins of 5 days. Black lines indicate the median duration and white diamonds indicate the mean duration.

5.4.5 Interactions between *Bartonella* species

Using multinomial and binomial LR tests on coinfection frequencies, there was evidence of both negative and positive interactions between *Bartonella* species over the period of the experiment (Figure 5.6). Bats infected with Ew were significantly less likely to be coinfected with E2, E3, and E5; a reciprocal negative effect on Ew from these species was not detected. Related to this, the proportion of Ew infections that were also coinfections was low (30%) considering its high relative abundance in the population over time (Figure 5.4A). Species E1 and Eh6 had a reciprocal negative effect on each other. Reciprocal positive effects (i.e., more coinfections than expected) were found between species E3 and E5 and species E1 and E5. Bats were also more likely to be coinfected with Ew if they were already infected with E1, but there was no significant reciprocal effect of Ew on E1 (Figure 5.6).

	E1	E2	E3	E4	E5	Ew	Eh6	Eh7	Multinomial P	Proportion coinfections
E1	**12**	3	7	2	11	8	0	0	0.11	0.72
E2	3	**16**	8	2	5	4	2	0	0.38	0.60
E3	7	8	**46**	9	23	20	6	0	0.084	0.61
E4	2	2	9	**30**	3	17	1	1	0.51	0.54
E5	11	5	23	3	**55**	14	2	0	**0.0014**	0.51
Ew	8	4	20	17	14	**165**	7	1	0.054	0.3
Eh6	0	2	6	1	2	7	**27**	0	0.15	0.4
Eh7	0	0	0	1	0	1	0	**5**	0.8	0.29

More than expected
Less than expected

Figure 5.6: Patterns of *Bartonella* species coinfection. Rows are the focal species and columns are the partner infections. Numbers in the boxes are counts of coinfections between each pair of species; single infection counts for each species are on the diagonal. Black boxes show coinfections that occurred more frequently than expected, grey boxes show those that occurred less frequently than expected, and white boxes showed no significant pattern. Expected counts were based on the frequency of single and double infections of each *Bartonella* species, and significance was based on multinomial and binomial tests. The proportion of infections by each *Bartonella* species that were also coinfections are shown in the last column.

5.5 Discussion

Parasites do not infect hosts in isolation, but can instead form diverse communities in hosts that vary over time. However, it is unclear if the same forces that affect diversity in communities of free-living organisms act in the same way or with different strength in parasite communities. This study tested how well predictions of community ecology theory apply to host-vector-parasite systems through a unique approach that manipulated parasite dispersal among hosts within the population by changing the population density of the putative vector. Restriction of parasite dispersal minimized the effect of among-host transmission on *Bartonella* communities within individual hosts, thereby allowing the effects of ecological drift and selection on parasite community diversity to be measured. At the same time, by observing trends in the prevalence and diversity of *Bartonella* infections within the colony over the course of vector population decline and reintroduction, bat flies were confirmed as biological vectors of *Bartonella* in bats. Overall, the experiment shows that *Bartonella* communities are affected by dispersal, drift, and selection in similar ways to free-living organisms, although numerous forms of ecological selection might be acting simultaneously.

I first hypothesized that *Bartonella* communities in the colony would respond to changes in among-host dispersal/transmission by bat flies. Specifically, I predicted that infection prevalence and diversity would decline concurrently with the bat fly population and then increase upon reintroduction of flies. The results indicate that *Bartonella* prevalence and infection load declined along with the bat fly population, then increased when flies were reintroduced in January 2012 (Figure 5.2). This effect was seen across the whole population, but had a stronger effect on young bats born in the colony, likely attributable to their lack of prior exposure to *Bartonella* while flies were absent. Only a few vectors of *Bartonella* bacteria have been confirmed through controlled exposure of hosts to infected vectors (Morick et al., 2013; Tsai et al., 2011). This study confirms that bat flies are likely vectors of *Bartonella* bacteria in bats.

Bartonella diversity also decreased over the corresponding period when flies were declining (Figure 5.3). This decline may be attributed to the stochastic loss of rare species and the increase in abundance of some species, specifically Ew, through persistent infection (Figures 5.4–5.5). Interestingly, all diversity measures actually increased prior to the reintroduction of flies, reaching a local peak in diversity in January 2012 before declining. This second decline could be attributed to the decline of the dominant Ew, allowing potentially latent infection of other species to emerge (E1, E2, E3, E5). The dominance of these species continued after flies were reintroduced and among-host transmission was restored, thus causing a short decline in diversity measures. These patterns indicate that dispersal of infections by flies is key to the long-term maintenance of *Bartonella* community diversity in bats.

While the experiment was originally designed to define bats as being cases versus controls during the reintroduction of flies, this was not successful. Bats that received flies were not more likely to become infected or change Bartonella species after reintroduction. This probably occurred because bat flies did not remain on the bat they were placed on and instead moved among individuals in the colony. This would produce the poor correlation between infection status of bats and flies, as seen in the results presented and those of Becker et al. (2018) in vampire bats. Nevertheless,

this study establishes that the loss and reintroduction of bat fly vectors is associated with changes in *Bartonella* infection and diversity at the host population level.

I also hypothesized that limitation of parasite dispersal would result in stochastic losses of rare *Bartonella* species and changes in community beta diversity via ecological drift, and shifts in the rank abundance of *Bartonella* communities due to local selection. The rarest species in the community, *Bartonella* species Eh7, was lost during the course of the study and was not restored when flies were reintroduced. This failure was likely due to a sampling effect, wherein flies carry only a subset of the most common *Bartonella* species (Figure 5.3D; Figure 5.4C,D), therefore limiting opportunities for effective dispersal of rare species. As noted above, beta diversity did not exhibit the expected increase when the fly population declined. Instead there was a decrease in beta diversity due to the dominance of species Ew (Figure 5.3). This dominance of Ew was the most conspicuous trend in the dynamics of the *Bartonella* community over most of the study, except for the end of the experiment when there was a shift towards the next most abundant species, E5, and other lower ranked species (Figure 5.4A). This shift towards E5 and decline in Ew occurred before the reintroduction of flies and was independent of the effects of among-host dispersal (due to the absence of flies at this time). I speculate that this is an emergent pattern due to within-host selection against Ew by the host immune system. Specifically, as Ew came to dominate within the population and in individual bats, it may have become the primary target of host immune responses. As Ew was eliminated, this allowed for the emergence of other latent infections within coinfected bats. Thus, without dispersal of Bartonella species by bat fly vectors, ecological drift and selection by the host immune system can cause observable changes in bacterial communities.

Finally, I expected that potential interactions among *Bartonella* species would be detectable based on coinfection frequencies, providing evidence of competition or facilitation in pathogen communities. While most interactions were not significant, species Ew has negative effects on several species and typically has few coinfections (Figure 5.6). In contrast, positive effects were observed between species E1, E3, and E5, which show a much higher frequency of coinfection

(Figure 5.6). These results indicate that parasitic bacteria like *Bartonella* do have measurable ecological interactions that are not uniformly competitive.

From just a single experiment, several inferences about the ecology of *Bartonella* infections in bats can be made. First, *Bartonella* infections in bats can be persistent, lasting potentially hundreds of days in the absence of reinfection by vectors. Other studies have alluded to the possibility of persistent *Bartonella* infection with periodic reactivation in rodents (Bai et al., 2011a; Kosoy et al., 2004) and bats (Becker et al., 2018), however these studies have been done in open populations where reinfection by vectors was possible. Second, *Bartonella* community diversity at these timescales can be driven by dispersal, drift, and selection. This study has shown that in the absence of dispersal, the effects of drift and selection can be more apparent. Two types of selection can occur in these parasite population, either through interactions with host immune system or through interspecies interactions. As noted above, the immune system may lead to periodic selection against the dominant infection, a negative frequency-dependent mechanism that might help maintain diverse parasite communities (Fallon et al., 2004).

Dominance also appears to be a facet of bacterial communities as it is in free-living organisms (Smith and Knapp, 2003). The dominance of Ew may thus stem from multiple facets of its ecology. First, it appears to be persistent within bats (Figure 5.5) and secondly, it is readily taken up by flies (Figure 5.4C,D). I note that Ew is also the most clonal, i.e., genetically homogenous, species in the community and might be a more recently evolved or introduced species in *E. helvum* (Bai et al., 2015). While there was no evidence that Ew caused higher infection loads (by Ct value or number of markers positive), future studies should inspect growth curves throughout the infection cycle to see if Ew has any growth advantage. Other forms of interference or resource competition must be explored further, perhaps through controlled infection experiments.

Future work within this system might involve controlled exposure of *Bartonella*-negative bats and confirmation of the exposure route. Alternative routes might include through bat fly bite, requiring tropism of the bacteria to the salivary glands, or contamination through feces, requiring replication in the fly gut and persistent shedding of viable bacteria in feces. Additional studies

could examine immune function in bats (Boughton et al., 2011) in response to *Bartonella* infection, to confirm the existence of frequency-dependent selection against *Bartonella* species and determine the appropriate epidemiological models to explain *Bartonella* infection dynamics (Brook et al., 2017).

This study has contributed to a more comprehensive understanding of the ecology of *Bartonella* species in bats and connects with broader community ecology theory developed in free-living and symbiotic organisms (Costello et al., 2012; Miller et al., 2018; Vellend, 2010). Limitation of dispersal in this experiment led to declines in local species diversity in individual bats, a pattern that fits well with predictions from patch dynamics or mass effects models of metacommunities (Leibold et al., 2004). The results also show that not all bacterial interactions are negative, even among species that presumably share the same niche. This parallels the recognized importance of positive species interactions in plant communities (Bertness and Callaway, 1994) and among bacterial taxa in animal microbiomes and aquatic habitats (Faust et al., 2012; Hegde et al., 2018; Ju and Zhang, 2015). A recent study by Gutiérrez et al. (2018a) on *Bartonella* infections in desert rodents has also shown a mixture of negative, neutral, and positive interactions similar to the present study. Theoretical and experimental studies suggest that communities remain stable through a predominance of neutral or weak species interactions that can attenuate large competitive or facultative effects (Aschehoug and Callaway, 2015; McCann, 2000). Weak interactions, paired with the frequency-dependent selection already discussed, could provide a model for understanding how *Bartonella* species and other parasitic microorganisms coexist in communities. These mechanisms could allow bacteria to share a niche or split it temporally, which could lead to periodic shifts in the dominant species but maintain the community as a whole. Future work using this system and similar longitudinal studies on other pathogens in natural host populations could lead to additional insights on the nature of microorganismal communities and the broad ecological processes that act across taxonomic and spatial scales.

Chapter 6

Concluding remarks

The research presented in this dissertation deepens our knowledge of the evolutionary history and ecological dynamics of blood-borne microparasites of bats. However, much research still remains, not only to characterize the diversity of parasites in bats and mammals generally, but also to develop a more mechanistic understanding of within-host infection dynamics and the forces maintaining parasite diversity in host communities. I identify three primary research needs for blood-borne microparasites: 1) comprehensive surveys of microparasites in potential reservoir hosts and vectors, 2) experiments to identify competent vectors and confirm alternative transmission routes beyond vector-borne transmission, and 3) efforts to model infection dynamics, phylogeography, and spillover risk.

Comprehensive surveys of reservoir hosts are critical for understanding fundamental aspects of microparasites in natural populations, including host range, infection prevalence, and any spatiotemporal variation that might exist in prevalence and diversity. Only through systematic surveys can we overcome issues of sampling bias and generate reliable predictions of parasite diversity globally. Collection of whole blood in vials or Whatman cards (or blood pellets after separation of serum) and ectoparasites should be done from as many hosts as possible in a given community to assess patterns of specificity among sympatric hosts. It is likely that untold numbers of blood samples are already available in freezers from previous survey efforts for other parasites or population genetic studies that could be reused in studies of blood-borne microparasites. However, it will be necessary for research groups to collaborate and share samples (Phelps et al., 2019). Once collections have been established, standardized methods of genotyping or barcoding will create parasite datasets that can be compared and merged with past studies (Dario et al., 2017b; Hutchinson and Stevens, 2018; Kosoy et al., 2018). Wherever possible, direct isolation of parasites should be attempted to allow for more complete characterization of novel species, through multi-locus genetic analysis or full genome sequencing (Gutiérrez et al., 2017). With these genetic data, 'ghost'

taxa of microparasites might be rediscovered and large gaps in the phylogenies of trypanosomes, haemosporidians, *Bartonella*, and other blood parasites can be filled in.

In addition to phylogenetic analyses, these systematic survey efforts will allow for comparisons of parasite taxonomic diversity among host orders and species using rarefaction curves or biodiversity estimators, potentially accounting for host sharing through power scaling relationships (Anthony et al., 2013; Carlson et al., 2019; Chao et al., 2014). Researchers will be able to build parasite sharing networks among hosts and identify ecological predictors of host specificity (Luis et al., 2015; McKee et al., 2019; Nieto-Rabiela et al., 2019; Pedersen and Davies, 2009; Pedersen and Fenton, 2007). Comprehensive estimates of microparasite diversity will allow for testing of correlates of parasite diversity and the risk of spillover into humans, such as latitude, host diversity, climate, land use, and human population density (Brierley et al., 2016; Gay et al., 2014; Maganga et al., 2014; Olival et al., 2017). Together, such studies would provide a more fundamental understanding of the macroecological forces that generate and maintain parasite diversity in nature.

There is also a need for experimental studies to test hypotheses that might be generated from past and future surveys of microparasites in animal populations. One thread that appears to exist in research on blood-borne microparasites is that few candidate vectors have been identified and even fewer have been experimentally confirmed. Therefore, the range of vectors that can transmit parasites like *Bartonella*, *Trypanosoma*, haemosporidians, and other blood-borne microparasites may be larger than is currently appreciated, with many potential vectors that vary in competence. Alternative transmission routes may exist, especially for *Bartonella* and *Trypanosoma*, including mechanical transmission by vectors (possibly including consumption of vectors by hosts), direct and indirect host-to-host transmission via shedding of parasites in host body fluids (feces, urine, saliva), and potentially vertical transmission in both hosts and vectors. These alternative routes must be investigated further to determine the relative importance of these alternative routes and incorporate them into models of infection dynamics and parasite evolution. Such investigations may take the form of artificial feeding experiments (Kernif et al., 2014) to determine the site of parasite

replication in vectors and to confirm viability of parasites in body fluids that lead to transmission (feces, saliva). Controlled infection experiments of hosts (Colton and Kosoy, 2013; Morick et al., 2013) could demonstrate within-host kinetics of infection and the potentially systemic nature of infections (e.g., colonization of reproductive or digestive systems). Within these controlled infection experiments, relevant measures of host immune responses to parasitic infection might be gained from metatranscriptomics (Lilley et al., 2019). Such experimental data would inform choices of heirarchical structure and parameter estimates for modeling studies.

Quantitative models are key to understanding how pathogens are maintained in nature, however few exist for blood-borne microparasite infections. This is partly because these infections can involve multiple reservoir hosts and vectors, and potentially interacting co-infections, which can severly overcomplicate modeling efforts (Buhnerkempe et al., 2015; Heesterbeek et al., 2015). Nevertheless, comprehensive data collection in natural host populations or from experimental studies might be able to identify key systems that are analytically tractable (i.e., a limited number of hosts, vectors, and infectious agents). With data from such systems, modeling could reveal mechanisms of infection persistence in populations and within individual hosts, and the effects of within-host competition between coinfections (Brook et al., 2017; Pedersen and Fenton, 2007; Ranaivoson et al., 2019; Sherlock et al., 2013; Telfer et al., 2010). Such modeling efforts may highlight unique aspects of host-parasite interactions that may explain the importance of certain host groups as infectious reservoirs (Schountz et al., 2017).

The work presented in this dissertation highlights African fruit bats as a potential model system for studying blood-borne microparasite diversity and infection dynamics. For example, the host-parasite associations in this system could also provide insights on the historic biogeography of *E. helvum*, *R. aegyptiacus*, and African bats more broadly. Previous studies have shown that different lineages of bats independently colonized Africa from Asia at least three and possibly four times (Almeida et al., 2016; Juste et al., 1999). *Eidolon* appeared to be the first genus to colonize, however it is unclear how *Eidolon* reached Africa. *E. dupreanum* is only on Madagascar and *E. helvum* is distributed across the mainland, so the colonization of the African mainland could have

been from Indian Ocean islands or vice versa. The presence of *Pteropus* species, which are more related to *Eidolon* than other African bats, on Indian Ocean islands suggests island hopping is possible (Almeida et al., 2014, 2016; Teeling et al., 2005). The genus *Rousettus* colonized later, most likely via island hopping through the Indian Ocean (Almeida et al., 2016; Juste et al., 1999; Stribna et al., 2019). While comprehensive phylogenetic analysis of African and Asian fruit bats would help to clarify these ancient movements, simultaneous analysis of their bat fly vectors, bacterial symbionts, and blood-borne microparasites could be a useful complement. The bat fly genera *Cyclopodia* and *Eucampsipoda* are associated with fruit bats across Asia, Australia, and islands in the Indian and Pacific Oceans (Maa, 1962, 1968; Olival et al., 2013; Theodor, 1973). *Bartonella* bacteria, haemosporidians, and trypanosomes have been detected in fruit bats or bat flies from these regions, however no comprehensive multi-agent surveys have been performed (Austen et al., 2015; Barbosa et al., 2016; Brook et al., 2015; Hou et al., 2018; Mackerras, 1959; McGhee, 1949; Miyata and Tsukamoto, 1975; Morse et al., 2012b; Olival et al., 2007; Schaer et al., 2018, 2019; Thompson et al., 2014; Van Peenen et al., 1968; Wilkinson et al., 2016). The evolutionary history of these microparasites could shed light on historical parasite transmission patterns during the diversification and geographic dispersal of fruit bats across continents.

The captive colony of *E. helvum* in Ghana may also produce additional studies on within-host infection dynamics and immune responses to *Bartonella* infections in bats. Bayesian occupancy models using the data from the experiment presented in Chapter 5 would be useful for identifying demographic predictors of infection, long-term persistence of infections, and competition between *Bartonella* species within coinfected hosts and in colonization of susceptible hosts, as has been done previously in rodent-*Bartonella* systems (Sherlock et al., 2013; Telfer et al., 2010). As one of only a few captive bat colonies used for pathogen research, additional experiments on this colony through controlled infections of *Bartonella*-free bats and measurement of immune responses following exposure (via bat flies or inoculation) would elucidate key aspects of bat-*Bartonella* interactions that could be compared with *Bartonella* infections in other systems and possibly to other bat parasites.

While much work lies ahead to understand the ecology and evolution of bat microparasites, and parasitism as a lifestyle generally, new methods available to parasitologists and disease ecologists will hopefully accelerate research efforts and reveal novel insights. In an era of affordable sequencing technologies and computational resources, there is tremendous potential to reveal heretofore poorly understood aspects of host-parasite interactions and macroecology; however the success of these efforts will depend on international, multidisciplinary collaborations.

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Appendix I

I.1 Sequencing of bat-associated *Bartonella* strains and molecular data validation

Genomic DNA was extracted from 129 bat-associated *Bartonella* cultures using a simple heat extraction protocol (incubation at 95°C for 10 min) and diluted 1:10 in extraction buffer (Qiagen, Valencia, CA). Amplification of targeted genetic loci used published primers and protocols (Bai et al., 2013, 2015; Buffet et al., 2013; McKee et al., 2017). Amplification of *groEL* was unsuccessful for many strains with the available primers (Zeaiter et al., 2002), so this locus was not sequenced for any bat-associated strains and was only available from MLSA and genomic data. Positive PCR amplicons were purified using the Qiagen QIAquick PCR Purification Kit and sequenced in both directions with the same primers on an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Reads were then assembled in Lasergene v14 (DNASTAR, Madison, WI). Repeated amplification or sequencing was performed for some missing genes, but for 28 strains there was one or more sequence that could not be obtained: *ftsZ* (2), *nuoG* (2), *ribC* (22), or *rpoB* (3). Preliminary phylogenetic analysis determined that seven strains showed evidence of homologous recombination with another bat-associated strain (even after repeated amplification and sequencing) and one showed highly discordant phylogenetic positions across sequenced loci, so these strains were removed from the database.

Previous analyses have shown that the protein-coding loci (*ftsZ*, *gltA*, *groEL*, *nuoG*, *ribC*, *rpoB*) are under purifying selection with low ratios of non-synonymous to synonymous substitutions (Bai et al., 2015; Buffet et al., 2013). The 16S rRNA locus is known for being highly conservative within a bacterial genus (Kosoy et al., 2018; La Scola et al., 2003). As a spacer sequence, ITS is unlikely to be under selection. I examined GC content across the full alignment for all 332 taxa using DAMBE v7.0.48 (Xia, 2018). The eubartonellae clade and *B. tamiae* exhibited a stationary GC content distribution between 0.38–0.48 while *B. apis*, *Candidatus* Tokpelaia hoelldoblerii, and *Brucella abortus* had progressively higher GC content values (Figure I.1). Previous studies have shown that the similarity in GC content between *B. tamiae* and eubartonellae can affect phylogenetic results. Specifically nucleotide alignments show that *B. tamiae* is a sister taxon to

eubartonellae while protein alignments or nucleotide alignments without the third codon position show that *B. tamiae* is a sister taxon to *B. apis* (Bisch et al., 2018; Segers et al., 2017). Since this inference was focused primarily on the eubartonellae clade and not on its putative sister taxa in arthropods, I determined that the stationary GC content distribution for eubartonellae was acceptable for phylogenetic analysis and required no correction.

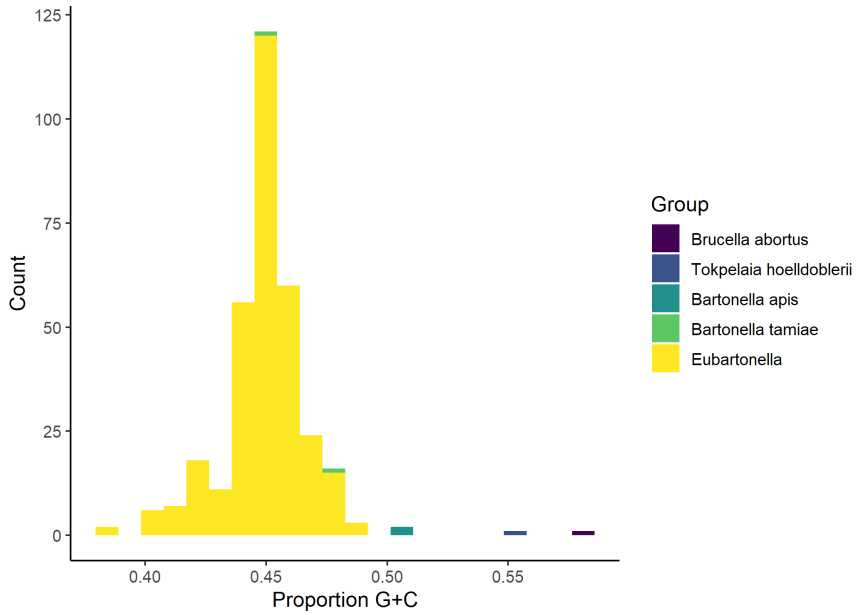

Figure I.1: Histogram of GC content across taxa. Nucleotide content was calculated across the concatenated alignment of nine loci for all 332 taxa.

To confirm the absence of homologous recombination within taxa in the database, I generated a network phylogeny in SplitsTree v4.14.8 (Huson, 2005) using the concatenated alignment and the Neighbor-Net method (Bryant and Moulton, 2003) on uncorrected pairwise distances. The network phylogeny showed a moderately tree-like structure (Figure I.2) with parallelograms connecting closely related taxa and basal splits indicative of shared evolutionary history. A pairwise homoplasy (PHI) test (Bruen et al., 2005) for recombination implemented in SplitsTree found no statistically significant evidence for recombination (P = 1) for the concatenated alignment or each locus separately (Table I.2).

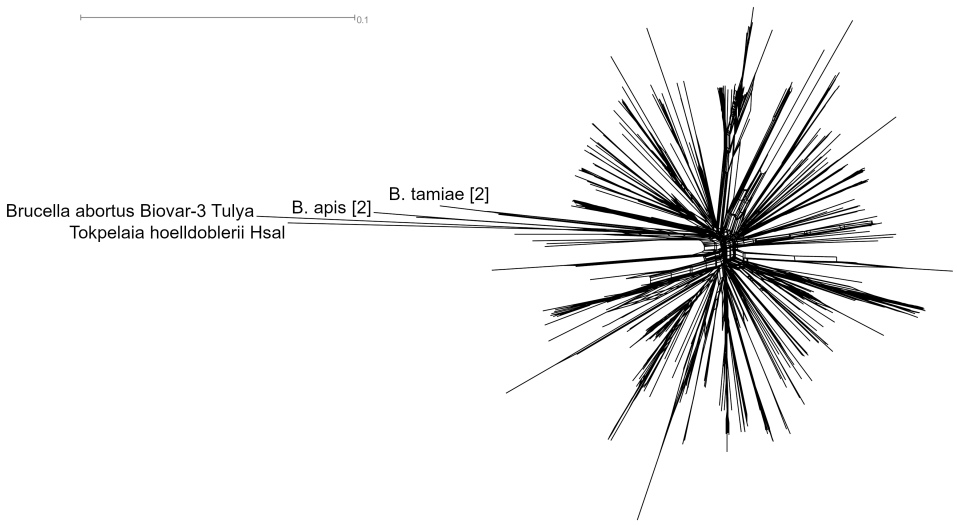

Figure I.2: Network phylogeny of *Bartonella* strains. The network was produced using the Neighbor-Net method on uncorrected pairwise distances calculated from an 8345 base pair alignment of nine genetic loci. Distances are show as the number of nucleotide substitutions per site.

Separate loci were tested for the presence of nucleotide substitution saturation by plotting uncorrected versus adjusted distances (Tamura-Nei model (Tamura and Nei, 1993)) and transitions/transversions versus adjusted distances using DAMBE and the R package `ape` (Paradis et al., 2004, 2016; R Core Team, 2019). Adjusted distances did not show substantial saturation, exhibiting a strongly linear relationship with only slightly asymptotic behavior at the farthest distances (Figure I.3). Transitions and transversions fell along a straight line (Figure I.4) and transitions largely outnumbered transversions for all loci except ITS (Xia, 2018), indicating no substantial evidence of saturation. The absence of significant saturation was confirmed for all loci (Table I.3) in DAMBE using the test developed by Xia (2018). Based on all the tests above, I determined that these molecular loci would be appropriate for phylogenetic analysis and accurate estimation of divergence times.

I.2 Testing alternative models in BEAST

To increase confidence in the robustness of my conclusions with respect to phylogenetic model choice, I performed additional runs in BEAST using alternative models and subsets of sequence data. The amount of data and the complexity of models led to long computational runtimes (up

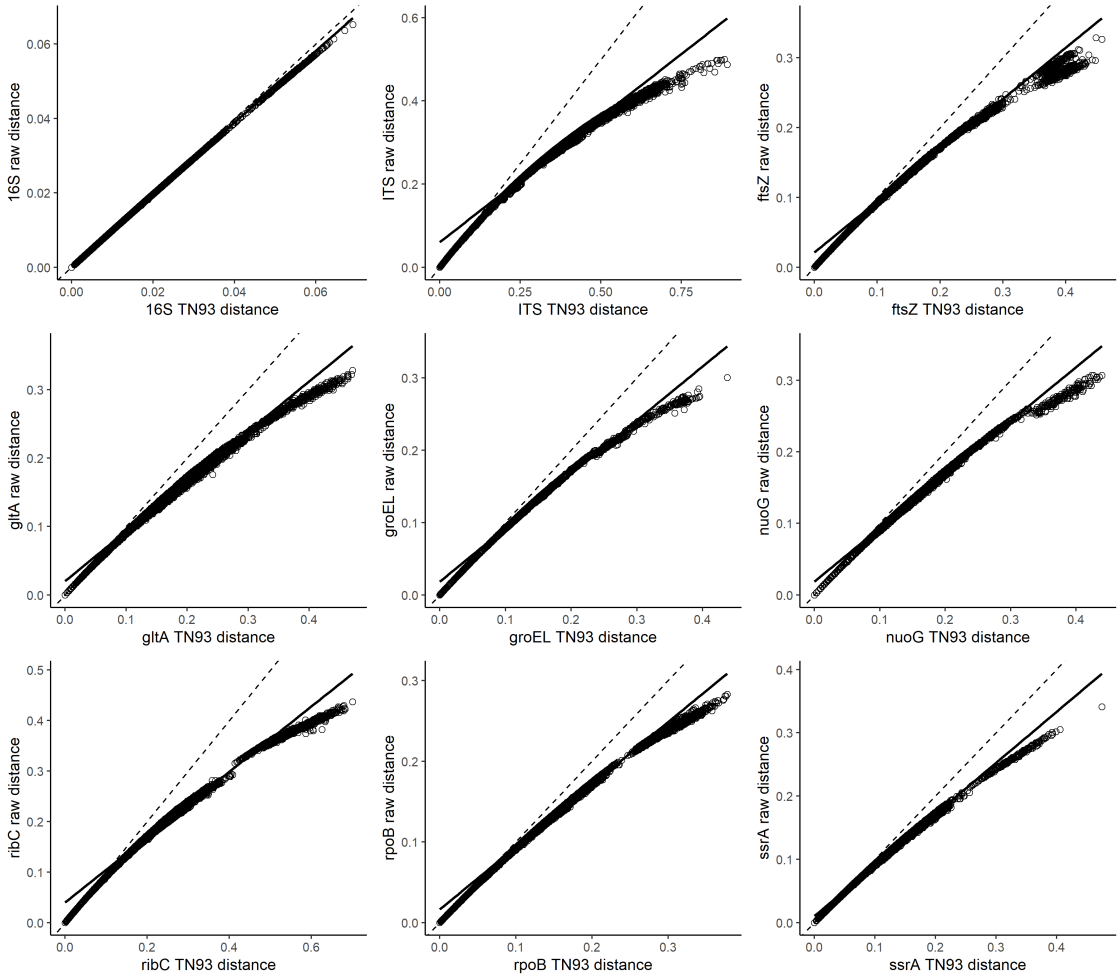

Figure I.3: Nucleotide substitution saturation across nine sequenced loci using uncorrected versus adjusted distances. Points represent pairwise distances for all taxa sequenced at each locus. Raw distances represent the uncorrected pairwise distances and adjusted distances were calculated using the Tamura-Nei model. The dashed line shows the 1:1 line for uncorrected versus adjusted distances and the solid line shows the best-fit line for linear regression.

to 7 days) that reached the limit permitted on CIPRES (Miller et al., 2010). For this reason, I did not pursue a formal model selection approach through estimation of marginal likelihoods (Baele et al., 2012, 2013) and instead chose to run a non-exhaustive series of models using combinations of alternative model settings to assess the combined effects on the topology and divergence times on the resulting tree.

For models that used a TN+I+G model for *ssrA* the two prior distributions for the kappa priors were chosen to be lognormal with log mean of one and a log standard deviation of 1.25 with an initial value of 2. For models that used an uncorrelated relaxed clock model with a lognormal

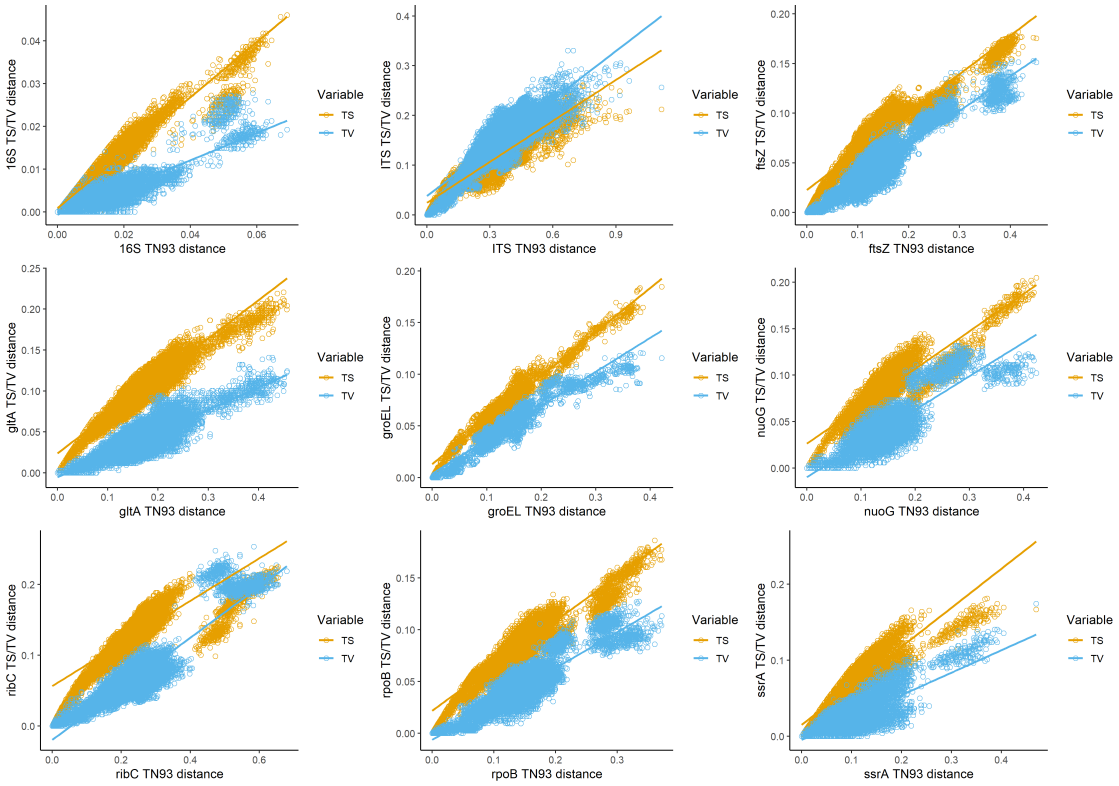

Figure I.4: Nucleotide substitution saturation across nine sequenced loci using adjusted distances versus transitions and transversions. Points represent pairwise distances for all taxa sequenced at each locus. Adjusted distances were calculated using the Tamura-Nei model. Transitions (TS) are colored orange and transversions (TV) are colored blue. The solid lines show the best-fit lines for linear regression for transitions and transversions.

distribution of clock rates along branches, the means for each locus were set as for the exponential distribution detailed in the main text. An additional prior was set for the standard deviation of the lognormally distributed clock rates using an exponential distribution with a mean of 0.33. All model combinations were run until parameters converged to stationary distributions as determined through visual inspection in Tracer v1.7.1 (Drummond and Rambaut, 2007). Burn-in iterations were removed and the maximum clade credibility (MCC) tree was selected using TreeAnnotator (Drummond and Rambaut, 2007; Drummond et al., 2012). I then compared the topology and divergence dates (particularly the estimated divergence date of eubartonellae) of the MCC trees.

Regardless of substitution, codon partitioning, clock, or tree models, I found only limited variation in the topology of the tree across all runs with no major changes in the position of large clades that would influence the results or conclusions in the main text. The divergence dates of eubar-

tonellae (Table I.4) and a posteriori defined clades (Table I.7) varied little across runs, indicating that the molecular data, taxon sampling, and choice of prior on the 16S rRNA clock were more important to phylogenetic inference than any other model settings. The only major differences observed in the topology and divergence dates of the tree were observed when a strict clock was used. These runs predicted a younger divergence date for eubartonellae (~57 mya) and showed a different arrangement of clades A–C and the clades that contain *B. bacilliformis*, *B. rochalimae*, and *B. clarridgeiae*. All runs using strict molecular clocks had lower likelihoods than runs using relaxed clocks, so the use of a strict clock was rejected. As long as variation in clock rates were allowed to be uncorrelated across the branches of the tree, the topology and divergence dates on the tree were stable.

I note that the exclusion of ITS sequences had little effect on tree topology and divergence dates, so this locus may have had limited phylogenetic signal. Nevertheless, I retained this locus for the final run used in the main text. Since substitution models had little effect on the trees, I chose to use the GTR+I+G substitution model for all loci for consistency with the maximum likelihood tree as written in the main text. Likewise since codon partitioning and relaxed clock models had little influence on results, I chose not to use codon partitioning and to use exponential distributions for the uncorrelated relaxed clocks rather than lognormal distributions for the final runs in the main text to reduce the number of independent parameters that needed to be estimated.

I.3 *Bartonella* lineages associated with arthropods

Several *Bartonella* lineages in the database were labeled as being associated primarily with arthropods for the ancestral state reconstruction analysis. *B. apis* was originally isolated from western honey bees (*Apis mellifera*) and has not been associated with any mammalian hosts (Kešnerová et al., 2016). While several strains of *B. apis* have been characterized from honey bees in North America and Europe (Kešnerová et al., 2016), I chose to associate this species with the Palearctic ecozone to reflect the hypothesized historical distribution of domesticated *Apis mellifera* in northern Africa or the Middle East (Cridland et al., 2017). *B. tamiae* was originally isolated from humans in Thailand (Kosoy et al., 2008), this likely represents an accidental association. Genetic

sequences identified as *B. tamiae* or closely related to this species have been obtained from several arthropod species including bat flies and bat ticks (Bai et al., 2018; Leulmi et al., 2016) and chigger mites collected from rodents (Kabeya et al., 2010). Given its basal position relative to the mammal-associated eubartonellae clade and closer affinities with *B. apis* (Kešnerová et al., 2016), I chose to associate *B. tamiae* primarily with arthropods for this analysis.

I labeled *B. bacilliformis* and its relatives *B. ancashensis* and *Candidatus* B. rondoniensis as being associated with arthropods instead of a particular mammalian order because a reservoir host has not been conclusively determined for these species. *Bartonella bacilliformis* causes severe morbidity and mortality in humans, and prevalence is generally low in human populations, so humans are unlikely to be the reservoir host (Sanchez Clemente et al., 2012). Furthermore, repeated attempts to isolate *B. bacilliformis* from alternative plant or animal reservoirs have been unsuccessful (Sanchez Clemente et al., 2012). Despite the uncertainty about the reservoir host, *B. bacilliformis* is known to be vectored by *Lutzomyia* spp. sandflies (Battisti et al., 2015; Billeter et al., 2008; Breitschwerdt and Kordick, 2000). A recent study also reported the presence of *B. bacilliformis* in ticks collected from tapirs and peccaries in Peru (del Valle-Mendoza et al., 2018). The phylogenetically related *Candidatus* B. rondoniensis was also described from the assassin bug *Eratyrus mucronatus* in French Guiana (Laroche et al., 2017). While the host or vector of *B. ancashensis* is unknown (Mullins et al., 2015) it is part of a clade that includes *B. bacilliformis* and *Candidatus* B. rondoniensis. Given the uncertainty of the mammalian hosts for this *Bartonella* clade, I chose to associate this group primarily with arthropods since it appears to be the ancestral trait (Neuvonen et al., 2016). Future work that conclusively determines the mammalian hosts of *B. bacilliformis* and its allies is clearly necessary and could improve the inference of ancestral hosts for *Bartonella* lineages.

Finally, the host origin of *B. senegalensis* is unclear since it was isolated from the soft tick *Ornithodoros sonrai* in Senegal (Mediannikov et al., 2013). Although the ticks were found in rodent burrows, the presence of the bacteria was not confirmed in any mammals, so I chose to associate this bacteria with arthropods. Similar to the clade that includes *B. bacilliformis*, future

studies involving this bacteria and its host associations will improve our knowledge of evolution within the *Bartonella* genus.

I confirmed that none of these choices had an effect on the results by repeating stochastic character mapping using alternative assignments of traits to these tips. Bats were always inferred to be the ancestral hosts of eubartonellae. Thus, I chose to retain these trait assignments for the analysis in the main text.

I.4 Additional figures and tables

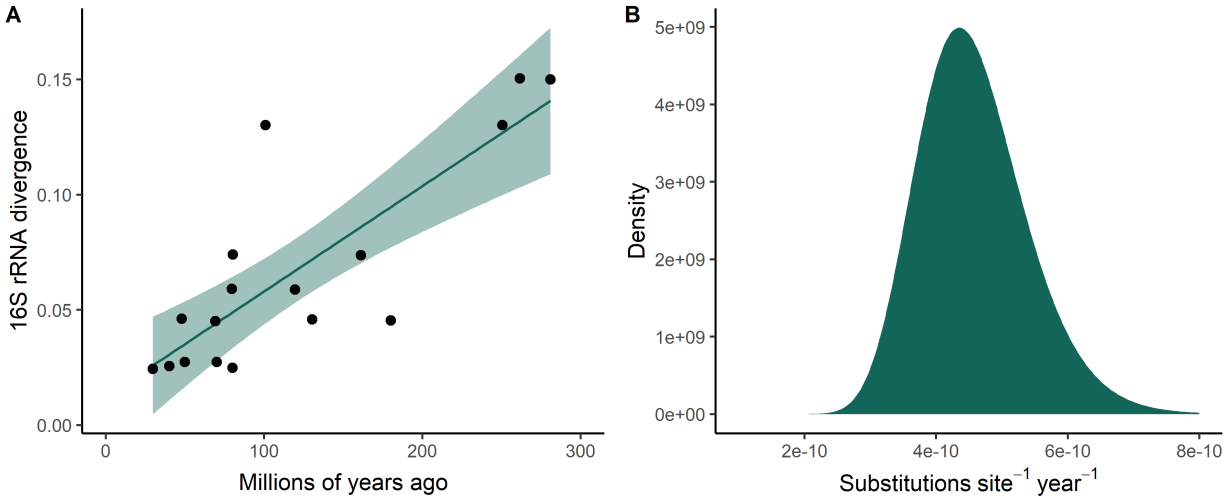

Figure I.5: Estimated molecular clock for 16S ribosomal RNA (rRNA). (A) Linear regression of 16S rRNA divergence and host divergence times for bacterial symbionts of arthropods from Kuo and Ochman (2009). (B) A lognormal distribution for the 16S rRNA molecular clock estimated by moment matching to the normal distribution of the fitted mean and standard error of the regression.

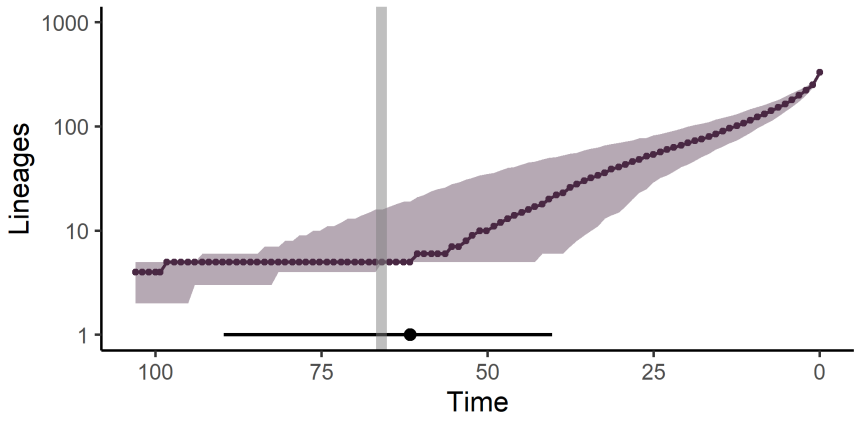

Figure I.6: Number of *Bartonella* lineages through time. Circles show the median number of lineages and shading representing the 95% HPD interval. The diversification date of eubartonellae is shown as a circle at the bottom of the figure with a line for the 95% HPD interval. Time is shown in millions of years. The Cretaceous-Paleogene extinction event is drawn as a gray line at 66 million years ago.

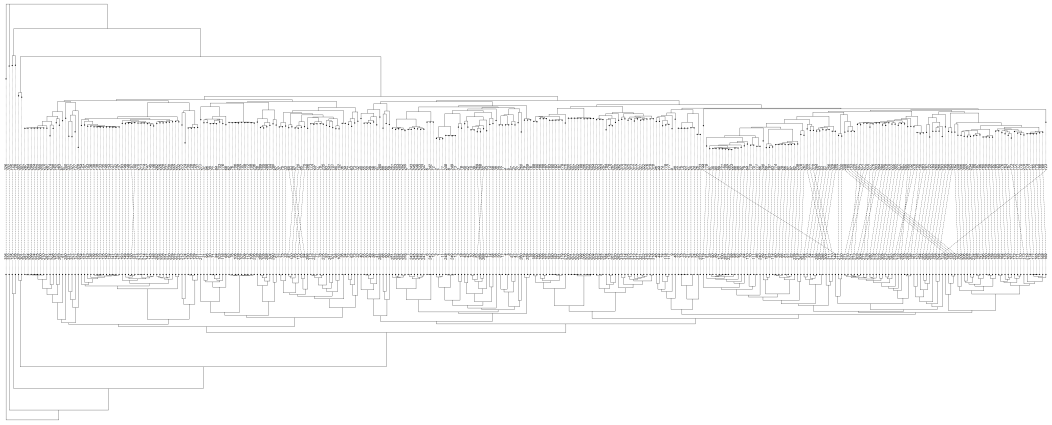

Figure I.7: Comparison of Bayesian and maximum likelihood trees. The Bayesian tree (top) used separate sequence evolution models for each of the nine partitioned loci. The maximum likelihood tree (bottom) used concatenated sequences of all nine loci.

Figure I.8: Timed maximum clade credibility tree of *Bartonella* lineages including ancestral reconstruction of (A) host orders and (B) ecozones. Posterior probabilities (PP) for nodes are indicated by the size of circles. Branch lengths are in millions of years. Branches are colored according to their most probable (PP > 0.5) host order or ecozone, with host or ecozone probability shown by the color of circles at each node.

Table I.1: The number of taxa with sequences for each locus and the coverage of taxa out of 332 are listed, as well as the number of sites (base pairs) included in the final database. The best DNA substitution model was chosen based on the Akaike information criterion (AIC) in jModelTest. The proportion of invariant sites and the substitution rate gamma shape parameter were estimated from the best model. GTR, generalized time-reversible; TN, Tamura-Nei; G, gamma distributed rate variation; I, proportion of invariant sites.

Locus	Name	Taxa	Coverage	Sites	AIC best model	Invariant sites	Gamma
16S	16S ribosomal RNA	289	0.87	1511	GTR+I+G	0.77	0.33
ITS	16S–23S internal transcribed spacer	251	0.76	1833	GTR+I+G	0.11	1.1
ftsZ	cell division protein	327	0.98	885	GTR+I+G	0.46	0.64
gltA	citrate synthase	332	1	348	GTR+I+G	0.32	0.71
groEL	heat-shock chaperonin protein	116	0.35	1632	GTR+I+G	0.47	0.81
nuoG	NADH dehydrogenase gamma subunit	227	0.68	342	GTR+I+G	0.48	0.74
ribC	riboflavin synthase	256	0.77	561	GTR+I+G	0.2	0.86
rpoB	DNA-directed RNA polymerase beta subunit	322	0.97	849	GTR+I+G	0.48	0.68
ssrA	transfer-messenger RNA	220	0.66	384	TN+I+G	0.31	0.6

Table I.2: Results of pairwise homoplasy index (PHI) tests for homologous recombination. The parameter k represents the number of informative sites within a window of 100 base pairs. P-values greater than 0.05 indicate that the observed PHI was outside of the expected distribution of PHI for the tree, thereby failing to reject the null hypothesis of no recombination.

Locus	k	Expected mean PHI	Expected variance PHI	Observed PHI	P-value
16S	8	0.42	$2.6\text{x}10^{-4}$	0.423	0.57
ITS	50	0.61	$3.6\text{x}10^{-5}$	0.78	1
ftsZ	43	1.81	$2.5\text{x}10^{-4}$	1.9	1
gltA	57	1.24	$4.9\text{x}10^{-4}$	1.21	0.067
groEL	39	1.1	$3.9\text{x}10^{-5}$	1.13	1
nuoG	45	1.62	$9.5\text{x}10^{-4}$	1.64	0.71
ribC	69	1.64	$2.5\text{x}10^{-4}$	1.7	1
rpoB	43	1.93	$2.9\text{x}10^{-4}$	1.97	0.98
ssrA	45	0.63	$2.2\text{x}10^{-4}$	0.7	1
Concatenated	40	0.95	$6.5\text{x}10^{-6}$	1.2	1

Table I.3: Results of tests for substitution saturation. The index of substitution saturation (Iss) was calculated for each locus across a series of subtrees randomly pruned to a number of taxa based on 100 iterations. The critical index (Iss.c) is the value at which the sequences will begin to fail to recover the true tree and was calculated for each locus across the series of sampled taxa. If Iss is smaller than Iss.c and the P-value is less than 0.05, then I can conclude that the sequences have not experienced severe substitution saturation and can be used for phylogenetic reconstruction. The Iss and Iss.c values shown here assume a symmetrical tree topology and use the proportion of invariant sites from Table I.1.

Locus	Taxa	Iss	Iss.c	T	DF	P-value
16S	4	0.15	0.8	8.1	24	0
	8	0.15	0.78	7.1		0
	16	0.16	0.59	4.7		0.0001
	32	0.16	0.78	6.5		0
ITS	4	0.014	1.3	76.8	21	0
	8	0.014	1.6	82.8		0
	16	0.017	0.61	26.9		0
	32	0.019	2.1	85.3		0
ftsZ	4	0.33	0.82	6.1	47	0
	8	0.33	0.82	5.6		0
	16	0.33	0.58	2.7		0.0086
	32	0.34	0.85	5.6		0
gltA	4	0.27	0.78	12.2	121	0
	8	0.25	0.74	11.4		0
	16	0.26	0.63	8.6		0
	32	0.27	0.7	10.2		0
groEL	4	0.27	0.8	17.4	265	0
	8	0.28	0.75	15.8		0
	16	0.28	0.72	14.5		0
	32	0.28	0.7	14		0
nuoG	4	0.3	0.78	10.7	117	0
	8	0.29	0.73	9.6		0
	16	0.29	0.65	7.9		0
	32	0.3	0.69	8.3		0
ribC	4	0.27	0.79	11.3	106	0
	8	0.26	0.76	10.3		0
	16	0.26	0.61	7.2		0
	32	0.27	0.74	9.6		0
rpoB	4	0.31	0.78	12.1	163	0
	8	0.3	0.74	10.8		0
	16	0.31	0.68	9.2		0
	32	0.32	0.68	9.2		0
ssrA	4	0.11	1.4	15.3	13	0
	8	0.11	1.9	18.5		0
	16	0.11	0.66	5.9		0.0001
	32	0.11	2.6	26.2		0

Table I.4: Robustness of mammal-infecting eubartonellae divergence date to model choice. RelTime divergence dates were estimated in MEGA using uniform prior distributions based on the confidence intervals of 15 host divergence dates listed in I.7 and a maximum likelihood tree based on a concatenated alignment of all nine loci. BEAST divergence dates were estimated using separate prior distributions for all nine genetic loci separately with a strong prior distribution on the 16S rRNA locus and diffuse continuous-time Markov chain priors on the remaining loci. Separate BEAST runs using alternative sequence evolution, tree, and clock models were run until parameters converged. Intervals in parentheses show either the 95% highest posterior density interval for BEAST analyses or the 95% maximum likelihood confidence interval for RelTime. The primary model used in the main text is in bold. All runs were performed with all nine loci except those marked with a dagger, which were run with ITS. Codon partitioning was added to the last two runs in the table (marked with a double dagger). GTR, generalized time-reversible; TN, Tamura-Nei; I, proportion of invariant sites; G, gamma distributed rate variation; BD, birth-death; BDI, birth-death with incomplete sampling.

Method	Sequence evolution model	Tree model	Clock model	Divergence date
RelTime	GTR+I+G	Concatenated maximum likelihood	Relative rates	66.3 (63.5–69.1)
BEAST	All loci GTR+I+G	Coalescent, constant size	Strict lognormal	57.6 (38.1–82.5)
BEAST	All loci GTR+I+G	BD	Strict lognormal	57.2 (37.2–81.7)
BEAST	All loci GTR+I+G	BDI	Strict lognormal	56.9 (35.6–80)
BEAST	All loci GTR+I+G	Coalescent, constant size	Relaxed lognormal	69.6 (45.1–103.1)
BEAST	All loci GTR+I+G	BD	Relaxed lognormal	65.4 (42–96.7)
BEAST	All loci GTR+I+G	BDI	Relaxed lognormal	63.5 (42.3–94.2)
BEAST	*ssrA* TN+I+G, other loci GTR+I+G	BDI	Relaxed lognormal	**63.4 (40.9–97.1)**
BEAST	All loci GTR+I+G	BDI	Relaxed exponential	61.6 (40.3–89.7)
BEAST	*ssrA* TN+I+G, other loci GTR+I+G	BDI	Relaxed exponential	64.5 (40.8–101.5)
BEAST	All loci GTR+I+G	BDI	Relaxed lognormal	63.4 (41.8–95.8)†
BEAST	*ssrA* TN+I+G, other loci GTR+I+G	BDI	Relaxed lognormal	64 (40.6–101.3)†
BEAST	All loci GTR+I+G	BDI	Relaxed exponential	59.9 (39.5–90.3)†
BEAST	*ssrA* TN+I+G, other loci GTR+I+G	BDI	Relaxed exponential	59.5 (37.2–84.1)†
BEAST	All loci GTR+I+G	BDI	Relaxed lognormal	67.2 (41.3–97.4)‡
BEAST	All loci GTR+I+G	BDI	Relaxed lognormal	65.3 (41.5–97.1)‡

Table I.5: Summary of *Bartonella* clades and host associations. Host clades above or below the order level associated with each *Bartonella* clade and any named *Bartonella* species or *Candidatus*-level species are listed. Clades A, D, G, L, and N are novel bat-associated clades described in this study. Clade O contains the predominantly rodent-associated clades H–N. Host clades are detailed in Table I.6.

Bartonella clade	Tips in clade	Host order(s)	Host clade	*Bartonella* species in clade
A	51	Chiroptera	Noctilionoidea	*Candidatus* B. rolaini
B	4	Dasyuromorphia, Diprotodontia, Peramelemorphia	Marsupialia	*B. australis*
				Candidatus B. antechini
				Candidatus B. bandicootii
				Candidatus B. woyliei
C	32	Artiodactyla	Pecora	*B. bovis*
				B. capreoli
				B. chomelii
				B. dromedarii
				B. melophagi
				B. schoenbuchensis
				Candidatus B. davousti
D	55	Chiroptera	Yinpterochiroptera	*B. naantaliensis*
E	19	Rodentia	Sciuridae	*B. jaculi*
				B. heixiaziensis
				B. washoensis
F	10	Carnivora	Felidae	*B. henselae*
				B. koehlerae
G	15	Chiroptera	Vespertilionoidea	
H	29	Rodentia	Murinae	*B. elizabethae*
				B. fuyuanensis
				B. grahamii
				B. queenslandensis
				B. rattimassiliensis
				B. mastomydis
				B. tribocorum
I	4	Rodentia	Gerbillinae	*B. pachyuromydis*
J	22	Rodentia	Arvicolinae	*B. birtlesii*
				B. doshiae
				B. taylorii
K	3	Rodentia	Neotominae	*B. vinsonii*
L	7	Chiroptera	Myotis	*Candidatus* B. mayotimonensis
M	2	Rodentia	Sigmodontinae	
N	30	Chiroptera	Phyllostomidae	
O	88	Rodentia	Muroidea	

Table I.6: Description of *Bartonella* clades and host associations. MRCA, most recent common ancestor.

Bartonella clade	Host clade	Description
A	Noctilionoidea	MRCA for families Noctilionidae, Mormoopidae, and Phyllostomidae in order Chiroptera
B	Marsupialia	MRCA for orders Dasyuromorphia, Diprotodontia, and Peramelemorphia in infraclass Marsupialia
C	Pecora	MRCA for families Bovidae and Cervidae in order Artiodactyla
D	Yinpterochiroptera	MRCA for families Rhinolophidae, Hipposideridae, and Pteropodidae in order Chiroptera
E	Sciuridae	MRCA for genera *Sciurus*, *Tamiasciurus*, *Glaucomys*, *Eutamias*, *Urocitellus*, *Spermophilus*, *Otospermophilus*, and *Cynomys* in family Sciuridae
F	Felidae	MRCA for genera *Panthera*, *Lynx*, *Felis*, *Puma*, and *Acinonyx* in family Felidae
G	Vespertilionoidea	MRCA for families Vespertilionidae and Molossidae in order Chiroptera
H	Murinae	MRCA for genera *Bandicota*, *Rattus*, *Niviventer*, *Melomys*, *Uromys*, and *Mus* in subfamily Murinae
I	Gerbillinae	MRCA for genera *Pachyuromys*, *Meriones*, and *Sekeetamys* in subfamily Gerbillinae
J	Arvicolinae	MRCA for subfamily Arvicolinae
K	Neotominae	MRCA for subfamily Neotominae
L	Myotis	MRCA for species *Myotis blythii* and *Myotis lucifugus* in family Vespertilionidae
M	Sigmodontinae	MRCA for genera *Hylaeamys*, *Akodon*, and *Sigmodon* in subfamily Sigmodontinae
N	Phyllostomidae	MRCA for family Phyllostomidae
O	Muroidea	MRCA for superfamily Muroidea (excluding genus *Typhlomys*)

Table I.7: Comparison of divergence date estimates for 15 *Bartonella* clades with divergence dates of the associated hosts within each clade collated from TimeTree. The number of published studies used to estimate host divergence dates is listed. Both host and *Bartonella* clade divergence dates are in units of millions of years. Intervals in parentheses show either the 95% highest posterior density interval for *Bartonella* clade dates or the 95% confidence interval for host clade dates. Intervals in brackets show the ranges. Details regarding *Bartonella* clades are found in Tables I.5–I.6.

Bartonella clade	TimeTree studies	Host clade date	*Bartonella* clade date
A	19	43 (41–46) [36.7–60.4]	45.9 (29.6–68.4) [24.1–110.1]
B	17	62 (58–67) [49.8–82]	35.1 (21.9–53.8) [16–84.7]
C	10	27.3 (23.1–31.5) [20.8–38.7]	15.4 (9.4–23.1) [6.9–36.2]
D	21	58 (56–61) [46–71.2]	49.6 (32.3–72.5) [25.2–110.1]
E	11	35 (29–40) [17.8–47.6]	18.8 (10.7–28.9) [8.7–52.9]
F	12	15.2 (12.3–18.1) [9.6–26.3]	8.4 (4.8–13) [3.3–27.8]
G	15	49 (45–52) [36–60.4]	36.9 (22.9–55.1) [18.7–84.3]
H	84	20.9 (18.3–23.4) [8.8–53.6]	20.8 (13.1–30.6) [9.7–47.5]
I	6	18.4 (10.3–26.4) [11–28.4]	16.4 (9.1–25.1) [6.9–38.5]
J	3	18.6 [15.2–20.9]	25.2 (15.5–37.1) [11.7–47.2]
K	8	19.3 (12.1–26.4) [8.6–32]	11.3 (6.1–18.6) [4.2–35.4]
L	6	18.1 (9.3–27) [10.8–32.8]	10.5 (5.7–17.2) [4.7–31.9]
M	5	19.8 (10–29.5) [11.6–29.7]	7.4 (2.7–15.2) [1.6–30.7]
N	16	31 (29–33) [25–35.3]	23.9 (15.5–35.2) [11.9–54.4]
O	16	45 (42–49) [35.9–60.1]	40.4 (26.4–59) [21.1–95]

Table I.8: Prior distributions for phylogenetic analysis in BEAST.

Parameter	Distribution	Initial value
A-C substitutions	gamma(0.05, 10)	1
A-G substitutions	gamma(0.05, 20)	1
A-T substitutions	gamma(0.05, 10)	1
C-G substitutions	gamma(0.05, 10)	1
G-T substitutions	gamma(0.05, 10)	1
Base frequencies	uniform(0, 1)	0.25
Gamma shape parameter	exponential(0.5)	0.5
Proportion of invariant sites	uniform(0, 1)	0.5
Birth-death birth rate	uniform(0, 1E5)	0.01
Birth-death relative death rate	uniform(0, 1)	0.5
Proportion of taxa sampled	beta(1, 1)	0.01
16S rRNA UCED clock rate	lognormal(-21.5, 0.18)	4.6×10^{-10}
Host state transition rates	gamma(1, 1)	1
Ecozone state transition rates	gamma(1, 1)	1

Table I.9: Results of stochastic character mapping of host orders and ecozones on 1000 posterior sampled trees. The posterior distribution of the number of transitions is given as the median and the 95% HPD interval (in parentheses).

Network	Transition	Count
Order	Arthropoda → Chiroptera	1 (0–1)
	Arthropoda → Outgroup	1 (0–1)
	Carnivora → Arthropoda	1 (0–1)
	Chiroptera → Arthropoda	1 (0–2)
	Chiroptera → Artiodactyla	1 (0–1)
	Chiroptera → Carnivora	1 (0–2)
	Chiroptera → Diprotodontia	1 (0–2)
	Chiroptera → Rodentia	2 (1–4)
	Diprotodontia → Dasyuromorphia	1 (0–1)
	Diprotodontia → Peramelemorphia	1 (0–1)
	Rodentia → Carnivora	3 (2–5)
	Rodentia → Chiroptera	2 (1–3)
	Rodentia → Eulipotyphla	4 (3–4)
	Rodentia → Lagomorpha	1 (0–1)
	All order transitions	26 (24–30)
Ecozone	Afrotropic → Indo-Malayan	2 (0–5)
	Afrotropic → Nearctic	3 (1–5)
	Afrotropic → Palearctic	3 (0–7)
	Australasia → Indo-Malayan	1 (0–2)
	Australasia → Palearctic	1 (0–3)
	Indo-Malayan → Afrotropic	2 (0–4)
	Indo-Malayan → Australasia	1 (0–2)
	Indo-Malayan → Nearctic	1 (0–3)
	Indo-Malayan → Neotropic	2 (0–3)
	Indo-Malayan → Palearctic	3 (1–5)
	Nearctic → Afrotropic	1 (0–4)
	Nearctic → Indo-Malayan	1 (0–3)
	Nearctic → Neotropic	1 (0–3)
	Nearctic → Palearctic	4 (2–7)
	Neotropic → Afrotropic	1 (0–3)
	Neotropic → Indo-Malayan	1 (0–3)
	Neotropic → Palearctic	2 (0–5)
	Outgroup → Indo-Malayan	1 (0–2)
	Palearctic → Afrotropic	8 (4–12)
	Palearctic → Australasia	3 (1–6)
	Palearctic → Indo-Malayan	12 (9–16)
	Palearctic → Nearctic	11 (8–14)
	Palearctic → Neotropic	6 (3–9)
	Palearctic → Outgroup	1 (0–3)
	All ecozone transitions	82 (71–92)

Appendix II

II.1 Additional figures and tables

Table II.1: Oligonucleotide primers used for genotyping of bat flies and bacterial detection with conventional PCR amplification. Sequences designated [F] are forward primers and those designated [R] are reverse primers.

Target	Locus	PCR round	Primer sequence	Primer name	Product size (bp)	Reference
Bartonella	*ftsZ*	1	ATTAATCTGCAYCGGCCAGA [F]	Bfp1	885	Zeaiter et al. (2002)
			ACVGADACACGAATAACACC [R]	Bfp2		
		2	ATATCGCGGAATTGAAGCC [F]	ftsZ R83	670	Colborn et al. (2010)
			CGCATAGAAGTATCATCCA [R]	ftsZ L83		
	gltA	1	GCTATGTCTGCATTCTATCA [F]	CS443f	767	Birtles and Raoult (1996); Gundi et al. (2012)
			GATCYTCAATCATTTCTTTCCA [R]	CS1210r		
		2	GGGGACCAGCTCATGGTGG [F]	BhCS781.p	356	Birtles and Raoult (1996); Norman et al. (1995)
			AATGCAAAAAGAACAGTAAACA [R]	BhCS1137.n		
	ITS	1	CTTCAGATGATGATCCCAAGCCTTCTGGCG [F]	325s	364-398	Diniz et al. (2007)
			GAACCGACGACCCCCTGCTTGCAAAGA [R]	1100as		
Arthropod mitochondrial DNA	16S rRNA	1	TACGCTGTTATCCCTAA [F]	LR-J-13007	411	Kambhampati and Smith (1995); Simon et al. (1994); Szalanski et al. (2004)
			CGCCTGTTTATCAAAAACAT [R]	LR-N-13398		
	cytb	1	AGGRCAAATATCATTTTGAG [F]	A5	387	Dittmar de la Cruz and Whiting (2003)
			AAATATCATTCTGGTTGAATATG [R]	B1.1		
Enterobacteriales	16S rRNA	1	GGGTTGTAAAGTACTTTCAGTCGT [F]	ArsF	575	Duron et al. (2008)
			CCTYTATCTCTAAAGGMTTCGCTGGATG [R]	ArsR3		

Table II.2: Thermocycler protocols used for conventional PCR amplification.

Target	Locus	PCR round	Thermal program
Bartonella	*ftsZ*	1	95°C 4:00, (95°C 0:30, 55°C 0:30, 72°C 1:00)x40, 72°C 10:00, 4°C ∞
		2	95°C 4:00, (95°C 0:30, 55°C 0:30, 72°C 1:00)x40, 72°C 10:00, 4°C ∞
	gltA	1	95°C 2:00, (95°C 0:30, 48°C 0:30, 72°C 2:00)x40, 72°C 7:00, 4°C ∞
		2	95°C 3:00, (95°C 0:30, 55°C 0:30, 72°C 0:30)x40, 72°C 7:00, 4°C ∞
	ITS	1	95°C 3:00, (95°C 0:30, 66°C 0:30, 72°C 0:30)x55, 72°C 5:00, 4°C ∞
Arthropod mitochondrial DNA	16S rRNA	1	95°C 3:00, (95°C 0:45, 46°C 0:45, 72°C 0:45)x55, 72°C 7:00, 4°C ∞
	cytb	1	95°C 12:00, (95°C 0:30, 40°C 0:30, 72°C 2:00)x55, 72°C 7:00, 4°C ∞
Enterobacteriales	16S rRNA	1	95°C 2:00, (95°C 0:30, 52°C 0:30, 72°C 1:30)x55, 72°C 5:00, 4°C ∞

Figure II.1: Neighbor-joining phylogenetic tree of concatenated *Bartonella ftsZ* and *gltA* sequences produced from a 1248 bp alignment (888 bp *ftsZ*, 360 bp *gltA*) of 175 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Bartonella* species/strains previously obtained from bats are colored blue, strains from *E. helvum* and *C. greefi* are colored brown, and strains from *R. aegyptiacus* and *E. africana* are colored green.

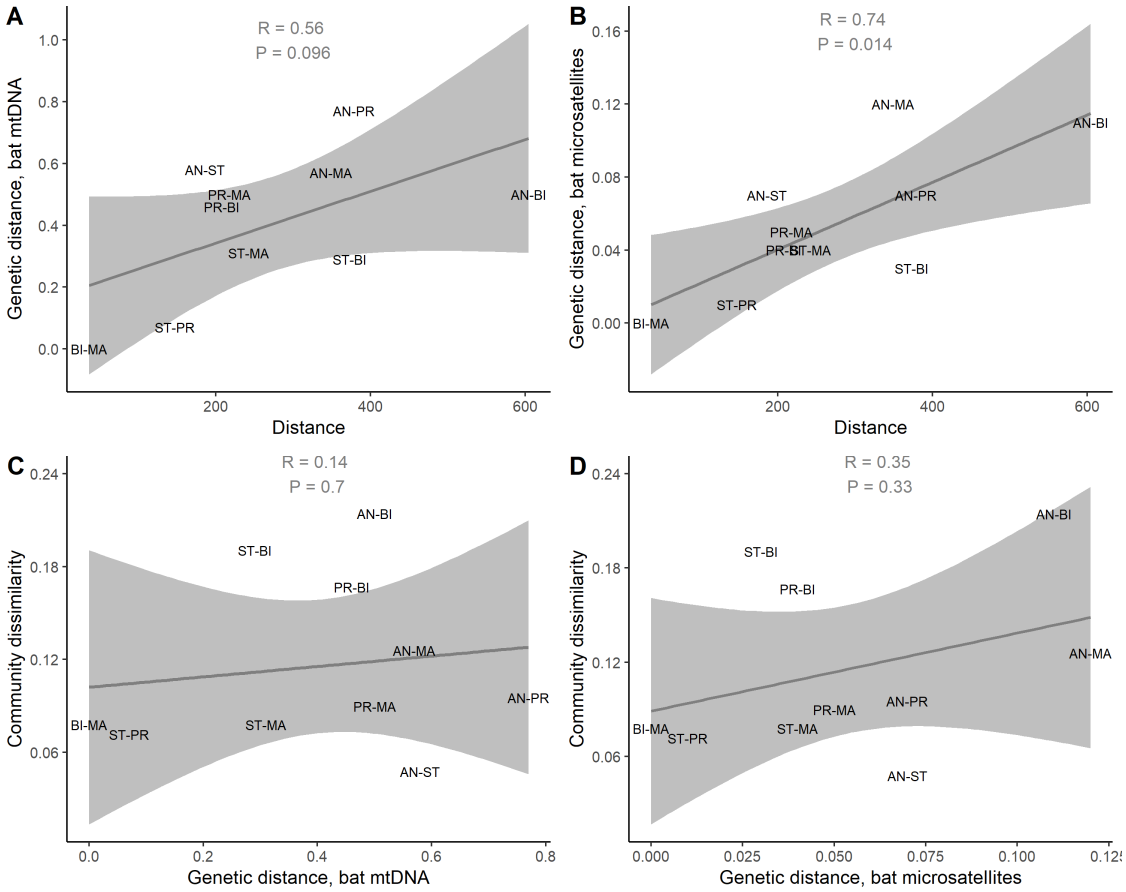

Figure II.2: Correlations between genetic data from *E. helvum* populations, physical distance between sampling locations, and *Bartonella* community dissimilarity. R is Pearson's correlation coefficient and P is the p-value for the linear regression. (A) Relationship between physical distance and mtDNA genetic distances, Slatkin's linearized $\phi_{ST} = \phi_{ST}/(1 - \phi_{ST})$ for *cytb* sequences. (B) Relationship between physical distance and genetic distances for microsatellites, Slatkin's linearized $F_{ST} = F_{ST}/(1 - F_{ST})$. (C) Relationship between genetic distances from mtDNA and *Bartonella* community dissimilarity. (D) Relationship between genetic distances from microsatellites and *Bartonella* community dissimilarity. All values are recorded in Table II.6. Locations are abbreviated AN – Annobón, BI – Bioko, MA – mainland (Ghana), PR – Príncipe, and ST – São Tomé.

Table II.3: Counts of bat fly and bat fly bacterial symbionts detected at each sampling location. Counts were used for calculation of relative abundance in Figure 4.3B,D,F.

Locus	Species	Genotype	Location	Count
Mitochondrial 16S rRNA	*C. greefi*	1	Annobón	81
			Bioko	38
			Ghana	41
			Príncipe	55
			São Tomé	93
	E. africana	1	Ghana	25
			Nigeria	3
		2	Príncipe	10
			São Tomé	1
	D. biannulata	1	Ghana	1
Mitochondrial *cytb*	*C. greefi*	1	Annobón	3
			Bioko	10
			Ghana	25
			Príncipe	9
			São Tomé	32
		2	Annobón	8
	E. africana	1	Ghana	15
		2	Ghana	4
		3	Ghana	3
		4	Ghana	18
		5	Príncipe	9
			São Tomé	1
Bacterial 16S rRNA	*C. greefi*	1	Annobón	1
			Ghana	12
			Príncipe	3
			São Tomé	5
	E. africana	1	Ghana	11
		2	Príncipe	3
			São Tomé	1

Table II.4: Counts of *Bartonella* species detected in bat flies across locations. Counts are based on the total number of confirmed sequences from any of three genetic loci used for detection (ITS, *ftsZ*, *gltA*). Counts were used for calculation of relative abundance in Figure 4.4A and community dissimilarity in Figure 4.5.

Bat host species	Bat fly species	Location	E1	E2	E3	E4	E5	Ew	Eh6	Eh7	*B. rousetti*
E. helvum	*C. greefi*	Ghana	3	21	11	49	61	126	0	0	0
		Bioko	7	3	10	45	63	110	1	2	0
		Príncipe	20	27	1	31	35	48	0	3	0
		São Tomé	25	68	7	44	96	85	0	4	0
		Annobón	19	33	8	11	106	151	0	1	0
R. aegyptiacus	*E. africana*	Ghana	0	0	0	0	0	0	0	0	37
		Príncipe	0	0	0	0	0	0	0	0	10
		São Tomé	0	0	0	0	0	0	0	0	4

Table II.5: Age distribution of *E. helvum* populations sampled for bat flies. Ages are abbreviated N – neonate, J – juvenile, SI – sexually immature, and A – adult. Counts were used for calculation of relative abundance in Figure 4.4C.

Location	Years sampled	N	J	SI	A	Total
Annobón	2010	1	0	69	132	202
Bioko	2010	84	0	4	17	105
Príncipe	2010	0	10	11	40	61
São Tomé	2010	26	0	15	61	102
Ghana	2009, 2012, 2016	20	63	406	1249	1705

Table II.6: Distance measures for sampled populations. Physical distance is measured in kilometers between islands and the mainland, considering Ghana as a representative population for the mainland as in Figure 4.2B. *Bartonella* community dissimilarity is calculated as one minus the Spearman rank correlation between counts of *Bartonella* species across locations. Genetic distances for *E. helvum* across locations are recorded as Slatkin's linearized $\phi_{ST} = \phi_{ST}/(1 - \phi_{ST})$ for mitochondrial cytb sequences and $F_{ST} = F_{ST}/(1 - F_{ST})$ for microsatellites taken from Peel et al. (2013).

Comparison	Physical distance	*Bartonella* dissimilarity	*E. helvum* genetic distance, mtDNA	*E. helvum* genetic distance, microsatellites
Bioko-Mainland	35.86	0.07	0.00	0.00
Príncipe-Mainland	217.23	0.08	0.50	0.05
São Tomé-Mainland	242.36	0.06	0.31	0.04
Annobón-Mainland	349.32	0.10	0.57	0.12
Príncipe-Bioko	207.61	0.12	0.46	0.04
São Tomé-Bioko	372.72	0.14	0.29	0.03
Annobón-Bioko	604.56	0.15	0.50	0.11
São Tomé-Príncipe	147.14	0.05	0.07	0.01
Annobón-Príncipe	378.78	0.07	0.77	0.07
Annobón-São Tomé	185.52	0.03	0.58	0.07

Table II.7: Bat fly specimens tested for bacterial symbionts by PCR. The number of positive specimens based on confirmed *Enterobacteriales* sequences is recorded.

Bat fly species	Location	Tested	Symbiont positive
C. greefi	Ghana	70	12
	Bioko	138	0
	Príncipe	74	3
	São Tomé	135	5
	Annobón	95	1
E. africana	Ghana	43	11
	Príncipe	10	3
	São Tomé	1	1

Appendix III

III.1 Supplementary methods

III.1.1 Sampling protocol

Captive bats (Table III.1) were collected using hand nets after cordoning bats into one quarter of the cage with a curtain system and then placed into a smaller cage until processing. Wild bats (the first three captive cohorts and those from 31 January 2012) were captured from roosts using 6–18 m mist nets or hand nets then placed in individual cloth bags until processing. While under manual restraint, the surface of the inner wing along the propatagial vein was wiped with 70% ethanol then 0.2–1.0 ml of whole blood was collected using a citrated 1 ml syringe and transferred into labeled microcentrifuge tubes. After bleeding had ceased, bats were released either into the main area of the enclosure or back to the wild roost. On two occasions bat flies were collected from bats, M10 from the captive colony and J12 from the wild source colony. Bat flies were collected from the pelage of bats and placed in individual sterile tubes labeled with the ID number of the host bat. Whole blood was immediately frozen at -80°C or blood clots were separated from serum then frozen at -80°C; bat flies were kept frozen at -80°C. All samples were shipped to the Centers for Disease Control and Prevention Division of Vector-Borne Diseases in Fort Collins, CO on dry ice where they were kept at -20°C or below until DNA extraction.

III.1.2 DNA extraction

Bat flies were rinsed in 70% ethanol then in sterile 1x PBS (0.15 M, pH 7.5, CDC, Atlanta, GA) before being transferred to a clean microcentrifuge tube and triturated in 500 µl brain heart infusion (BHI) broth (CDC, Atlanta, GA) using a sterile pestle. Genomic DNA was extracted from bat blood samples and triturated bat flies either by hand using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) or with a QIAxtractor automated instrument (Qiagen) following the manufacturer's protocols for tissues (flies) and blood. Extraction controls (blank wells) were included to ensure no cross-contamination occurred during extraction. Extracted DNA was stored in clean microcentrifuge tubes at -20 or 4°C during the duration of pathogen testing.

Table III.1: Demographics of bats entering the captive colony (n = 112). Age classes follow Peel et al. (2016): neonate (NEO), juvenile (JUV), sexually immature subadult (SI), and sexually mature adult (A). NEO and JUV classes were born in captivity (BIC) in 2010 and 2011 as cohorts in 4 and 5.

	Time (days) since		Cohort number	A		SI		BIC (NEO/JUV)	
Date	Study start	Last sampling		F	M	F	M	F	M
2009-07-28	0	0	1		11		1		
2009-11-05	100	100	2	3	5	3	2		
2010-01-28	184	84	3	29	12	7	5		
2010-03-06	221	37							
2010-04-01	247	No sampling	4					7	4
2010-05-21	297	76							
2010-07-14	351	54							
2010-09-23	422	71							
2010-11-05	465	43							
2011-03-04	584	119							
2011-04-01	612	No sampling	5					14	9
2011-07-13	715	131							
2012-01-17	903	188							
2012-01-31	917	14							
2012-02-14	931	14							
2012-03-15	961	30							
				32	28	10	8	21	13

III.1.3 Bacterial detection and sequencing

Bartonella spp. were detected via conventional PCR targeting the 16S–23S intergenic spacer region (ITS) via single-step PCR (Diniz et al., 2007),and the citrate synthase (*gltA*) and cell division protein (*ftsZ*) genes via nested PCR (Bai et al., 2016). Quantification of *Bartonella* infection load was performed using real-time PCR targeting the *Bartonella* transfer-messenger RNA gene (*ssrA*) (Diaz et al., 2012). *Rickettsia* DNA was detected using a separate real-time PCR assay targeting the 23S ribosomal RNA subunit (Kato et al., 2013). Samples positive for *Rickettsia* by real-time PCR were confirmed using conventional PCR targeting the *Rickettsia gltA* gene using a nested protocol (Choi et al., 2005; Lee et al., 2014; Regnery et al., 1991). Primers and thermocycler protocols for all real-time and conventional PCR are listed in Tables III.2–III.3.

All PCR amplifications were run in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) with the addition of a CFX96 Real-Time System (Bio-Rad) for real-time PCRs. For all PCRs, positive (*Bartonella doshiae*, *Rickettsia felis*) and negative (RNase-free water only) controls were included to determine correctly sized amplicons and to detect potential cross-contamination, re-

Table III.2: Oligonucleotide primers used for bacterial detection via real-time and conventional PCR amplification. Sequences designated [F] are forward primers, those designated [R] are reverse primers, and those designated [P] are TaqMan probes. FAM is 6-Carboxyfluorescein (maximum fluorescence at 518 nm).

Bacteria	Locus	PCR type	PCR round	Primer sequence	Primer name	Product size (bp)	Reference
Bartonella	*ftsZ*	conventional	1	ATTAATCTGCAYCGGCCAGA [F]	Bfp1	885	Zeaiter et al. (2002)
				ACVGADACACGAATAACACC [R]	Bfp2		
		conventional	2	ATATCGCGGAATTGAAGCC [F]	ftsZ R83	670	Colborn et al. (2010)
				CGCATAGAAGTATCATCCA [R]	ftsZ L83		
	gltA	conventional	1	GCTATGTCTGCATTCTATCA [F]	CS443f	767	Birtles and Raoult (1996); Gundi et al. (2012)
				GATCYTCAATCATTTCTTTCCA [R]	CS1210r		
		conventional	2	GGGGACCAGCTCATGGTGG [F]	BhCS781.p	356	Birtles and Raoult (1996); Norman et al. (1995)
				AATGCAAAAAGAACAGTAAACA [R]	BhCS1137.n		
	ITS	conventional	1	CTTCAGATGATGATCCCAAGCCTTCTGGCG [F]	325s	364–398	Diniz et al. (2007)
				GAACCGACGACCCCCTGCTTGCAAAGA [R]	1100as		
	ssrA	real-time	1	GCTATGGTAATAAATGGACAATGAAATAA [F]	ssrA-F	301	Diaz et al. (2012)
				GCTTCTGTTGCCAGGTG [R]	ssrA-R		
				(FAM)-ACCCCGCTTAAACCTGCGACG-(BHQ1) [P]	ssrA-P		
Rickettsia	23S rRNA	real-time	1	AGCTTGCTTTTGGATCATTTGG [F]	PanR8-F	Not reported	Kato et al. (2013)
				TTCCTTGCCTTTTCATACATCTAGT [R]	PanR8-R		
				(FAM)-CCTGCTTCTATTTGTCTTGCAGTAACACGCCA-(BHQ1) [P]	PanR8-P		
	gltA	conventional	1	GGGGGCCTGCTCACGGCGG [F]	RpCS.877p	381	Regnery et al. (1991)
				ATTGCAAAAAGTACAGTGAACA [R]	RpCS1258n		
		conventional	2	GGCTAATGAAGCAGTGATAA [F]	RpCS896p	338	Choi et al. (2005); Lee et al. (2014)
				GCGACGGTATACCCATAGC [R]	RpCS1233n		

Table III.3: Thermocycler protocols used for bacterial detection via via real-time and conventional PCR amplification.

Bacteria	Locus	PCR type	PCR round	Thermal program
Bartonella	*ftsZ*	conventional	1	95°C 4:00, (95°C 0:30, 55°C 0:30, 72°C 1:00)x40, 72°C 10:00, 4°C ∞
			2	95°C 4:00, (95°C 0:30, 55°C 0:30, 72°C 1:00)x40, 72°C 10:00, 4°C ∞
	gltA	conventional	1	95°C 2:00, (95°C 0:30, 48°C 0:30, 72°C 2:00)x40, 72°C 7:00, 4°C ∞
			2	95°C 3:00, (95°C 0:30, 55°C 0:30, 72°C 0:30)x40, 72°C 7:00, 4°C ∞
	ITS	conventional	1	95°C 3:00, (95°C 0:30, 66°C 0:30, 72°C 0:30)x55, 72°C 5:00, 4°C ∞
	ssrA	real-time	1	60°C 1:00, 95°C 10:00, (95°C 0:15, 60°C 1:00)x45, 60°C 1:00, 4°C ∞
Rickettsia	23S rRNA	real-time	1	95°C 8:00, (95°C 0:05, 60°C 0:30)x45, 4°C ∞
	gltA	conventional	1	95°C 2:00, (95°C 0:20, 48°C 0:30, 60°C 2:00)x35, 4°C ∞
			2	95°C 10:00, (95°C 0:30, 55°C 0:30, 72°C 1:00)x30, 72°C 5:00, 4°C ∞

spectively. PCR products were inspected for the presence of positive amplicons of the correct size by gel electrophoresis using 1.5% agar and GelGreen stain (Biotium, Hayward, CA). Positive amplicons were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse reads were assembled and edited using the SeqMan Pro program in Lasergene v14 (DNASTAR, Madison, WI). Sequences obtained from bat blood or bat flies were initially confirmed to the bacterial genus using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

III.1.4 Phylogenetic analysis of bacterial sequences

Bacterial sequences obtained from bats and bat flies were aligned with reference sequences for named species from each detected bacterial genus and with sequences for each bacterial genus

that have been detected previously in bats (Supplementary Data). Alignments for each genetic locus were performed separately using MAFFT v7 (Katoh and Standley, 2013). Alignments were trimmed to a common length eliminating poorly aligned positions using Gblocks v0.91b (Castresana, 2000). Alignments were then visually inspected for errors and manually corrected. Concatenation of multiple loci for phylogenetic analysis was performed after alignment and trimming using Phyutility v2.2 (Smith and Dunn, 2008). Neighbor-joining trees were generated using the Tamura-Nei DNA evolution model in MEGA v7 (Kumar et al., 2016; Tamura and Nei, 1993). Branch support for each tree was estimated from 1000 bootstrap samples from the respective alignment.

III.1.5 Regression analyses

Linear regression was performed to determine demographic factors that influence *Bartonella* infection status for bats sampled from the colony in March 2010. Two data sets were used: a set containing data from all bats and a set containing data from females only. In the full data set, covariates included bat sex, age class (neonate, juvenile, sexually immature subadult, and sexually mature adult) following Peel et al. (2016) and the presence/absence of bat flies on each bat. In the females only data set, covariates were the same as the full dataset (excepting sex) but also included pregnancy status (pregnant or not). Data were fit to covariates in a generalized linear model (GLM), treating infection status as a binomial variable with a logit link. Model selection was then performed based on Akaike's information criterion corrected for finite sample sizes (AICc) using the dredge function in the R package `MuMIn` (Bartoń, 2016; R Core Team, 2019). The model with the smallest AICc was chosen unless another model was less than two AICc points away from the top model (Burnham and Anderson, 2004), in which case the simpler model was chosen.

Segmented regression was performed to detect breakpoints in measures of *Bartonella* prevalence and diversity over the course of the experiment. Separate GLMs were fit for each measure in R. Bartonella single infection and coinfection prevalence were both treated as binomial variables with a logit link. Real-time PCR cycle threshold (Ct) values, number of sequenced *Bartonella* markers, *Bartonella* species richness, Shannon number, inverse Simpson index, number of *Bartonella* species in a sample, and *Bartonella* beta diversity were all treated as gamma-distributed

variables. For Ct values, an inverse gamma model was fit. Segmented regression was performed on fitted GLMs using the R package `segmented` (Muggeo, 2017) with initial breakpoints at 6 March 2010 (M10, day 221) and 17 January 2012 (J12, day 903) based on visual inspection of the data.

III.1.6 Likelihood ratio tests

I tested whether *Bartonella* species coinfected bats with other species more than expected by chance using a multinomial test adapted from a previous study analyzing patterns of influenza A transmission in birds (Pepin et al., 2013). Following Pepin et al., only double infections (two coinfecting species) were included because higher order infections were rare and challenging to interpret. The null hypothesis for the test was that *Bartonella* species i would coinfect with any other species j with equal probability and the expected counts for partner coinfections of species i would be proportional to the frequency of each partner in all single (s) and double (d) infections. Therefore, the expected counts for each j partner of subtype i, $\mathrm{E}[X_j]$ are $\mathrm{E}[X_j] = (X_j^{s+d}/N_j^{s+d}) * X_i^d$, where X_j^{s+d} is the total number of single and double infections for partner j, N_j^{s+d} is the total number of single and double infections for all j partners, and X_i^d is the total number of double infections for species i. The maximum likelihood estimates for the parameters in the null multinomial model for each species i are then: $\pi_j = \mathrm{E}[X_1/\sum(\mathrm{E}[X_j]), ..., \mathrm{E}[X_n]/\sum(\mathrm{E}[X_j])$. The probabilities under the null and alternative models are: $\mathrm{P}(X)_0 = N_j^d! \prod(\pi_j^{xi}/X_i!)$ and $\mathrm{P}(X)_A = N_j^d! \prod(p_j^{xi}/X_i!)$ and the likelihood ratio statistic D is $-2\ln(\mathrm{P}(X)_0/\mathrm{P}(X)_A)$, which is approximately distributed χ^2_{n-1}. The likelihood ratio statistic was divided by the correction factor $1 + \sum(\pi_j^{-1} - 1)/6N_j^d(n-1)$ to decrease type I error inflation due to the difference between the moments of the likelihood ratio statistic and the chi-square distribution. Differences between the observed and expected counts of coinfections were tested using binomial likelihood ratio tests, using the same correction factor as above. Functions for multinomial and binomial likelihood ratio tests were written in R. These functions were first used to test for differences in observed and expected counts of coinfections for the whole course of the experiment (961 days). Additional tests were performed on two partitions of the experiment: bats sampled before J12 and bats sampled after J12. This was based on visual

observation of a change in the frequency of *Bartonella* species starting around this point in the experiment.

In addition to the tests of the observed and expected counts of coinfections, these same likelihood ratio test functions were used to perform tests on changes in the frequency of single infections and coinfections in the captive colony over time and differences in the relative frequency of infections between bats and bat flies. Specifically, I performed likelihood ratio tests on the relative frequency of *Bartonella* species before versus after J12, using the before-J12 frequencies as the expected frequencies to calculate the likelihood ratio statistic. I calculated the differences in the frequency of *Bartonella* species in bats versus bat flies sampled on M10 and J12, using the frequencies in bats as the expected frequencies. I also calculated the differences in the frequencies of *Bartonella* species in bats after J12 versus bat flies sampled on J12, again using the frequencies in bats as the expected frequencies.

III.1.7 Assumptions

Within this system, *Bartonella* infection does not cause obvious signs of disease in bats or flies (Kosoy, 2010), so it is assumed that there are no parasite-mediated mortality effects. Hosts are considered as discrete patches containing parasite species and the dynamics of these infections are linked through transmission by bat flies as they disperse among hosts. *Bartonella* species are assumed to be static and not measurably evolving over the current study, an assumption supported by the very low mutation rates (Gutiérrez et al., 2018b). Finally, although vertical transmission of *Bartonella* from dam to offspring is possible (Kosoy et al., 1998), it has not been demonstrated in bats, so I assume that bats are born uninfected, and the primary transmission route is through vector transmission.

III.2 Supplementary results

III.2.1 Phylogenetic analysis of detected *Bartonella* and *Rickettsia* species

*Bartonella*sequences from *E. helvum* and *C. greefi* predominantly grouped closely with six *Bartonella* species previously described from *E. helvum*: *Bartonella* spp. E1–E5 and Ew (Bai

et al., 2015; Kosoy et al., 2010). The phylogenetic distinctiveness of these species can be observed based on all loci sequenced: *gltA*, *ftsZ*, and ITS (Figures III.1–III.3). In addition to these six species, two novel *Bartonella* genogroups were observed in both *E. helvum* and *C. greefi*, denoted *Bartonella* spp. Eh6 and Eh7. *Bartonella* sp. Eh6 was detected at all three sequenced loci whereas species Eh7 was only detected at *gltA* and *ftsZ* (Figures III.1–III.3). Sequences representing these two potentially novel *Bartonella* species have been submitted to GenBank with the following accession numbers: MN250730–MN250774 (*gltA*), MN250775–MN250788 (*ftsZ*), and MN249715–MN249720 (ITS).

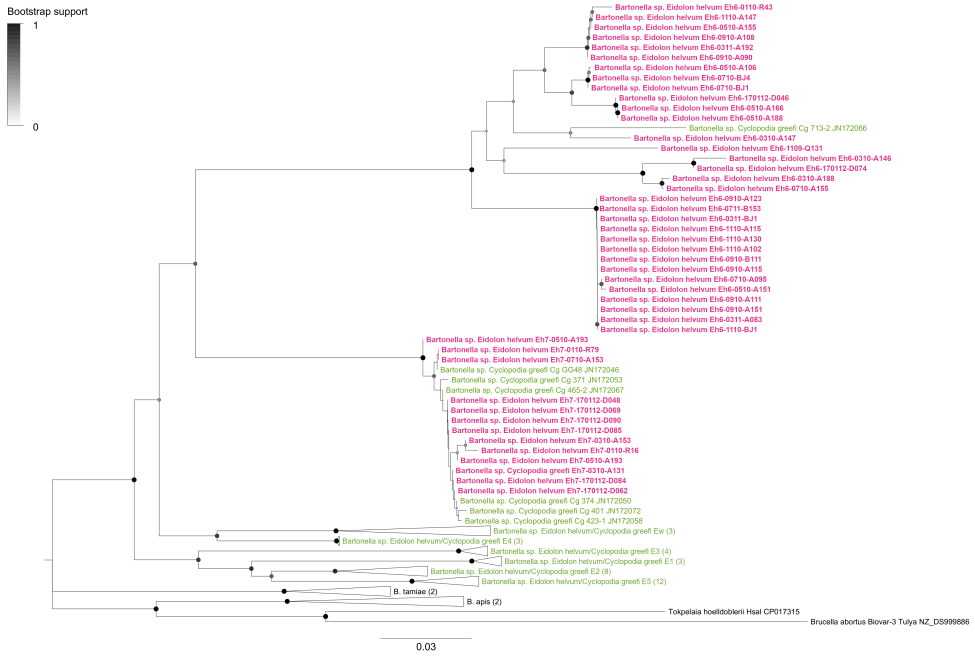

Figure III.1: Neighbor-joining phylogenetic tree of *Bartonella gltA* sequences produced from a 366 bp alignment of 91 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Bartonella* sequences previously obtained from *E. helvum* or *C. greefi* are colored green while names of new sequences from these species are colored pink.

All *gltA* sequences from species Eh6 were found to be similar to each other (88.5–100% sequence identity), and according to BLAST search, similar (88.2–94.7% sequence identity) to a sequence obtained from *C. greefi* collected from *E. helvum* on Bioko island in the Gulf of Guinea

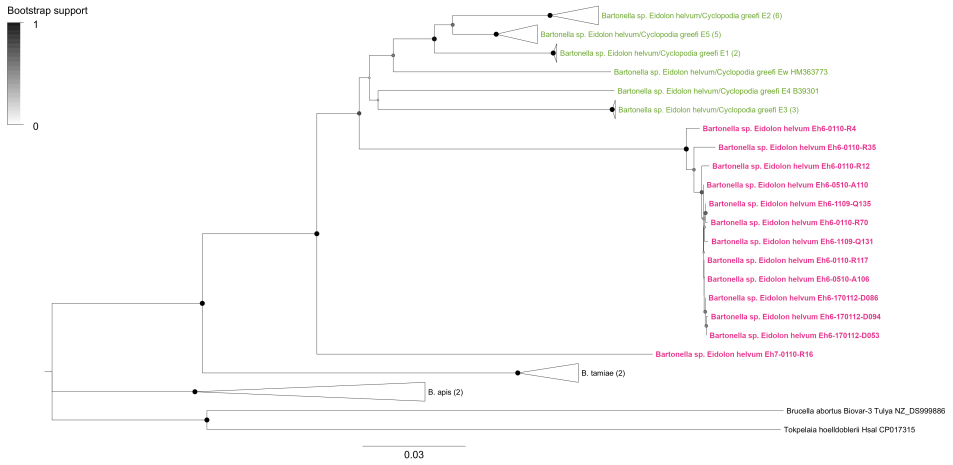

Figure III.2: Neighbor-joining phylogenetic tree of *Bartonella ftsZ* sequences produced from an 879 bp alignment of 37 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Bartonella* sequences previously obtained from *E. helvum* or *C. greefi* are colored green while names of new sequences from these species are colored pink.

(GenBank accession number JN172066) by Billeter et al. (2012). All *ftsZ* sequences were highly similar to each other (98.5–100% sequence identity), as were ITS sequences (99.4–100% sequence identity). All *gltA* sequences from species Eh7 were similar to one another (99.7–100% sequence identity) and similar (99.7–100% sequence identity) to six sequences (GenBank accession numbers JN172046, JN172050, JN172053, JN172058, JN172067, and JN172072) from *C. greefi* collected from *E. helvum* on Annobón and Bioko islands in the Gulf of Guinea and in Ghana (Billeter et al., 2012). Sequenced loci grouped species Eh6 and Eh7 as monophyletic groups distinct from other *E. helvum*-associated *Bartonella* species with unanimous bootstrap support (Figures III.1–III.3).

A neighbor-joining tree (Figure III.4) produced from concatenated *ftsZ* and *gltA* sequences from known *Bartonella* species and *Bartonella* strains detected in bats (Supplementary Data) demonstrates that *Bartonella* species from *E. helvum* and *C. greefi* are broadly distributed in the *Bartonella* phylogeny. *Bartonella* sp. Ew strongly clusters (97% bootstrap support) with three other *Bartonella* strains isolated from *Myotis blythii* and *Rhinolophus ferrumequinum* in Georgia (Urushadze et al., 2017). This clade is more distantly related to *Bartonella* species infecting rodents, carnivores, and primates (*B. washoensis*, *B. quintana*, *B. henselae*, and *B. koehlerae*).

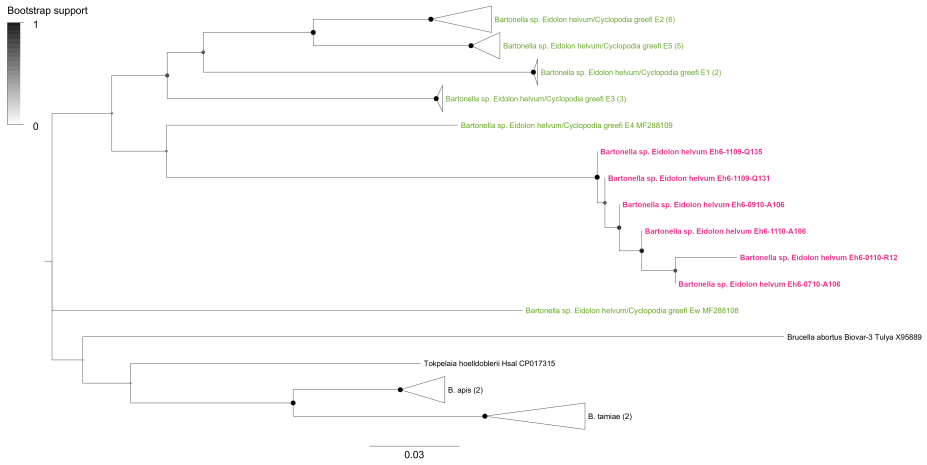

Figure III.3: Neighbor-joining phylogenetic tree of *Bartonella* ITS sequences produced from a 436 bp alignment (including gaps) of 30 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Bartonella* sequences previously obtained from *E. helvum* or *C. greefi* are colored green while names of new sequences from these species are colored pink.

Bartonella spp. E3, E1, E2, and E5 are part of a large and distinct clade of bat-associated *Bartonella* strains isolated from hosts in several bat families including Hipposideridae, Rhinolophidae, Miniopteridae, and Vespertilionidae in Africa and Eurasia (Kosoy et al., 2010; Lilley et al., 2015; Lin et al., 2012; McKee et al., 2017; Urushadze et al., 2017). While this clade only received 35% bootstrap support in the current tree using concatenated *ftsZ* and *gltA*, a previous analysis using three additional loci and a Bayesian phylogenetic approach found 100% posterior support for this clade (McKee et al., 2017). *Bartonella* sp. Eh6 is contained with a clade (53% bootstrap support) including strains from *Pipistrellus pipistrellus* and *Myotis blythii* from Georgia (Urushadze et al., 2017). The Bayesian analysis by McKee et al. (2017) showed that this smaller clade is included as a subclade within the larger Old World bat-associated clade mentioned above with 100% posterior support. Thus, it is likely that sequencing of additional loci from *Bartonella* sp. Eh6 is part of this large clade. *Bartonella* sp. E4 is part of a clade that includes several *Bartonella* strains associated with rodents and carnivores, including *Bartonella rochalimae* and *B. clarridgeiae*, as well as another bat (*Myotis emarginatus*) from Georgia (Urushadze et al., 2017). While the bootstrap support for this clade is low (17%), sequencing of additional markers may result in higher support (McKee

et al., 2017; Urushadze et al., 2017). Finally, *Bartonella* sp. Eh7 was positioned as ancestral to all *Bartonella* species infecting mammals but derived from *B. tamiae* and *B. apis* with 99% bootstrap support.

Figure III.4: Neighbor-joining phylogenetic tree of concatenated *Bartonella ftsZ* and *gltA* sequences produced from a 1248 bp alignment (888 bp *ftsZ*, 360 bp *gltA*) of 175 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Bartonella* species/strains previously obtained from bats are colored blue while names of new strains from *E. helvum* or *C. greefi* are colored orange.

Only one bat and one fly were positive for *Rickettsia* sp. DNA, both sampled on M10; however the positive fly was not collected from the positive bat. The two *Rickettsia gltA* sequences obtained from *E. helvum* and *C. greefi* in March 2010 were identical to one another (313/313 bp). Both sequences have been submitted to GenBank with accession numbers MN255799 and MN255800. A neighbor-joining tree generated from *gltA* sequences (Figure III.5) showed that

these *Rickettsia* sequences are distinct from those previously obtained from bats or bat ectoparasites. Our sequences were most closely related to *Rickettsia akari* (61% bootstrap support) within the transitional group rickettsiae clade that also includes *R. felis*, *R. hoogstraalii*, *R. lusitaniae*, and *R. australis* (Sánchez-Montes et al., 2016; Weinert et al., 2009). Other bat-associated *Rickettsia* strains have been detected from this clade in mainly insectivorous bats or their associated soft ticks in Africa, Eurasia, and North America (Hornok et al., 2018, 2019; Sánchez-Montes et al., 2016).

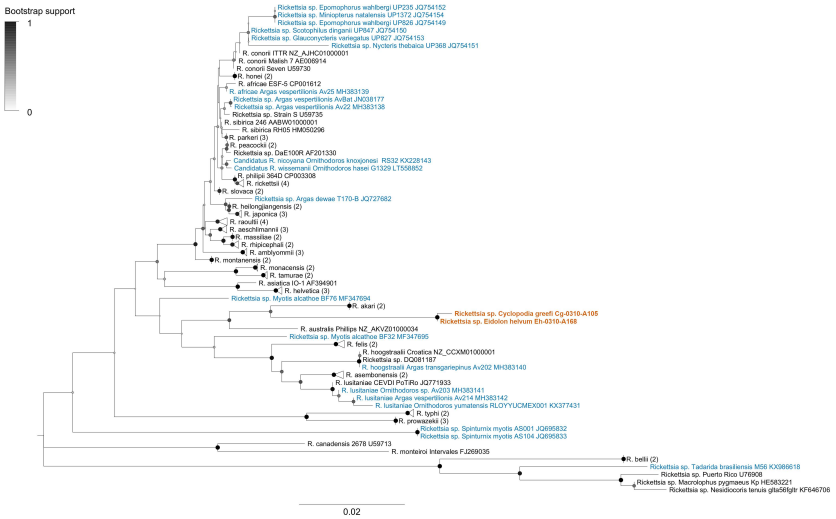

Figure III.5: Neighbor-joining phylogenetic tree of *Rickettsia gltA* sequences produced from a 1230 bp alignment of 96 sequences. Branch support values based on 1000 bootstrap replicates are indicated by the size and color of circles drawn at each branch. Evolutionary distances were computed using the Tamura-Nei method and are in units of number of base substitutions per site. Names of *Rickettsia* species/strains previously obtained from bats are colored blue while names of new strains from *E. helvum* or *C. greefi* are colored orange.

III.2.2 Demographic patterns of *Bartonella* prevalence during experiment

No significant differences were observed in the proportion of males and females infected (Figure III.6B) at the start of the study (χ^2 = 1.1, df = 1, P = 0.29) or by the end of the study (χ^2 = 0.17, df = 1, P = 0.68). However, there was a significant increase in the proportion infection between the start and end for males (χ^2 = 19, df = 1, P < 0.001) and females (χ^2 = 10.5, df = 1, P < 0.001), although this is linked to the increases observed in neonates/juveniles and adults.

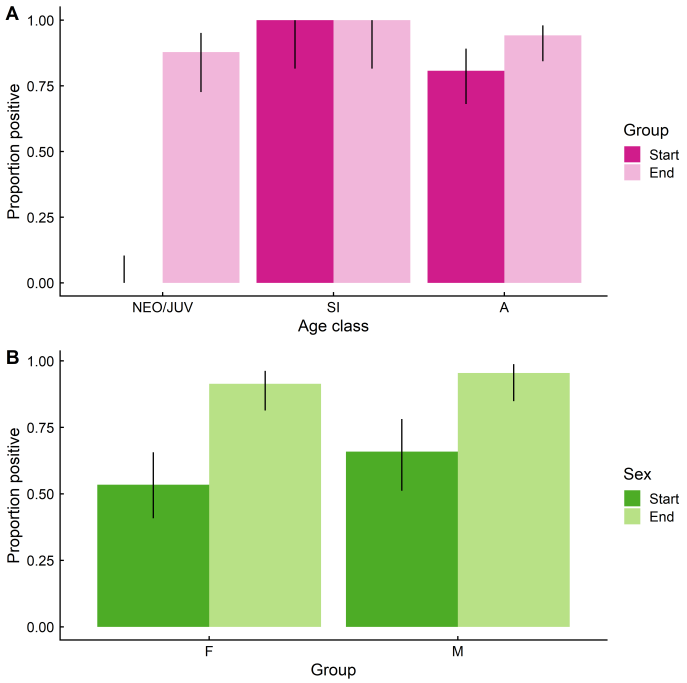

Figure III.6: Change in the proportion of individuals positive for *Bartonella* at the start (upon entry into colony) and end (15 March 2012) of the experiment according to (A) age class and (B) sex. Wilson score 95% confidence intervals are drawn at the top of each box.

III.2.3 Effects of bat fly reintroduction on treatment versus control bats

Despite the significant changes in prevalence and infection load observed after J12, the reintroduction of flies into the colony was intended to be a randomized treatment/control study to compare bats receiving flies to those that did not receive flies in terms of their change in infection status. For all bats that tested negative for *Bartonella* on J12, bats that received flies were not more likely to become infected than bats that did not receive flies (χ^2 = 0.012, df = 1, P = 0.54). This pattern remains even if bats were split into two groups: subadults and adults (χ^2 = 0.25, df = 1, P = 0.31) and neonates and juveniles (χ^2 = 2.5x10^{-31}, df = 1, P = 0.5). Including the bats that were positive for *Bartonella* on J12, bats that received flies were slightly more likely to become infected or change *Bartonella* species than bats without flies, but this difference was not significant when all age groups were combined (χ^2 = 1.5, df = 1, P = 0.11). However, subadult and adult bats that received flies were more likely to become infected or change *Bartonella* sp. than bats that did not

receive flies (χ^2 = 3.2, df = 1, P = 0.036). A similar pattern was not observed in neonates and juveniles (χ^2 = 0.38, df = 1, P = 0.27).

Additionally, there was poor correspondence between the *Bartonella* species found in the colony bats that received flies with the *Bartonella* species found in the bats that were the donors for the flies or other flies that were removed from the donor bats. The frequency of finding the same *Bartonella* species in the recipient bat and either the donor bat or a sampled fly taken from the donor bat (13/27, 48.1%) was no better than random ($\chi^2 = 5x10^{-31}$, df = 1, P = 0.5). This was again true if bats were subdivided into subadults and adults (5/13, 38.5%; χ^2 = 0.039, df = 1, P = 0.58) and neonates and juveniles (8/14, 57.1%; $\chi^2 = 2.8x10^{-32}$, df = 1, P = 0.5). Using the additional data from the collection of bat flies on M10, no correlation was observed between the presence of a fly and whether a bat was positive (Pearson's R = -0.067, t = -0.52, df = 59, P = 0.61). The frequency of finding the same Bartonella species in the bat and the sampled bat fly (9/26, 34.6%) was no better than random (χ^2 = 0.71, df = 1, P = 0.8).

III.2.4 Differences in *Bartonella* prevalence and diversity between bats and bat flies

Bartonella prevalence in bat flies (93%) collected from the colony on M10 was similarly high as in the colony bats (Figure 5.2A). The flies collected on J12 from the wild bat population had a slightly lower infection prevalence (89%) compared to the wild bats (94%), and both the wild flies and wild bats had higher prevalence than the bats in the colony (31%) on the same date. Average infection loads in flies on M10 were less than in the colony bats, indicated by higher Ct values (Figure 5.2B). Similarly, wild bat flies had higher Ct values on J12 than the wild bats but were lower than in the colony bats. Bat flies had a higher average number of positive markers but lower coinfection prevalence compared to bats from their respective populations in the colony on M10 and from the wild population on J12 (Figure III.9).

On M10, all *Bartonella* diversity measures (species richness, Shannon index, inverse Simpson index, number of species an individual sample, beta diversity) in flies were lower than in the bat population at that time (Figure 5.3; Figure III.10). On J12, all diversity measures except inverse

Simpson index in wild bats were higher than in the captive colony. Diversity measures in flies sampled from wild bats at this time were lower than in the wild population.

There were significant differences in the relative abundance of *Bartonella* species in the bats and flies sampled on M10 ($D = 43.7$, $df = 7$, $P < 0.001$) with significant differences observed in species E4 and E5 using binomial LR tests (Figure 5.3C; Table III.6). Differences between the relative abundance of *Bartonella* species after the reintroduction of flies on J12 and the wild flies that were introduced into the colony were observed ($D = 16.3$, $df = 6$, $P = 0.012$), with substantially higher abundance of Ew and lower abundance of E1 in the flies than the colony bats (Figure 5.3D; Table III.6). Similarly, differences were observed in distribution of species between the wild bats and wild flies sampled on J12 ($D = 16.7$, $df = 7$, $P = 0.019$), with a higher abundance of E5 and lower abundance of Eh6 and Eh7 in the flies than in the wild bats (Figure 5.3D; Table III.6). As detailed below, similar results were observed if the relative counts were used instead of relative abundance.

III.2.5 Shift in *Bartonella* community diversity using relative counts

Tests for changes in *Bartonella* diversity were initially performed using the relative abundance of *Bartonella* species based on the individual number of sequences acquired for each *Bartonella* species in a sample across the three different genetic markers. Using just the presence of a *Bartonella* species in a sample by any one of the different markers, what I term relative counts, very similar patterns were observed in the change in the distribution of *Bartonella* species over time (Figure III.13) and similar statistical test results for the comparison of species distributions before and after the reintroduction of flies (Table III.7). In fact, there is a very strong positive correlation (Pearson's $R = 0.99$, $t = 20.9$, $df = 6$, $P < 0.001$) between the abundance and counts for each Bartonella species over the entire study and at each sample time point (Figure III.14).

III.2.6 Individual infection histories and duration of infections

Individual infection histories for all 112 identified bats and relevant statistics for their histories are included in the Supplementary Data. Out of the 112 individual bats sampled during this study

period, 102 (91.1%) were sampled at least two time points in a row. The remaining 10 bats were either euthanized after first sampling (n = 1), were found dead between sampling time points (n = 5), or had disappeared and were presumed dead (n = 4).

There was considerable individual variation among bats in their infection histories, with some bats never becoming infected, bats with intermittent infections throughout the study, bats clearing infection soon after entry into the colony, and other bats with highly persistent infections. Of the 112 bats that were sampled, 100 (89.3%) tested positive at least once during the study and 65 (58%) bats were positive at entry into the colony. Of the 102 bats that were sampled more than once, 95 (93.1%) tested positive at least once: 80/95 (84.2%) were positive at more than one time point and 15/95 (15.8%) were positive only once. Of the 15 positive only one time, 12 (80%) were bats born into the colony in April 2010 (n = 2) or April 2011 (n = 10) and 10/12 (83.3%) became positive only after the flies were reintroduced on J12. The three adult bats only infected once were from the cohort that entered the colony in January 2010. One of these bats was positive on entry and was found dead in May 2010, another became positive shortly after entry in March 2010, and the third adult did not become positive until after the fly reintroduction.

III.3 Additional figures and tables

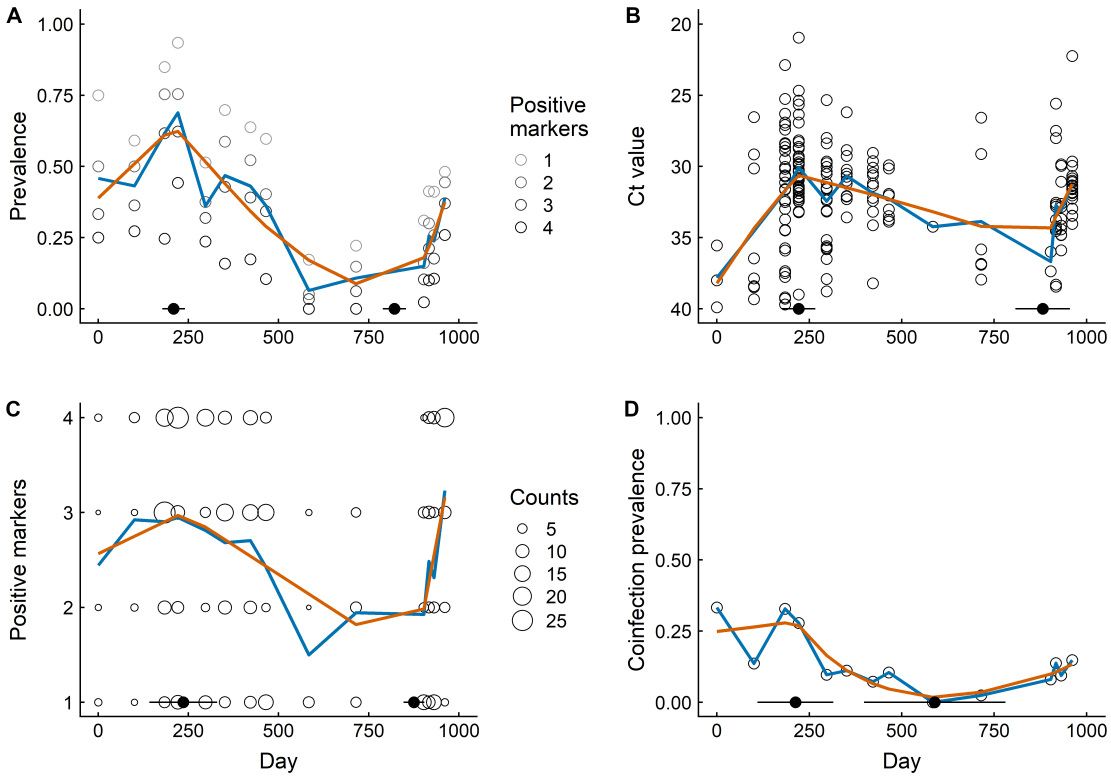

Figure III.7: Segmented regression analysis of *Bartonella* prevalence and load. (A) Points for *Bartonella* prevalence are shown considering one or more, two or more, three or more, or all four markers positive (including RT-PCR). (B) Only points with RT-PCR Ct values < 40 are shown. (C) Points show the number of markers that were positive for each individual with the width proportional to the number of individuals positive at that many markers. (D) Coinfection prevalence was measured by the number of individuals that were positive for two or more *Bartonella* species at each time point. For each measure, lines for the calculated mean trend (blue) and the predicted trend from segmented regression (orange) are drawn over the data points. Breakpoints and 95% confidence intervals estimated by segmented regression are shown above the x-axis.

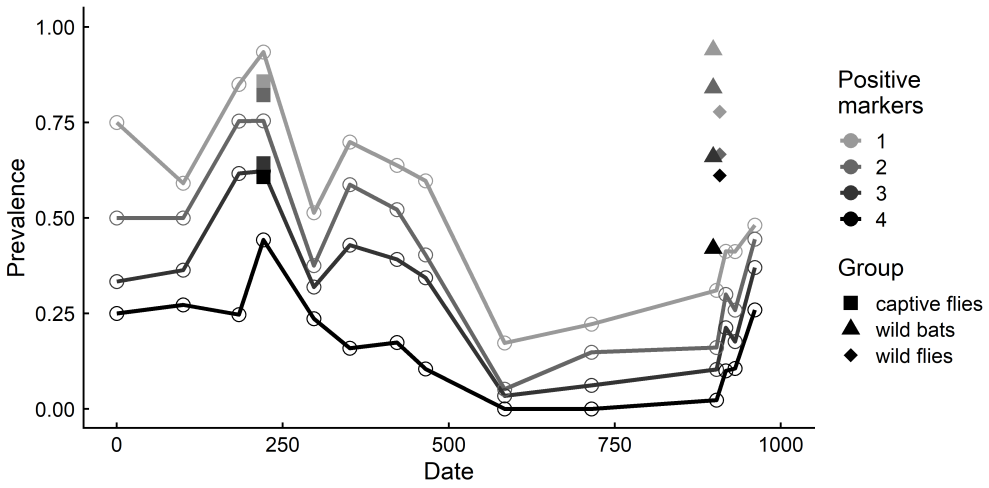

Figure III.8: *Bartonella* infection prevalence according to the number of markers positive. Separate lines are drawn for prevalence estimates in the *E. helvum* colony over time considering one or more, two or more, three or more, or all four markers positive (including RT-PCR). Points for sampled bat flies and wild bats are shown as unique symbols. A light grey bar on the x-axis shows the period after bat flies were reintroduced.

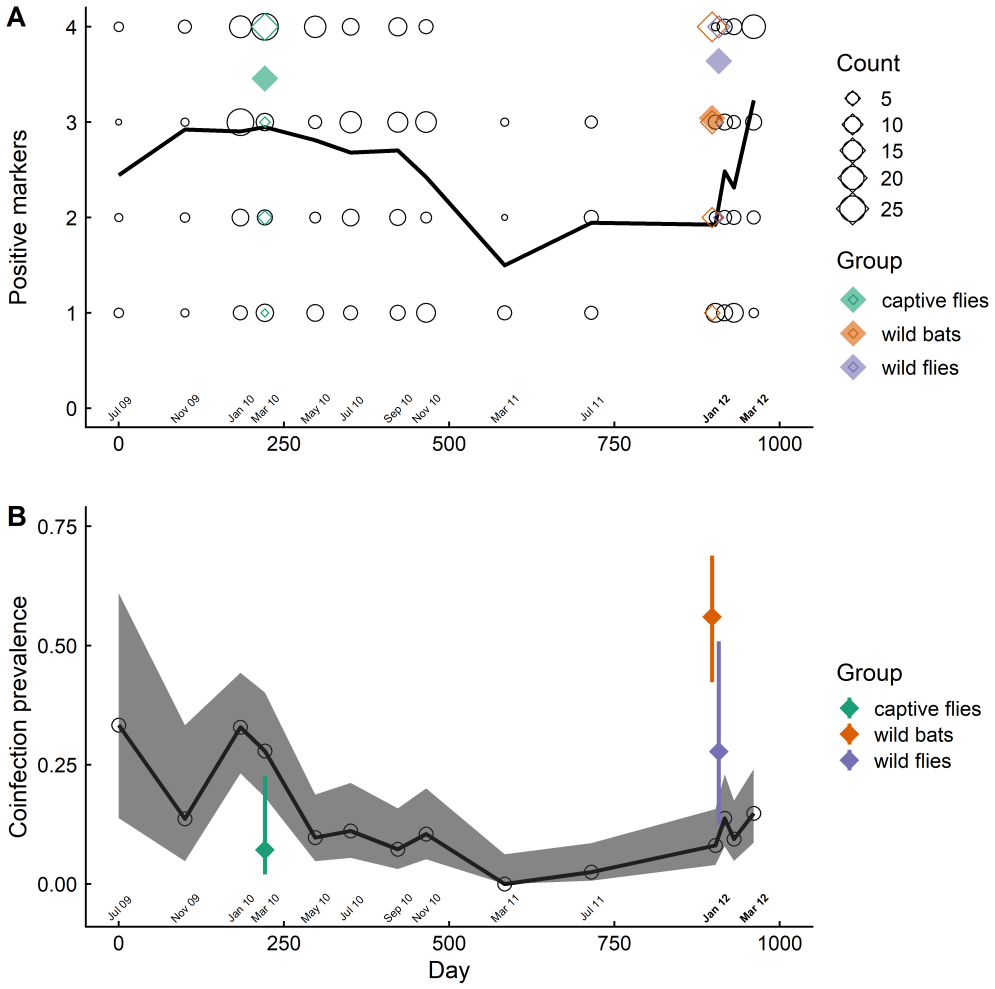

Figure III.9: *Bartonella* infection load according to number of positive markers from each positive bat and coinfection prevalence in the *E. helvum* colony. (A) Points show the number of markers that were positive for each individual with the width proportional to the number of individuals positive at that many markers. Points for sampled bat flies and wild bats are shown as colored points with calculated mean values (filled symbols). (B) Coinfection prevalence was measured by the number of individuals that were positive for two or more *Bartonella* species at each time point. Wilson score 95% confidence intervals (dark grey) were drawn around prevalence estimates at each sampling time point. Coinfection prevalence and confidence intervals for sampled flies and wild bats are shown as colored points.

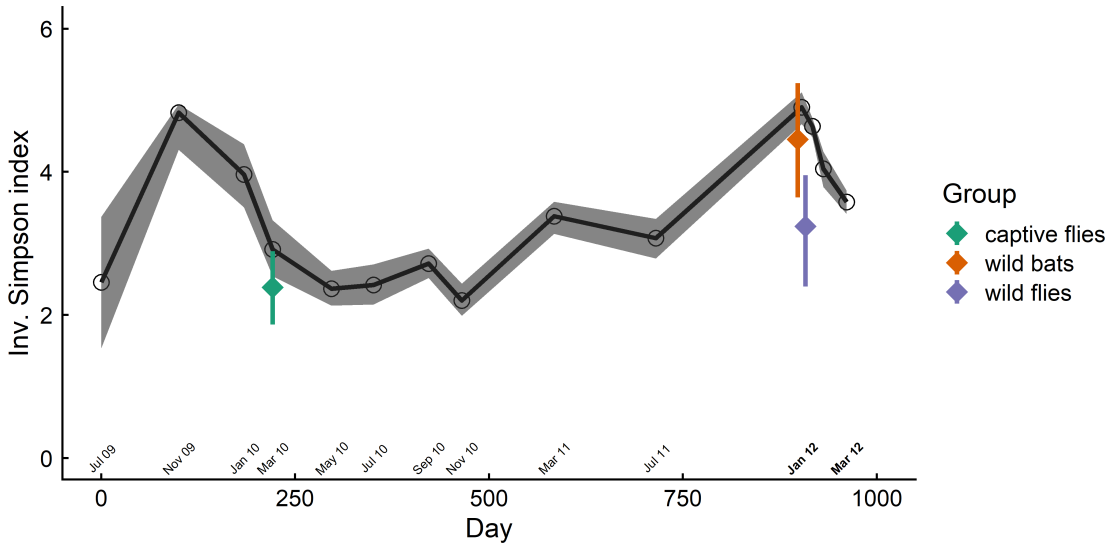

Figure III.10: Inverse Simpson index of *Bartonella* species evenness. Dark gray intervals around are bootstrap 95% confidence intervals from 1000 samples from the observed multinomial distribution of *Bartonella* species relative abundances. Diversity and confidence intervals for sampled *C. greefi* flies and wild *E. helvum* are shown as colored points. Months labeled in bold font on the x-axis show the period after bat flies were reintroduced.

Table III.4: Segmented regression analysis of *Bartonella* prevalence, load, and diversity. Coefficients and confidence intervals were estimated for change in slope at breakpoints. Statistical significance of parameters is indicated as true (1) or false (0) based on whether the confidence intervals overlap zero.

Regression variable	Model family (link)	AICc	Coefficient	Estimate	95% CI, lower	95% CI, upper	Significant
Infection prevalence	Binomial (logit link)	708.6	Change1-2	-1.1×10^{-2}	-1.4×10^{-2}	-7.3×10^{-3}	1
			Change2-3	2.3×10^{-2}	1.7×10^{-2}	2.9×10^{-2}	1
			Point1-2	209.1	177.6	240.6	1
			Point2-3	821.3	789.2	853.3	1
Ct value	Gamma (inverse)	1029.2	Change1-2	-3.6×10^{-5}	-5.0×10^{-5}	-2.2×10^{-5}	1
			Change2-3	5.6×10^{-5}	1.2×10^{-5}	1.0×10^{-4}	1
			Point1-2	221.1	175.9	266.3	1
			Point2-3	881.4	807.5	955.3	1
Positive markers	Gamma (identity)	1458	Change1-2	-4.3×10^{-3}	-7.7×10^{-3}	-8.5×10^{-4}	1
			Change2-3	2.3×10^{-2}	1.4×10^{-2}	3.2×10^{-2}	1
			Point1-2	235.9	141.8	330	1
			Point2-3	875.5	846.6	904.5	1
Coinfection prevalence	Binomial (logit link)	85.6	Change1-2	-9.1×10^{-3}	-1.8×10^{-2}	2.0×10^{-4}	0
			Change2-3	1.4×10^{-2}	5.7×10^{-3}	2.2×10^{-2}	1
			Point1-2	212.4	110	314.8	1
			Point2-3	589.2	397.9	780.5	1
Species richness	Gamma (identity)	56.1	Change1-2	-1.9×10^{-2}	-3.6×10^{-2}	-1.6×10^{-3}	1
			Point1-2	184.2	29.8	338.7	1
Shannon number	Gamma (identity)	43.2	Change1-2	4.8×10^{-3}	-1.0×10^{-3}	1.1×10^{-2}	0
			Point1-2	467.1	66.7	867.5	1
Inv. Simpson index	Gamma (identity)	44.2	Change1-2	7.0×10^{-3}	1.4×10^{-3}	1.2×10^{-2}	1
			Point1-2	463	252.3	673.8	1
Species in sample	Gamma (identity)	550.8	Change1-2	1.9×10^{-3}	1.0×10^{-3}	2.7×10^{-3}	1
			Point1-2	581.1	443.6	718.6	1
Beta diversity	Gamma (identity)	7764.2	Change1-2	3.3×10^{-3}	2.9×10^{-3}	3.6×10^{-3}	1
			Change2-3	-4.2×10^{-3}	-6.1×10^{-3}	-2.2×10^{-3}	1
			Point1-2	373.3	357.0	389.7	1
			Point2-3	907.2	880.4	933.9	1

Table III.5: Multinomial and binomial likelihood ratio (LR) tests for changes in *Bartonella* relative species abundances before and after the reintroduction of bat flies. The period before flies were reintroduced covers July 2009 to July 2011. The period after flies were reintroduced covers 17 January 2012 to March 2012.

Date	E1	E2	E3	E4	E5	Ew	Eh6	Eh7
2009-07-28	0	0	11	4	1	3	0	0
2009-11-05	0	0	7	6	5	4	5	0
2010-01-28	3	4	37	16	19	60	7	4
2010-03-06	3	6	18	17	11	72	3	2
2010-05-21	0	0	4	19	1	47	7	1
2010-07-14	1	1	9	11	12	64	5	1
2010-09-23	0	0	7	4	33	54	8	0
2010-11-05	1	3	6	5	9	57	6	0
2011-03-04	0	0	3	2	0	6	3	0
2011-07-13	4	0	2	4	2	14	1	0
2012-01-17	8	9	10	2	15	6	0	0
2012-01-31	14	10	9	0	19	17	0	0
2012-02-14	10	4	5	6	26	15	0	0
2012-03-15	2	11	29	0	39	19	1	0
Total abundance	46	48	157	96	192	438	46	8
Sum total abundance	1031							
Before abundance	12	14	104	88	93	381	45	8
Before total abundance	745							
Before frequency	0.016	0.019	0.14	0.12	0.12	0.51	0.06	0.011
After abundance	34	34	53	8	99	57	1	0
Expected abundance	4.6	5.4	39.9	33.8	35.7	146.3	17.3	3.1
After total abundance	286							
After frequency	0.12	0.12	0.19	0.028	0.35	0.2	0.0035	0
Multinomial adjusted LR	350.1							
Multinomial P	0							
Binomial adjusted LR	78.7	69.8	4.5	30.5	91.1	116.5	27.3	6.1
Binomial P	0	1.1×10^{-16}	0.034	3.4×10^{-8}	0	0	1.8×10^{-7}	0.014

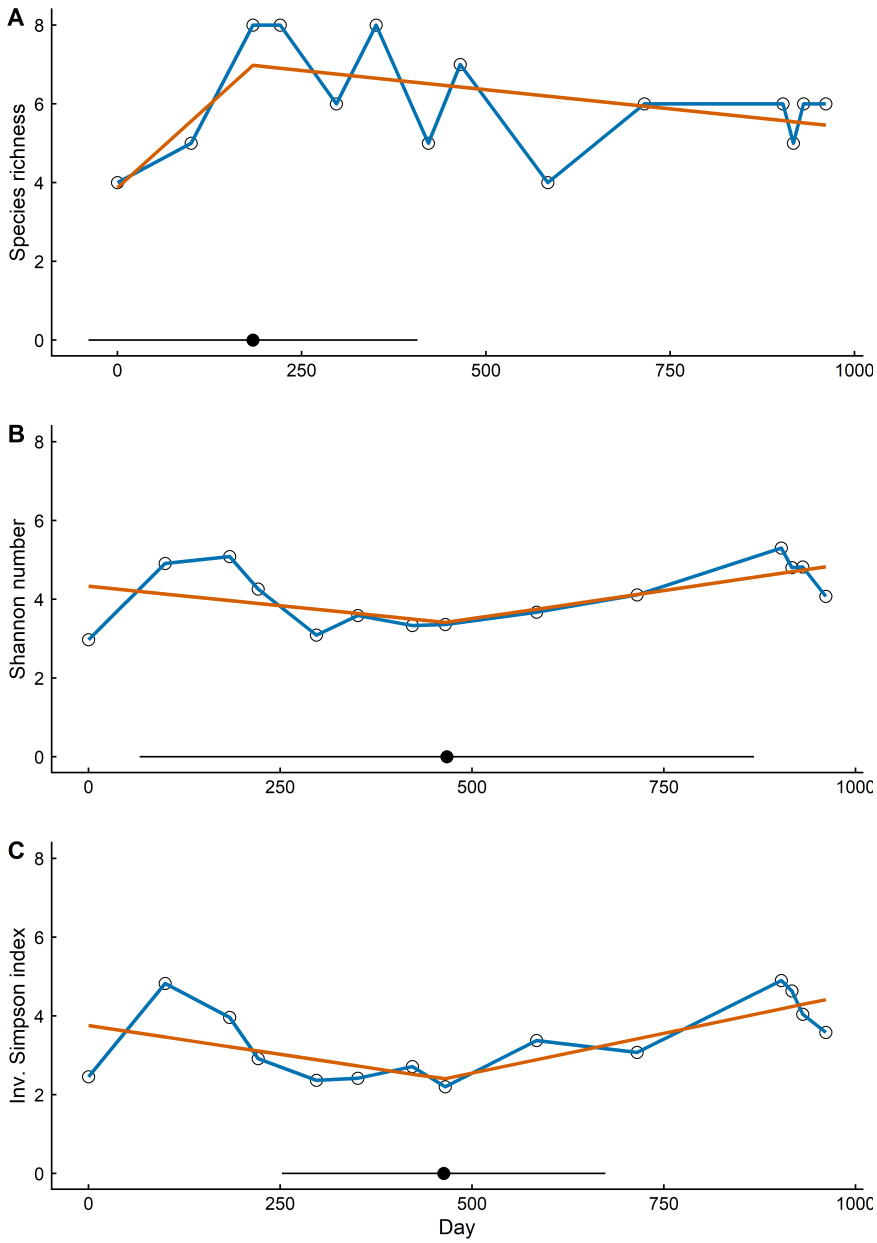

Figure III.11: Segmented regression analysis of colony-level *Bartonella* diversity measures: (A) species richness, (B) Shannon index of species evenness, and (C) inverse Simpson index of species evenness. For each measure, lines for the calculated mean trend (blue) and the predicted trend from segmented regression (orange) are drawn over the data points. Breakpoints and 95% confidence intervals estimated by segmented regression are shown above the x-axis.

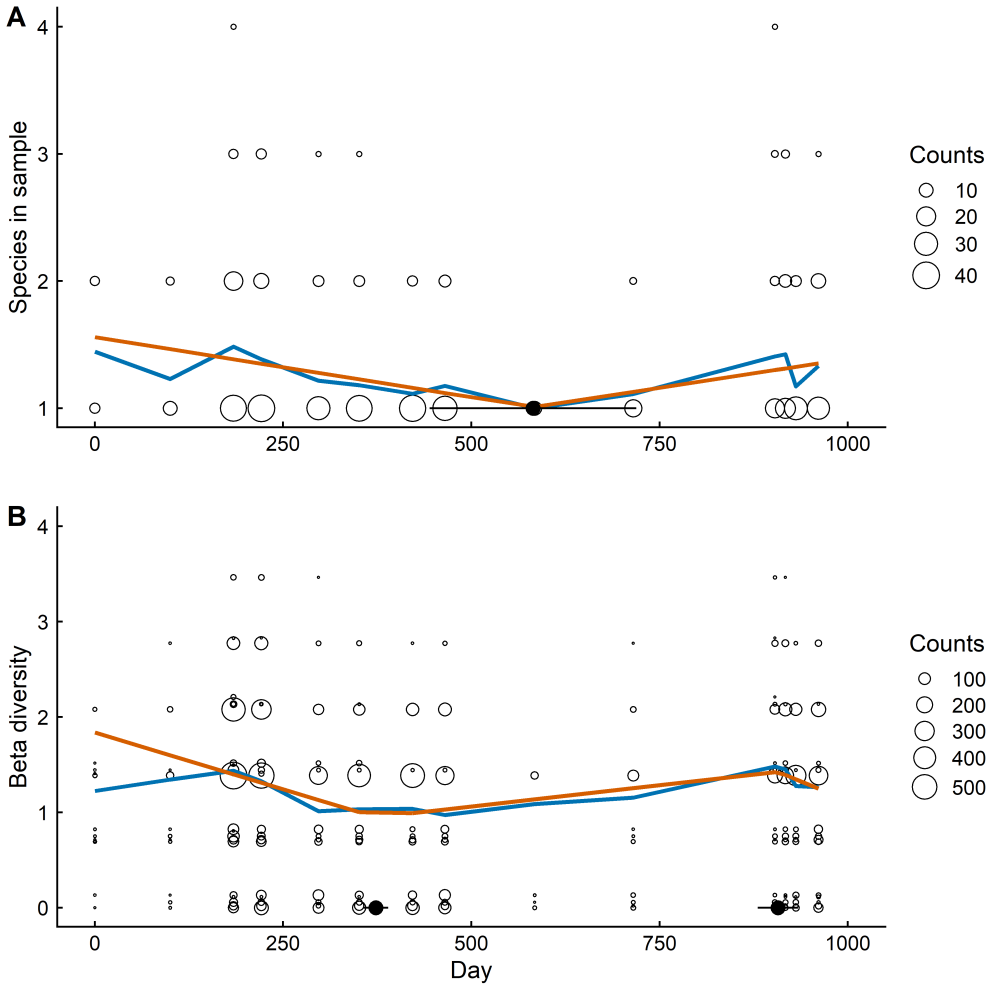

Figure III.12: Segmented regression analysis of individual-level *Bartonella* diversity measures. Points show the number of *Bartonella* species observed in an indvidual sample (A) and the binomial index of beta diversity (B; compared to all other bats in the colony) for each individual with the width proportional to the number of individuals with that same diversity value. For each measure, lines for the calculated mean trend (blue) and the predicted trend from segmented regression (orange) are drawn over the data points. Breakpoints and 95% confidence intervals estimated by segmented regression are shown above the x-axis.

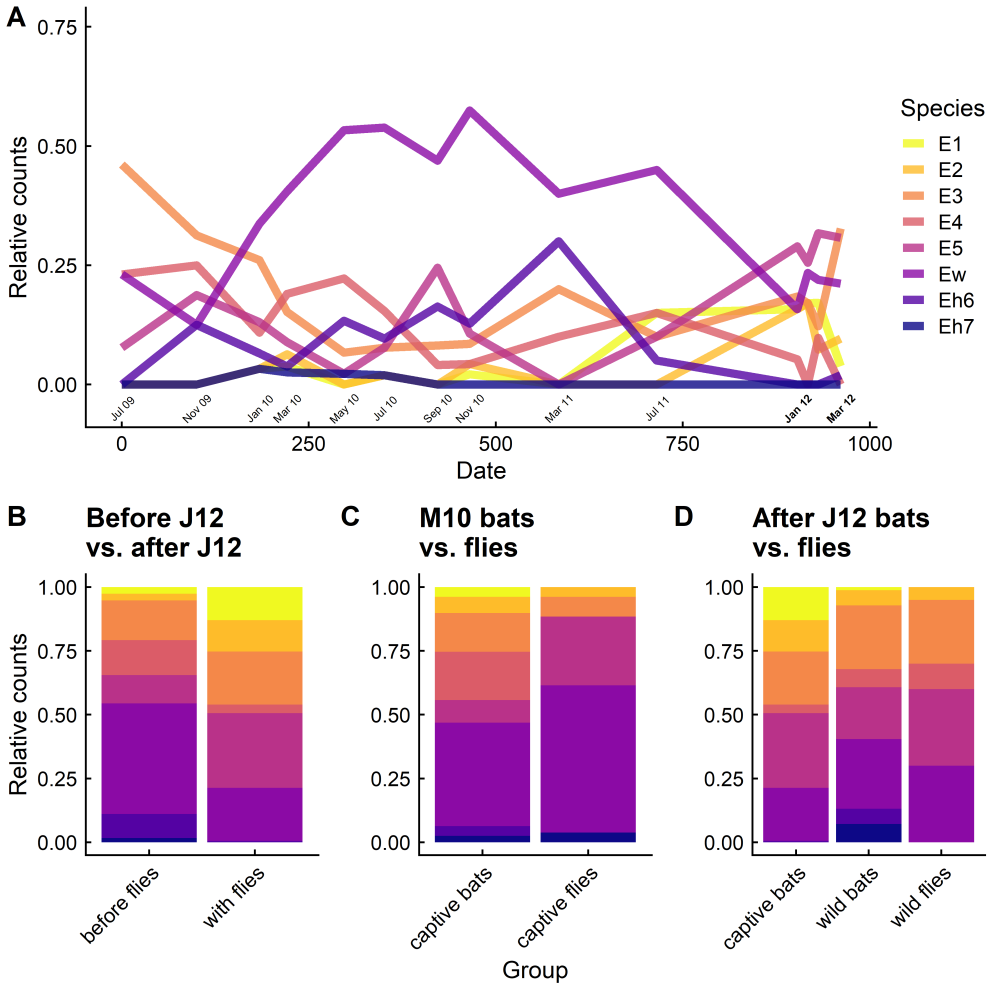

Figure III.13: Relative counts of *Bartonella* species in the captive colony over time (A–B) and between sampled bat flies and their respective bat populations (C–D). Relative counts (A) at each time point were estimated from presence *Bartonella* species based on any positive sequence from ITS, *gltA*, and *ftsZ*. Months labeled in bold font on the x-axis show the period after bat flies were reintroduced. Tests for differences in the relative counts of species were performed between bats in the captive colony before and after bat flies were reintroduced on 17 January 2012 (B); between bat flies sampled from the colony and the captive bat population in March 2010 (C); and between bat flies and wild bats sampled on 17 January 2012 and the captive colony population after flies were reintroduced (D).

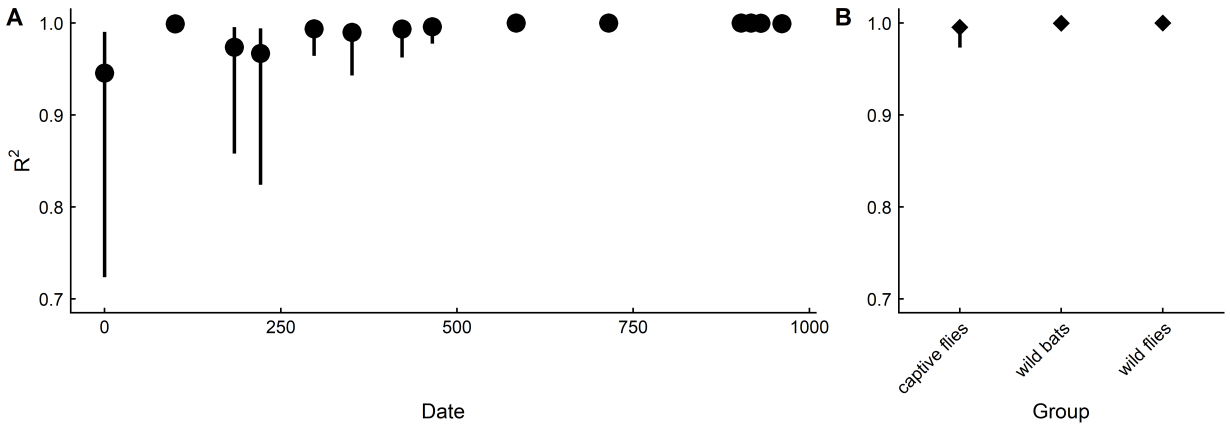

Figure III.14: Correlation between relative abundance and relative counts of *Bartonella* species. Pearson correlation values and 95% confidence intervals are shown for each time point in the experiment (A) and for bat flies sampled on M10, wild bats sampled on J12, and wild bat flies sampled on J12 (B).

Table III.6: Multinomial and binomial likelihood ratio (LR) tests of *Bartonella* species abundance changes between groups of sampled bats and bat flies: (A) tests between captive bats and captive flies in March 2010, (B) tests between wild bats and wild flies on 17 January 2012, and (C) tests between captive bats (aggregated over the period after flies were introduced) and sampled wild flies on 17 January 2012.

A									
Date	Group	E1	E2	E3	E4	E5	Ew	Eh6	Eh7
2010-03-06	captive bats	3	6	18	17	11	72	3	2
2010-03-06	captive flies	0	3	4	0	19	35	0	1
Bats total abundance		132							
Bats frequency		0.023	0.045	0.14	0.13	0.083	0.55	0.023	0.015
Expected abundance		1.4	2.8	8.5	8	5.2	33.8	1.4	0.9
Flies total abundance		62							
Flies frequency		0	0.048	0.065	0	0.31	0.56	0	0.016
Multinomial adjusted LR		43.7							
Multinomial P		2.5E-07							
Binomial adjusted LR		2.7	0.015	3.3	16.1	23.8	0.049	2.7	0.0049
Binomial P		0.10	0.9	0.069	6.1×10^{-5}	1.1×10^{-6}	0.82	0.1	0.94

B									
Date	Group	E1	E2	E3	E4	E5	Ew	Eh6	Eh7
2012-01-17	wild bats	1	5	21	6	17	23	5	6
2012-01-17	wild flies	0	1	5	3	13	14	0	0
Bats total abundance		84							
Bats frequency		0.012	0.06	0.25	0.071	0.20	0.27	0.06	0.071
Expected abundance		0.43	2.1	9	2.6	7.3	9.9	2.1	2.6
Flies total abundance		36							
Flies frequency		0	0.028	0.14	0.083	0.36	0.39	0	0
Multinomial adjusted LR		16.7							
Multinomial P		0.019							
Binomial adjusted LR		0.78	0.73	2.4	0.067	4.4	2	4	4.9
Binomial P		0.38	0.39	0.12	0.8	0.036	0.15	0.045	0.028

C									
Date	Group	E1	E2	E3	E4	E5	Ew	Eh6	Eh7
2012-01-17	captive bats	34	34	53	8	99	57	1	0
2012-01-17	wild flies	0	1	5	3	13	14	0	0
Bats total abundance		286							
Bats frequency		0.12	0.12	0.19	0.028	0.35	0.2	0.0035	
Expected abundance		4.3	4.3	6.7	1	12.5	7.2	0.13	
Flies total abundance		36							
Flies frequency		0	0.028	0.14	0.083	0.36	0.39	0	
Multinomial adjusted LR		16.3							
Multinomial P		0.012							
Binomial adjusted LR		7.2	3.1	0.44	2.1	0.028	5.4	0.2	
Binomial P		0.0073	0.076	0.51	0.15	0.87	0.02	0.66	

Table III.7: Multinomial and binomial likelihood ratio (LR) tests for changes in *Bartonella* relative species counts before and after the reintroduction of bat flies. The period before flies were reintroduced covers July 2009 to July 2011. The period after flies were reintroduced covers 17 January 2012 to March 2012.

Date	E1	E2	E3	E4	E5	Ew	Eh6	Eh7
2009-07-28	0	0	6	3	1	3	0	0
2009-11-05	0	0	5	4	3	2	2	0
2010-01-28	3	3	24	10	12	31	6	3
2010-03-06	3	5	12	15	7	32	3	2
2010-05-21	0	0	3	10	1	24	6	1
2010-07-14	1	1	4	8	4	28	5	1
2010-09-23	0	0	4	2	12	23	8	0
2010-11-05	1	2	4	2	5	27	6	0
2011-03-04	0	0	2	1	0	4	3	0
2011-07-13	3	0	2	3	2	9	1	0
2012-01-17	6	6	7	2	11	6	0	0
2012-01-31	8	8	8	0	12	11	0	0
2012-02-14	7	3	5	4	13	9	0	0
2012-03-15	2	5	17	0	16	11	1	0
Total counts	34	33	103	64	99	220	41	7
Sum total counts	601							
Before counts	11	11	66	58	47	183	40	7
Before total counts	423							
Before frequency	0.026	0.026	0.16	0.14	0.11	0.43	0.095	0.017
After counts	23	22	37	6	52	37	1	0
Expected counts	4.6	4.6	27.8	24.4	19.8	77	16.8	2.9
After total counts	178							
After frequency	0.13	0.12	0.21	0.034	0.29	0.21	0.0056	0
Multinomial adjusted LR	183.3							
Multinomial P	0							
Binomial adjusted LR	38.2	34.9	3.3	21.6	42.2	39	26.9	5.8
Binomial P	$6.4\text{x}10^{-10}$	$3.5\text{x}10^{-9}$	0.07	$3.3\text{x}10^{-6}$	$8.2\text{x}10^{-11}$	$4.2\text{x}10^{-10}$	$2.1\text{x}10^{-7}$	0.016