Symbiotic systems of bats, bat flies, and gut bacteria in a fragmented forest

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Statement of originality

I, Omar Khalilur Rahman, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Thesis abstract

The effects of habitat disturbance on parasites and microbes is poorly understood despite implications for host health and conservation. In this thesis I identify landscape properties that impact epidemiological patterns, bipartite interactions and metacommunity structure of ectoparasitic arthropods of bats and their endosymbiotic bacteria across a fragmented region from the Atlantic Forest of Brazil and I compare the performance of routine molecular identification methods using conventional laboratory procedures, to a mobile laboratory including the MinION DNA sequencer. My results show that *in situ* experiments on portable sequencing platforms are a viable alternative to Illumina and Sanger sequencing, although there are trade-offs between sequence quality and speed that precluded immediate uptake. I found the prevalence of the bat fly *Trichobius joblingi* on the bat *Carollia perspicillata* to be higher in forest fragments than continuous forests. I also found the infection intensity of the bat fly *Paratrichobius longricus* on the bat *Artibeus lituratus* in continuous forests to be higher in females than males. Network analyses shows modularity to be positively correlated to habitat area for ectoparasitic networks, but negatively correlated to isolation in endosymbiont networks. Metacommunity structure analysis suggests bat fly assemblages in fragmented forests follow a quasi-Gleasonean structure with respect to habitat area. Overall, my research reveals that the effects of habitat fragmentation can be observed in parasitic and endosymbiotic communities, and the persistence of specialist symbiotic species rely on the presence of specific hosts, for example ectoparasitic and endosymbiotic communities in the smallest fragments remain

connected by interactions with disturbance tolerant hosts. My findings have implications for conservation through monitoring trends in potential disease vectors in wild populations.

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Chapter 1:
Introduction

Fragmentation of natural habitats

Anthropogenic changes to the environment are causing lasting detrimental effects such as biodiversity loss and disruption to ecosystem services (McKinney, 2008; Memmott et al., 2007). Habitat fragmentation is considered a primary threat to natural landscapes and has been shown to cause declines in biodiversity (Coelho et al., 2020; Tilman et al., 2017). In simple terms, habitat fragmentation is the breaking apart of terrain, and an increase in the number of patches as a result (Curtis, 1956; Moore, 1962). This process typically involves the splitting of continuous natural environments into a larger number of smaller environments, which often includes habitat loss but distinctly also habitat reconfiguration (Fahrig, 2003). In addition to anthropogenic sources of habitat fragmentation such as logging and urbanisation (Hein et al., 2011), natural events including forest fires (Wright, 1974; Wright and Heinselman, 2014), lava flow from volcanoes (Vandergast et al., 2004), and flooding (Perotto-Baldivieso et al., 2011) result in communities of species becoming isolated and confined to smaller habitat patches.

Habitat fragmentation is often examined through the theory of island biogeography, which focuses on the size and isolation of habitat patches rather than the fragmented landscape as a whole (Brühl et al., 2003; Prugh et al., 2008). The theory of island biogeography is generally attributed to Kirkby et al. (1968), who observed that larger and less isolated oceanic islands had higher species richness than smaller and more

isolated ones. Similar patterns have been observed in other terrestrial (Charles and Ang, 2010) and aquatic systems (Miyazono and Taylor, 2013) where habitat fragmentation has taken place and where each patch represents a figurative 'island', and communities within each habitat are considered distinct from neighbouring ones. From here on I refer to these habitats in a fragmented landscape as patches.

Patch-based fragmentation studies tend to measure habitat area and isolation to infer community processes such as extinction risk and colonisation rates (Steffens and Lehman, 2018). Larger patches are able to support higher population numbers than smaller patches, decreasing the likelihood of extinction through stochastic events and inbreeding depression (Gilpin and Diamond, 1976; Hanski and Ovaskainen, 2000). Patches that are less isolated are able to replenish their population through species immigrating from the mainland or larger habitats (Brown and Kodric-Brown, 1977; Simberloff and Wilson, 1969). However, the benefits of larger patch size and smaller isolation are not independent and can intersect, for example large patches may experience higher immigration rates than smaller patches of equal degrees of isolation by virtue of being more visible colonization targets (Gilpin and Diamond, 1976).

Fragmentation study design

One criticism sometimes raised against patch based fragmentation studies is the difficulty in disentangling the effects of habitat fragmentation and habitat loss, to paraphrase Fahrig (2019), large patches have more habitat intact and patches become more isolated as more habitat is lost around them. Landscape based studies can generate observations of the effects of fragmentation *per se* by controlling for habitat when making comparisons. In one model for this MacDonald et al. (2018) used a

nested set sampling design, splitting 30 islands into groups of 1-8, and comparing groups with similar total habitat, whilst considering different configurations (e.g. number of islands in a group, inter-island distance). There are also long-term fragmentation experiments that are able to control for habitat area of affected patches alongside other factors such as climate change (Wilson et al., 2016), habitat corridors (Metatron; Legrand et al., 2012, Savannah River Site (SRS); Brinkerhoff et al., 2005), land use and cover patterns (Stability of Altered Forest Ecosystems (SAFE); Ewers et al., 2011). However, unlike experimental or curated circumstances, finding a fragmented landscape with the right configuration of patches to control for area may not be always feasible in natural settings.

A review of habitat fragmentation literature suggests that in recent years 45.7% of studies use individual patches as units of inference, 47% use patches and their surrounding matrix, and only 7.3% adopt the whole landscape approach though this is becoming more popular (Fardila et al., 2017). The choice of scale (e.g. landscape vs patch) may depend on the process or pattern of interest and species being studied, but should not be considered mutually exclusive methods. Thornton et al., (2011) reviewed species responses to patch and landscape level variables in birds, invertebrates, reptiles, and mammals; finding that some species were highly responsive of both patch-level variables (e.g. habitat area and habitat quality), but landscape-level variables (e.g. isolation) tended to be more important for large bodied species (i.e. mammals); nonetheless they stress that a multi-level approach (considering both patch and landscape level variables) is appropriate for many taxa. In the following chapters of this thesis, I use a combination of patch and landscape level metrics (e.g. area, isolation, habitat complexity) to describe several habitat patches

within a fragmented forest system, and I use the term ‘landscape metrics’ to refer to all variables collectively.

Habitat fragmentation, as discussed in this thesis, refers to a landscape-level process where historical changes in land use within the Atlantic Forest resulted in the division of continuous forest into smaller forest patches that are isolated from each other by a matrix of modified landscape (e.g., farmland, pastures, logged forest). I examine the long-term effects of habitat fragmentation on communities of bats and their ectoparasites and discuss the implication for host health and pathogen transmission within this natural community.

Parasite ecology

Parasitism is one of the most common ecological strategies found in nature, but is generally underrepresented in ecological community studies (e.g. food webs; Lafferty et al., 2008). Parasites inflict a cost to hosts they infect by stealing resources that could otherwise be used by the host for growth, maintenance, or reproduction (Poulin et al., 2002). Examples of parasitism can be seen across many taxa, but two non-exhaustive groups include ‘macroparasites’ that can be seen with the naked eye (e.g. insects; Spielman, 2017), worms; Elsasser et al., 2009), and ‘microparasites’ or parasitic microorganisms (e.g. bacteria; Breitschwerdt, 2014), viruses; Le Goff et al., 2011). Examining the host-parasite relationship when faced with ecological change continues to be a complex subject especially in the context of public health (Macnab and Barber, 2012; Thongsripong et al., 2018). For example, a mammal could be host to a parasitic arthropod that vectors a bacterial pathogen. In such a context, understanding the composite effects that ecological changes such as habitat fragmentation can inflict on

each trophic layer (i.e. animal host, parasite, and pathogen) allows us to predict the risks of disease emergence and persistence (Breitschwerdt, 2014; Wood et al., 2014). For many parasites, their exclusion from ecological studies can be attributed to the difficulty in accurately identifying them (Bower et al., 2019). Identifying parasites based on morphology (i.e. examining physical features) is a relatively inexpensive, but some groups of taxa have limited variance in morphological features, and some sister taxa can be indistinguishable through morphology alone (McManus and Bowles, 1996). Molecular approaches involving DNA sequencing (Perkins, 2000), and protein profiling (Müller et al., 2013) provide a more reliable form of identification that is able to discern between morphologically similar cryptic species. Unlike morphology, DNA-based methods avoid problems in species with complex life stages (Chibwana et al., 2015) or that exhibit phenotypic plasticity (Jaakola et al., 2015).

DNA barcoding

Species identification through DNA barcoding is based on the premise that intraspecific variation at a particular locus is smaller than interspecific variation, and those short genetic markers can then be used to differentiate species (Hebert et al., 2003). Barcoding projects to build reference databases typically involve gathering many specimens, identifying them through conventional methods such a morphology, and archiving their DNA barcodes alongside supplementary data such as locality and photographs (Hajibabaei et al., 2005). Organisations such as the Consortium for the Barcode of Life (CBOL; Costa and Carvalho, 2007) and the Barcode of Life Data Systems (BOLD Systems, 2013) provide platforms that aid in managing DNA barcode records globally. There are several genes that have been used for DNA barcoding, but for

animals, a 657-bp fragment at the 5' region of the mitochondrial gene cytochrome *c* oxidase (COI) has been adopted as a 'global standard' (Hajibabaei et al., 2007). Examples of popular barcoding regions in other taxa include the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (*rbcL* in plants and fungi; Hollingsworth et al., 2009), 16s-rDNA (rRNA in bacteria; de Oliveira Martins et al., 2020), and the internal transcribed spacer region (ITS in several taxa; Cheng et al., 2014; Kress et al., 2005; Seifert, 2009). Reasons for deviating from the 'global standard' COI barcode may include exceptional cases where COI sequences have not been produced robustly, or shown to be too divergent within species (Vences et al., 2005a), or in some groups such as plants, mitochondrial genomes have not evolved rapidly enough to differentiate species (Kress et al., 2005). DNA taxonomy is a related technique that can be used to assess diversity of samples when involving understudied taxa, which lack a robust DNA barcode database (Blaxter et al., 2004). The DNA barcoding workflow for identification begins by creating a reference library of 'barcodes', for a variety of species of interest. A barcode from an unidentified specimen can then be compared and a hypothesis about identity can be made based on highly similar sequences.

In the absence of a full reference library, barcodes can be compared to each other and patterns of inter and intra specific divergence can be used to identify likely species. Similar to DNA barcoding, this form of "DNA taxonomy" involves genetic markers with limited variation, but rather than relying on existing reference sequences for specimen identity, sequences are grouped together based on a defined threshold of variation, and designated molecular operational taxonomic units (MOTUs) rather than conventional Linnaean taxonomy (Floyd et al., 2002). Amplicon Sequence Variants

(ASVs) or Exact Sequence Variants (ESVs) are another alternative method for resolving sequence diversity, where each unique set of sequences is recognised as its own MOTU, effectively having haplotypes as units of diversity (Callahan et al., 2017). In these cases, the resulting MOTUs are not equivalent to 'species', and do not correspond to any other form of OTU (Blaxter, 2004) or taxonomic threshold but permit basic comparisons of diversity.

Throughout my thesis, several forms of DNA barcoding are used in conjunction with morphological keys. In chapter 2, I utilise the portable and compact MinION sequencer developed by Oxford Nanopore Technologies (ONT, Mikheyev and Tin, 2014) to adapt a routine DNA barcoding experiment and sequence COI barcodes from bats in Belize *in situ*. Unlike parallel platforms such as Sanger sequencing, the MinION does not require imaging equipment to detect nucleotides, and can be scaled down to a portable level (Kono and Arakawa, 2019). MinION devices are powered from USB (Universal Serial Bus) connections typically from laptop computers, allowing them to be deployed in remote locations (e.g. rainforests; Pomerantz et al., 2010, the Artic; Goordial et al., 2017; Space stations; Burton et al., 2020). *In situ* DNA sequencing pipelines accelerate the process of running molecular analysis tied to field work, by bypassing the need for transport and long-term preservation of organic material. Express and on demand DNA sequencing has also been applied in the diagnosis of pathogens during a disease outbreak (Hoenen et al., 2016; Wölfel et al., 2015). While this technology is still developing it is promising for eventual analysis on site. In Chapter 3 I employ both the conventional DNA barcoding of individual specimens, and DNA metabarcoding of more complex samples using lab-based processes. DNA metabarcoding combines DNA barcoding with high throughput sequencing to detect traces of multiple species in a

single mixed sample. It is as an efficient way to identify mixtures of unknown sources (e.g. detecting DNA of degraded or microscopic organisms from soil; Taberlet et al., 2012). I employed a metabarcoding approach for samples of cotton swabs retaining a mixture of parasites' early stages (e.g. juveniles) taken from the fur of bats in the Atlantic Forest, whereas the relatively larger adult stages could be isolated and sequenced individually via Sanger sequencing of DNA barcodes. Gut microbes from bat flies were also sequenced using a metabarcoding approach.

Networks

A central concern in conservation biology is the loss of biodiversity due to habitat fragmentation (Morris, 2010). Biodiversity is a measure of the variety of living organisms, typically referring to individual species within a community (van der Plas, 2019), but it can also encompass genomic variation within a population (Pollock, 2002). Additionally biodiversity can be extended to mean the number and types of interactions which species are engaged in within a community (Dyer et al., 2010). Species interactions, are often depicted as networks which depict predation (Kondoh, 2008), mutualism (Bascompte and Jordano, 2007), and parasitism (Marcogliese, 2004). Networks can be characterised by the number of links (interactions) each species has, and the combination of species united by links (Newman, 2003). In chapter 4 of this thesis, I use network theory to compare the structure of host-symbiont interactions involving ectoparasites and bacterial endosymbionts across fragmented habitat patches. Each isolated habitat patch contains separate communities of interacting species, and I compare how the distribution of interactions in these host-symbiont networks relate to patch-level habitat variables (e.g. area and isolation). I describe the

network topology using connectance, nestedness, and modularity (see chapter 4 for calculation methods). Connectance is an inverse measure of specialisation (De Araújo et al., 2015), in that when comparing between different networks, a highly connected network has lost specialists or acquired generalists (Heleno et al., 2012). Modularity relates to the presence of compartments or modules, where a group of species tend to interact more with each other (ingroup) than species outside of the compartment (outgroup) (Dormann et al., 2009). Nestedness is a measure of redundancy in the network, where the interactions experienced by species with fewer links are a complete subset of interactions experienced by species with more links (Bascompte et al., 2003).

Metacommunity

Each habitat patch resulting from fragmentation can represent a distinct local community linked to other such patches by dispersal (HANSKI and GILPIN, 1991), sometimes referred to as a metacommunity (Leibold et al., 2004). One ambiguity in this definition is the nature of the discrete boundaries that constitutes a 'local community' and Leibold et al. (2004) posits at least three broad types of metacommunities have been considered within this framework: (1) discrete, permanent habitat patches (e.g. oceanic islands forming terrestrial metacommunities, pond and lakes separated by land form aquatic metacommunities (Mehranvar and Jackson, 2001), (2) temporary habitat, distinct from external matrix, but varying in position and frequency (e.g. pitcher plants rely on dispersal to maintain metacommunity of insects and bacteria; (Kneitel and Miller, 2003), (3) permanent habitat with ambiguous boundaries due to a large variance in dispersal ability among species (e.g. invertebrates and fish in coral reefs have different dispersal ability, and

can have different boundaries for a 'local community'; Alzin et al., 2009). Heino et al., (2015a) points out a critique that some studies make comparisons between metacommunities with vastly different dispersal abilities (e.g. diatoms versus insects versus fish), when the limits of a local community for a species with high dispersal may be the same as the limits for a metacommunity for a species with low dispersal ability.

Leibold et al., (2004) mentions four theoretical models of how metacommunities might form: (1) the *neutral perspective* where the environment has no bearing on community composition and instead only community processes such as random migration, extinction, and immigration contributes to species demographics, resulting in community similarity decreasing as patch distance increases (Rosindell et al., 2011); (2) *patch dynamics* involves a coloniser-competitor trade-off, where better colonisers dominate more isolated patches, but better competitors dominate less isolated patches (Holyoak et al., 2005); (3) in *species sorting*, biotic interactions and abiotic environmental conditions filter out unsuitable species at each patch (Soininen, 2014); (4) and with *mass effects* species may persist in patches with suboptimal conditions if their dispersal rates from source patches are high enough (Shmida and Wilson, 1985). However it has been argued that these theoretical mechanisms are not mutually exclusive and researchers should instead focus on the relative roles of biotic interactions, environmental conditions and dispersal in determining the distribution of communities (Winegardner et al., 2012).

In chapter 5 of this thesis, I use the Elements of Metacommunity Structure (Leibold and Mikkelson, 2002) to describe the distribution of species in a metacommunity. Leibold and Mikkelson, (2002) proposed that when a metacommunity is presented as a

site-by-species matrix (presence/absence), three aspects of metacommunity structure can be observed: coherence, turnover, and boundary clumping (see chapter 5 for details on accompanying statistics for EMS analysis). A species range is considered to be completely coherent when, in a presence/absence matrix, the species is present in all sites (Figure 1.1), absences found towards extremes in either rows or columns are termed embedded absences and counting these interruptions in species ranges provides a measure of 'coherence' of the metacommunity. Species turnover is the frequency of one species replacing another across the array of sites, for each possible site-species pair, and a metacommunity exhibiting no turnover is nested, where species richness at less diverse sites are complete subsets of more diverse sites (Figure 1.2). Boundary clumping measures the extent to which species ranges are clustered together (Figure 1.3). The three aspects examined by EMS are applied to judge whether the metacommunity expressed by the matrix fits an idealized metacommunity pattern (Leibold and Mikkelson, 2002). For example, a matrix that is coherent, experiences high turnover, and has clumped boundaries is said to exhibit a Clementsian structure. This is based on ideas by Clements, (2012) where species with shared evolutionary history also share similar tolerances and ecological requirements, and experiences turnover together along a common gradient (Figure 1.3a). Whereas a matrix that is coherent, experiences high turnover, but does not exhibit clumped boundaries has a Gleasonean structure. This is derived from Gleason, (1926) description of a community where species show individual responses to environmental gradients, but still share similarities in tolerances leading to more continuous gradient of species distribution than observed in a Clementsian metacommunity (Mihaljevic et al., 2018). Some of the idealised patterns obtained through EMS can be related back

to three of the four models on the formation of metacommunities mentioned by Leibold et al., (2004): patch dynamics, neutral perspective, and species sorting (Figure 1.4). Meynard et al., (2013) complemented EMS analysis with variance partitioning to separate the importance of environmental and spatial factors in structuring metacommunities and detect effects of dispersal (mass effects model). EMS analysis was chosen for my analysis as it only requires a presence-absence data type, and does not make assumptions on which factors, be they environmental or spatial, are driving metacommunity structure.

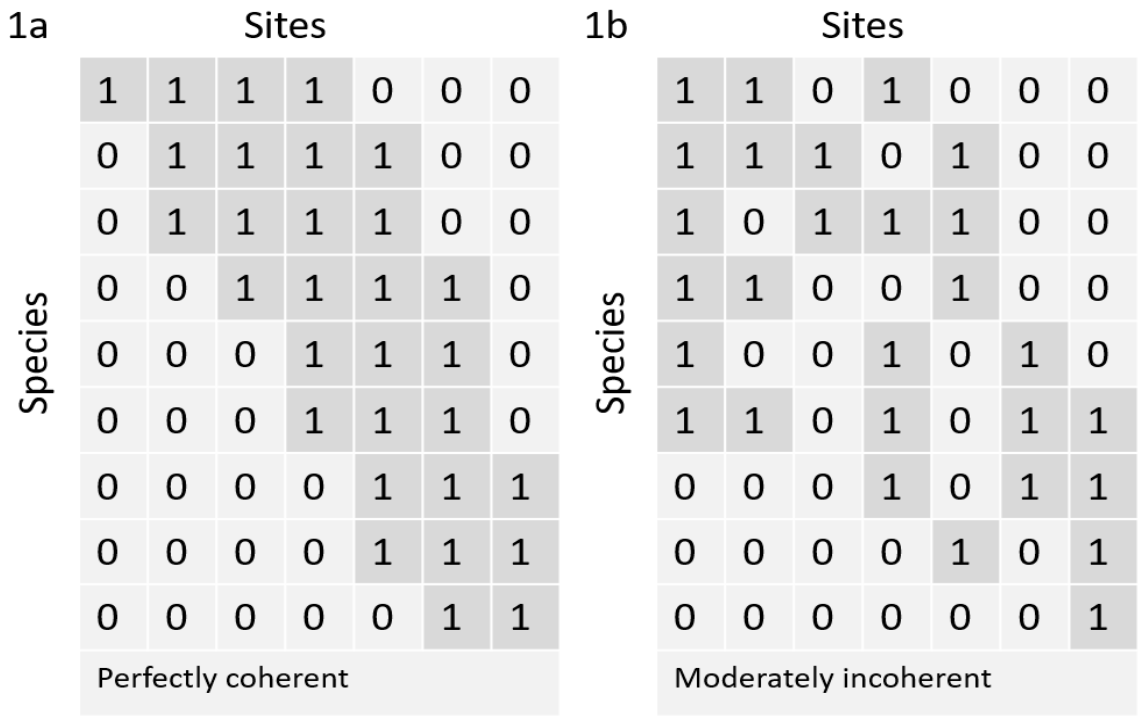


Figure 1.1: Metacommunity coherence as indicated by the occurrence of embedded absences. Entries denote presence (1) or absence (0) of species (rows) at sites (columns). The perfectly coherent metacommunity has zero embedded absences (1a), the moderately incoherent metacommunity has 12 embedded absences (1b).

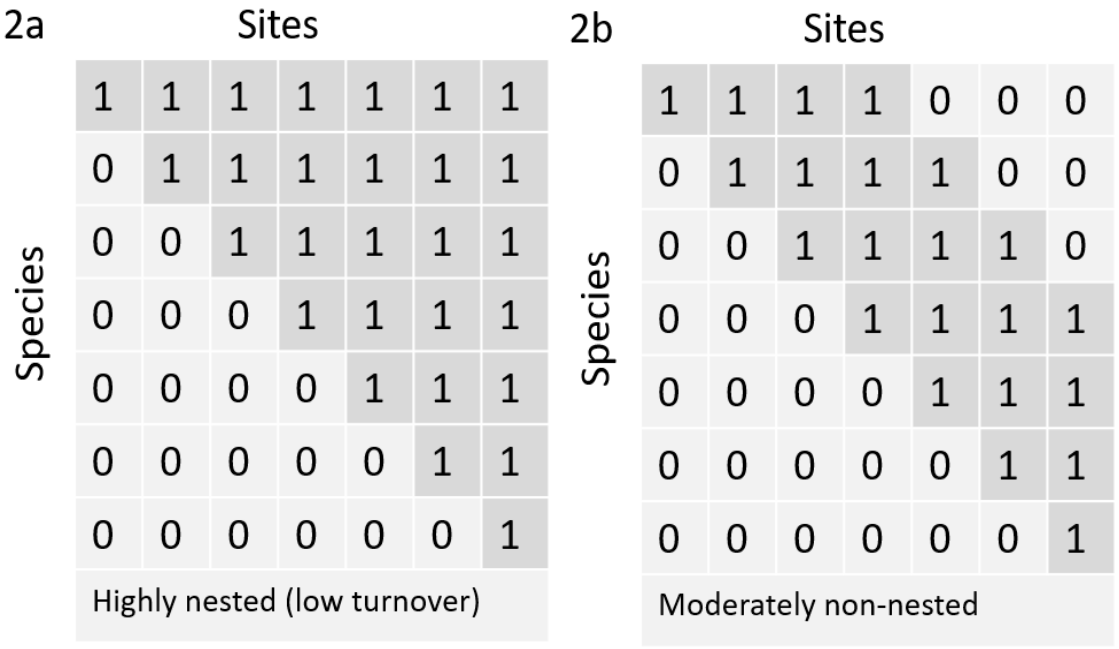


Figure 1.2: Species turnover as indicated by the number of times species are replaced between two sites. Entries denote presence (1) or absence (0) of species (rows) at sites (columns). A highly nested metacommunity experiences no turnover (2a; only either gains/losses species), a moderately non-nested metacommunity both gains and losses species between sites (2b).

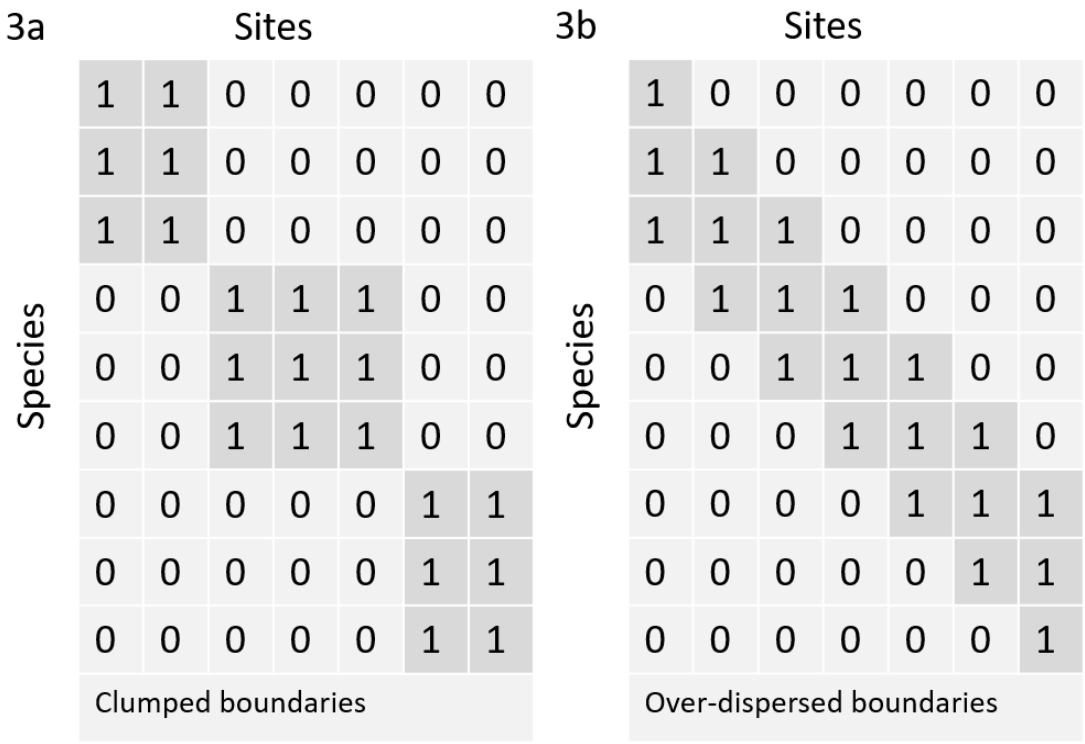


Figure 1.3: Clumped boundaries characteristic of Clementsian distributions (3a), and over-dispersed boundaries characteristic of Gleasonean distributions (3b)

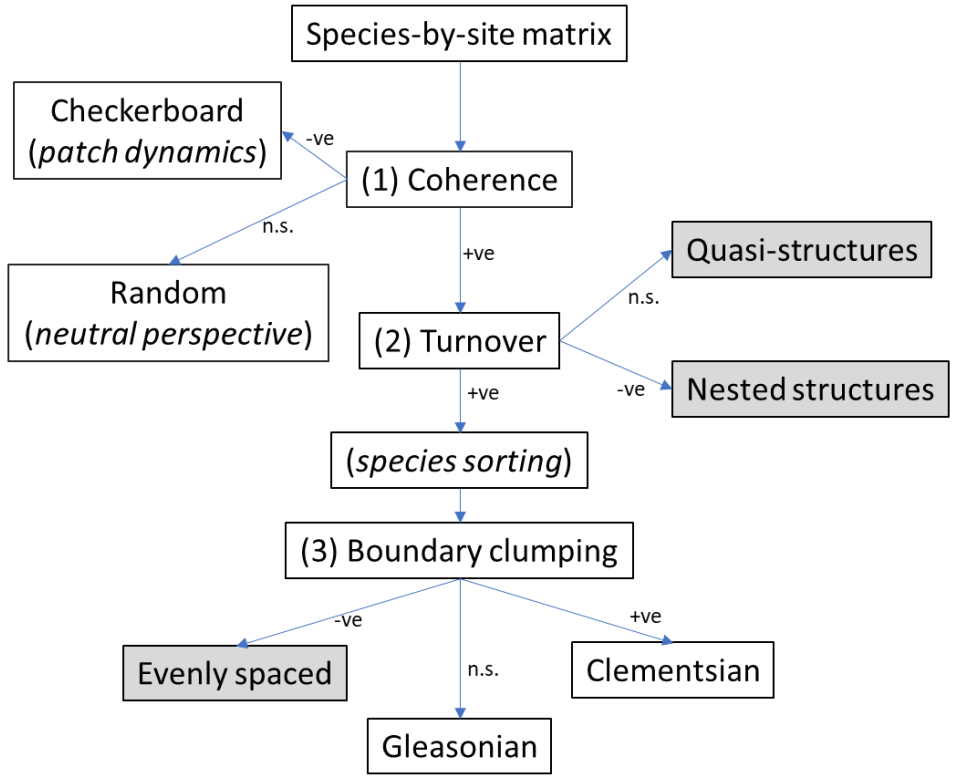


Figure 1.4: Idealised metacommunity structures and possible causes: neutral perspective (random/non-significant coherence), patch dynamics (checkerboard/negative coherence), species sorting (coherent with positive turnover). Structures not clearly linked to the four models of metacommunity formation are in grey boxes. n.s., non-significant.
Figure adapted from Meynard et al., (2013)

The Atlantic Forest of Brazil

The data from my thesis are primarily collected from the Atlantic Forest of Brazil. The Atlantic Forest stretches across the coast of Brazil from the northern state of Rio Grande do Norte to the southern state of Rio Grande do Sul (Figure 1.5), and contains high biodiversity and endemism across its tropical and subtropical biomes (da Fonseca, 1985). The Atlantic Forest was once the largest rainforest in the Americas and originally covered 150 million ha of land (Ribeiro et al., 2009). Since the European colonization and massive deforestation activity over the intervening 500 years, estimates for remaining forest cover now range from 7.6% of original primary forest (Morellato and Haddad, 2000) to 28% when including secondary, successive and edge-affected forests (Rezende et al., 2018) distributed as archipelagos of small, isolated forest fragments (<100 ha; Ranta et al., 1998) embedded within a matrix of pastures and monospecific agricultural fields (Ribeiro et al., 2009). Within the past decade, the Atlantic Forest continues to be an important economic engine for Brazil, contributing 70% of global domestic product and 2/3 of the industrial economy (Martinelli and Moraes, 2013). The Atlantic Forest meets the criteria for a biodiversity hotspot and conservation target as it has a high number of endemic plants (i.e. at least 1500 species; Martini et al., 2007), and is threatened by habitat loss (i.e. has lost >70% of primary vegetation; Myers et al., 2000), but despite anti-deforestation policies put in place in 2006, habitat continues to be cut down (SOS Mata Atlântica and INPE, 2019). Current projects to rejuvenate the Atlantic Forest include reforestation (Rodrigues et al., 2011), reintroducing wild populations of fauna (Galetti et al., 2017), and connecting fragments through habitat corridors (Lees and Peres, 2008). The long history of disturbance makes the Atlantic Forest an ideal candidate to model the chronic effects

of human activity and land use change (Izqulerdo et al., 2008; Lira et al., 2012a), habitat loss and fragmentation (Lôbo et al., 2011; Tabanez and Viana, 2000), and climate change (Carnaval and Moritz, 2008).

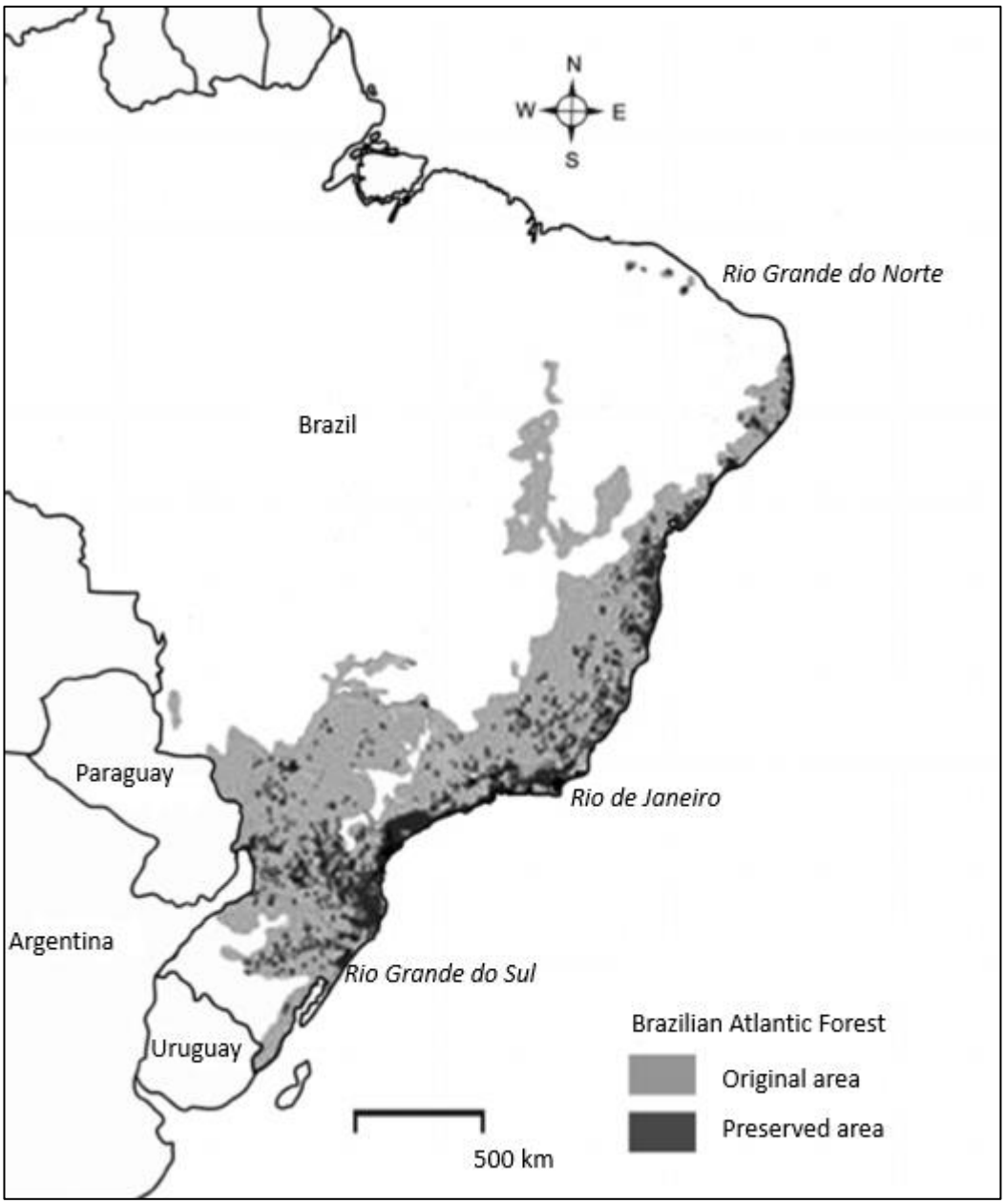


Figure 1.5: Distribution of original (light grey) and preserved forest (dark grey) in the Atlantic Forest*, figure adapted from Thompson et al., (2011).*

Bats and their parasites as a model system

In this thesis I focus on a model multi-trophic level system of bats, their parasites and the microbes living in the parasites. Bats (order Chiroptera) are nocturnal mammals present in all biomes except for the Artic, Antarctic, and a few oceanic islands (Mehlhorn, 2014). Bats are the only mammals capable of powered flight, and are found in a range of sizes, some large species in the new world can have forearm length >106 mm (e.g. *Vampyrum spectrum;* Peterson and Kirmse, 1969), while smaller species can be found with forearms <30 mm (e.g. *Rhinophylla pumilio;* Rinehart and Kunz, 2006), where forearm is a reasonable proxy for body size. Globally the size range covers orders of magnitude from giant pteropodids to tiny bumble bee bats in Asia. Bats vary greatly in terms of sociality, some species of bats are solitary or form small groups or harems (Chruszcz and Barclay, 2002; Reckardt and Kerth, 2009a), while others form colonies of several million individuals (Betke et al., 2008). Colony sizes within the same species can also vary greatly (e.g. from 12 to 803 individuals per colony in *Myotis*; (Zahn, 1999).

Generally, society has an ambivalent view of bats (Ghanem and Voigt, 2012). Bats feature prominently in cultures around the world. Some beliefs surrounding bats include bringing good fortune and happiness (e.g. in China; Li, 2010), being a bad omen (Suwannarong et al., 2020) and symbols of death and rebirth (e.g. in Madagascar; (Asante et al., 2013). Bats provide great utility to humans as they pollinate crops (Tremlett et al., 2020), and control insect pest populations (Wordley et al., 2014). Conversely, some bats are infamous for causing damage to fruit crops, by biting pieces of fruit or eating them outright (Korine et al., 1999), although more study is needed to evaluate the actual damage caused by bats rather than incidental fruit loss (Tollington

et al., 2019). Three species in South and Central America are famously known for drinking the blood of other animals. The common vampire bat (*Desmodus rotundus*) for example is notorious for grazing on mammals, and their prey suffer from bite wounds, loss of blood, and may become infected by viruses introduced during feeding (Mehlhorn, 2014).

Bats are a major focus in public health for their ability to vector pathogens, which is especially important to consider in agricultural landscapes with an increase in human-bat contact (Leroy et al., 2005; Wong et al., 2007). Besides getting an infection through biting, passive forms of infections can occur through aerosol viral particles derived from the urine and faeces of bats (Kim *et al.*, 2016), and from traces of saliva left on bitten and half-eaten fruit (Hughes et al., 2006). Bats roosting near or in human settlements poses a risk in transmitting pathogens to livestock, pets, and humans (Delpietro et al., 2001; Williamson et al., 1998). Certain features of bats are thought to contribute to their efficacy as pathogen carriers and spreaders, such as their propensity to aggregate into large colonies and occasionally switch roosts (D'Auria et al., 2010; Lourenço and Palmeirim, 2007). Some species are thought to suppress immune responses during hibernation (Bouma et al., 2010; Lučan et al., 2016a), and their diverse feeding ecology allows opportunities to traverse and come into contact with many other animals (Han et al., 2015).

Identifying changes in general patterns of immune response due to habitat change can be difficult because of the variation caused by differences in landscape structure and matrix surrounding the habitat, especially in a taxonomic group such as bats, that feature a wide range of dispersal abilities and home ranges (Voigt *et al*., 2016). The

ability to fly can give bats access to a range a habitats and mitigate the risks due to habitat change and fragmentation (Fenton, 1997). However, their dispersal ability can be dependent on the type of matrix surrounding the habitat, as well as the connectivity of neighbouring habitat (Brändel et al., 2020), and so they can still be at risk from even small changes in habitat and landscape (Meyer et al., 2009). For example, an aquatic landscape surrounding the habitat has shown to heavily restrict bat dispersal, even for those commonly regarded as disturbance tolerant (e.g., *Carollia, Dermanura*) (Meyer *et al.*, 2016). Brändel et al., (2020) found that forest fragments surrounded by agricultural landscapes could support high local bat diversity due to an abundance of food and roosting resources, but agricultural areas with low crop diversity (e.g., monocultures) reduced the connectivity of surrounding fragments producing a dispersal response more similar to island systems.

A review of published home range data for bats suggests that responses to disturbance such as habitat loss are highly dependent on the species and habitat type, rather than physical traits such as body size (Bernard and Fenton, 2003). The differences in mobility between species can be detected through differences in genetic structure across a fragmented landscape, where populations of less mobile bat species are more structured (i.e. genetically distinct) than more mobile bat species with show a more homogenous metapopulation (Meyer et al., 2009). In fragmented landscapes, the distance between suitable roosts and foraging has direct implications for energetic costs of daily activities and dispersal (Estrada et al., 1993). The absence of certain bat species in patches may point to that patch having insufficient resources to maintain a population, or immigration of new populations being restricted due to behavioural

(e.g., aversion to open spaces) or ecological (e.g., isolation) processes (Cosson et al., 1999).

Radiotelemetry data from several species of neotropical bats suggests home ranges vary between 65 to 530 ha, with some species limiting activity to 500 m from roosts while a few species travel larger distances (Bernard and Fenton, 2003). Home ranges for bats sampled for this thesis was not measured directly, however published studies have included home ranges for the common species examined in this thesis including:

Carollia perspicillata with ranges between of 5.5 - 32 ha and 489- 500 m nightly commuting distance (Bonaccorso et al., 2007; Trevelin et al., 2013), *Artibeus lituratus* with ranges between 124.4 ha and 1158.8 m commuting distance (Trevelin et al., 2013), *Glossophaga soricina* with ranges 430-890 ha and 500 m – 3 km commuting distance (Aguiar et al., 2014), *Desmodus rotundus* with ranges of 35 ha (Wilkinson, 1985), and *Sturnira lilium* with ranges of 36-90 ha with 50 m- 1.4 km commuting distance (Loayza and Loiselle, 2008). Some bats undertake long-range flights for migration outside of home ranges (e.g. *C. perspicillata* for about 2.5 km at a time; Bernard and Fenton, 2003, and *A. lituratus* for about 20-35 km at a time; Arnone et al., 2016), but these are less frequent. This suggests a highly variable use of landscape with nightly movements sometimes restricted to only tens of meters in some species but many kilometres in others.

Bats and disease reservoirs

A review by López-Baucells et al., (2018a) suggests 51% of virological studies (from 1936 to 2016) frame bats as a public health concern while only 4% simultaneously discuss the important ecosystem services bats provide. Tuttle, (2017) points to the

discovery of SARS coronavirus in 2002 causing a spike in negative sentiment towards bats in the past decade. The more recently discovered strain of coronavirus SARS-CoV-2 (COVID-19) around December 2019 continues to fuel public fear of bats as sequence analysis suggests they are likely key reservoirs for SARS-CoV-2 or its precursor (Shereen et al., 2020), although alternative zoonotic origins in other animals are being investigated (Hamid et al., 2020) and an intermediate vector is likely.

Attempts to limit the spread of viruses typically involved targeted destruction of bat roosting sites (O'Shea et al., 2016), although it has been shown that vaccination programs targeting livestock (Sohi et al., 2020), pets (Wera et al., 2017), and wild bat populations (Stading et al., 2017) are more effective at reducing the risk of viruses spilling over to humans. As a case in point, (Streicker et al., 2012) reported that attempts at culling *Desmodus rotundus* to reduce rabies infection not only failed to eliminate the virus in the area but inadvertently increased its prevalence in disturbed colonies in comparison to undisturbed ones. In general, while bats are notorious for collectively harbouring more kinds of zoonotic pathogens than other mammals (Ranjan et al., 2016), this is likely due to the sheer diversity (species richness) represented by bats overall (Brook and Dobson, 2015). Bats also tend to be resistant to viral infections (Brook et al., 2020), with some infections not pathogenic to bats themselves (Leroy et al., 2005).

Parasite persistence in host communities involves individual to landscape-level factors (Wilber et al., 2020). Habitat fragmentation as a consequence of abiotic or biotic factors can affect parasite persistence, for example by changing local host densities, or altering the frequency of transmission among hosts (Greer and Collins, 2008). Habitat

fragments are likely to be degraded due to higher competition for reduced resources, increasing stress and susceptibility to disease among individual hosts (Lafferty and Gerber, 2002). Reduced population sizes and migration induces genetic erosion and reduces disease resistance through increased inbreeding (Belasen et al., 2019; Lyles and Dobson, 1993). At the landscape level, the connectivity of habitat fragments can influence the likelihood of parasite invasion as well as the colonisation of hosts into habitat (Renwick and Lambin, 2013; Wilber et al., 2020).

Changes in land use associated with habitat fragmentation, such as urbanisation and agricultural activity between patches increases risk of disease emergence and zoonoses from increased contact among wildlife, humans, and livestock (Morand et al., 2019). Parasite richness has been shown to decrease with habitat disturbance, likely due to the host species they depend on becoming scarce in degraded patches (Wilkinson et al., 2018), however some parasites (e.g. hookworm, malaria, scrub typhus; Shah et al., 2019) benefit from human altered landscapes due to synergistic effects (e.g. irrigation, increased agricultural cover) that either benefit their transmission or their hosts survival (Hanski, 1997). Predicting effects of landscape change on parasite persistence and abundance must take their life history into consideration. For highly host-specific parasites, environmental effects on parasite abundance are more likely to be attributed to changes in local densities of their hosts, especially if they do not spend an extensive amount of time off-host (Merino and Potti, 1996; Pilosof et al., 2012a) .

Dynamics of infectious diseases is dependent on the distribution of vectors and the presence of viable hosts (Jones et al., 2011), and spillover risks worsens when

landscape modification increase contact rates between reservoir and domestic hosts (Martin et al., 2015). Ecological interventions can involve disrupting the interface between wildlife and humans or domestic animals, and include medical approaches such as disinfection and vaccination (Sokolow et al., 2019), or physical barriers such as using bed nets to prevent mosquitoes from spreading malaria (Martin et al., 2015) and curbing bat-to-human transmission of viruses by restricting their access to shared food (Salah Uddin Khan et al., 2010). Understanding how hosts navigate their environment in response to landscape change such as habitat fragmentation can help better allocate resources to managing disease spread by deploying interventions towards regions at greatest risk (White et al., 2018). Combining spatial elements associated with disease risk and geographic datasets can improve the accuracy of predictive disease risk modelling (Hay et al., 2004), on condition that datasets include sufficient spatial heterogeneity to account for variation in the ecological conditions that drive disease risk (Wimberly et al., 2008). This approach has allowed more understanding of the precise processes and factors that modulate disease dynamics (e.g. edge effects; Jones et al., 2011, forest density; Gillespie and Chapman, 2006), rather than broad effects of habitat disturbance.

Parasite specificity

Blood-feeding arthropods are important vectors of pathogens that commonly feed on humans and animals, and in the process can pick up infectious agents and pass these on to other hosts during subsequent feedings (Marcondes, 2016). Many haematophagous arthropods have adaptations to be more effective parasites such as flattened bodies that facilitate movement and hiding, or strong claws allowing a better grip to avoid detachment from the host (Burkett-Cadena, 2019). Some parasites can be

behaviourally, morphologically, or physiologically adapted to a few or a single host species such that they cannot survive on a novel host (Bush and Clayton, 2006). Host specificity measures the degree to which a particular parasite species is limited to a particular host species (Poulin, 2010). Terms to describe host specificity used by (Wenzel, 1975) include 'monoxenous' to mean a single host species, 'stenoxenous' to mean a range of congeneric hosts, 'oligoxenous' to mean hosts within the same family, and 'polyxenous' to mean a wide range of unrelated hosts. Host specificity is typically dictated by the host-parasite evolutionary relationship (Bruyndonckx et al., 2009; Poulin, 2010), and other ecological factors such as the parasite's life history (Bellay et al., 2013), host mobility, and social structure (Dick and Patterson, 2007a; Poulin et al., 2011). In this thesis I focus on two highly specialised blood-feeding parasites that are exclusively found on bats: bat flies and wing mites.

Bat flies and wing mites

Bat flies (Diptera: Hippoboscoidea consist of two families: the monophyletic Nycteribiidae and the paraphyletic Streblidae (Dittmar et al., 2006). Bat flies live in the fur and on the wing membrane of bats, feeding exclusively on the host's blood. The bat fly life cycle begins with the egg being fertilized within the female, where the resulting larva continues to develop up to the third instar stage, which then the female bat fly deposits. The larva pupates and when the adult emerges it then seeks a host (Marshall, 1970). They are obligate ectoparasites and the adult forms can only survive without a host for a few hours (Fritz, 1983). However, there are two periods within the bat fly life cycle that feature obligatory decoupling from the host: firstly this involves gravid female bat flies that leave the host bat briefly to deposit prepupae larvae onto the roost substrate, and secondly the non-motile pupal stages continue to develop while

attached to roost substrate (Dick and Dittmar, 2014). For obligatory parasites such as bat flies, the host is often considered to be the "habitat" for the parasite; bat flies being sensitive to host variables such as species and host size, and also responding to the number of conspecifics on potential hosts (Dick and Dick, 2006). However, given that bat flies undergo pupation on roost substrate (that can last a few weeks; Fritz, 1983), the type of roost that bats utilise can affect the quantity and quality of bat flies, and thus the bat fly's capacity to act as vectors (Dick and Dittmar, 2014). Bat flies are permanent parasites and cannot survive prolonged periods without a host (Overal, 1980). Only female bat flies are required to leave the host to deposit prepupae, and as mating occurs on the host, males do not normally have to leave the host at all (Dick and Patterson, 2007b). Among bat flies (streblids and nycteribids), only 78% of streblids have developed wings capable of short and rapid flight (Kunz, 2013). Streblids use flight as a means to reach a nearby host (either from an adjacent bat, or roost substrate), and a means to escape from a distressed host (Whitaker et al., 1988). Experiments observing bat fly *Trichobius major* suggests that positive host seeking stimuli include heat, and carbon dioxide, whereby the parasites will walk and hop towards the source, rather than rely on flight to close the distance; and in the absence of positive stimuli, they remained stationary cleaning themselves until positive stimuli were introduced (Caire et al.,1985). Dispersal and distribution of bat flies is entirely dependent on the travel and roosting behaviour of their respective bat hosts (Bolívar-Cimé et al., 2018a), and the decoupling of prepupae from the host onto roost substrate allows bat flies to have high inter-colony transmissibility as bat colonies switch and rotate roosting sites (Reckardt and Kerth, 2009b).

Wing mites (Acari: Mesostigmata) belong to the family Spinturnicidae, and are generally found on the wing and tail membranes of bats (Rudnick, 1960). The life cycle of wing mites consists of 5 stages: egg, larva, protonymph, deutonymph, and adult;

where the egg and larval stages develop within the female mite, which gives birth directly to a protonymph on the bat host. Protonymphs, deutonymphs, and adults are then dependent on the bat host for blood meals, each stage separated by a moulting stage; the total duration for a standard life cycle is not known (Colín-Martínez and García-Estrada, 2016; Rudnick, 1960). As obligate blood-feeding parasites, both bat flies and wing mites are regarded as excellent candidates for vectoring pathogens. Wing mites are possible mechanical vectors for the White Nose Syndrome fungus *Pseudogymnoascus destructans*, that increases mortality rates in bats during hibernation (Lučan et al., 2016b). Evidence of *Bartonella sp.* and *Rickettsia sp.* infection in bat flies (Do Amaral et al., 2018) and wing mites (Szubert-Kruszyńska et al., 2019) strongly suggests these endosymbionts can be shared between the ectoparasites and their bat hosts, however their pathogenicity in the ectoparasites or the capacity for the ectoparasites to act as vectors in not currently known.

The high level of host specialisation exhibited by bat flies are thought to lead bat fly communities to mirror the variation exhibited by host bat communities. For example, bat roosting dynamics and crowding in degraded habitats was observed to modulate parasite prevalence and intensity of infestations as well as promote novel host-parasite interactions (Cottontail et al., 2009a; Hernández-Martínez et al., 2019a). Environmental variables such as temperature and humidity in bat roosts are thought to directly affect bat fly development and mortality rates (Pilosof et al., 2012a). Bolívar-Cimé et al., (2018a) found that relative prevalence of two species of bat flies on cave-dwelling *Desmodus rotundus* differed between fragmented and continuous forests, postulating that the two ectoparasites had contrasting tolerances to temperature and humidity in roosts. Bat-wing mite infestations are not known to be affected by

environmental factors within the bat roost (Sheeler-Gordon and Owen, 1999), and their distribution is thought to only depend on the presence and well-being of their hosts (Lourenço et al., 2016; Reckardt and Kerth, 2009b).

Thesis organisation and aims

In this thesis, I investigate the multi-trophic level metacommunity structure of bats, their parasites and parasite microbes. I employ a combination of metacommunity and site-level techniques to show how host-parasite interactions change when faced with varying degrees of habitat fragmentation. Chapter 2 introduces technology and methods for performing DNA barcoding *in situ*, where I tested a routine procedure to barcode COI from neotropical bats while in a remote field location. I discuss the benefits of novel technologies and the convenience of *in situ* DNA amplification and sequencing when compared to conventional lab-based methods but opted to use the later for the remaining studies in this thesis. Chapters 3 to 5 investigates the metacommunity of bats and their ectoparasites in forest fragments in the Atlantic Forest. In chapter 3, I describe the infection of bat flies using epidemiological statistics and compare communities in fragmented and continuous forests. I initially analysed both bat flies and wing mites and opted to use the bat fly data on further analyses based on the excellent taxonomic resolution obtained. Chapters 4 and 5 pertain to both bat-bat fly and bat fly-endosymbiont interactions. In chapter 4, I use bipartite network analysis to show how the topology of host-symbiont interactions changes with varying landscape measures. I also show that some taxa play a key role in connecting different parts of the network. In chapter 5 I use EMS to describe the best fit pattern shown by the bat fly and endosymbiont metacommunities, as well as subsequent modelling techniques to suggest which landscape property best

determines metacommunity patterns. I found the distribution of bat flies and endosymbionts both appear to be compartmentalised (e.g. the bat fly metacommunity in fragmented sites were distinct from ones in continuous forest sites) and that habitat area was a likely factor in their metacommunity structure. The Clementsian and Gleasonean structures found suggest that species sorting (filtering based on environmental conditions) limited the presence of certain species. I discuss the implications of my thesis in chapter 6, as well as provide suggestions to supplement future work involving symbiont metacommunities.

Chapter 2:
Mobile laboratories and DNA barcoding in the field

Abstract

Molecular methods of species identification such as DNA barcoding have provided important breakthroughs in identifying cryptic species and degraded material, but their use in the field has been limited by the need for dedicated laboratory equipment. The advent of portable thermocyclers promises to move the technology into the field, with far-reaching consequences for ecological and evolutionary biologists. Here I describe my testing of these technologies by using DNA collected from blood samples to supplement morphological identification of bats species in Lamanai, Belize and using FTA® card technology, miniPCR™ thermocyling, and MinION™ nanopore sequencing to generate DNA barcodes, based on the cytochrome *c* oxidase subunit 1 gene (*COI*), in the field. I contrast these data to traditional Sanger sequences produced from the same individuals. Despite success identifying these species from DNA samples in the field, demonstrating that the technique is possible, the sequences were of poor quality and several potential options in the procedure were unrealistic given current technologies. In this chapter I describe the approach and data I was able to obtain and provide suggestions to improve performance.

Introduction

DNA based species identification

Species identification and monitoring in the field typically relies on morphological identification (Ashrafi et al., 2010; Moratelli and Oliveira, 2011), and may employ techniques including live capture and trapping (Wells et al., 2004), camera trapping (Wearn et al., 2013), and visual counts (Volpato et al., 2009). These approaches rely on the species displaying externally distinct features, and all are complicated by cryptic species (Hajibabaei et al., 2006b; Mitchell, 2008). In cases where species cannot be easily distinguished morphologically, definitive diagnoses may not be possible in the field or without sacrificing the animal, which is often undesirable or impossible. Molecular approaches to species identification require only a small amount of tissue, which allows for non-invasive methods of biomonitoring from trace materials (e.g. scats, saliva, hairs) (Nichols et al., 2012; Pearson et al., 2014; Wultsch et al., 2014). In addition, morphological identification requires a high level of expertise for specific taxa, whereas molecular techniques can be applied using a more general approach (Cook et al., 2010; Gaston and O'Neill, 2004). DNA barcoding has been established as the primary method of routine species identification and delimitation complementing morphology (Hebert et al., 2003; Mitchell, 2008). Among animal life DNA barcoding is primarily based around a 657bp region of the mitochondrial cytochrome *c* oxidase subunit 1 gene. Comparing unknown sequences to existing DNA voucher sequences (Hebert et al., 2003) can be used to retrieve an identification. Because most DNA barcoding takes advantage of PCR amplification even trace materials contained in environmental DNA samples (eDNA) can be utilised. The use of high throughput

sequencing (i.e., simultaneous analysis of millions of sequences; HTS) in conjunction with DNA barcoding has broad applications including rapid barcoding of museum collections (Janzen et al., 2005), assessing phylogeographic patterns (Christiansen et al., 2018), and revealing cryptic diversity (Hajibabaei et al., 2007, 2005; Ivanova et al., 2006).

Molecular approaches for field data

To date, molecular technologies have been largely restricted to laboratory settings (Borisenko et al., 2008) resulting in significant delays between field research and DNA analysis. Samples collected in the field must be preserved to avoided degradation during storage and transport. Getting samples from field to lab is often a laborious process involving permits from multiple agencies, careful packaging, specialised (and expensive) transport, and international shipments from remote locations (Guevara et al., 2017).

DNA extraction kits are frequently tailored for specified sample types (e.g. blood, tissue, water, faeces;(Bohmann et al., 2014), however most extractions kits require additional large, heavy table-top equipment for centrifugation, a water bath to facilitate lysis, and additional consumables. While these methods can be cumbersome and time consuming, studies have successfully demonstrated that extraction kits can be used outside a laboratory, even when electrical power was inconsistent (Hoenen et al., 2016; Pomerantz et al., 2010). Alternatives to equipment and reagent-heavy extraction kits have been explored, such as Whatman® FTA® cards which consist of chemically treated paper which both lyse cells on contact and preserve the released DNA, which is then bound to the card until released by elution. FTA® cards are tailored

for virtually any cell type including blood (Ivanova et al., 2009; Pearson et al., 2014), muscle tissue (Ivanova et al., 2012), and stool (Lalani et al., 2015; Nechvatal et al., 2008). In additional to DNA extraction in minutes, FTA® cards and similar technology easily preserve DNA at room temperature for prolonged periods.

Mobile laboratory technology

Rapid technological innovations in portable miniaturised machinery have allowed biologists to go beyond standard DNA extractions and preservation in the field. The MinION nanopore sequencer developed by Oxford Nanopore Technologies is a sequencing platform that involves stringing strands of nucleotides through 'nanopores' in a membrane (Jain et al., 2015). The module (MinION flow cell) is small (10 x 3.2 x 2 cm), light (90 g), and powered by a USB connection to a standard computer (Mikheyev and Tin, 2014). The sequencer has been deployed in viral outbreaks (Hoenen et al., 2016; Quick et al., 2017) and had its physical and functional constraints tested on the International Space Station alongside the miniPCR™ thermocycler (Castro-Wallace et al., 2016), which has also been designed for mobile laboratory set-ups, being small (5 x 12 x 10 cm) and light (400 g) (Lin, V. 2015). Portable sequencers and thermocyclers provide the potential for complete molecular processing in the field, negating problems of preservation, and transport of samples prior to getting them into the lab (Johnson et al., 2017; Pomerantz et al., 2010). However, there are limitations of various mobile laboratory toolkits including the need to freeze some reagents not in use and additional required table-top equipment or batteries when lacking access to a reliable power grid. It may also be difficult to avoid ambient contamination without a controlled lab environment (see (Wölfel et al., 2015) for their foldable glovebox

design). Finally, there must be a way to analyse the data within a reasonable timeframe.

Bioinformatics on local machines

Data processing and analysis requires computational resources. Large amounts of data often rely on high-performance computational clusters (HPCCs) for processing power (Glenn, 2011), but access requires a stable network connection making them unsuitable for remote locations. Several sequencing platforms (454, Ion Torrent) produce relatively small amounts of sequence data, allowing data analysis to be done locally on commercial laptops. For example, generating an assembly from >600 000 reads from an *E. coli* genome took about 13 mins on a dual quad-core MacPro laptop from 2008 (Glenn, 2011). With the MinION™ sequencer, the amount of data being produced can be monitored in real-time, and halted by the user to minimise requirements for computer storage and processing power (Cao et al., 2016; Juul et al., 2015).

Scope and objectives

Here I tested field sequencing methods for species identification by DNA barcoding bats at a field site adjacent to the Lamanai Archaeological Reserve in Orange Walk, Belize. Mammals, particularly Neotropical bats are well-represented in DNA barcoding reference libraries (Clare et al., 2007; Ivanova et al., 2012) making them an ideal model system. I targeted a 450 bp segment of the cytochrome *c* oxidase subunit 1 (*COI*), which can be amplified from blood even if some degradation exists and easily distinguishes species in mammals (Borisenko *et al*. 2008). I also evaluate the cost, time and quality of mobile lab components for DNA extraction, amplification and

sequencing, and address whether I could provide a molecular verification of field identifications *in situ.*

Methods

Field Sequencing

Preparing thermosensitive reagents for travel

Several temperature sensitive components of the methods in this study had to be carried in cooling wallets for several hours when travelling to preserve their viability. I used FRIO® Duo Wallets, pre-chilled at suitable temperatures for my reagents (4°c and -20°c), conveniently keeping them at recommended storage levels when travelling. While these did not remain at temperature, they were cool on arrival and the reagents were viable. On site I used a small bar fridge to maintain cold temperatures though these were variable over the period of the sampling due to ambient temperature fluctuations and variability in the electrical generation.

Belize site and available infrastructure

The field work was carried out at the Lamanai Field Research Center (17.75° N, 88.65° W) over a 12-day period (24th April - 5th May 2018). A communal classroom on site served as a base of operations for examining captured bats as well as conducting lab work. It was not feasible to create a sterile environment suited for molecular biology work, apart from limiting my work to one area that was not used for other activities. A generator supplied electricity to the classroom, allowing the use of electric equipment.

Bat sampling and identification

Field procedures followed guidelines for safe and humane handling of bats published by of the American Society of Mammalogists (Sikes, 2016) and were approved by the Institutional Animal Care and Use Committees of the University of Georgia (A2014 04-016-Y3-A5) and American Museum of Natural History (AMNHIACUC-20170403 and AMNHIACUC-20180123). Fieldwork was authorized by the Belize Forest Department under permits WL/2/1/17(16), WL/2/1/17(19), and WL/2/1/18(16).

Bats were captured in and around the Lamanai Archaelogical Reserve, a protected site bordered by the New River Lagoon, forest, and agricultural sites. A team of researchers used both mist nets and harp traps as part of a daily effort. Bats were captured with mist nets at the exits of roosts or along flight paths from 19:00 until 22:00. Nets were tended continuously, and bats were removed immediately upon capture. Harp traps were set from 18:00 to 05:00 and checked at regular intervals. After capture, bats collected were held individually in clean cotton bags and brought back to the classroom where they were identified by expert taxonomists based on body size, external morphology, and dental morphology using expert knowledge and descriptions in field guides (Reid, 1997).

I selected eight of these provisional species for taxonomic confirmation via DNA barcoding: *Saccopteryx bilineata, Pteronotus mesoamericanus, Natalus mexicanus, Desmodus rotundus, Glossophaga soricina, Uroderma bilobatum, Sturnira parvidens,* and *Molossus rufus*. I selected these species to represent the different families captured during the field campaign which are captured in enough frequency to ensure

N=3 individuals for each species. I avoided congeners to maximize the potential for unambiguous species ID.

Blood collection and preservation

Depending on mass, 3–30 µL of blood was collected by lancing the propatagial vein with a sterile needle. Blood was collected with heparinized capillary tubes and stored on Whatman FTA cards to preserve DNA. I air-dried all blood spotted FTA cards for at least 20 minutes. To minimize contamination during this process, I placed them in open zip-lock bags facing away from other work being conducted in the shared facility. Once dried, I sealed the FTA cards in zip lock bags alongside desiccant pouches.

DNA extraction and COI fragment amplification

For each bat sampled, I cut out a 1.5mm^2 portion of the blood spot on the FTA card using a Miltex® Biopsy Punch. Each disc was put in a 0.2mL PCR tube filled with 20µL molecular grade water. I extracted DNA by submerging the disc in sterile water in a PCR tube and incubating it at 90°C for 15 minutes using a miniPCR™ thermocycler. Once extraction was complete, I discarded the used disc.

I performed PCRs in 15µL reactions using the QIAGEN Multiplex PCR kit. Each reaction contained 2µL DNA eluate, 7.5µL QIAGEN Master Mix, 0.75µL of each primer, and 4µL of molecular grade water. The primers used were as previously published (Ivanova et al., 2006; Pfunder et al., 2004) but modified to include a 5' universal tail sequence for the Oxford Nanopore Technologies barcoding kit. The primer and tail sequences are as follows:

Forward, RonM [5'-GGMGCMCCMGATATRGCATTCCC-3'] (Pfunder et al. 2004)

Reverse, VR1 [5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'] (Ivanova et al. 2006)

Forward tail [5'-TTTCTGTTGGTGCTGATATTGC- forward primer- 3']

Reverse tail [5'-ACTTGCCTGTCGCTCTATCTTC- reverse primer- 3']

I performed the PCR cycling using the miniPCR™ with the following cycle program: Initial denaturation at 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes.

Library preparation for MinION™ sequencing

Once I obtained the 5' tagged *COI* amplicons from the initial PCR, I followed the 1D PCR barcoding amplicons protocol (SQK-LSK108) produced by Oxford Nanopore Technologies for the MinION™ platform. Minor changes and omissions to the protocol were made to simplify the procedure for the field, namely 1) all DNA quantification steps were omitted, and 2) all volumes relating to PCR product and AMPure® XP beads are smaller because of my initial PCR volume of 15µL. The workflow was otherwise as follows:

i) I cleaned the first round PCR using AMPure® XP beads (Beckman Coulter, Inc). I added 13µL of PCR product to 13µL of beads; the 1:1 ratio was

chosen to remove unused primers and small fragments (<200 bp). I resuspended the DNA in 18µL molecular grade water.

ii) I conducted a second PCR to attach the 'PCR Barcode', a molecular identification tag (MID) which provides a recognisable index for each sample during sequencing. Each reaction contained 5µL purified DNA, 7.5µL QIAGEN Master Mix, 1µL of MID (Molecular ID sequences), and 1.5µL of molecular grade water. The indices and sequences of the 8 MIDs used are available in the Appendix (1.1). I performed the thermocycling using the miniPCR™ with the following cycle program: Initial denaturation at 95°C for 15 minutes followed by 12 cycles of 95°C for 15 seconds, 62°C for 15 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. I cleaned the 2nd round PCR products using AMPure® XP beads as in step (i).

iii) From each of the now uniquely labelled 2nd round PCR products, I took 2µL and pooled these together to perform end repair and dA-tailing. This mixture consisted of 48µL DNA, 7µL NEBNext ® Ultra II End-prep reaction buffer (NEB), 3µL NEBNext® Ultra II End-prep enzyme mix (NEB), and 2µL DNA CS (a positive control provided by ONT); and I incubated these at 20°C for 5 minutes, and 65°C for 5 minutes. This was then subjected to another bead clean up but with 60µL AMPure® XP beads to maintain a 1:1 ratio and resuspended with 31µL molecular grade water.

iv) I performed MinION™ adapter ligation and tethering by mixing 30µL end-prepped DNA, 20µL Adapter mix, and 50µL Blunt/TA Ligation Master Mix. I then purified this adapter ligated DNA library with 65µL AMPure® XP beads;

a 0.65 ratio retains fragments of >400bp. I washed the beads with 140µL ABB buffer (ONT), and finally incubated and eluted these in 15µL elution buffer (ONT).

Priming and running the MinION™ sequencer

I primed an R9 flow cell with 1000µL of priming mix (ONT) via the priming port. I prepared the loading library by mixing 12µL of DNA library with 35µL RBF (ONT), 25.5µL LLB (ONT), and 2.5µL molecular grade water; I then added this to the flow cell via the SpotON sample port.

I started the sequencing run using the MinION™ software, MinKNOW™. The software selected the protocol to use based on my given parameters: MinION™ Flow cell FLO-MIN 106, library preparation SQK-LSK108, no live-basecalling, and a 48-hour run-time. After 8 hours, additional running buffer was added to prolong the sequencing run up until a total of 12 hours. A brief "ping" is required to activate the software thus I did not conduct this experiment entirely offline. Offline use is possible but requires pre-authorisation from the company. Some security settings can prevent software activation and should be tested.

Bioinformatics

I used Albacore 1.2.5 (https://github.com/dvera/albacore) to process the FAST5 files generated by MinKNOW™. This included base-calling, demultiplexing, and conversion to Fastq files. Additional details on the sequencing run performance were produced on the cloud-based Metrichor/EPI2ME platform after returning from the field study. I used Porechop (https://github.com/rrwick/Porechop) to cross-check the demultiplexing by Albacore, and then trim nanopore adapters from the fastq files. The remaining bioinformatics were done on Galaxy, an online scientific analysis platform

(Afgan et al., 2016) which can also be used offline using command line options. First, I removed both forward and reverse primers from the fastq files using Cutadapt (Galaxy version 1.16.1), and converted these to fasta format, collapsed them to unique sequences, and length filtered these to remove shorter reads (<300 bp). In general, I used the simplest and most limited bioinformatics procedures so that high performance computing would not actually be required to replicate these procedures. I used local BLAST searches to compare the surviving reads to reference sequences from the barcode of life database (BOLD; Ratnasingham and Hebert, 2013) generated from bats previously sampled at the same location in Belize augmented by the addition of several records from nearby areas. The reference database contained 3 reference sequences for 9 bat species (*Desmodus rotundus, Eptesicus furinalis, Glossophaga soricina, Pteronotus mesoamericanus, Sturnira parvidens, Uroderma bilobatum, Molossus rufus, Natalus mexicanus,* and *Saccopteryx bilineata*). When using blastn, I included the option '-outfmt' to request the output to be in a tabular format. The tabular blast output could then be manipulated and analysed in R (R Development Core Team, 2011) in csv format.

Comparison of alternate methods and software

To assess the data quality of the MinION™ output, I compared it to data collected using conventional high-quality sanger protocols after returning from the field. DNA was extracted from newly cut discs from the same bloodspots on FTA cards used in the field. I followed the same extraction method as above and amplified using the unmodified forward RonM and reverse VR1 primers. Positive and negative PCR results was verified on an agarose gel. The PCR amplicons were subjected for conventional Sanger sequencing at Source BioScience , and the resulting amplicons were

respectively basecalled and assembled using Phred and Phrap software (Ewing and Green, 1998) , and were manually edited in BioEdit v7.2.6 (Hall, 1999). Variability between the individual representatives of each species were visualised in WebLogo (Crooks et al., 2004).

Results

Overview of workflow from capture to sequence

I conducted this experiment during a field campaign in April 2018. Over the sampling period more than 100 bats were caught daily, but the target of 3 specimens per species was only obtained after 10 sampling days. There were no issues with the equipment until running the MinION™ sequencer. The operating temperature of the Min-FLOW 107 ranged from 30 to 34°C, and the ambient temperature exceeded that even in the evening, causing the sequencing run to halt repeatedly. I solved this problem by putting the MinION™ inside the refrigerator with its door open, cooling it for the duration of the run but this caused a series of delays between sample loading and operation. This was exacerbated by problems with connectivity to receive the "ping" required for the MinKNOW™ software. In the end the sequencing run did not begin until 8 hours after sample loading, which likely reduced yield. At the end of 12 hours, the MinKNOW™ summary page reported a total of 22011 reads across 5783839 base events with 326 of 512 pore channels still available. However, when basecalled using Albacore, only 7202 of those reads passed its default quality settings. Performance data from EPI2ME revealed a steep decline in bases detected per hour towards the 8-hour mark, but slightly improvement after the addition of buffer (Figure 2.1a). The average read length was also reported as 544 bases, with about one third of reads coming short of the expected 590 bases in length (Figure 2.1b).

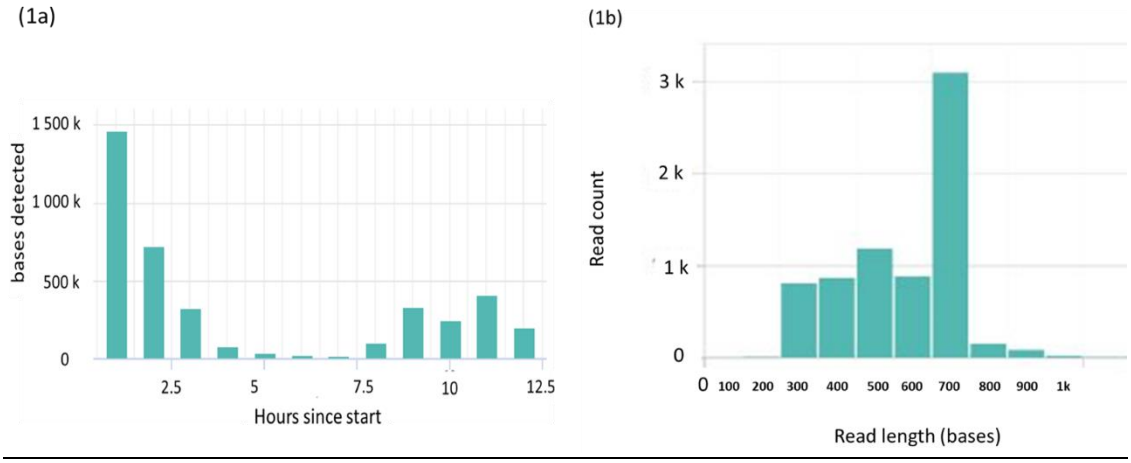


Figure 2.1: Performance diagnostics of the MinION sequencing run. *(1a) shows the number of bases detected per hour and (1b) shows the lengths of reads throughout the run*.

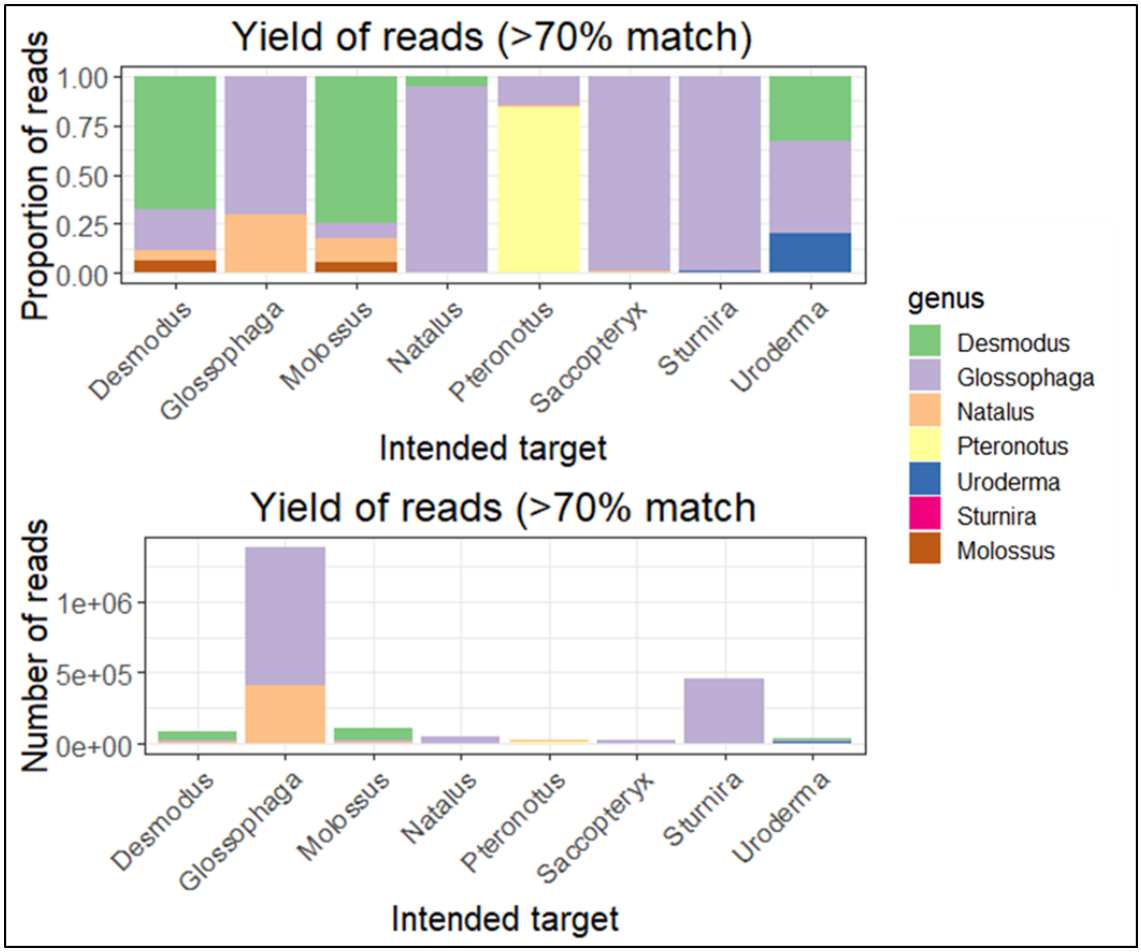


Figure 2.2: Distribution of reads across all 8 MIDs. Each column represents individual MIDs, labelled by the bat species assigned to it. The coloured stacks in each column represents the BLAST matches assigned to reads.

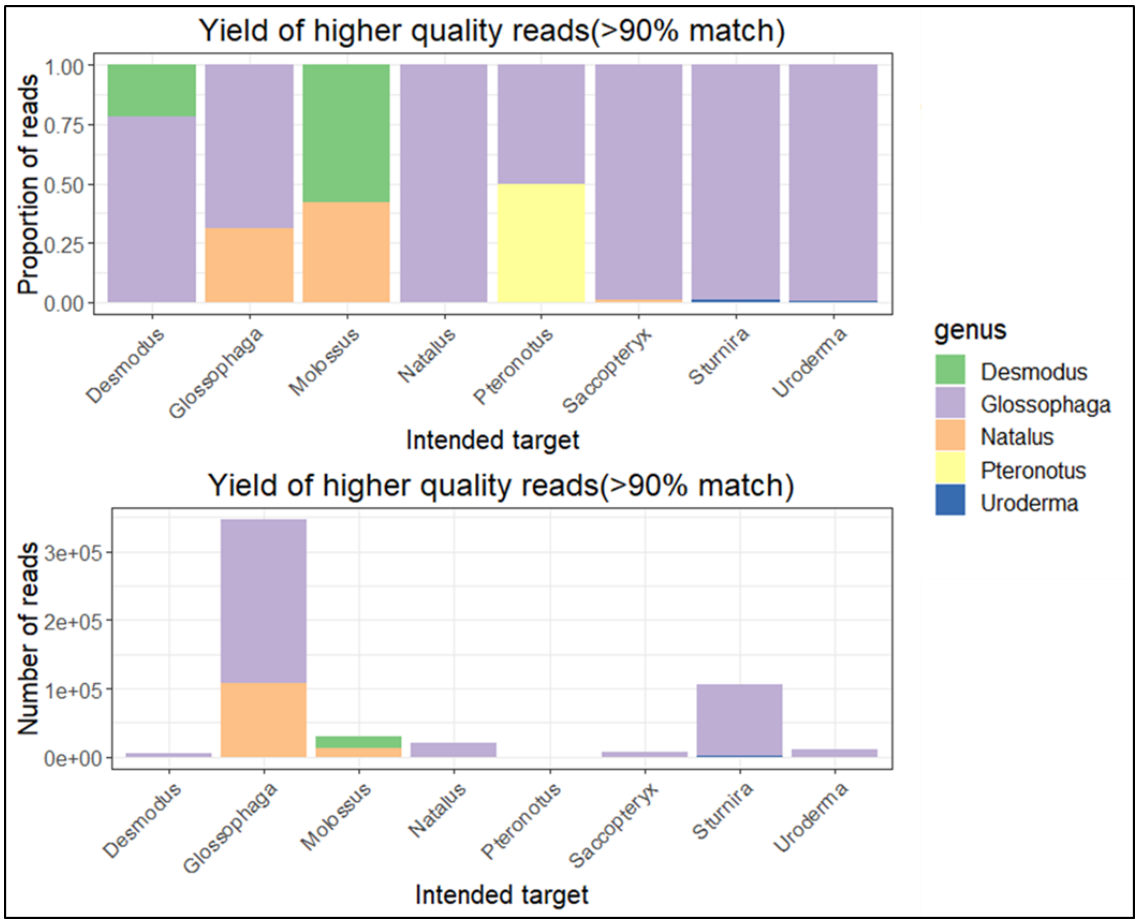


Figure 2.3: Distribution of reads across all 8 MIDs. Each column represents individual MIDs, labelled by the bat species assigned to it. The coloured stacks in each column represents the BLAST matches assigned to reads. Only reads having at least a 90% match to an identity are included.

Bioinformatics and sequence identification

Demultiplexing the reads with Albacore suggested 33 additional nanopore MIDs besides the 8 that were used. Each of these spurious MIDs had 1 or 2 reads assigned to them. Cross-checking the demultiplex outcome with Porechop reduced the number of anomalous MIDS to 1, for a total of 9 MIDs compared to the expected 8. After trimming the reads, comparing all haplotypes from the 8 expected MIDs to BOLD references showed that 7 of the 8 targeted species were detected, *Saccopteryx* was not detected (Figure 2.2). However, the nanopore MIDs failed to separate the species correctly, instead having multiple bat species appearing within each MID, with *Glossophaga* dominating most of the reads. When I only included reads that had >90% BLAST match, the number of detected species fell to 5, with *Molossus*, *Sturnira*, and *Saccopteryx* now missing (Figure 2.3).

Except for *Glossophaga*, the top matching haplotypes for the bats that could be placed to genus were singletons, with *Natalus* notably achieving a 100% match (Table 2.1A). Furthermore, except for *Glossophaga* and *Natalus*, all other bats had their top matching haplotype associated to the correct MID (Table 2.1B). However, this apparent correct assignment was obstructed by the common flooding of higher quality *Glossophaga* reads into other MIDs.

Many bioinformatics pipelines for HTS data remove low frequency haplotypes as likely errors. To test the hypothesis that more common haplotypes represent the "true" sequence, I plotted a regression between haplotype copy number and BLAST match percentage (as a measure of similarity between my unknown amplicon sequences and any entry in the custom known reference database). I found haplotype copy number to be unrelated to BLAST match percentage (P=0.883, Figure 2.4). The number of

haplotypes for each genus that survived the 90% match benchmark is summarized in Table 2.2.

Figure 2.4: Regression plot showing little interaction between haplotype copy number and match percentage. Each point represents a unique haplotype. No regression line was generated for Pteronotus as each of its remaining haplotypes were singletons.

Sanger sequencing from FTA cards

Each of the 8 genera of bats sampled were represented in the Sanger sequencing run, although both *Glossophaga sp.* and *Natalus sp.* had only 2 successful samples rather than 3 (Table 2.3); the *Natalus sp.* sample failed to amplify (gel not shown), and the *Glossophaga sp.* sample was found to be contaminant (omitted from results). Each of the remaining sequences were between 395-421bp and showed high matches (>99%)

to their species in the NCBI database (*Sturnira parvidens* reads were matched to *Sturnira lilium* as a result of a recent taxonomic revision; Sánchez-Hernández and Romero-Almaraz, 2003).

Table 2.1: Summary of BLAST results according to highest % matches alongside the copy number related to that haplotype. 1A shows the highest % match achieved by the 7 detected genera and their associated MIDs. 1B shows the highest % match obtained by each MID and the intended target from that MID.

(1A)

Genus	% match	MID	copy
Uroderma	94	BC01	1
Pteronotus	92	BC03	1
Sturnira	77	BC13	1
Molossus	88	BC38	1
Desmodus	93	BC49	1
Glossophaga	96	BC73	349
Natalus	100	BC85	1

(1B)

MID	Genus	% match	target
BC01	*Glossophaga*	95	*Uroderma*
BC03	*Glossophaga*	94	*Pteronotus*
BC13	*Glossophaga*	96	*Sturnira*
BC25	*Natalus*	95	*Saccopteryx*
BC38	*Glossophaga*	94	*Molossus*
BC49	*Glossophaga*	95	*Desmodus*
BC73	*Natalus*	100	*Natalus*
BC85	*Glossophaga*	95	*Glossophaga*

Table 2.2: Number of haplotypes and genera retained after the 90% match filtering.

Genus	*Uroderma*	*Desmodus*	*Glossophaga*	*Natalus*	*Pteronotus*
Haplotypes	5	40	451	129	3

Note: The maximum number of haplotypes expected for each target species is 3, the number of individual bats sampled.

Table 2.3: Blast hits of Sanger sequence reads obtained from FTA-blood samples from bats.

Sample ID	GenBank match	Accession	% iD	%Cover
Des1	*Desmodus rotundus*	JF435339.1	100	100
Des2	*Desmodus rotundus*	JF435339.1	99.76	100
Des3	*Desmodus rotundus*	JF435339.1	100	100
Ptero1	*Pteronotus mesoamericanus*	JF448278.1	99.76	100
Ptero2	*Pteronotus mesoamericanus*	JF499030.1	100	100
Ptero3	*Pteronotus mesoamericanus*	JF448265.1	99.76	100
Glos2	*Glossophaga soricina*	JF499022.1	100	100
Glos3	*Glossophaga soricina*	JF499022.1	99.75	100
Sac1	*Saccopteryx bilineata*	JF435741.1	99.76	99.52
Sac2	*Saccopteryx bilineata*	JF435741.1	99.72	90.88
Sac3	*Saccopteryx bilineata*	JF435741.1	100	100
Uro1	*Uroderma bilobatum*	MG191883.1	100	100
Uro2	*Uroderma bilobatum*	MG191905.1	99.76	100
Uro3	*Uroderma bilobatum*	MG191883.1	99.02	97.14
Sturn1	**Sturnira lilium*	JF446856.1	100	100
Sturn2	*Sturnira lilium*	JF447338.1	91.16	99.52
Sturn3	*Sturnira lilium*	JF447338.1	100	100
Mol1	*Molossus rufus*	MH185176.1	99.76	100
Mol2	*Molossus rufus*	JF448089.1	100	100
Mol3	*Molossus rufus*	MH185176.1	99.76	100
Nat1	*Natalus stramineus*	JF447283.1	99.76	100
Nat2	*Natalus stramineus*	JF447280.1	99.52	100

Note: Sturnira lilium was recently revised and the correct name for the local population is now *S. parvidens*. Blast hits reflect previous taxonomic designations.

Table 2.4 Contrasts between examples of mobile and conventional lab technology. Asterisk (*) denotes methods used in other chapters in this thesis. Citations used: Camacho-Sanchez et al., 2013; Chacon-Cortes and Griffiths, 2014; Santos, 2018; Green et al., 2019; Quick et al., 2017

Process	Selection criteria	*In-situ* barcoding	Lab-based barcoding
Sample preservation from the field	Method	FTA® Elute® cards	e.g. tubes with preservative*
	Additional requirements	Temperature and humidity to facilitate drying	Liquid nitrogen required for long term storage
	Compatible sample types	Designed for cell suspensions (e.g. blood, saliva), up to 40 µl	Wider range of tissues, depending on preservative
DNA extraction	Method	FTA® Elute® cards	e.g. QIAGEN DNA Extraction kits*
	Additional requirements	Sterile water to rinse card, Sterile distilled water and heating apparatus to elute DNA	Many reagents and single-use consumables per sample. requiring heat blocks, vortexes, centrifuge
	Performance	Lower yield than extracting from samples directly. Greatly diminished for degraded material.	Greater yield, adjustable protocols to handle degraded material.
PCR	Method	MiniPCR®	e.g. Biorad T100 thermocycler
	Max. Capacity	8 samples	384 samples
	Additional requirements	No program memory. Requires laptop or phone to install thermocycling program. Simple programs.	Up to 400 programs. Built-in touch interface. Complex programs (e.g. thermal gradient)

	Dimensions	<500 g, 50 x 100 x 130 mm	9 kg , 250 x 450 x 230 mm
Sequencing	Method	MinION (nanopore)	Sanger
	Dimensions	87 g, 105 x 23 x 33 mm	180 kg, 100 x 73 x 89 cm
	Capacity	One “experiment” at a time. (e.g. one genomic library, or one pooled metabarcoding library)	386 or 96 well plates
	Additional requirement	18-37 operating temp	N/A
	Library preparation time	Versatile. Simplest non-pcr experiment needs 10-20 minutes. Most complex metabarcoding experiments need 3 hours.	Minimum 1-2 hours

Discussion

Despite recovering sequencing reads suitable to identify these species, the reads from the field-based sequencing using the MinION™ sequencer were truncated and of poor quality, raising questions about whether species-level identifications would be possible in a more diverse sample which included congeners. PCR tags failed to resolve reads to specific samples, likely due to poor quality of the recovered MIDs.

These results demonstrate that the methods work, although the end-results were extremely error prone, limiting their current application. The use of the RonM and VR1 primers for amplifying mammalian *COI* has been demonstrated to be reliable for generating short barcodes (Borisenko et al., 2008; Ivanova et al., 2012) which are adequate to identify mammals, and this has been particularly well tested for neotropical bats (Borisenko et al., 2008) under laboratory conditions. Though I successfully amplified the correct region, a minority of the recovered reads were of the expected 421bp length. The barcodes I obtained through Sanger sequencing the FTA samples were mostly the expected 421bp . Previous studies using FTA cards to extract DNA have noted lower DNA yields compared to conventional kits but were still able to generate reliable DNA barcodes using lab based sequencers (Borisenko et al., 2008; Mbogori et al., 2006) suggesting FTA sample preservation and extraction is not a cause of barcode quality reduction. There were no obvious signs of reagent degradation, but this does not rule out the possibility that problems with transporting and storing reagents in the field may have contributed to poor results in this study. The longest period where components were kept at inconsistent temperatures was the 24 hours, during transit to the field site. Other studies report storing reagents 10 - 15 days at inconsistent or ambient temperatures with successful PCR amplification, though

certain enzymes had reduced performance towards the end of that period (Guevara et al., 2017; Pomerantz et al., 2010). Access to a refrigerator and freezer is exceptional for a field study, and, despite generator cycling causing power fluctuations, the temperature remained reasonably consistent during the field sequencing. Electricity provided via a large generator allowed us to operate the microcentrifuge and miniPCR™, where others have had to rely on batteries (Guevara et al., 2017). Significant issues with temperature regulation of the MinION™ occurred when the ambient temperature (37°C) exceeded the operating threshold (34°C) and, combined with issues surrounding the need for a clear internet signal to initiate sequencing, there was a delay in sequencing which affected the quality of reads generated. The loaded library spent 20 hours in the flow cell, with only one top up of running buffer. ONT protocols cautions against flow cell drying which can damage the membrane or clog the nanopores with loading beads. This delay was exacerbated by a firewall issue with the MinKNOW™ software 'ping' which I had not previously encountered but could be avoided in future work.

The detection of multiple bats associated with each MID was surprising since one species per MID was expected. Sanger sequencing from the same FTA samples once returned from the field showed no signs of cross-contamination making the probability low at the sample collection stage. *Glossophaga soricina* dominated most MIDS and the run in general. Broad environmental contamination of all samples is possible given the location. A lack of a fume-hood might have contributed to *Glossophaga* DNA compromising every sample on the FTA card, but this issue has not previously been observed using FTA cards or miniPCR™. The more likely explanation is that the MIDs, which are supposed to act as unique tags for each sample were recovered in low

quality reducing the accuracy of MID assignment. Upon initial editing rather than detecting 8 MIDs, I recovered 33 possible codes (most never used) and even with the stricter quality filtering of Porechop, one impossible MID remained. If MID codes were poorly recovered during sequencing it is likely reads were mis-assigned. The correct assignment of abundant haplotypes from some bats to their MID supports this.

Sequence quality obtained from MinION™ runs is known to vary greatly, with error rates between 12-35% previously reported (Ashton et al., 2015; Jansen et al., 2015; Malla et al., 2017). Wang et al., (2015) were able to produce >99% genome coverage of an influenza virus using the Genomic DNA sequencing kit which allows 2D reads (two directional sequencing). Other 2D kit users sequenced 17 bacterial genera but only retrieved 6 at an 80% assignment threshold, and 8 at a 60% threshold (Benítez-Páez et al., 2016). This variability in sequence quality has also been observed in 1D kits. Pomerantz et al., (2010) reported *de* novo assemblies of *16S*, cytochrome *b*, and *NADH* dehydrogenase 4 genes, but success ranged from >99-91.7% identity to Sanger-generated consensus sequences; with several attempts failing due to reads that wouldn't assemble. 2D kits are not currently compatible with protocols requiring PCR, thus PCR multiplexing used here was limited to 1D kits. Five out of eight species in my study generated reads with >90% identity to reference sequences, which is within the expected boundaries for a nanopore sequencing run. However, the design of my study deliberately made identification simple by only including one species per genus using a custom database containing those exact species. The error rate suggests that including congeners with similar sequences would have made it difficult to correctly assign taxonomic designations. The error rate might be mitigated by targeting large fragments for amplification (>4000 bp) (Krehenwinkel et al., 2018) and by using BLAST

parameters such as: longest alignment length, and lowest E-values (Parker et al., 2017). While the latter approach has potential, it is outside the scope of DNA barcoding (which typically uses short fragments), and thus would not be suitable with the existing libraries.

Our results and those of others suggest several ways to improve the methods for future use of this technology in the field. First, Clare et al., (2014) demonstrated using custom primers to amplify the target *COI* region and including a MID tag for library preparation in metabarcoding removes the need for a second PCR step and bead clean-up, saving time and reagents, and preserves PCR products. Each sample needs its own custom primer, increasing costs, but for small-scale studies this would be a feasible shortcut for multiplexing protocols minimising cross contamination of MIDs. PCR mixes could also be prepared ahead of time under more stringent lab conditions so that only DNA would need to be added in the field, further limiting potential contamination. One aspect of the ONT protocols omitted in the current study was DNA concentration checking, which was skipped because of difficulty transporting some reagents. Low DNA concentrations of some samples could contribute to explaining the discrepancy in relative read assignment to different species. At the final step of preparing the sequencing library, I pooled together equal volumes for each MID-tagged sample library but had no indication of concentration nor whether any samples had simply failed at the extraction, PCR, or clean up stages. DNA quantification methods such as QuBit (Life Technologies, Grand Island, NY) and Nanodrop (Desjardins and Conklin, 2011) are not easily carried to the field, but it may be sufficient to gauge relative concentrations using gel electrophoresis. Like miniPCR™, the BluGel™ (Amplyus) is a simple small sample (n=9 -13) gel electrophoresis (23 x 10 x 7 cm)

system operated using a mobile phone. Pre-casting gels can lessen the added burden of carrying liquids and agarose, though this needs to be balanced against whether equal representation is needed or whether technical replicates with occasional PCR failure would suffice. When running the MinION™, the forced shut-down due to overheating was fixed by placing the device inside of a refrigerator. An alternative solution would be to place the MinION™ on a cooling pack, although runs can proceed for hours and cooling packs would need to be replaced.

The bioinformatics steps in the current study were not performed in the field due to time constraints. However, the only component that required internet-access was the Galaxy platform for adapter trimming, collapsing, and filtering and this was for convenience only. All tools can be run via command line on a laptop using the same protocols or any one of a dozen other similar programs. The key component to making the bioinformatics procedure tractable without high performance computing is the use of a limited reference database. Because I knew the species' identities *a priori*, the reference database in a BLAST could be small (e.g. just the focal bats, just bats in these genera, just Neotropical bats) but the more "unknown" the sample, the greater the task of identification will be both computationally and technologically.

The emergence of mobile laboratory technologies is being driven by the increasing interest in developing and testing capabilities of these systems for applications in human health surveillance (Hoenen et al., 2016; Schmidt et al., 2017; Wölfel et al., 2015) as well as evolutionary and conservation applications (Menegon et al., 2017; Pomerantz et al., 2010). The use of FTA cards as a safe and convenient alternative for collecting and preserving samples is being increasingly employed for a range of

material (e.g. stool smears; Lalani et al., 2018, sputum; Linhares et al., 2012) and taxa (e.g. fish; Tsutsumida et al., 2009, insects; Harvey, 2005). An important benefit is provided by FTA cards is that samples become non-infectious once dried (Linhares et al., 2012), which can facilitate export/import of samples that might contain pathogens. Several "PCR in a box" options for portable thermocyclers are available such as the battery-powered Palm PCR™ (Ahram Biosystems Inc., Seoul, Korea), or the smartphone-interfaced Biomeme™ (Biomeme Inc., 2015). For experiments that also require gel electrophoresis the Bento (www.bento.bio) offers a centrifuge, thermocycler, gel rig, and illuminator all in one unit (330mm x 214mm x 81mm). I opted for the miniPCR™ because it is a modular system allowing the PCR to be carried independently of gel rig and centrifuge. As a primary component of this study, and the only real option for field sequencing at the time of our field work, Nanopore sequencing platforms provide additional tools, such as VolTRAX™ (ONT), a device to automate library preparation and alternative flow cell and sequencer options such as Flongle™ and SmignION™ respectively, both suitable for smaller, simpler protocols using low powered devices. These are designed to be simple and quicker (e.g. 10-minute library preparation kits, small sample sizes) and currently lack kits to accommodate PCR or multiplexing/barcoding. While sequencing platform options for simpler experiments are available, the 'standard' MinION™ has been demonstrated to still be an accessible teaching tool in teaching field genomics (Watsa et al., 2019). The complexity of questions that can be answered will likely depend on the combination of components that make up a mobile laboratory. For example, a 10-minute library preparation kit lacking multiplexing options would not be ideal for identification of multiple specimens but could be used to confirmation the presence of a species of

ecological or economic interest. In this case, Flongle™ and SmidgION™ may be viable as the equivalent of a sophisticated dipstick, providing a general molecular scan of a sample with minimal effort. Compact diagnostic technology will be valuable in characterising disease outbreaks or in border surveillance for invasive species or illegal trade. These applications require faster, more streamlined protocols suitable for the level of urgency and needed resolution. My protocol is intended to augment morphological identification but there is a soft limit on the number of samples for a sequencing run, assuming a single MID is assigned to each. That said, this protocol is viable for small-scale studies requiring rapid information.

In this analysis I tested a field-friendly protocol for DNA barcoding and demonstrate how accessible mobile laboratory technologies such as the miniPCR™ and MinION™ nanopore sequencer can allow rapid and reproducible express species identification outside dedicated laboratories, albeit with some difficulties. While the data generated was of low quality, I was able to provide field confirmation of the bat identifications while demonstrating the technological potential. The setbacks encountered can be avoided in future implementations to increase sequence quality and successful identification. My analysis suggests that relatively low-cost investment in field-viable laboratory alternatives can be used in tandem with classical morphological identification to rapidly catalogue species and answer ecological questions in scenarios including challenged in tissue preservation, permitting, transportation, and time-sensitive operations.

Chapter 3:
Ectoparasite epidemiology in the Atlantic Forest

Abstract

Ectoparasites from 17 species of neotropical bats were sampled from continuous and fragmented host habitat within the Atlantic Forest of Brazil. A combination of DNA barcoding and metabarcoding of *COI* (Cytochrome Oxidase I) was used in conjunction with morphology to identify 25 species of bat flies (*Streblidae* and *Nycteribidae*), 8 potential clades of mites, and 1 species of tick (*Dermancentor nitens*). While a more complete DNA database of ectoparasitic mites in Brazil would be required to identify mites to lower taxonomic levels, bat flies were successfully identified using a combination of taxonomy and DNA. Bat flies were found to be aggregated in their host populations, where most individuals had few parasites, and few individuals had many parasites. Bat fly intensity was highest in hosts with moderate levels of body condition. In this analysis, I identified contrasting patterns of parasitism in different habitat types. For example, the bat fly *Trichobius joblingi* was twice as prevalent on *Carollia perspicillata* in fragmented sites as in continuous forest. This may reflect roosting restrictions in disturbed habitat causing larger roosting colonies of mixed host species. In contrast, bat fly *Paratrichobius longricus* infections on *Artibeus lituratus* was higher in intensity in continuous than fragmented forests, but this was only true for female bats, which might hint at female exclusive roosting behaviour in more heterogenous habitat. This study demonstrates that patterns of parasitism can be affected by changes in habitat, and emphasizes that host traits such as species, body condition,

and sex should be considered jointly for a holistic approach to identifying vulnerable host groups.

Introduction

Bat Ecology

Bats (Chiroptera) are one of the most widely distributed terrestrial mammals, found in almost all unglaciated regions of the world (Procheş, 2005). With close to 1500 described species, bats account for one fifth of known mammalian biodiversity (Frick et al., 2019). According to IUCN red lists, anthropogenic activities such as logging, mining, and urbanisation represent the top threats to endangered and critically endangered species of bats (IUCN, 2020). Forests and subterranean structures (i.e., caves and mines) represent important bat habitats with considerable conservation value to protecting existing populations (Garbino and Tavares, 2018). For example, caves provide more permanent roosting sites which tend to attract large multi-species colonies, while in contrast, plant structures such as tree hollows or foliage are preferentially occupied by some species (Kühnert et al., 2016). Most bats rely on forests for foraging, particularly in the tropics (Voigt and Kingston, 2015), with specific features (e.g. rivers, fruiting trees) being used by different species.

As a group, the mobility and dietary breadth covered by bats affords them an important role in ecosystem services such as pest control (Ghanem and Voigt, 2012), pollination (Hodgkison et al., 2003), and seed dispersal (Sarmento et al., 2014). As such, the ongoing and residual consequences of habitat disturbances such as forest fragmentation on bat populations are a matter of concern for conservationists worldwide (Frick et al., 2019). Habitat fragmentation can influence bat population

density by affecting dispersal (Cushman, 2006), mortality, and reproductive rates (Andrén and Andren, 1994). Additionally, increasingly heterogenous distribution of resources across fragmented habitats can cause bat populations to experience stress due to insufficient or patchy distribution of food and shelter (Williams and Kremen, 2007) and reduction in habitat size (Brühl et al., 2003).

Responses to habitat fragmentation

Even at small scales, forest fragmentation has been connected to lower bat diversity and activity levels, thought to be caused by reduced vegetation cover and understory (Costa et al., 2019). As forest regeneration relies on seed-dispersal, the loss of seed-dispersing species and the disruption of animal-plant mutualistic interactions compounds problems of extinction in regions with strong fragmentation pressure (Marjakangas et al., 2020). Species more tolerant to disturbance still remain but have reduced dietary breadth, and have to share key resources, leading to simplified interaction networks (Laurindo et al., 2019). The limited or altered diet available in fragmented habitats has been shown to cause shifts in the heterogeneity of core microbiota in bats, which have downstream consequences for host health and innate immunity (Ingala et al., 2019a). Reduced forest and vegetation cover is correlated with increased prevalence in viral (Hiller et al., 2019) and filarial (Cottontail et al., 2009b) infection. (Seltmann et al., 2017) suggests a positive feedback loop between the decrease in body condition of bats in dilapidated habitats, exacerbated by chronic stress from habitat disturbance, and the burden of parasitic infection and this has implications for disease transmission, but this is often considered separately form the ecological impacts. For example, (López-Baucells et al., 2018b) reports that the

increase in interest in bat community health research tends to emphasise bats as a major threat to public health, and disregard their key roles in ecosystem stability and susceptibility to perturbations, which counterintuitively makes bat conservation more difficult when interest spikes (MacFarlane and Rocha, 2020a).

Parasites of bats

Bats are hosts to a range of arthropod ectoparasites belonging to Acari (ticks and mites), Dermaptera, Diptera, Hemiptera, and Siphonaptera (Bertola et al., 2005). Of these parasites, bat flies (Diptera: Nycteribiidae and Streblidae) and wing mites (Acari: Spinturnicidae) are examples of families that are exclusive to bats. Both bat flies and wing mites are highly specialised hematophagous inhabitants of bat fur, wing, and tail membrane (Juliana C Almeida et al., 2011; ter Hofstede et al., 2004), and both exhibit some form of viviparity, where the eggs or larvae develops within the female. In bat flies, all larval stages develop within the female until they are ready to pupate, whereby the female leaves the host momentarily to deposit the prepupae onto the roost substrate (Graciolli et al., 2019; Marshall, 1970). Less is known about the general life cycle of wing mites, but examples from genus *Spinturnix* exhibit internal development of the egg and larval stages within the female, which then gives birth to a protonymph whilst still on the host (Colín-Martínez and García-Estrada, 2016; Orlova et al., 2018). There is evidence to show that parasitism can have strong effects on bat communities (Warburton et al., 2016) and escalate during nursing season where female bats are more heavily parasitized than males (Frank et al., 2016a; Patterson et al., 2008a), and can experience up to a 10% reduction in mass as a result (Lourenço and Palmeirim, 2007). Generally, parasitism and disease are thought to be a

contributing factor in population decline in many species (De Castro and Bolker, 2005). When evaluating emerging diseases in wildlife, (Tompkins et al., 2015) suggest an emphasis on microparasites (e.g. viruses, bacteria, fungi) when compared to macroparasites (e.g. helminths, arthropods) since the former tend to show greater virulence. However, some parasitic arthropods have been suspected to be vectors of more virulent diseases. Parasitic flies and ticks harbour pathogenic strains of *Bartonella spp.* (Stuckey et al., 2017; David A Wilkinson et al., 2016), *Rickettsia spp.* (Dietrich et al., 2016; Do Amaral et al., 2018), and are possible mechanical vectors for the fungus *Pseudogymnoascus destructans*, which causes white-nose syndrome (Lučan et al., 2016a). Transmission of pathogens directly from bat ectoparasites to humans and other wildlife has yet to be proven (Bai et al., 2018), and bat ectoparasites biting or parasitizing humans is considered to be a rare occurrence (Piksa et al., 2013).

It is unclear how habitat fragmentation impacts the dispersal of bat ectoparasites and their interactions with their hosts, which has potential implications for prevalence and spill-over of zoonotic diseases (Rulli et al., 2017). (Bolívar-Cimé et al., 2018b) found a higher prevalence of bat flies in continuous forest than the habitats embedded in pastures, but this trend was only true for one of the three bats species they examined; they also found that fragmentation had no significant effect on bat fly infestations for bats that use permanent roosts such as caves. (Frank et al., 2016b) reported a dilution effect for parasitism in female bats, where a habitat with higher bat species richness (associated with less disturbed habitat) had a lower prevalence of bat flies. (Saldaña-Vázquez et al., 2013) found no difference in the prevalence of bat fly infections in bat populations from forest fragments compared to coffee plantations and suggested two host traits to explain the observation; the first being the tendency to switch roosts

(low roost fidelity), and the second being solitary or paired social organisation; both traits limiting the acquisition and horizontal transfer of bat flies within a roost. Together, these studies suggest the effects of fragmentation on ectoparasite interactions can depend on multiple factors across host traits and landscape properties and may require both species and community-level analyses.

In this chapter, I document the prevalence, intensity and aggregation of bat flies and wing mites found on bats sampled from continuous forest and forest fragments within the Atlantic Forest, Brazil. Then, for two of the most common bat-bat fly pairs, I examine how their infection dynamics may vary across levels of habitat disturbance.

Methods

Sampling: Atlantic forest

Data for this study was collected from patches of forest surrounding the Reserva Ecológica de Guapiaçú (REGUA), one of several reserves in the Atlantic Forest of Brazil. All fieldwork, sampling, and bat identification was carried out by Tiago Teixeira (Teixeira, 2019). Altogether, 10 fragmented forest sites and 3 continuous forest sites within REGUA (control) were sampled (Figure 3.1). This habitat consists of a mixture of natural and secondary forest (Ribeiro et al., 2009). Fragmented sites were confined to forests on hilltops and steep cliffs and ranged from 20-243ha in area and were located 60-600m from the nearest alternative habitat (not necessarily sampled). All 13 sites were sampled for 6 nights each, between May 2016 and January 2017. Mist-nets (heights of 6, 9, and 12 metres) were set along anticipated bat-flight routes on trails, corridors, near streams and plant resources and were moved each night. Each site was

sampled using 7-10 nets (net-effort of 275.94 m^2h) and monitored every 30-45 minutes from sunset to midnight.

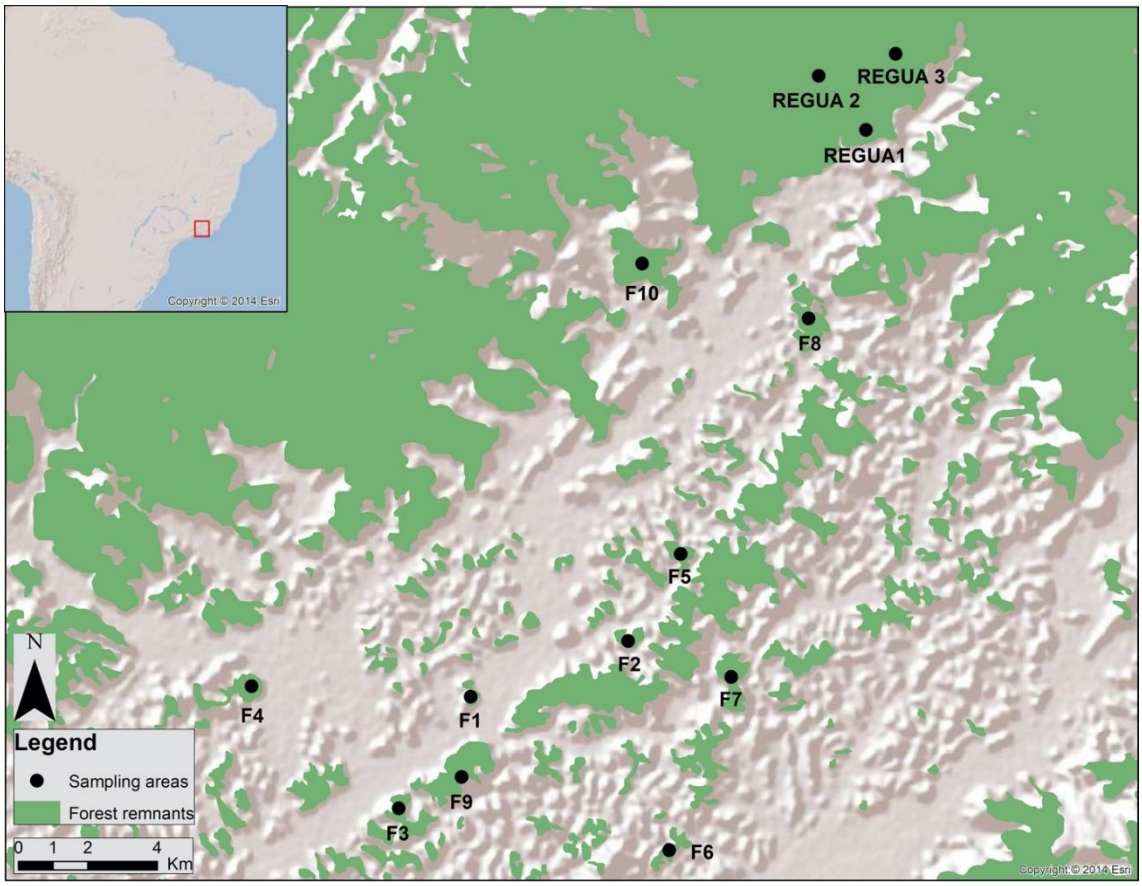


Figure 3.1: Map of sampling area of REGUA sites. Forest fragments are numerically sorted according to area size (F1 being smallest, F10 being largest). Black nodes mark approximate site locations. Figure taken from Teixeira (2019).

Bat capture, ectoparasite collection

Captured bats were kept in individual cotton bags until they were examined. Bats were identified using morphological keys based on Neotropical and Brazilian bat field guides (dos Reis et al., 2007; L Emmons, 1991; Reis et al., 2013). Morphometric measurements such as body length and mass, in addition to gender and age group (adult or juvenile)

were recorded. The wings and fur of each bat were thoroughly combed for ectoparasites with forceps for at least 90 seconds. All visible ectoparasites (typically bat flies) were collected and stored in 95% ethanol; cotton swabs of ethanol were used to collect ectoparasites too fine to be picked using forceps.

Processing Bat flies

Tubes containing bat flies were sent to the American Museum of Natural History (AMNH) for identification and endosymbiont analysis by Kelly Speer (Speer et al., 2020). Bat flies were first morphologically identified under a Leica S9i microscope using keys from literature and comparing material from the Field Museum's collections (DeLong, 2004; Graciolli and Carvalho, 2009; Wenzel, 1975). In preparation for downstream molecular analyses, each bat fly was dried, and then vortexed in 500µL PBS (1x) to wash-off external bacteria. The abdomen of each bat fly was split from its thorax to be used in DNA extraction.

DNA identification of bat flies

DNA extraction was conducted at the AMNH using the Zymo Research ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research). Each bat fly abdomen was digested overnight in proteinase K (digestion solution: 95uL Zymo Research Solid Tissue Buffer Blue, 95uL sterile water, 10uL Zymo Research proteinase K) at 55°C. The remaining steps in the extraction followed the manufacturers protocol but were adapted as follows: digested samples were homogenized in a bead beater for 20 minutes at 3000rpm; beaten samples were stored at -80°C until all samples were at this step; the DNA elution step was increased to 5 minutes, and the sterile water was heated at 55°C prior to application onto the filter; and the elution step was repeated using the first eluate. Negative controls using sterile water were used during DNA extraction.

Bat fly COI: PCR reactions consisted of 7.5 µL of 2x TopTaq Master Mix (Qiagen), 0.2 µM of each primer, 1.5 µL coral load, 1 µL template DNA, and sterile water adding up to 15 µL. The thermocycling program is as follows: an initial denaturation of 94 °C for 1 minute, 5 cycles of 94 °C for 1 minute, 45 °C for 90 seconds, and 72 °C for 90 seconds, followed by 35 cycles of 94 °C for 1 minute, 50 °C for 90 seconds, and 72 °C for 1 minute, with a final extension of 72 °C for 5 minutes.

Success of COI amplification was verified using gel electrophoresis (1.5% agarose gel), and then the PCR products cleaned using AMPure XP beads (Clarke, 2014); and prepared for cycle sequencing on the ABI 3730xl DNA Analyzer. Reagents and thermocycler conditions for this sequencing procedure are listed in table 3.1. Sequences were trimmed to 645bp segments, checked for quality, and aligned using ClustalW in Geneious v.10.2.4 (Kearse et al., 2012). A phylogeny was built using RAxML v.8 using a model of GTR+G evolution based on AIC scores taken from jModelTest 2.1, with 1000 bootstrap replicates on the CIPRES Science Gateway (Miller, Pfeiffer and Schwartz, 2010; Darriba et al., 2012). The formed clades were examined on FigTree v1.4.2 to confirm clade membership matched the morphological identifications of bat flies (Stamatakis, 2014; Rambaut, 2018).

Table 3.1 PCR mix recipes and thermocycling conditions for amplifying, tagging, quantifying, and Sanger sequencing mite and cotton samples.

PCR Objective	Target *COI*	Sanger PCR	MID PCR	qPCR
Reaction volume (µL)	15	10	20	10
Master Mix (µL)	7.5 (Qiagen multiplex)	4 (Big Dye)	10 (Qiagen multiplex)	8 (SYBR FAST)
Primer goal	Amplicon	Terminator dye	Unique tagging	Quantifying
Each primer (µL)	0.75	1	1	-
Template DNA (µL)	2	2 (exo-sapped)	8	2
sdH20 (µL)	4	3	-	-
Program source	(Hajibabaei et al., 2006a)	NBAF	NBAF	KK4873
Initial denaturation (temp °C/s)	94-900 (15 minutes)	96-60	95-900 (15 minutes)	95-300 (5 minutes)
Denaturation (temp °C/s)	94-60	94-10	98-10	95-30
Annealing (temp °C/s)	56-90	50-5	65-30	60-45
Extension (temp °C/s)	72-90	60-240 (4 minutes)	72-30	-
Final extension (temp °C/s)	72-600 (10 minutes)	-	72-300 (5 minutes)	-
Number of cycles	35	39	10	35
Total time (min)	130	166	32	48

DNA identification of mites: primer evaluation

I performed all molecular work to identify wing mites at the NERC Biomolecular Analysis Facility at the Sheffield University (NBAF). I conducted preliminary PCR primer testing with 4 pairs of primers which target COI and have been applied across a variety of invertebrate taxa: 1) MLepF1 and C_LepFolR (Hebert et al., 2013; Hernández-Triana et al., 2014), 2) C_LepFolF and MLepR2 (Hajibabaei et al., 2006b; Hernández-Triana et al., 2014), 3) Uni-MinibarF1 and Uni-MinibarR1 (Meusnier et al., 2008), and 4) ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale et al., 2011). I tested each primer set using DNA extracts from cat fleas (*Ctenocephalides felis*) provided by NBAF Sheffield. I selected primer set 1 (MLepF1 and C_LepFolR) as the primary set for this work as it amplified well (see appendix 2.1), primer set 2 (C_LepFolF and MLepR2) performed similarly and was retained as a secondary primer pair for any amplification failures. Additionally, I consulted morphological keys from (Tipton and Wenzel, 2011; Wenzel, 1975) to identify wing mites under light microscopy (See appendix 2.2 and 2.3) but I failed to consistently confirm species by this approach without reference material and I relied exclusively on DNA metabarcoding for all mite identification for the remainder of this chapter.

DNA identification of bat flies: DNA extraction and PCR

Sample preparation : The number of mites included in each extraction tube depended on how they were collected and stored. Those collected using forceps were processed as individuals while those collected on cotton swab were processed as a single sample and this also dictated downstream sequencing approaches (Sanger sequencing vs. metabarcoding via Illumina MiSeq respectively). For Sanger sequencing, 172 mites

taken from 64 bats were extracted in individual tubes. Mite samples split this way were given a short (5 seconds) wash in fresh ethanol to minimize contamination pooling during field collection. Cotton-samples tended to be numerous and smaller (possible juveniles and eggs; images available in appendix 2.2) and could not easily be manipulated using forceps. I processed a total of 31 cotton-samples, each containing swabs from individual bats, as 31 individual samples. In order to remove biological material from the swabs as much as possible, I spread each cotton-sample in a petri dish and allowed them to dry, I used a x100 microscope and forceps to scrape organic material off the swab for 2-3 minutes and removed the cotton swab from the petri dish afterwards. I then added 400 µL of 95% ethanol to the petri dish and used pipettes to transfer this new pool to extraction tubes. I placed these tubes in a heated centrifuge (30°C for 10 minutes) at 100 rpm to both remove excess ethanol and collect organic material at the bottom, for a pooled extraction.

Qiagen lysis : For extracting DNA from the mites, I followed a protocol described in (Desloire et al., 2006) which included mechanical disruption of mites in 20-25 µL PBS and the DNeasy Blood and Tissue extraction kit (Qiagen, 2006) with modified reagent volumes. The lysis mix included 80-100 µL ATL, 10 µL proteinase K, 260 µL AW1 and AW2 buffers, and 50 µL AE elution buffer. The mites were incubated (56 °C) in proteinase K for at least 5 hours. I mechanically disrupted the mites by crushing them against the sides of the extraction tubes with PBS using disposable plastic pestles; while I was able to obtain some DNA without damaging the specimen, the mites frequently either float above the lysis buffer or adhere to the sides of the tube when whole, failing to be submerged during the extraction process making mechanical disruption an optimal approach.

Initial PCR : DNA extracts from individual and pooled mites were subject to the same procedure to amplify a 407-bp fragment from their mitochondrial *COI* using primer pair MLepF1 (5' GCTTTCCCACGAATAAATAATA 3'; Hajibabaei et al., 2006a) and C_LepFolR which is an even mixture of two reverse primers LepR1 (5'GGTATAACTATRAARAAAATTAT3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA3') (Hernández-Triana et al., 2014). For DNA extracts from cotton swaps intended for Illumina MiSeq, tagged versions of the primers were used; the 5' tag for MLepF1 was (5'TCTACACGTTCAGAGTTCTACAGTCCGACGATC-) and the 5' tag for both reverse primers were (5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-). The recipe for PCR mix and settings for the PCR program is listed in Table 3.1.

Sanger sequencing preparation

Inactivate product: I visualised PCR products, I ran 4 µL of product on a 2% agarose gel to estimate its relative amplification success. I incubated 6 µL of product (adjusted to 5ng/µL with dH20 if necessary) with 2 µL ExoSap-IT (ThermoFisher) for 15 minutes at 37°C, then 15 minutes at 80°C to remove free nucleotides and inactivate enzymes in the product.

Sequencing PCR: For each exo-sapped product, I ran two sequencing PCR reactions, one forward primer reaction and one reverse primer reaction. The master mix for each reaction consists of 0.5 µL Big Dye V3.1 and 3.5 µL 2.5x sequencing dilution buffer (ThermoFisher). Reagent volumes and the PCR program are listed in Table 3.1 as Sanger PCR. I prepared the sequencing PCRs in 96-well plates alongside positive controls of 2 µL plasmid pGEM-3Zf(+) and 2 µL primer M13-21.

Ethanol precipitation: I precipitate each product by adding 2 µL 235mM EDTA, 2 µL 3M sodium acetate (pH 5.2), 10 µL ddH20 and 52.5 µL 95% ethanol, and incubating them for 15 minutes in the dark. I centrifuged plates at full speed for 15 minutes, to pellet PCR product at the bottom of the wells. I then removed the precipitating solution from the wells by placing the plates upside down, on folded paper towel, and spun these at 190g for 30 seconds. I add 76.5 µL of ethanol to the wells to wash the PCR pellets and then spun them upright for 5 mins and then removed the ethanol using the same method. The ethanol washing was done twice for each pellet. I dried plates for 1 minute and added 10 µL of formamide to each pellet and denature it by incubating at 95°C for 3 minutes. The resulting plates were sequenced on an ABI3730.

Illumina sequencing preparation

Tagging PCR product: I assigned each cotton derived sample PCR product identifiers based on unique combinations of dual-plexed Fi5 and Ri7 primers. I used these primers in a tailed PCR to add Molecular Identifiers (MIDs) and Illumina sequencing sites to the amplicon products (master mix and PCR program listed in Table 3.1 as MID PCR).

Purifying tagged product: I used a fluorimeter to check the concentration of 2 µL of each tagged product. I took 150 ng from each product to create 3 pools of tagged products (2 pools of 8 products, and 1 pool of 7 products). I used a speed vac to concentrate the pools to 50 µL each and purified the pools of product using AMPure XP beads following manufacturer's instructions (Ronaghi et al., 2011). I mixed 50 µL of product with 25 µL of beads, as a 0.5x bead concentration will bind large fragments (>600bp) to be discarded. I used 67.5 µL of beads for a 0.9x bead concentration to bind

smaller fragments (>200bp) and discarding the supernatant, retaining our tagged amplicon library.

Quantification with qPCR: for each amplicon library, I made 3 sets of 100-fold, 1000-fold, and 10000-fold dilutions. These dilutions were run in a qPCR using 8 µL SYBR FAST master mix (KK4873, KAPA Biosystems) and 2 µL diluted libraries. The reaction was set up on ABI's Quantstudio software, the program is listed in Table 3.1 as qPCR. I used the known concentrations to pool all amplicon libraries in equimolar amounts at 4 nM to be sent for Illumina MiSeq sequencing.

Bioinformatics

Sanger trimming sequence processing: I converted ABI format sequences to fasta files, filtered for quality and trimmed adapters using Phred and Phrap (Ewing et al., 1998). I manually edited sequences using CodonCode Aligner (CodonCode Corporation, www.codoncode.com) and Bioedit (Hall, 1999). Edited sequences were uploaded to the Biodiversity of Life Database (BOLD) under the project code 'BCOKR'.

Illumina MiSeq data processing: bioinformatic analyses was conducted on the *iceberg* high performance computing system hosted by Sheffield University. I used Trimmomatic to remove low quality sequences (Phred quality score <30) and any Illumina adapters from my raw data (Bolger et al., 2014). I paired the trimmed reads and aligned these using FLASH (Magoč and Salzberg, 2011), and then converted from fastq to fasta format. I used Mothur to trim off primer sequences and demultiplex the data, distinguishing sequences originating from separate cotton-samples (Schloss et al., 2009). I used Usearch and Unoise to remove chimeric sequences and cluster groups of highly similar sequences (at least 97% similar) (Edgar, 2016, 2010). I treated clusters

as mOTUs (molecular operational taxonomic units) and blasted each against the NCBI nucleotide database (Agarwala et al., 2017). Any mOTU not assigned to a known ectoparasite was removed. The remaining sequences were aligned and edited using to voucher sequences. Representatives of each mOTU were used to generate a neighbour joining tree using the kimura 2 parameter model of sequence evolution in MEGA 5 to compare MOTU similarities.

Epidemiological statistics of bat fly infection

I calculate several infection indices for every bat-bat fly pair using the Quantitative Parasitology (QP) online platform (Reiczigel et al., 2019). These indices are as follows:

- Prevalence: The proportion of infected hosts, expressed as a percentage. A 95% confidence interval (CI) is provided to express the uncertainty of sample prevalence to estimate population prevalence. This CI is calculated based with Sterne's method as it has been shown to be suitable for small numbers of parasite counts (Sakakibara, 2014).
- Mean (μ) Intensity: Average number of parasites found on an infected host. Uninfected hosts are excluded when calculating population totals this way. A bias-corrected and accelerated bootstrap of 2000 times was used to calculate the 95% CI for mean intensity.
- Aggregation: expressed by the index of discrepancy (*D*) (Poulin, 1996), measures the difference between the observed distribution of parasites when compared to a theoretical distribution where all hosts are equally infested. *D* ranges from 0 (total equality) to 1 (maximal inequality).

For two bat-bat fly pairs, *Carollia perspicillata* - *Trichobius joblingi* (CT) and *Artibeus lituratus* - *Paratrichobius longicrus* (AP) I compare how prevalence and mean intensity vary between habitat type (fragmented or continuous forest) and host sex. I focus on these host-parasite pairs because they were the most abundant bats captured and their primary bat fly association (Komeno and Linhares, 1999; Tipton and Wenzel, 2011; Wenzel, 1975). For between group comparisons, I used Fisher's Exact Test for comparing prevalence, and two-sample *t* test for mean intensity.

To examine the relationship between ectoparasite intensity and host traits (for CT and AP) I used Generalised Linear Models (GLM) with parasite intensity as the responding variable and host mass and sex as predictors. Negative binomial distribution was used to account for the aggregated distribution of parasites (Wilson and Grenfell, 1997) using the *glm.nb* function from the MASS package in R (Riplley et al., 2018). Models with predictors were compared to the null model (containing only the intercept) using likelihood-ratio chi-square (LR) in the *anova.glm* function (Hastie and Pregibon, 2017).

Results

Sample collections and identifications

Bat flies

The original field collections included 988 bats representing 26 species, 343 (34%) of which were parasitized by at least one bat fly. Site F10 had the highest prevalence (44% of bats were hosts). The most abundant host species was also the most abundant bat collected *Carollia perspicillata*, with 176 hosts from 383 captures, followed by *Artibeus lituratus*, with 55 hosts from 190 captures. A total of 842 bat flies were collected from 17 bat species, and 813 of these parasites were morphologically

identified to species level, the remaining 29 could only be identified to genus using morphology (Table 3.2). However, molecular analysis with *COI* barcoding supports the allocation of these parasites to 20 described species, and 5 species-level mOTUs (e.g *Trichobius sp.1, Trichobius sp.2, etc.)*. About half (436) of the total bat fly collection was obtained from samples of *C. perspicillata*.

Ectoparasitic mites

For mites processed by metabarcoding, 36 ectoparasite mOTUs were obtained from 31 bats sampled using cotton swabs. These mOTUs formed at least 12 clades (Figure 3.2): 3 clades, consisting of 20 mOTUs had >95% blast matches at a species level, although these only belong to 3 species: bat flies *M. aranea* and *T. joblingi*, and mite *Dermacentor nitens* (Table 4) indicating significant MOTU over-splitting. Four clades had low identity matches (<94%) to unclassified mite vouchers collected from Canada (Blagoev et al., 2016), these matches were to *Trombidiformes sp*. BOLD:ACJ2331, *Digamasellidae sp*. BOLD:ACI5517, *Mesostigmata sp.* BOLD:ACI6949, and *Stigmaeidae sp*. BOLD:ABV1836. The remaining 5 clades had < 90% match to a known reference. Sequence length of mOTU representatives was unrelated to match similarity. The 143 Sanger sequences uploaded to BOLD were identified as belonging to family Spinturnicidae. Of these, 31 were assigned to existing BINs (Barcode Index Numbers) AAF9245 or AAF9243, the remaining sequences remain unassigned, which typically happens to sequences that are under 500 bp and contain >1% ambiguous bases (BOLD Systems, 2013)

Table 3.2 Captured bats and associated bat flies identified . Species names are used when able. Nh = number of bat hosts (and number of parasitized host), inf = number of a host species infected by that parasite, N_p = number of parasites infesting that host species, P (% prevalence), I_μ (mean intensity), D (index of discrepancy). Common hosts (n>50) are in bold.

Bat	N_h	Bat fly	Inf	N_p	P	I_μ	D
Artibeus fimbritatus	16 (3)	*Aspidoptera phyllostomatus*	2	4	12.5 [2.3-37.2]	2 [1-2]	0.853 [0.683-0.882]
		Megistopoda aranea	1	1	6.2 [0.3-30.5]	1	0.882 [0.647-0.882]
Artibeus geoffroyii	13 (9)	*Anastrebla modestini*	7	14	53.8 [26-77.6]	2 [1.29-2.43]	0.531 [0.324-0.736]
		Exastinion clovisi	7	32	53.8 [26-77.6]	4.57 [2.43-7.57]	0.629 [0.473-0.821]
Artibeus lituratus	190 (55)	*Basilia juquiensis*	1	3	0.5 [0-3]	3	0.99 [0.969-0.99]
		Megistopoda proxima	1	1	0.5 [0-3]	1	0.99 [0.963-0.99]
		Metelasmus pseudopterus	1	1	0.5 [0-3]	1	0.99 [0.969-0.99]
		Paratrichobius longicrus	50	77	26.3 [20.5-33.1]	1.54 [1.34-1.8]	0.796 [0.744-0.845]
		Speiseria ambigua	1	2	0.5 [0-0.3]	2	0.99 [0.958-0.99]
		Trichobius joblingi	2	5	1.1 [0.2-3.8]	2.5 [1-4]	0.987 [0.97-0.99]
Artibeus obscurus	63 (8)	*Aspidoptera phyllostomatus*	5	11	7.9 [3.2-17-3]	2.2 [1.2-3.8]	0.932 [0.88-0.969]
		Megistopoda aranea	1	1	1.6 [0.1-8.5]	1	0.969 [0.906-0.969]
		Paratrichobius longicrus	2	3	3.2 [0.6-10.9]	1.5 [1-1.5]	0.958 [0.905-0.969]
		Strebla guajiro	1	1	1.6 [0.1-8.5]	1	0.969 [0.906-0.969]
Carollia perspicillata	383 (176)	*Paratrichobius longicrus*	1	1	0.3 [0-1.5]	1	0.995 [0.982-0.995]
		Paraeuctenodes similis	4	5	1 [0.4-2.7]	1.25 [1-1.5]	0.989 [0.974-0.995]
		Speiseria ambigua	29	36	7.6 [5.2-10.7]	1.24 [1.07-1.45]	0.934 [0.909-0.954]
		Strebla guajiro	37	47	9.7 [7-13]	1.27 [1.11-1.51]	0.919 [0.892-0.942]
		Trichobius dugesioides	43	55	11.2 [8.3-14.8]	1.28 [1.14-1.42]	0.904 [0.876-0.93]
		Trichobius joblingi	132	292	34.5 [29.7-39.4]	2.21 [1.96-2.55]	0.778 [0.744-0.81]
Dermanura cinerea	9 (1)	*Neotrichobius delicatus*	1	3	11.1 [0.6-44.4]	3	0.8 [0.4-0.8]
Desmodus rotundus	72 (23)	*Paratrichobius longicrus*	1	1	1.4 [0.1-7.4]	1	0.973 [0.918-0.973]

		Speiseria ambigua	1	1	1.4 [1-7.4]	1	0.973 [0.932-0.973]
		Strebla mirabilis	1	1	1.4 [1-7.4]	1	0.973 [0.904-0.973]
		Strebla wiedemanni	18	26	25 [15.8-36.2]	1.44 [1.17-1.89]	0.99 [0.958-0.99]
		Trichobius dugesioides	1	2	1.4 [0.1-7.4]	2	0.987 [0.97-0.99]
		Trichobius furmani	3	4	4.2 [1.1-11.6]	1.33 [1-1.67]	0.932 [0.88-0.969]
		Trichobius joblingi	3	4	4.2 [1.1-11.6]	1.33 [1-1.67]	0.969 [0.906-0.969]
Glossophaga soricina	29 (4)	*Strebla guajiro*	1	1	3.4 [0.2-16.9]	1	0.958 [0.905-0.969]
		Trichobius sp1	1	2	3.4 [0.2-16.9]	2	0.969 [0.906-0.969]
		Trichobius sp4	4	5	13.8 [4.9-30.8]	1.25 [1-1.5]	0.995 [0.982-0.995]
Myotis nigricans	21 (4)	*Basilia juquiensis*	4	12	19 [6.8-40.3]	3 [1.25-5.75]	0.989 [0.974-0.995]
Myotis riparius	15 (4)	*Basilia juquiensis*	4	10	26.7 [9.7-53.4]	2.5 [1-3.25]	0.762 [0.56-0.875]
Phyllostomus hastatus	11 (7)	*Trichobius longipes*	7	44	63.6 [33.3-86.5]	6.29 [3.71-10.6]	0.553 [0.386-0.749]
Platyrrhinus lineatus	27 (5)	*Paratrichobius sp.*	2	5	7.4 [1.3-23.7]	2.5 [2-2.5]	0.9 [0.771-0.929]
		Strebla guajiro	1	1	3.7 [0.3-18.1]	1	0.929 [0.786-0.929]
		Trichobius angulatus	2	2	7.4 [1.3-23.7]	1	0.893 [0.679-0.929]
Sturnira lilium	71 (33)	*Aspidoptera falcata*	19	52	26.8 [17.4-38.7]	2.74 [2-3.68]	0.817 [0.745-0.883]
		Megistopoda proxima	22	30	31 [2.1-42.9]	1.36 [1.14-1.59]	0.739 [0.646-0.826]
		Trichobius joblingi	1	1	1.4 [0.1-7.5]	1	0.972 [0.917-0.972]
Sturnira tildae	12 (5)	*Aspidoptera falcata*	5	11	41.7 [18.1-70.6]	2.2 [1-4.2]	0.692 [0.523-0.846]
		Megistopoda proxima	4	7	33.3 [12.3-63]	1.75 [1-2.5]	0.714 [0.523-0.846]
Tonatia bidens	1 (1)	*Strebla wiedemanni*	1	1	100 [5-100]	1	*NA*
		Trichobius joblingi	1	1	100 [5-100]	1	*NA*
		Trichobius sp.3	1	12	100 [5-100]	12	*NA*
Trachops cirrhosis	1 (1)	*Trichobius sp.2*	1	5	100 [5-100]	5	*NA*
Vampyressa pusilla	18 (4)	*Neotrichobius delicatus*	4	6	22.2 [8-47.1]	1.5 [1-1.75]	0.772 [0.587-0.895]

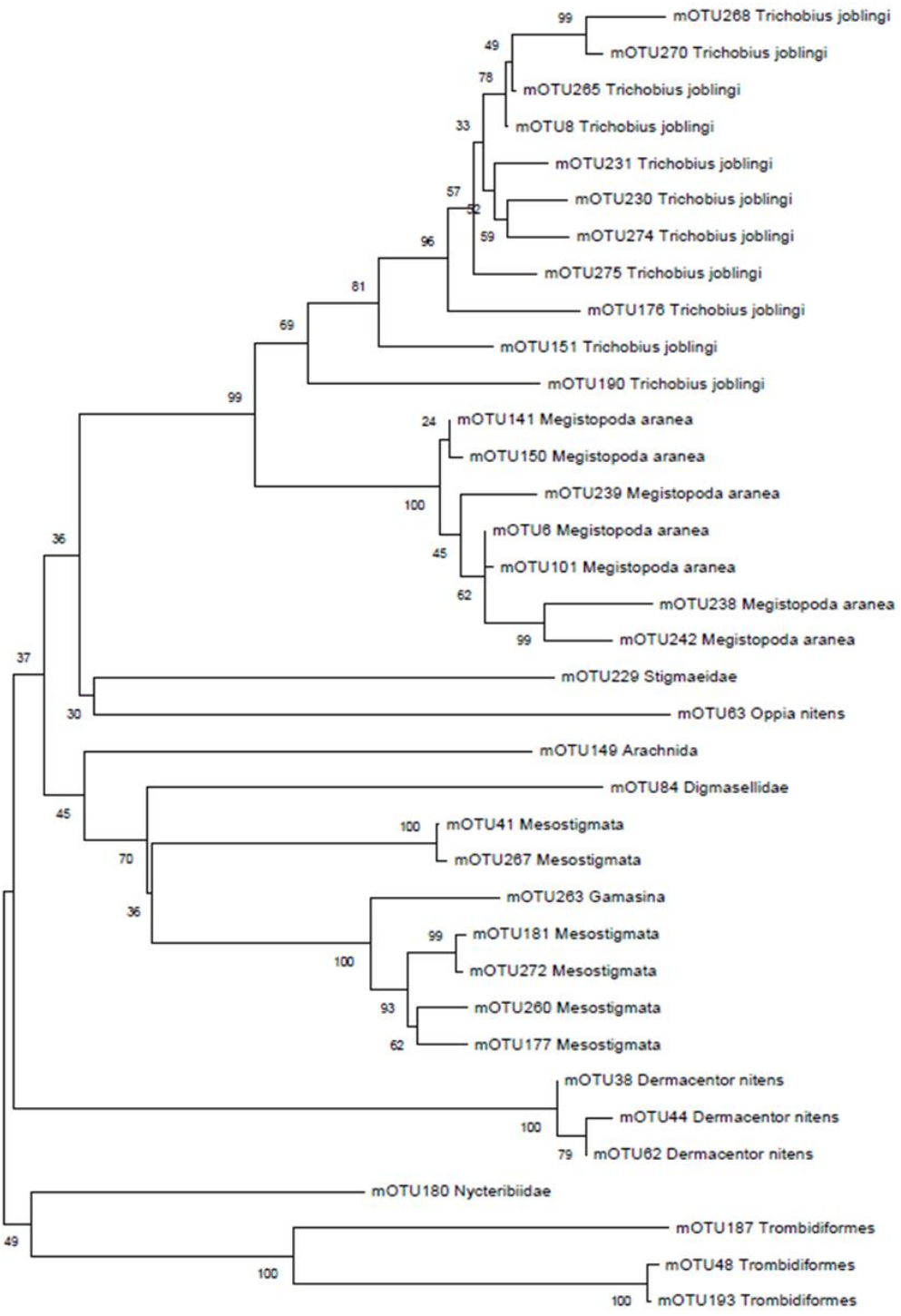


Figure 3.2: Phylogenetic tree of 31 mOTUs generated through metabarcoding fur and wing swabs from bats.

Bat fly associations

The most abundant bat flies collected were *Trichobius joblingi* (n= 303), followed by *Paratrichobius longicrus* (n=82), and *Aspidoptera falcata* (n=63). Only 1 species from the family Nycteribidae, identified as *Basilia juquiensis,* was collected (n=25). Several bat species were caught in low numbers (n< 50), limiting the possible analyses for these species (e.g. 100% infestation for the bat *Tonatia bidens* represents a single individual). Similarly, some bat-bat fly associations were only detected once (e.g., *Basilia juquiensis* on *Artibeus lituratus*) and were excluded from further analyses. For the common bat species (n> 50), the highest parasite prevalence observed was *Trichobius joblingi* on host *Carollia perspicillata* (34.5% of individuals), followed by *Paratrichobius longicrus* on host *Artibeus lituratus* (26.3% of individuals), and *Aspidoptera falcata* on host *Sturnira lilium* (26.8% of individuals) (Table 3.2). Highest mean intensity for the common bat species was found in *A. falcata* on *S. lilium* (2.74), followed by *T. joblingi* on *A. lituratus* (2.5) and *Aspidoptera phyllostomatus* on *Artibeus obscurus* (2.2). Bat flies with the highest aggregation for common hosts were *T. joblingi* on *A. lituratus* (D = 0.987), *P. longicrus* on *A. obscurus* (D = 0.958), and *Trichobius furmani* on *Desmodus rotundus* (D = 0.932).

Bat fly epidemiology

When fragments and continuous forests were considered together, there were no differences in overall prevalence (CT= 0.743; AP =0.449) or mean intensity (CT=0.203; AP=0.47) between the sexes of the two bat-bat fly pairs (Table 3.3). When considering differences between site types, there were no differences in prevalence (*P*= 0.627; Fisher's exact test) or intensity (*P*=0.596; Fisher's exact test) for AP. In contrast, *T.*

joblingi on C. perspicillata was more than twice as prevalent in fragments than in continuous forest (*P* <0.0001; Fisher's exact test), although no difference was seen in their intensities (*P*=0.075; Fisher's exact test).

When considering both host sex and site type, the prevalence of *T. joblingi* on *C. perspicillata* was higher in fragments than continuous forest, and this was true for both male and female hosts (*P*= 0.002, and *P*=0.006 respectively; Fisher's exact test), however *T. joblingi* infection on female *C. perspicillata* was twice as intense in fragments than in continuous forests (*P*=0.001; Fisher's exact test). In contrast, there was no significant difference in prevalence (*P*= 0.1411; Fisher's exact test) or intensity (*P*= 0.26; Fisher's exact test) of *P. longicrus on* male *A. lituratus* across site types, however in continuous forests, *P. longicrus on A. lituratus* intensity in females was higher than males (*P*=0.029; Fisher's exact test), although this was only a 0.6 difference in mean intensity. *P. longicrus on A. lituratus* in females in continuous forests appeared to be twice as prevalent as fragments, but this was not seen as statistically significant (*P*=0.065; Fisher's exact test).

For both *T. joblingi* on *C. perspicillata* and *P. longicrus* on *A. lituratus* interactions, the highest infestations were found on individuals with moderate body condition (Figure 3.3 and 3.4). The highest infestation for *T. joblingi* on *C. perspicillata* was shared amongst 3 females with 8 bat flies each, whereas for *P. longicrus* on *A. lituratus* the highest infestation was on a single male with 5 bat flies, although most *P. longicrus* on *A. lituratus* infestations tended to be low (n<2). Neither host mass nor sex were found to predict bat fly intensity in for either CT or AP, with all tested models (mass, sex, mass + sex) not being significantly different to the null model (results not shown).

Table 3.3 Prevalence and mean intensity for bat flies Trichobius joblingi on Carollia perspicilata , and Paratrichobius longricus on Artibeus lituratus based on host sex and sampling location. P-values for paired group comparisons are listed in grey rows; comparisons for prevalence were made with Fisher's exact test, two-sample t-test for mean intensity. SD = standard deviation for mean intensity.

Group	Infected	Captured	Prevalence	Mean intensity	SD
Trichobius joblingi on *Carollia perspicilata*					
Males	54	163	33.10%	2	1.414
Females	76	216	35.20%	2.382	1.883
Between sexes			0.7432	0.203	
Fragments	120	303	39.70%	2.273	1.742
REGUAs	10	76	14.30%	1.545	0.934
Between site type			**<0.0001**	0.075	
Males, Fragments	48	120	40.00%	2.021	1.451
Males, REGUAs	6	43	14.00%	1.833	1.169
Females, Fragments	71	182	39.00%	2.465	1.919
Females, REGUAs	5	34	14.70%	1.2	0.447
Males, between site type			**0.0022**	0.707	
Females, between site type			**0.006**	**0.001**	
Fragments, between sexes			0.9045	0.161	
REGUAs, between sexes			1	0.343	
Paratrichobius longricus on *Artibeus lituratus*					
Males	21	93	22.60%	1.429	0.926
Females	29	96	30.20%	1.621	0.775
Between sexes			0.449	0.470	
Fragments	29	104	27.90%	1.483	0.829
REGUAs	21	86	24.40%	1.619	0.865
Between site type			0.6277	0.596	
Males, Fragments	14	48	29.20%	1.571	1.089
Males, REGUAs	7	45	15.60%	1.143	0.378
Females, Fragments	15	56	26.80%	1.4	0.507
Females, REGUAs	14	40	35.00%	1.857	0.949
Males, between site type			0.1411	0.260	
Females, between site type			0.4994	0.1350	
Fragments, between sexes			0.8289	0.596	
REGUAs, between sexes			0.0653	**0.029**	

Table 3.4. GLM output for infection intensities against host traits. Models were compared using AICc.

Trichobius joblingi on *Carollia perspicilata*							
Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
~1	2	300.13	0	0.36	0.36	-148.03	**1**
Sex	4	301.61	1.47	0.17	0.52	-146.69	**2.11**
Forearm	3	301.91	1.77	0.15	0.67	-147.88	**2.4**
Mass	3	302.15	2.02	0.13	0.8	-148.01	**2.76**
Mass + Sex	5	302.33	2.19	0.12	0.92	-145.99	**3**
Mass + Forearm	5	303.61	3.48	0.06	0.98	-146.63	**6**
Sex + Forearm	3	306.05	5.92	0.02	1	-145.7	**18**
	variable	Estimate	Std. Error	z value	Pr(>|z|)		
Mass	(Intercept)	-1.15829	1.03654	-1.117	0.264		
	Mass	0.00341	0.01427	0.239	0.811		
Sex	**Female**	**-0.7397**	**0.2009**	**-3.681**	**0.000232**		
	Male	-0.3817	0.3021	-1.264	0.206393		
Forearm	(Intercept)	-3.04062	3.88483	-0.783	0.434		
	Forearm	0.02984	0.05442	0.548	0.583		
Artibeus lituratus and Paratrichobius longricus							
Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
Mass (g)	3	784.04	0	0.29	0.29	-388.99	**1**
~1	2	784.68	0.63	0.21	0.5	-390.32	**1.38**
Sex	4	785.22	1.18	0.16	0.67	-388.55	**1.81**
Forearm	3	785.57	1.53	0.14	0.8	-389.75	**2.07**
Sex + Forearm	3	786.58	2.54	0.08	0.88	-390.26	**3.62**
Mass + Sex	5	787.05	3	0.07	0.95	-388.43	**4.14**
Mass + Forearm	5	787.49	3.44	0.05	1	-388.65	**5.8**
	variable	Estimate	Std. Error	z value	Pr(>|z|)		
Mass	(Intercept)	0.87723	0.72669	1.207	0.2274		
	Mass	-0.08312	0.04921	-1.689	0.0912		
Sex	Female	-0.235	0.1429	-1.645	0.1		
	Male	-0.2337	0.2184	-1.07	0.285		
Forearm	(Intercept)	-1.44217	2.65962	-0.542	0.588		
	Forearm	0.0276	0.06613	0.417	0.676		

Figure 3.3: Infestation profile across body condition for Trichobius joblingi on Carollia perspicilllata.

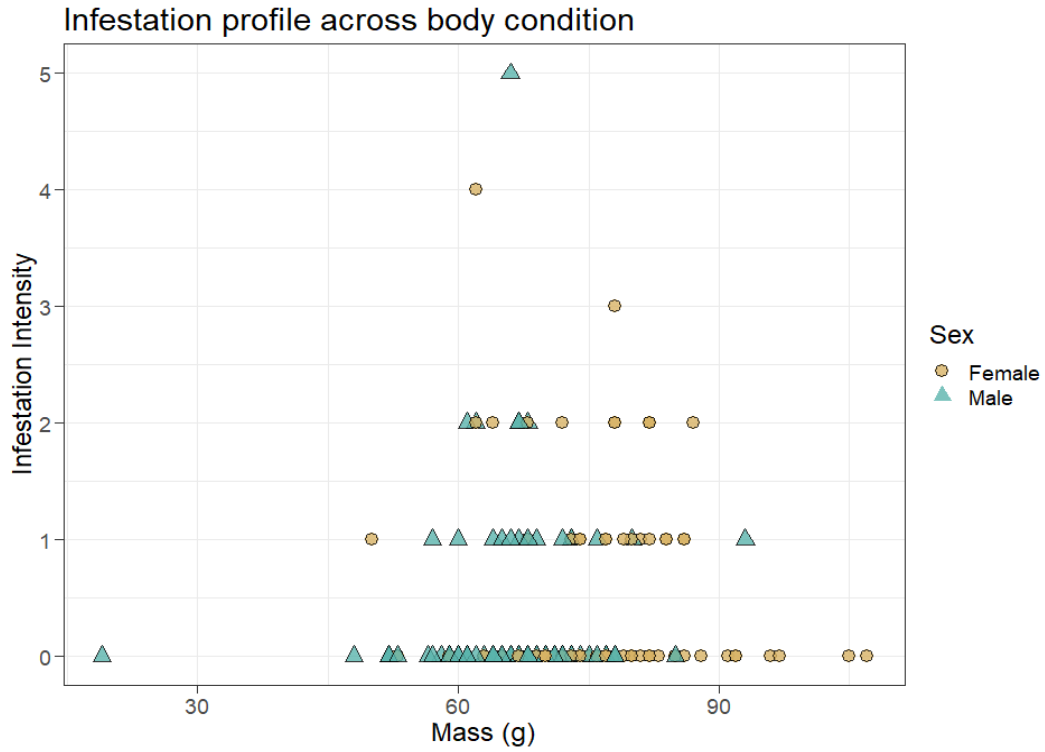


Figure 3.4: Infestation profile across body condition for Paratrichobius longicrus on Artibeus lituratus.

Discussion

In this study, I report on the prevalence of ectoparasites associated with bats across fragmented habitat in the Atlantic forest, and for two species-level associations, examine host and habitat related factors associated with this infection. My analysis suggests that bat fly parasitism can differ between continuous and fragmented habitats, but responses varied based on host species and host sex. My results also support the conclusion that parasites tend to be aggregated on their hosts (Leung, 1998), although these estimates may only be valid for the more commonly sampled bat-ectoparasite pairings (E R Morgan et al., 2005; Wilson et al., 2001). In this study, I group 3 "control" sites from continuous forest together and do the same to the 10 fragmented forest sites, although there the 13 sites do vary across several landscape metrics, I show in Chapter 3 that my grouping method is justified at a metacommunity level.

Previous studies of ectoparasites on Brazilian bats have included ticks (Muñoz-Leal et al., 2016), flies and mites (Almeida et al., 2016; Juliana C. Almeida et al., 2011; Santos et al., 2013). However, these studies identified their specimens using morphological keys (e.g. for mites; Herrin and Tipton, 1967, for bat flies; Tipton and Wenzel, 2011; Torres et al., 2019). As far as I am aware, COI data for ectoparasitic mites on bats for this region remains scarce. This scarcity of data makes some levels of identification, particularly for mites, challenging. Bruyndonckx et al., (2009) overcame the problem of an incomplete sequence database by complementing COI and 16S sequences of mites with morphological keys. This same approach was taken when identifying the bat flies

analysed here. For mites we used a combination of barcoding for larger specimens and metabarcoding for the microscopic specimens with mixed success. Our data suggest that a MOTU approach using any standard thresholds significantly overestimates mite diversity. When arranged phylogenetically or compared to reference material (figure 3.2) this over splitting becomes obvious where multiple MOTU are matched to the same reference material. While most sequences could not be classified to species, the overall ectoparasite diversity obtained from metabarcoding material from cotton swabs highlights the parasite richness potentially missed when considering larger, more easily captured ectoparasites. Improved genetic target regions and databases for mites should be developed to more easily identify this group.

I found some evidence that bat fly infections differ between continuous and fragmented habitats, and these responses appear to be species specific. The prevalence of *T. joblingi* on *C. perspicillata* was higher in fragments than in continuous forests, but the same was not true for *P. longicrus* on *A. lituratus*. Plausible reasons for this may have to do with roost conditions and colony sizes seen with each bat species. Bat fly populations are known to be larger in permanent roosts such as caves and buildings than transient arboreal based roosts (Dittmar et al., 2009; Patterson et al., 2007). Colony sizes can vary drastically among bat species, *A. lituratus* are known to form small harem colonies (one male, several females; Muñoz-Romo et al., 2008), reported to range between 3-15 individuals (Zortea and Chiarello, 1994), and remain in small colonies in fragmented habitats (Evelyn and Stiles, 2003). In contrast, while *C. perspicillata* may also form small harems (Porter, 1978), they also roost in mixed species colonies as large as 200 bats (Deleva and Chaverri, 2018). A combination of reduced viable roosts in fragmented habitats and the propensity for *C. perspicillata* to

group in larger colonies may contribute to higher bat fly prevalence. Studies comparing parasite infection in other taxa from disturbed vs undisturbed habitats point to factors such as low habitat quality affecting stress levels (Lenihan et al., 2001; Ramírez-Hernández et al., 2019), and habitat heterogeneity and vegetation cover affecting host density and movement (Ruiz-Sánchez et al., 2017; Thamm et al., 2009; Young et al., 2015), which in turn affect a hosts susceptibility to parasite infection.

It is notable that in my two most captured species, bat fly infestation on female *C. perspicillata* females from fragments have higher infestations than those in continuous forests, and female *A. lituratus* in continuous forests had a higher infestation that males. Bat flies and other parasites have been known to sometimes favour female hosts, often attributed to reproductive status, where bat flies on pregnant and lactating females have access to juveniles, thereby increasing horizontal and vertical transmission of bat flies (Patterson et al., 2008a; Warburton et al., 2016) this may also be related to roost preference with females spending greater periods of time in dense roosting maternity colonies. However, while I did include pregnant and lactating females in the analyses, only a small number were infected with bat flies (5 infected out of 19 pregnant or lactating *C. perspicillata;* 15 out of 41 pregnant or lactating *A. lituratus*). An alternate explanation for the bias towards female hosts may be related to sexual dimorphism exhibited in some bat species where females are larger than males and can host a larger number of parasites (Presley and Willig, 2008). However, this sexual dimorphism is only seen in *A. lituratus* and not *C. perspicillata* in my sample, in line with previous analyses of sexual dimorphism in bats (Ulian and Rossi, 2017). (Rocha et al., 2017) observed more female than male neotropical bats in edge and matrix habitats, and this was more evident during the reproductive season. This

may point to less overlap between roosts utilized between the males and female bats, which could result in different infestation severity. Sex differences in parasitism is sometimes ascribed to behavioural and immunological differences between sexes in vertebrates (Klein, 2004). For example, androgens such as testosterone are thought to suppress the immune system (Hou and Wu, 1988) causing male-biased parasitism in some mammals (Schalk and Forbes, 1997). Sex hormones can also regulate behaviours that increase exposure to parasites from conspecifics such as aggression (Barnard et al., 1998; Folstad et al., 1989) or roaming (Altizer et al., 2003) during mating season. In contrast, (Rosso et al., 2020) found that males in the sexually dimorphic lizards *Anolis apletophallus* had more severe mite infestations due to their enlarged dewlap providing a nourishing attachment site, rather than solely due to immunological suppression.

Past studies on the effects of body condition on parasite infection have drawn varied conclusions. Patrício et al., (2016) and (Patterson et al., 2008b) found that body condition of bats had no effect on the degree of parasitism, while (Linhares and Komeno, 2006) saw bats with reduced body condition harbouring more parasites, and (Presley and Willig, 2008) found parasitism rate were positively correlated with body condition in some bat species, but negatively correlated in others. My models suggest that host mass and sex did not influence bat fly intensity in this study. Bize et al., (2008) observed highest intensity for ectoparasitic louse fly on Alpine swift hosts with moderate body condition, reasoning that while hosts in poor body condition had reduce immune defence, they also represent an inadequately nutritious resource, suggesting that a trade-off is made between nutrition and immunity. It is unknown to what extent bat flies can assess the immunocompetence of potential hosts, whether

they make that kind of evaluation prior to host selection in the wild or whether immunocompetence might be heritable along with parasite vertical transmission. However, a host-choice experiment conducted by (Witsenburg et al., 2015) found that bat flies had the ability to detect bats carrying non-infected stages of malaria-like parasites, and showed a preference for these hosts suggesting some level of host choice; although they admit that this behaviour is not seen in the wild, where host size and body condition were factors in host choice. In addition, Dick and Dick, (2006) found bat flies preferred clean hosts to those previously infested, but would avoid overcrowding on a single host, acknowledging an upper threshold to limit intraspecific competition. Admittedly, it is difficult to determine the cause-effect relationship between host body condition and infection intensity. For example, Fairn et al., (2008) found that insect host body condition was negatively correlated with parasitic mite intensity in wild populations and suggested that mite infection lowered host body condition, but also posited that hosts with poor body condition could simply be more susceptible to infection. However controlled infection experiments on guppies showed that guppies with relatively high body condition often become the most infested, as well as facilitate the spread of parasites within a population (Tadiri et al., 2013).

The distribution of parasites on host populations has empirically been described as a negative binomial distribution, where a small fraction of the host population carries a large portion of parasites (Wilson et al., 2006). I used the index of discrepancy (*D*) to measure parasite aggregation in the host population (Poulin, 1996), and find that most of the bat-bat fly pairings have values of *D* close to 1 (maximal inequality). The presence of this inequality in a host population has been linked to different host experiences, namely their exposure to parasites and their susceptibility to infection

(Wilson et al., 2001). Factors that have been linked to heterogenous levels of parasite exposure and susceptibility in other host-parasite systems include host personality (i.e. more social and active hosts risk exposure; Ezenwa et al., 2016), diet and infection history (i.e. nutrition and immunological memory;(Beechler et al., 2017),and host and parasite genotype compatibility (Morand et al., 1996; Tinsley et al., 2006), and chance encounters with 'high-risk environments' (e.g. a rodent host can become highly infested after encountering a cluster of nymphal ticks; (Calabrese et al., 2011). As mentioned previously, body condition can be linked to host susceptibility and distribution of parasites within a population (Fairn et al., 2008; Tadiri et al., 2013), however unlike the trend reported in those studies, bat flies here appear to be aggregated on hosts with moderate body condition.

Epidemiological estimates can be influenced by several sampling biases introduced by sampling method, study design, and sample size (Marques and Cabral, 2007). As an example of possible source of bias arising from sampling methods in this study, the parameters of streblid infestation of bats captured through netting alone may differ from bats remaining in roosts. These forms of observational biases (e.g. encountering, capturing, detecting certain groups of individuals) adds to the uncertainty in general conclusions made in disease ecology studies to wider communities (Lachish and Murray, 2018).

Poulin (2011) reported that observational bias can greatly affect the calculation of epidemiological indices in parasites showing aggregated populations, especially when sample sizes are small. Since aggregated parasite distributions means that most parasites are found on a few hosts, the likelihood of underestimating true values of

infestation intensity and abundance becomes more severe when fewer hosts are sampled as highly infected hosts are rarer (Jovani and Tella, 2006; Marques and Cabral, 2007). Morgan et al. (2005) observed that sample size bias is common when examining host classes such as age; where individuals from the oldest age classes tend to be underrepresented; however, they also note that mean burdens and infection prevalence are less sensitive to undersampling aggregated distributions. In this chapter, I observed higher bat fly intensities in host with moderate mass (figure 3.4). However, hosts with moderate mass were sampled much more frequently than hosts of low or high mass. Due to aggregated bat fly distributions, it is possible that infection intensities were underrepresented in hosts of low and high body mass to be compared on this basis.

Bats are a major focus for monitoring the emergence of infectious zoonotic diseases (López-Baucells et al., 2018b; Rabozzi et al., 2012; Webber and Willis, 2016). Blood-feeding arthropods are thought to be important potential vectors in the maintenance of diseases in wild bat populations (Melaun et al., 2014), although the capacity for these arthropods to spread zoonotic infections to humans directly is speculated to be small (Dick and Dittmar, 2014). Many genetic studies have shown that bat flies and wing mites carry the same strains of pathogens that infect their bat hosts (e.g. Bartonella; Morse et al., 2012a, Rickettsia; Wilkinson et al., 2016a, White-Nose-Syndrome; Lučan et al., 2016a), and habitat disturbance has been shown to increase the prevalence of vector-transmitted diseases (Cottontail et al., 2009c). Habitat disturbances such as deforestation and urbanisation can affect disease risk in wildlife

and human populations by impacting biodiversity, sanitation, and population density (Wood et al., 2017). High biodiversity has been shown to reduce disease risk in wild populations by diluting the population of susceptible hosts and sustaining populations of less competent host species within the community (Rohr et al., 2020; Wood et al., 2014). See chapter 6 for discussion on biodiversity-disease hypothesis.

Taken together, this study suggests additional consideration of host-parasite communities need to be taken to grasp the extent to which habitat disturbances can affect host ecology. Additionally, these effects may only be present in certain subcommunities, and the underlying ecological consequences need to be understood. Future work into surveying parasitic communities should consider both the visible and microscopic parasites, not only to better represent the potential pressures experienced by their hosts, but also illuminate possible interactions between the parasites themselves.

Chapter 4:
The impact of habitat fragmentation on host-symbiont networks

Abstract

Habitat fragmentation is a widespread disruptive force in ecological systems, and considerable effort has focused on understanding the impact on charismatic megafauna. In contrast, relatively little attention has been given to symbionts, particularly parasites, and how host-parasite relationships are affected by changing environmental conditions. Studies focusing on parasites are typically concerned with epidemiological metrics and how changes in host population affects parasite population dynamics. In contrast, I will focus on ectoparasitic bat flies which have a direct lifecycle with their bat hosts, and an obligatory pupal development stage on roost substratum and may thus respond directly to the environment. I also examine the gut microbiota of these parasites to provide insight on pathogen vector potential. I use bipartite network analysis and AIC-based model selection to examine the structure of bat-ectoparasite and ectoparasite-endosymbiont interactions across varying landscape metrics in a fragmented system in the Atlantic Forest. Bat fly richness was positively correlated with bat host richness, whereas endosymbiont richness was correlated with landscape properties rather than bat fly richness. I found that for both bat-ectoparasitic and ectoparasite-endosymbiont networks, differences in connectance and nestedness were not explained by landscape. Network modularity increased with habitat area in ectoparasitic networks, and modularity decreased with

habitat isolation in endosymbiont networks. However, both effects were present only when continuous forest sites were included in the analysis. My finding suggests that parasitic and microbial networks may not be sensitive to the environmental factors that affect hosts, but that microbial richness may respond to changes in landscape. A better understanding of how parasites and microbes respond to landscape change should be encouraged to predict changes in vectors of emerging infectious diseases.

Introduction

Habitat fragmentation

Ecosystems are comprised of species which exist within a complex network of interactions among organisms set within the biotic and abiotic environment. These systems can be slow to adapt to perturbations, especially when compared to the rapid changes occurring due to climate change (Erasmus et al., 2002; Walther et al., 2002) and landscape conversion (Lomolino and Perault, 2004; Meijer et al., 2011). In response to species loss, ecological networks have been observed to become simpler (Gonzalez et al., 2011), and often less connected (Gilbert, 2009), and more prone to secondary extinctions (Eklöf and Ebenman, 2006). The stability of ecological networks is frequently discussed alongside measurements of network complexity. In general, more complex networks composed of an abundance of connected prey species tend to be more stable (Uchida and Drossel, 2007), and a large number of generalist species have a strong role increasing the connectance of networks (Dunne et al., 2002). In contrast, systems dominated by specialists are susceptible to disturbance (Memmott et al., 2007). (McCann, 2000) suggests that it is not species diversity *per se* that drives ecosystem stability, but the diversity of functional groups able to differentially respond to changes in the ecosystem, and some studies highlight the fact that an abundance of

species exhibiting weak-interactions can safeguard a network from destabilising fluctuations due to the loss of strongly connected species (Amarasekare, 2003; Kokkoris et al., 1999).

Large-scale habitat fragmentation decreases the viability of many populations by affecting dispersal (Cushman, 2006), mortality, and reproductive rates (Andrén and Andren, 1994). This is often thought to be because fragmentation normally coincides with reduced habitat sizes which may not contain sufficient resources (Brühl et al., 2003). However, habitat size and fragmentation are not always linked and when there is no actual decrease in overall habitat size, resources may still be unevenly distributed among fragments (Williams and Kremen, 2007) thus fragmentation can have consequences independent of any change in overall habitat availability. The distinction between the impacts of habitat fragmentation and habitat loss is often subtle (Teixeira, 2019), and detangling these confounding effects is complex (Fahrig, 2007; Mortelliti et al., 2010; Thrush et al., 2008). For example, Bonin et al., (2011) reported time-dependent contrasting effects of fragmentation and habitat loss on coral reefs, with species richness and abundance of fish increasing initially but eventually tapering off in fragmented habitats, whereas species decline due to habitat loss only increased over time.

A distinct feature associated with habitat fragmentation is the often-increased isolation of remaining habitat patches. In these cases, population numbers may not be replenished by immigration from neighbouring communities (Magrach et al., 2011). While there is a general trend towards population decline due to habitat fragmentation, some species thrive in disturbed environments, particularly if they are good colonizers (Lynam and Billick, 1999), and can exploit situational resource

abundance (Benstead and Pringle, 2004), and escape from natural enemies (Roland, 1993a) which cannot themselves move between fragments. (Antongiovanni and Metzger, 2005) were able to classify birds in Manaus, Amazonia as either highly sensitive, moderately sensitive, or positively affected by fragmentation based on their persistence in habitat with different levels of disturbance, finding that some species were more abundant in secondary fragments than primary forests. However, in a study of beetles in fragmented habitats (Didham et al., 1998) acknowledge the usefulness of classifying species responses, but discuss the importance of examining complexity in life histories, providing examples of beetles designated as small-area, edge avoiders or large area, edge specialists, but speculating that these beetles may utilize different landscapes during their adult and juvenile stages, making such designations subject to change.

Measuring species interactions

While a large body of research has considered the response of biodiversity to habitat loss (Fahrig, 2003; Hanski, 2015), much less attention has been given to how species interaction networks are affected (but see for example, Ferreira et al., 2013; Hagen et al., 2012) and almost none have considered multiple trophic levels of interactions simultaneously. In network analysis interactions are represented as a graphical model. Many levels of biological hierarchy have been represented in networks, from genes and proteins in transcription regulation (Ramaprasad et al., 2012), to interacting species in communities (Sarmento et al., 2014). In ecosystem ecology, ecological networks consist of collections of potentially interacting species represented by nodes connected by edges (observed interactions). These networks typically represent predation (Allesina and Pascual, 2008), pollination (Vázquez et al., 2009), or parasitism

(Rigaud et al., 2010); and measuring changes in these structures over time or space allows us to quantify dynamic systems. For example, to assess the flow of energy through the ecosystem, to measure the stability of the community in the face of environmental changes (Laliberté and Tylianakis, 2010a) or to identify key species maintaining ecosystem structure (Hagen et al., 2012; Morris, 2010). They also provide a predictive model allowing us to quantify the robustness of an ecosystem against future species loss (Kaiser-Bunbury et al., 2010) and pinpoint species responsible for the preservation of ecosystem services (García-Algarra et al., 2017).

One way to assess ecological systems is by measuring mathematical properties, or 'metrics' across multiple networks generated from measuring species interactions. Node-level metrics such as degree (number of links a node has; Jordano et al., 2003a) and centrality (proximity to other nodes in the system; Freeman, 1978) determine which nodes functionally bind the system together (e.g. in food webs, herbivores can act as bottlenecks for energy moving towards higher trophic levels Proulx et al., 2005); whereas network-level metrics such as connectance (proportion of potential links that are observed; Jordano, 2000) and nestedness (tendency of 'specialist' nodes to share interaction partners with 'generalist' nodes, Bascompte et al., 2003) can be used to compare the robustness of different systems to disturbance (e.g. a more connected and nested system would be less prone to collapse after initial extinction events Dunne et al., 2002).

Analysing species interactions has improved our understanding of the causes and consequences of biodiversity loss in disturbed habitats. For example, the loss of habitat complexity (homogenisation) through deforestation has been observed to simplify predation networks (food webs) by exposing prey to predation (Laliberté and

Tylianakis, 2010b). Similarly species loss reduces the compartmentalisation (modularity) of some networks making them more vulnerable to invasive species and the spread of infectious agents (Marichal et al., 2010; Morris et al., 2016). However, network ecology is still an emerging field, with most existing literature focused on examining plant-mutualistic networks (Fortuna and Bascompte, 2006; Jordano et al., 2003b; Nielsen and Totland, 2014). Host-parasitoid (Lill et al., 2002; Macfadyen et al., 2011; MacFadyen et al., 2009), and other antagonistic networks or food webs (Mokross et al., 2013) have not received the same emphasis. Where antagonistic food webs have been explored, these studies tend to feature larger free-living species, with fewer examples of how networks of smaller, more cryptic organisms such as obligatory parasites and bacterial symbionts are affected by habitat disturbance. Where parasitism and habitat disturbance has been explored, the focus is on parasite prevalence driven by interest in regulating emerging infectious diseases (Bordes et al., 2015a; Hahn et al., 2014a; Lafferty, 2009; Sebaio et al., 2010) with minimal focus on ecological processes.

Host-parasite interactions and bacterial symbiosis

Despite a ubiquitous presence, parasitism is often overlooked in ecological networks, partly because of the small size of most parasites relative to their hosts which makes them difficult to study, and, particularly for microparasites, even more difficult to quantify (Gómez and Nichols, 2013; Lafferty et al., 2008; Marcogliese, 2004). Parasites are classified as symbionts bearing a non-lethal but negative effect for their hosts (Esch and Poulin, 2006), usually stealing nutrients (Schwanz, 2006), and limiting host growth and reproduction rate (Gorrell and Schulte-Hostedde, 2008). Studies of parasites in networks acknowledge their role as strong evolutionary forces against host fitness

(Lindholm et al., 2006), impacting populations directly (Hersh et al., 2014), as well as through their role as vectors of disease (Bradley and Altizer, 2007; Patz et al., 2000). However, there is increasing interest in understanding the role parasites play in shaping the structure and function of ecosystems by contributing to species diversity (Dunne et al., 2013), biomass (Marcogliese, 2004), and as food sources for other organisms (Johnson et al., 2010). Even in the context of endangered species conservation, there is a move against complete eradication of parasites even within vulnerable host populations, as they appear to have a role keeping immune systems primed against novel pathogens (Gompper and Williams, 2010).

Parasites are often investigated as potential vectors of infectious diseases, however the microbial endosymbionts of these parasites are also of increasing interest as they mediate behavioural and physiological traits of their vector hosts (Feldhaar, 2011). For instance, within Tsetse flies (*Diptera* : *Glossinidae*) which transmit African sleeping sickness, obligate mutualist bacteria they harbour have been shown to greatly reduce their vector competence (Aksoy et al., 2008). Pathogenic and mutualistic microbes often share the same environment in insect vectors (arthropod midgut), and beneficial endosymbionts can provide protection to the host from pathogen infection either through immune system priming (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Gehrer and Vorburger, 2012), or competitive exclusion (Dillon et al., 2005; Hamdi et al., 2011). The complex interactions of vector microbiomes and their potential impact on the spread of infectious diseases is driving research in bacterial symbiont diversity and tolerance to environmental change (Becker et al., 2017; Palumbi et al., 2017; Ribes et al., 2016) but very little is known about how such interactions may vary with landscape level factors.

Atlantic Forest

The Atlantic Forest of Brazil is one of the planet's most diverse rainforests, originally spanning 150 million ha (Pardini et al., 2010; Ribeiro et al., 2009). It is thought to include 1-8% of the world's total biodiversity, but faced heavy fragmentation and land conversion for agricultural exploitation starting in the 16th century (Cezar Ribeiro et al., 2011; Silva and Casteleti, 2003). Existing nature reserves protect 9% of remaining forests, which encompass only 1% of the original forest (Ribeiro et al., 2009). Most of the remaining forest is found in small (<50 ha) fragments of secondary forests, with an average of 1440m between patches and diameters as small as 100m (Lira et al., 2012b). This long history of fragmentation has driven an interest in research on the long term impacts on ecosystem functioning in terms of edge effects (Pardini, 2004), habitat loss (Tabarelli et al., 2005), and isolation (Lira et al., 2012b).

Bats (Chiroptera) are the second most species rich order of mammals (after rodents) and collectively fulfil important ecosystem services such as pollination, seed dispersal, and insect control (Ghanem and Voigt, 2012; Kunz et al., 2011). Their influence in shaping ecosystems coupled with their mobility makes them an important conservation target (Teixeira et al., 2014). Bats are hosts to obligate parasitic bat flies (Diptera: Nycteribiidae and Streblidae), which are highly specialised haemophagous arthropods. Bat flies are typically ectoparasitic and adapted to live in the bats' fur, the only known exception to this is the genus *Ascodipteron* with females embedding themselves within the bat's skin (Jobling, 1949). Bat flies can cause their hosts to suffer weight loss (Linhares and Komeno, 2000) and engage in excessive grooming (ter Hofstede et al., 2004), and bat flies have been linked to smaller brain size (Bordes et al., 2008). In addition, there has been interest in bat flies as potential reservoirs of

zoonotic pathogens such as *Bartonella* (Reeves et al., 2007), *Rickettsia* (Tahir et al., 2016; David A Wilkinson et al., 2016), and the dengue virus (Abundes-Gallegos et al., 2018).

Bat flies reproduce via adenotrophic viviparity, whereby a single larva hatches and develops within the female, and females deposit and attach prepupae (the mature larval stage preceding pupation) onto bat roost substratum, where they develop into pupae (Aguiar and Antonini, 2011). This obligatory interface with the bat roost suggests these ectoparasites are sensitive to changes in habitat structure beyond their direct interaction with host bats. These ectoparasites are, in turn, hosts to a consortium of microbes; some commonly studied pathogens mentioned previously (e.g. *Bartonella, Rickettsia*), and some only recently recognized as belonging to a rich and diverse clade of insect-symbiotic bacteria: *Arsenophonus* (Lack et al., 2011). *Arsenophonus*-strains and *Arsenophonus*-like organisms (henceforth referred to as ALOs) are exceptionally common in bat flies, and also found in numerous arthropod orders including hemipterans, and hymenopterans (Duron et al., 2014; Patterson et al., 2013a). Currently, not much is known about the exact function or impact each of these microbes have on bat flies, but the wide distribution of ALOs in insects likely hint to an important role; some speculated functions include sex ratio distortion (Dale et al., 2006), nutritional supplementation (Santos-Garcia et al., 2018), and parasitoid defence in some taxa (Duron, 2014).

For the purposes of this thesis, I use the term “ectoparasite” to refer to the dipteran ectoparasite and “microbe” to refer to the microbial symbiont of the ectoparasite, though it is acknowledged that these terms do not completely reflect the ecology of either group; for example, some microbial symbionts are intracellular parasites (e.g.

Wolbachia and *Bartonella*) (Solon F Morse et al., 2012). For simplicity I use 'ectoparasitic network' when referring to the interaction between bat flies and their bat hosts, and 'endosymbiont network' for bacteria associated with bat flies. This chapter follows on from existing work on bat-resource networks in the same fragmented habitats to create a multi-trophic level approach to investigating disturbed ecosystems (Teixeira, 2019a). The previous study of bat-resource networks has shown that smaller, more isolated habitats tended to have more nested networks, but other changes in network structure were unexplained by landscape variables (Teixeira, 2019a).

In this chapter I investigate bat fly-bat and endosymbiont-bat fly interactions in fragmented habitat of the Atlantic Forest. I use DNA barcoding and DNA metabarcoding to identify ectoparasites and microbes respectively and construct networks of interactions in each of 10 forest fragments and three control sites within a reserve. Using these data and a collection of environmental variables I then test the following predictions: 1) As communities occupy smaller and more isolated habitats, then they will contain simpler, less diverse networks for bat flies, but the impact on microbes will be less pronounced; 2) These networks should be more connected, and less nested for both layers of the networks; and 3) landscape gradients that affect the structure of bat fly-bat networks should affect endosymbiont-bat fly networks in similar patterns.

Methods

Methods for field collections and identification of bat flies are described in Chapter 3.

PCR Amplification and Sequencing of Microbes

Methods described in this section were performed at the American Museum of Natural History by Dr. Kelly Speer (Speer et al., 2020). Following bat fly identification, DNA extracts from bat fly abdomens were used to amplify microbial DNA by targeting the hypervariable region 4 (V4) of bacterial 16S rRNA. 16S rRNA PCR reactions consisted of 10 µL 5PRIME HotMasterMix (Quantabio), 0.2 µM of each primer, 5 µL template DNA, and sterile water adding up to 25 µL. The thermocycling program is as follows: initial denaturation at 94 °C for 15 minutes, 30 cycles of 55 °C for 30 seconds and 65 °C for 30 seconds, and a final extension of 65 °C for 5 minutes. PCRs were cleaned using SPRI magnetic beads (Beckman Coulter) and had their concentrations estimated using the Qubit 2.0 fluorometer dsDNA HS Assay Kit (Invitrogen). The Bioanalyzer 2100 DNA High Sensitivity chip (Agilent) was used to verify aliquots (10%) of samples for quality and amplicon size. Samples then had indexing primers attached via PCR. The concentration and quality of Indexed libraries were verified again and concentrated to at least 2nM. Library concentration was done by recleaning with SPRIselect beads, desiccating samples via vacufuge, and re-hydrating with 4-6µL of sterile water, depending on initial concentration. 206 samples were found to be low-yield and were pooled equimolarly into a 3.4nM pool. A final pool of 229 libraries was sequenced using an Illumina MiSeq v3 Reagent Kit with 2x300bp reads and 18% PhiX spike-in. 16s Reads were de-multiplexed and processed using the microbiome bioinformatics QIIME2 pipeline (https://docs.qiime2.org/2018.2/). Chimeras and reads containing PhiX were removed using DADA2, and clustered into amplicon sequence variants (ASVs) (Callahan et al., 2016). 16S references were obtained by trimming the GreenGenes Database v13.5 to the 16S rRNA V4 region (DeSantis et al., 2006). All reads were filtered to remove low resolution data and contaminants (using the R

package *decontam*) (Davis et al., 2018); additional detail on filtering criteria is available in Speer (2019). A coverage depth filter was then implemented to exclude all ASVs with fewer than 5 detected copies within individual bat flies. The MAFFT plug-in in QIIME2 was used to align the remaining reads (Katoh et al., 2005; Katoh and Toh, 2008). Highly variable regions of the alignment were masked using default options, and a midpoint-rooted phylogeny was built using the FastTree plug-in (Price et al., 2010). Tables of ASVs found in each sample, the taxonomic identification of those ASVs, and the created phylogeny were exported from QIIME2 and reformatted to be input into the *phyloseq* package in R (McMurdie and Holmes, 2013, 2012).

Constructing Bipartite Networks

Using the assigned taxonomy for bats, bat flies, and bacteria described in Chapter 3, I constructed 2 different bipartite networks for each of the 13 sites in this study. For ectoparasites I created a matrix where I recorded an interaction for each species of bat fly found on each species of bat, regardless of infestation intensity. For endosymbionts, I similarly measured the frequency of each bacterial genus found to associate with each species of bat fly, regardless of their relative abundances. For each of the 13 sites, I generated a frequency-based adjacency matrix tallying up the interactions. I then created a total of 26 bipartite networks from these matrices using the *bipartite* package (Dormann et al., 2009) in R v3.5.1 (R Core Team, 2019).

I calculated three network metrics using the *networklevel* function from *bipartite*, namely (weighted) connectance, niche overlap, and (weighted) NODF; and one metric, modularity was calculated using the *fast.greedy* function from the *igraph* package in R (Csárdi and Nepusz, 2006). Both unweighted and weighted connectance describe the ratio of realised links in the whole network relative to the total number of possible

links, but with weighted connectance, each link is weighed depending on the interaction strength (frequency) (Tylianakis et al., 2007; van Altena et al., 2016). Nestedness measures the degree to which specialists interact with subsets of species which generalists also interact with (Almeida-Neto et al., 2008). NODF (nestedness with overlap and decreasing fill) outputs a value from 0 to 100 (no nestedness to completely nested), and is known to be less sensitive to fluctuating species numbers than other measures of nestedness (e.g. nestedness temperature), so is more suitable for datasets which have varying sample sizes across treatments or sites (Almeida-Neto et al., 2007). Modularity estimates the clustering in a network, where nodes interact more with each other within clusters than nodes outside of it (Thébault, 2013).

Calculating Landscape Metrics

Several measurements to describe each of the 13 forest sites were calculated by Teixeira (2019). He used a combination of forest cover maps from Instituto Brasileiro de Geografia (IBGE), SOS Mata Atlântica (www.sosmataatlantica.com.br), and ESRI base maps available from ArcGIS software v10.1 to obtain a map of the forest remnants in this study that was then exported into the software Fragstats v3.1 in a *geotiff* format. Fragstats is a spatial pattern analysis program that takes in image files of landscapes and calculates landscape metrics. For this study I used fragment area (in hectares) and isolation (shortest distance to another patch of forest) as metrics for landscape spatial structure.

To measure habitat complexity, Teixeira (2019) followed methods described in (Delciellos et al., 2016) to select nine habitat variables (vertical overstory density, horizontal vegetation density, predominant tree size, presence of water courses, *Cecropia* trees, lianas, grasses or bamboos, palms of *Astrocaryum aculeatissimum* and

number of fallen logs). These variables were combined and reduced to two components via Principal Component Analysis under the *prcomp* function in R; the first component (PCAhab1) was positively associated with watercourses and *Cecropia* trees, but negatively associated with lianas, whilst the second component (PCAhab2) was positively associated with overstory, understory, and fallen logs.

Statistical Analysis and Model Selection

I measured the sampling completeness of my networks using the SC_W2 metric as described in (Macgregor et al., 2017) which scales the weighting of species depending on their number of interactions, favouring generalists, and does not heavily penalise a dataset for unobserved rare interactions. Chao2 was used as the richness estimator because of its robustness to small sample sizes (Colwell and Coddington, 1995). I built sets of candidate linear models to represent plausible a priori hypotheses reflecting how the network metrics connectance, nestedness, and modularity might be influenced by landscape metrics. From the landscape measures taken from Teixeira (2019), I chose a small subset of variables that have been reported to be correlated with properties of interaction networks, namely habitat size (Prugh et al., 2008; Sugiura, 2010), isolation or distance between neighbouring habitat (Miyazono and Taylor, 2013), and habitat complexity or heterogeneity (Laliberté and Tylianakis, 2010b). Before listing candidate models, I used the *vif* function from *car* package v3.0.6 to test for multicollinearity among the 4 variables : logArea, Isolation, PCAhab1 and PCAhab2. I found that logArea and PCAhab1 both had *vif* scores of >5, suggesting these predictors are highly correlated (Table 4.1). To avoid inflated coefficient estimates in my models, I opted to removed PCAhab1 from my analysis, but retained logArea as habitat size is one of the defining aspects of habitat fragmentation.

Table 4.1: vif scores of predictor variables including PCAhab1 (A), and without PCAhab1(B).

A	logArea	Isolation	PCAhab1	PCAhab2	B	logArea	Isolation	PCAhab2
	44.9817	3.678691	56.76394	1.165223		1.507312	1.579803	1.120711

I use the second-order bias-corrected Akaike Information Critera (AICc) to select models that best describe the relationship between network structure and landscape. AIC and AICc are derived from the Kullback-Leibler information (K-L) approach for model selection, which calculates the *information lost* when applying a candidate model to approximate real data, allowing users to identify the models in the candidate model set that lose the least information (Beier et al., 2001; Burnham and Anderson, 2001). The Akaike Information Criterion (Akaike, 1973) then links K-L information and maximised log-likelihood (abbreviated as LL here), as defined in the following formula: *AIC = -2(log-likelihood) + 2K*; where log-likelihood is the measure of model fit (the higher the number, the better the fit), and K is the number of model parameters including the intercept and functions to penalize overfitted models. In practice, the AIC values for each model in a set is calculated, and the model with the lowest AIC is chosen as the "best" for losing the least K-L information (Burnham and Anderson, 2002). AICc is a distinct variant of AIC that comparatively provides a more accurate estimate when sample sizes are small, and converges with AIC as sample size increases (Hurvich and Tsai, 1989; Sugiura, 1978).

For each network metric, I listed a set of 1-2 variable linear models and an intercept-only model, ranking them based on AICc. AICc tables were generated using the *aictab* function from *AICcmodavg* package v2.2.2. I chose to set an upper limit of 2-variables for my candidate models based on the number of data points available to include in

my regressions, which is 13 sampling sites. Testing complex models without *a priori* reasoning is generally discouraged, as the frequency of type-I errors (false positives) increases with number of predictor variables in model selection (Forstmeier and Schielzeth, 2011). A n/k ratio of 10 has been suggested as a conservative guide for predictor numbers, where n is the number of data points available and k is the number of predictors (Harrison et al., 2018).

My interpretation of AICc comparison tables are based on methods suggested by (Mazerolle, 2004) and technical recommendations for using AIC by (Burnham and Anderson, 2002). I use both ΔAICc and Akaike weights (AICcWt) to evaluate the candidate models. ΔAICc is the difference of AICc of a given model and the model with the lowest AICc, and the Akaike weights represent the ratio of ΔAICc for each model in a set given that the sum of weights equals 1. Akaike weights can also be used to compute an 'evidence ratio' to compare support between pairs of models (Burnham and Anderson, 2002). For example, if given the AICcWt for model *q* is 0.50 and AICcWt model *t* is 0.35, then the evidence ratio is calculated as 0.50/0.35 is 1.43, and indicates that model *q* has 1.43 times more support than model *t.*

For each set, the models with ΔAICc between 0-7 were used to make inference about the relationship between landscape and network structure. Generally, models with ΔAICc < 2 are considered having as good a support as the best model, and models with ΔAICc 2-7 have enough support to be considered for inference (Burnham and Anderson, 2002; Richards, 2008). I calculate the evidence ratio using for every candidate model in a set with respect to the top model of that set. I also use the nesting rule (Richards, 2008, 2005) to eliminate complex models from my candidate

set when there are simpler nested models with lower AIC_c. For example, if model A contains all the parameters of model B and at least one additional parameter, model B is considered to be "nested" within model A, and so if model B has a lower AIC_C than model A, the latter nested model will be excluded from the candidate set under the nesting rule. I then report the adjusted R^2 (variance explained by the model) for the remaining models as a measure of how useful the model is (Miles, 2014).

These methods for model selection were repeated to include only the 10 sites represent fragmented forest sites. This was done to detect patterns emerging due to the implicit differences between relatively pristine (REGUA 1,2, and 3) and disturbed habitats (F1-F10), rather than the explicit landscape measurements that was being tested. I refer to these distinct datasets as the complete dataset (n=13), and the fragmented dataset (n=10). I also compared the means of each network metric belonging to the fragmented and continuous forest datasets using a Welch two-sample t-test to determine their independence.

Results

Sample collections and identifications

Teixeira (2019) collected 988 bats representing 26 species, 343 (34%) of which were parasitized by at least one bat fly. Bat fly epidemiology is explored in more detail in Chapter 3, but in general, site F10 had the highest prevalence (44% of bats were hosts). The most abundant host species was also the most abundant bat collected *Carollia perspicillata*, with 176 hosts from 383 captures, followed by *Artibeus lituratus*, with 55 hosts from 190 captures. A total of 842 bat flies were collected from 17 bat species. 813 of these bat flies were morphologically identified to species level and the

remaining 29 were only identified to genus (association visualised in Figure 4.1). However, molecular analysis using DNA barcoding via *COI* (Hebert et al., 2003) supports the allocation of these bat flies to 20 described species, and 5 species-level MOTUs (molecular operational taxonomic unit (Floyd et al., 2002); e.g. denoted *Trichobius sp.1, Trichobius sp.2, etc.*). About half (n=436) of the total bat fly collection was obtained from samples of *C. perspicillata*, and the most abundant bat fly throughout was *Trichobius joblingi,* representing 303 individuals, 292 of which were found on *C. perspicillata*. Site F7 had the highest incidence (201 bat flies collected). See Chapter 3 for full details.

From this sample, 196 bat flies were used for endosymbiont analysis, this subsample only included bat flies whose species identities were morphologically confirmed. These were subjected to molecular analysis and bioinformatics following (Speer, 2019). In total, 189 sequencing libraries remained after quality filtering, for an average of 4247 reads per library and these data formed 1101 ASVs. The three most abundant bacterial endosymbionts found were *Arsenophonus sp.* (family Enterobacteriaceae), *Bartonella sp.* (family Bartonellaceae)*, and Wolbachia sp.* (family Rickettsiaceae), an overview is available in Figiure 4.2. There were differences in the prevalence of *Wolbachia sp.* across streblid and nycteribid bat flies; with it being always present in the Nycteribidae (n=22) and detected in only 27% of the Streblidae (n=170).

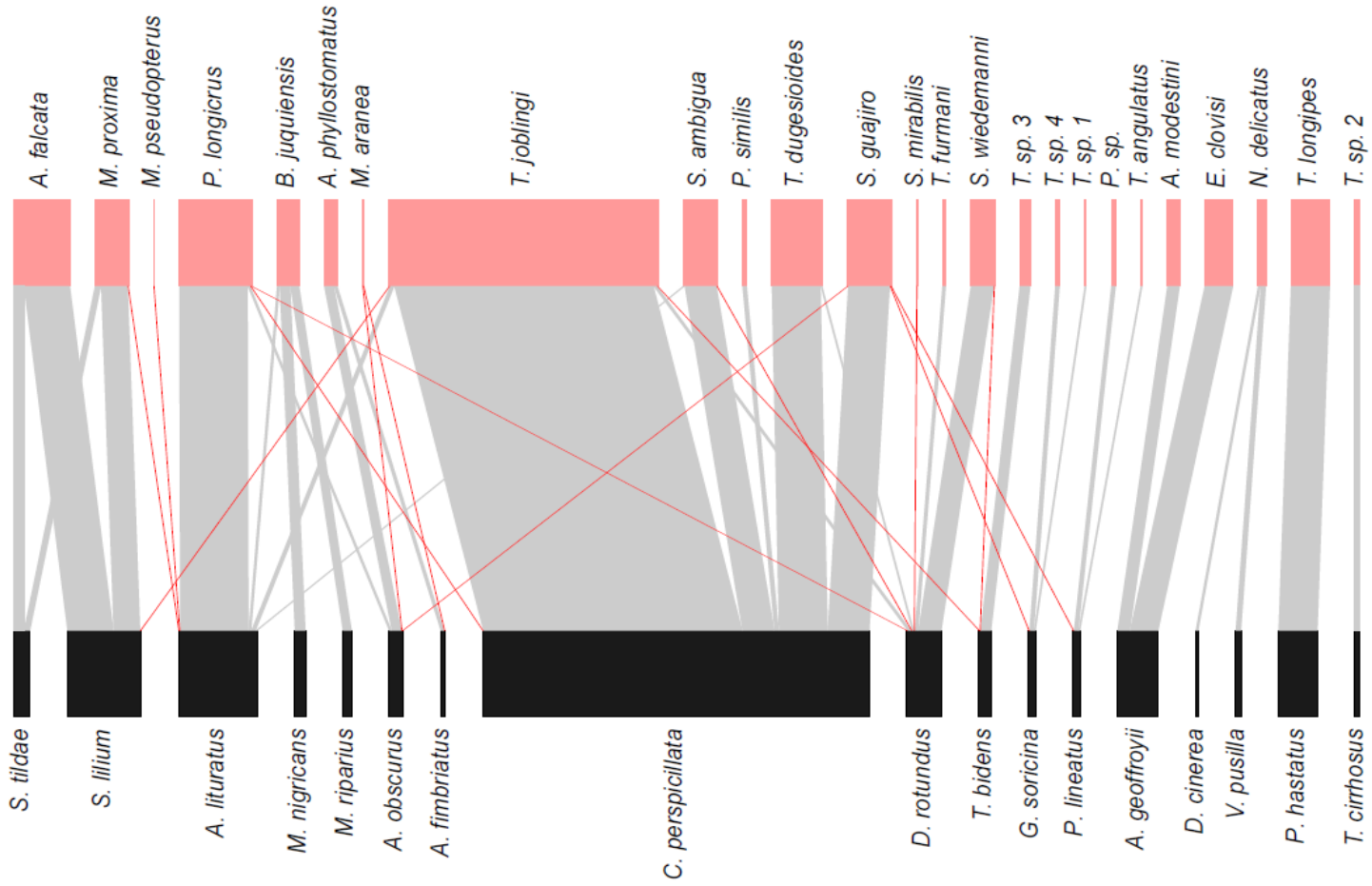


Figure 4.1. All infested bats (bottom row) and associated bat flies (top row) identified and used in ectoparasitic network ana lysis. Width of bars represent relative frequencies and red lines denote singleton interactions.

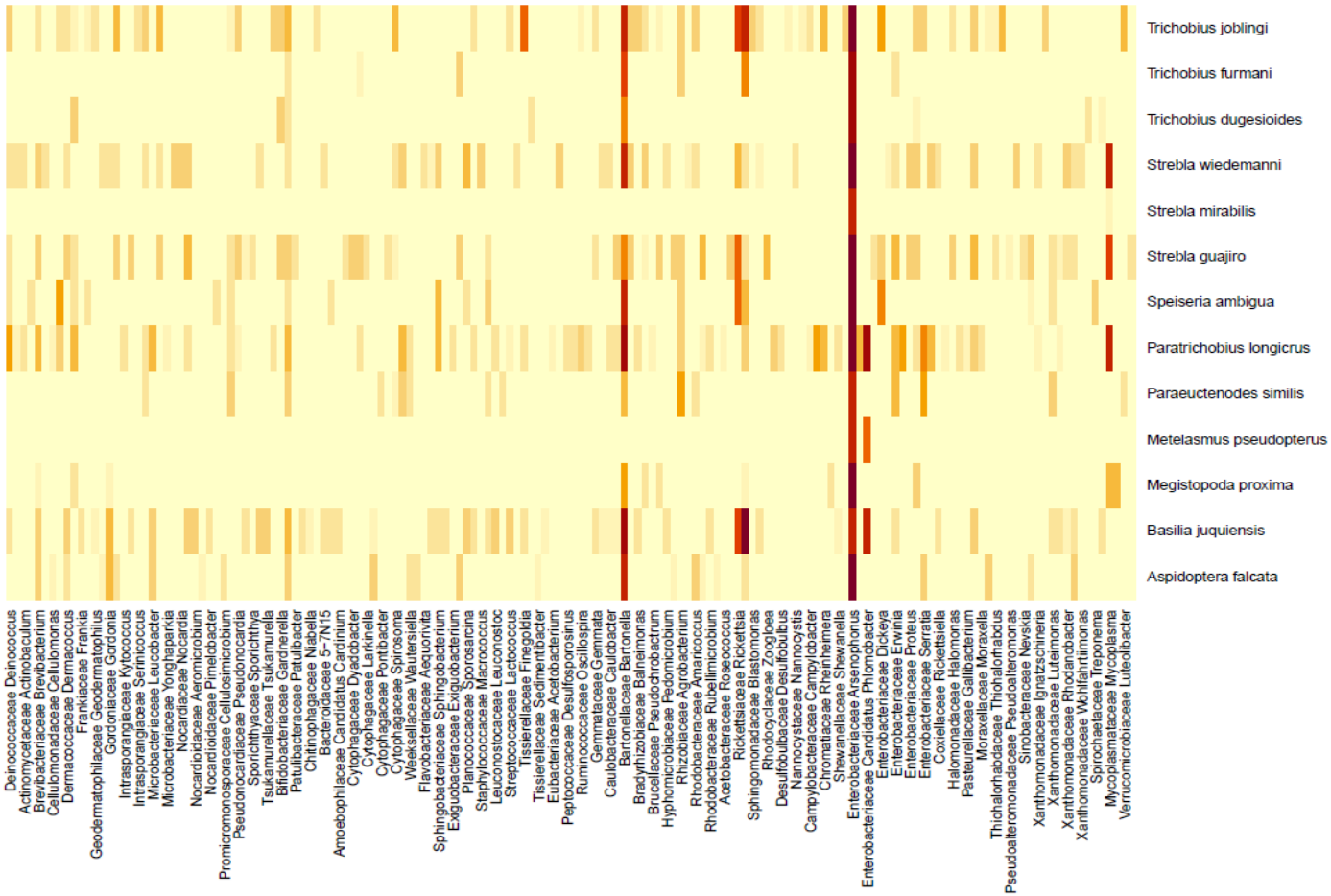


Figure 4.2 heat map of bacterial genus (columns) found in bat fly species (rows). Darker colours indicate higher intensity.

Population characteristics across sites

Bat fly richness across sites ranged from 5 species in fragment F3 to 16 species in control site REGUA 1 (Table 4.2) and was positively correlated with the richness of bat hosts collected (coefficient= 1.169, adj. R^2 = 0.5735, ΔAIC_c= 10.21 compared to the next best model). The prevalence of observed bat fly infection across sites was explained by habitat complexity, where prevalence decreased in more heterogenous habitat (coefficient= -0.021, adj. R^2 = 0.1146, ΔAIC_c=-39.60 compared to the next best model). Endosymbiont richness ranged from 16 genera in fragment F8 to 50 genera in both control sites REGUA 1 and 3 and was found to be best explained by area, isolation, and habitat heterogeneity rather than bat fly diversity (adj. R^2 =0.6962, ΔAIC_c=-13.22 compared to the next best model).

Table 4.2. Richness and sampling completeness for networks of both trophic layers. Sampling completeness was calculated using Chao2 estimator. Values for richness indicate species except for Bacteria where it is bacterial genus

	Ectoparasitic layer			Endosymbiont layer		
	Bats	Bat flies	SCw2	Bat flies	Bacteria	SCw2
F1	3	8	74.07	5	41	66.91
F2	4	7	92.84	6	25	55.02
F3	3	5	93.02	5	23	70.99
F4	7	9	83.76	8	37	63.97
F5	5	8	75.68	4	21	52.17
F6	3	6	78.05	3	30	20.91
F7	7	10	100.00	7	20	53.95
F8	7	13	97.56	7	16	72.43
F9	7	8	100.00	5	27	69.99
F10	6	10	93.62	7	40	64.54
REGUA1	8	16	85.82	10	50	33.47
REGUA2	8	10	97.50	5	39	44.31
REGUA3	8	12	94.38	6	50	34.62

AICc-based model selection

Network metrics for each sampling site are listed in Table 4.3 and tables 4.4 and 4.5 show the ranking of candidate models for all sets tested when data from all 13 sampling sites were included (Table 4.4a, 4.5a), and when continuous forest sites were excluded (Table 4.4b, 4.5b). There are 6 sets of models for each trophic layer organised as follows: one set for each network metric (connectance, nestedness, modularity), and two ways the data was analysed (the complete and fragmented datasets). Almost all model sets have multiple candidates within ΔAICc < 2, and very few models being ΔAICc > 7. Table 4.6 lists the summaries for the top models in every set (the top 2 models when possible) that ranked higher than the intercept-only models and were not excluded under the nesting rule.

For the ectoparasitic layer the best fit models (Δ AICc < 2) describing the connectance-landscape relationship include a logArea model (AICcWt = 0.46, adj.R^2 = 0.278), followed by both logArea + PCAhab2 (AICcWt = 0.25, adj.R^2 = 0.356) and the intercept-only model weighted at 0.18. The first two models here are nested, both having logArea in common and the second model additionally having PCAhab2, and the evidence ratio between them is 1.84. The best fit models describing the nestedness-landscape relationship included PCAhab2 (adj.R^2 = 0.166) followed very closely by the intercept-only model (Δ AICc = 0.03), with both models each weighing at 0.37. The next best model in the set was logArea (Δ AICc = 2.88, AICcWt = 0.09, adj.R^2 = -0.04). The logArea model (AICcWt = 0.58, adj.R^2 = 0.371) was the only best fit model in the modularity-landscape set (Figiure 4.3), the model that followed was Isolation (Δ AICc = 3.26, AICcWt = 0.11 , adj.R^2 = 0.191), with an evidence ratio of 1.94 between them.

Table 4.3: Network metrics for both trophic layers. Metrics include weighted connectance (wConn), weighted nestedness with overlap and decreasing fill (wNODF), and modularity (Mod) .

	Ectoparasitic Layer			Endosymbiont Layer		
	wConn	wNODF	Mod	wConn	wNODF	Mod
F1	0.16	0.00	0.54	0.15	16.61	0.077
F2	0.15	0.00	0.57	0.19	23.91	0.16
F3	0.20	0.00	0.46	0.24	26.55	0.24
F4	0.10	1.75	0.67	0.14	19.38	0.31
F5	0.11	0.00	0.56	0.22	27.98	0.37
F6	0.23	5.56	0.42	0.26	19.74	0.041
F7	0.10	0.00	0.42	0.16	21.45	0.065
F8	0.10	5.89	0.58	0.17	20.45	0.085
F9	0.10	2.04	0.55	0.19	19.10	0.13
F10	0.13	5.83	0.33	0.14	16.84	0.16
REGUA1	0.09	3.60	0.71	0.14	17.09	0.2
REGUA2	0.08	1.37	0.75	0.18	26.71	0.15
REGUA3	0.08	2.66	0.71	0.14	18.76	0.4

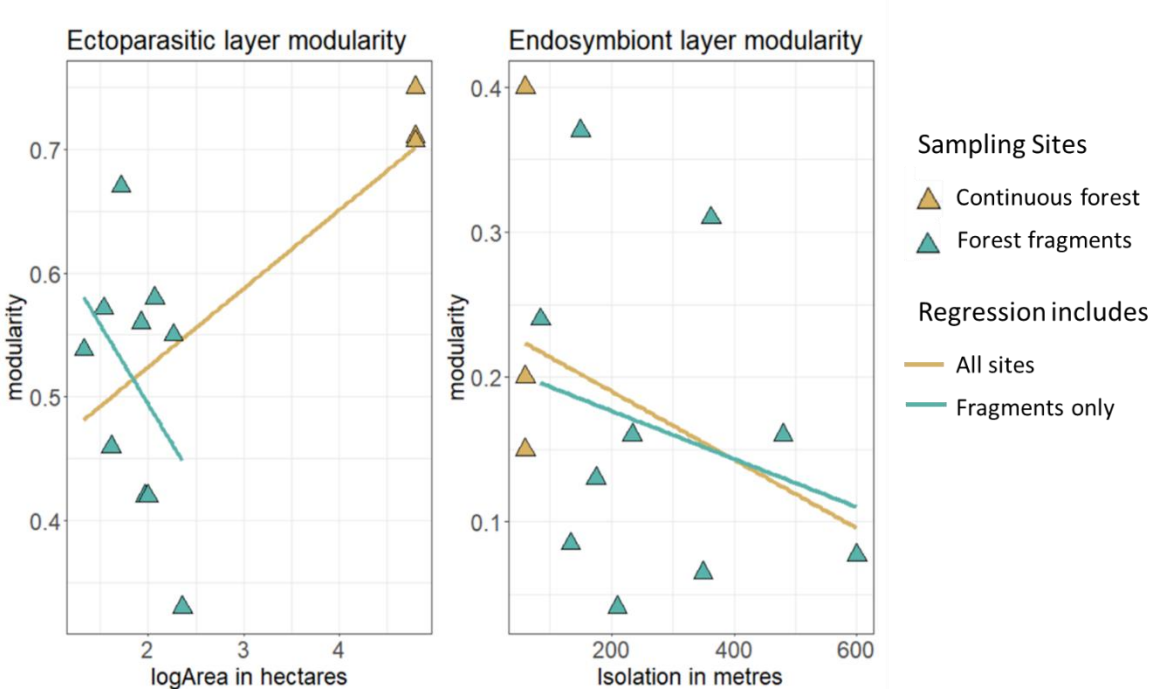


Figure 4.3 Modularity of endosymbiont networks across habitat isolation, and ectoparasitic networks across habitat area. Regressions show trend when including or excluding control sites.

Table 4.4. Support for candidate models predicting the effect of landscape variables on structure of ectoparasitic networks for the complete (A) and fragmented (B) datasets. The number of parameters (K), $AICc_c$ values, $\Delta AICc_c$, model weights (AICcWt), cumulative weights (C.Wt), Log-likelihood (LL), and evidence ratio (ER) are reported. ER is always calculated based on the top model of that set. Landscape variables are as follows: logArea (LArea), Isolation (Iso), and PCAhab2 (PCA2). Models in bold indicate *Δ AICc < 2. Models in grey indicate C.Wt < 0.95*

(A) Complete ectoparasitic set (n=13)							
Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
Weighted Connectance							
LArea	**3**	**-41.33**	**0**	**0.46**	**0.46**	**25**	**1.00**
LArea + PCA2	**4**	**-39.75**	**1.58**	**0.25**	**0.67**	**26.38**	**1.84**
~1	**2**	**-39.43**	**1.9**	**0.18**	**0.84**	**22.32**	**2.55**
LArea + Iso	4	-37.26	4.07	0.06	0.90	25.13	7.66
Iso	3	-36.75	4.58	0.05	0.95	22.71	9.20
PCA2	3	-36.51	4.82	0.04	0.99	22.59	11.50
Iso + PCA2	4	-33.69	7.64	0.01	1	23.34	46.00
Weighted NODF							
PCA2	**3**	**63.06**	**0**	**0.37**	**0.37**	**-27.2**	**1.00**
~1	**2**	**63.09**	**0.03**	**0.37**	**0.74**	**-28.95**	**1.00**
LArea	3	65.95	2.88	0.09	0.83	-28.64	4.11
Iso	3	66.5	3.43	0.07	0.9	-28.92	5.28
LArea +PCA2	4	67.23	4.16	0.05	0.94	-27.11	7.40
Iso + PCA2	4	67.25	4.19	0.05	0.99	-27.13	7.40
LArea + Iso	4	70.22	7.15	0.01	1	-28.61	37.00
Modularity							
LArea	**3**	**-16.17**	**0**	**0.58**	**0.58**	**12.42**	**1.00**
Iso	3	-12.9	3.26	0.11	0.7	10.79	5.27
~1	2	-12.48	3.69	0.09	0.79	8.84	6.44
LArea + Iso	4	-12.45	3.72	0.09	0.88	12.72	6.44
LArea + PCA2	4	-12.13	4.04	0.08	0.96	12.57	7.25
PCA2	3	-9.99	6.17	0.03	0.99	9.33	19.33
Iso + PCA2	4	-8.79	7.37	0.01	1	10.9	58.00

(B) Fragmented ectoparasitic set (n=10)							
Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
Weighted Connectance							
~1	**2**	**-29.19**	**0**	**0.57**	**0.57**	**17.45**	**1.00**
PCA2	3	-26.79	2.4	0.17	0.74	18.4	3.35
LArea	3	-26.56	2.63	0.15	0.89	18.28	3.80
Iso	3	-24.95	4.24	0.07	0.96	17.48	8.14
LArea + PCA2	4	-22.53	6.66	0.02	0.98	19.27	28.50
LArea + Iso	4	-20.82	8.37	0.01	0.99	18.41	57.00
Iso + PCA2	4	-20.8	8.39	0.01	1	18.4	57.00
Weighted NODF							
LArea + PCA2	**4**	**50.89**	**0**	**0.39**	**0.39**	**-17.44**	**1.00**
LArea	**3**	**51.49**	**0.61**	**0.29**	**0.69**	**-20.75**	**1.34**
~1	**2**	**52.41**	**1.52**	**0.18**	**0.87**	**-23.35**	**2.17**
PCA2	3	53.92	3.03	0.09	0.96	-21.96	4.33
Iso	3	56.69	5.8	0.02	0.98	-23.34	19.50
LArea + Iso	4	57.32	6.43	0.02	1	-20.66	19.50
Iso + PCA2	4	59.83	8.95	0	1	-21.92	NA
Modularity							
~1	**2**	**-12.98**	**0**	**0.62**	**0.62**	**9.35**	**1.00**
LArea	3	-10.62	2.36	0.19	0.81	10.31	3.26
Iso	3	-9.05	3.94	0.09	0.89	9.52	6.89
PCA2	3	-8.84	4.15	0.08	0.97	9.42	7.75
LArea + Iso	4	-5.59	7.4	0.02	0.99	10.79	31.00
LArea +PCA2	4	-4.72	8.26	0.01	1	10.36	62.00
Iso + PCA2	4	-3.11	9.87	0	1	9.56	NA

Table 4.5. Support for candidate models predicting the effect of landscape variables on structure of endosymbiont networks for the complete (A) and fragmented (B) datasets. The number of parameters (K), $AICc_c$ values, $\Delta AICc_c$, model weights (AICcWt), cumulative weights (C.Wt), Log-likelihood (LL), and evidence ratio (ER) are reported. Landscape variables are as follows: logArea (LArea), Isolation (Iso), and PCAhab2 (PCA2). Models in bold indicate Δ AICc < 2. Models in grey indicate C.Wt < 0.95.

(A) Complete endosymbiont set (n=13)

Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
Weighted Connectance							
~1	**2**	**-48.81**	**0**	**0.35**	**0.35**	**27.01**	**1.00**
LArea + Iso	**4**	**-48.62**	**0.19**	**0.32**	**0.67**	**30.81**	**1.09**
LArea	3	-46.77	2.05	0.13	0.8	27.72	2.69
Iso	3	-46.49	2.32	0.11	0.91	27.58	3.18
PCA2	3	-45.38	3.44	0.06	0.97	27.02	5.83
LArea + PCA2	4	-42.68	6.13	0.02	0.99	27.84	17.50
Iso + PCA2	4	-42.19	6.62	0.01	1	27.6	35.00
Weighted NODF							
LArea	**3**	**96.86**	**0**	**0.38**	**0.38**	**-44.1**	**1.00**
~1	**2**	**97.51**	**0.65**	**0.27**	**0.65**	**-46.16**	**1.41**
PCA2	3	98.98	2.13	0.13	0.79	-45.16	2.92
LArea + PCA2	4	99.95	3.09	0.08	0.87	-43.47	4.75
Iso	3	100.24	3.38	0.07	0.94	-45.79	5.43
LArea + Iso	4	101.08	4.22	0.05	0.98	-44.04	7.60
Iso + PCA2	4	103.09	6.24	0.02	1	-45.05	19.00
Modularity							
Iso	**3**	**-52.07**	**0**	**0.53**	**0.53**	**30.37**	**1.00**
~1	2	-49.85	2.22	0.18	0.71	27.52	2.94
LArea	3	-48.69	3.38	0.1	0.81	28.68	5.30
Iso + PCA2	4	-48.44	3.63	0.09	0.89	30.72	5.89
LArea + Iso	4	-47.85	4.22	0.06	0.96	30.42	8.83
PCA2	3	-46.38	5.69	0.03	0.99	27.53	17.67
LArea + PCA2	4	-44.47	7.6	0.01	1	28.73	53.00

(B) Fragmented endosymbiont set (n=10)

Model	K	AICc	Δ AICc	AICcWt	C.Wt	LL	ER
Weighted Connectance							
~1	**2**	**-34.14**	**0**	**0.42**	**0.42**	**19.93**	**1.00**
Iso	3	-33.27	0.87	0.27	0.69	21.63	1.56
LArea + Iso	4	-31.79	2.36	0.13	0.82	23.89	3.23
LArea	3	-31.5	2.64	0.11	0.93	20.75	3.82
PCA2	3	-30.01	4.13	0.05	0.98	20.01	8.40
Iso + PCA2	4	-27.27	6.87	0.01	0.99	21.63	42.00
LArea + PCA2	4	-25.61	8.53	0.01	1	20.81	42.00
Weighted NODF							
~1	**2**	**75.78**	**0**	**0.68**	**0.68**	**-35.03**	**1.00**
PCA2	3	79.13	3.35	0.13	0.81	-34.56	5.23
LArea	3	79.94	4.16	0.09	0.9	-34.97	7.56
Iso	3	80.02	4.24	0.08	0.98	-35.01	8.50
Iso + PCA2	4	84.92	9.13	0.01	0.99	-34.46	68.00
LArea + PCA2	4	84.93	9.15	0.01	1	-34.47	68.00
LArea + Iso	4	85.85	10.07	0	1	-34.92	NA
Modularity							
~1	**2**	**-37.34**	**0**	**0.5**	**0.5**	**21.52**	**1.00**
Iso	3	-35.42	1.92	0.19	0.69	22.71	2.63
LArea	3	-34.93	2.41	0.15	0.84	22.46	3.33
LArea + Iso	4	-33.55	3.78	0.08	0.91	24.78	6.25
PCA2	3	-33.24	4.09	0.06	0.98	21.62	8.33
Iso + PCA2	4	-30.2	7.13	0.01	0.99	23.1	50.00
LArea + PCA2	4	-29.27	8.07	0.01	1	22.63	50.00

Table 4.6 Landscape variables and estimates of the top candidates in each model set that ranked higher than the null model based on AICc . Model names indicate associated network metric: connectance (C), nestedness (N), and modularity (M). Variables are LArea (log habitat area), PCA2 (habitat heterogeneity), and Iso (habitat isolation). Δ AICc = 0 indicates it was the top model in the set.

Model	n	variable(s)	estimate	StdE	F	d.f.	adj. R2	*P*	Δ AICc
Ectoparasitic layer									
C1	13	LArea	-0.02	0.008	5.621	1	0.278	0.037	0
N1	13	PCA2	0.702	0.381	3.393	1	0.166	0.09	0
N2	10	LArea	5.412	1.688	7.897	2	0.605	0.01	0
		PCA2	0.7873	0.307					
N3	10	LArea	5.122	2.193	5.455	1	0.331	0.047	0.61
M1	13	LArea	0.063	0.022	8.071	1	0.371	0.016	0
Endosymbiont layer									
N5	13	LArea	3.489	4.899	4.103	1	0.205	0.067	0
M5	13	Iso	1.04e-4	4.25e-5	6.04	1	0.295	0.03	0

Table 4.7 t-test results comparing network metrics of fragmented and continuous forest ectoparasitic and endosymbiont layers. Metrics are w.Conn (weighted connectance), w.NODF (weighted NODF), and Mod (Modularity). Number of sites included for each habitat type are denoted by n. Comparisons in bold indicate a significant difference in means.

		Fragmented (n=10)		Continuous (n=3)				
Metric	Layer	Mean	SD	Mean	SD	t	df	p-value
w.Conn	**Ectoparasitic**	**0.138**	**0.047**	**0.083**	**0.005**	**3.616**	**9.828**	**0.004**
	Endosymbiont	0.186	0.042	0.153	0.023	1.73	6.55	0.129
w.NODF	Ectoparasitic	2.1	2.633	2.54	1.119	-0.413	8.776	0.688
	Endosymbiont	21.2	3.836	20.8	5.14	0.108	2.707	0.921
Mod	**Ectoparasitic**	**0.51**	**0.1**	**0.723**	**0.023**	**-6.21**	**10.92**	**$6.8e^{-5}$**
	Endosymbiont	0.163	0.11	0.25	0.132	-1.026	2.892	0.382

For the endosymbiont networks, there was no evidence that connectance is influenced by the measured environmental variables as the null model had the lowest AIC (AICcWt = 0.35). The best fit models describing nestedness-landscape included the logArea model (AICcWt = 0.38, adj.R^2 = 0.2) followed by the null model (Δ AICc = 0.65, AICcWt = 0.27), resulting in an evidence ratio of 1.4 between them. The sole best fit model in the modularity-landscape set was the Isolation model (AICcWt = 0.53, adj.R^2 = 0.29; see Figiure 4.3), with the next best model being the null model (Δ AICc = 2.22, AICcWt = 0.18), resulting in an evidence ratio of 2.9.

When comparing the fragmented and complete datasets, all model sets from both layers resulted in either the null model having the lowest AIC, or within Δ AICc < 2. Comparing the means of each network metric from the fragmented and continuous forest sites shows that for connectance (t = 3.61, df = 9.82, p-value = 0.004) and modularity (t = -6.21, df = 10.9, p-value = 6.8e-05), the ectoparasitic networks are significantly different between the habitat types (Table 4.7). No significant difference was found in endosymbiont networks between habitat types.

Discussion

While many studies have looked at the long-term effects of habitat fragmentation across varying taxonomic contexts including macroparasites (Bordes et al., 2015b; Sebaio et al., 2010) and microbiota (Amato et al., 2013; Dheilly et al., 2017), to my knowledge, this study is the first to consider how interactions between host, ectoparasites and microbes vary across an environmental gradient. In this analysis I tested the prediction that smaller and more isolated fragments would lead to less

diverse networks of bat flies with reduced impacts on microbes. My data suggests that the network structure of both trophic layers responds to changes in landscape, but not always the same way suggesting independence in the response of even obligate parasitic microbes.

My study included data from 842 infested bats across 13 sampling sites. While this number is comparable to similar studies involving parasitic interactions of bats (de Vasconcelos et al., 2016; Hernández-Martínez et al., 2019b) and rodents (Bordes et al., 2015a; Cardoso et al., 2018), and employed a standardised sampling effort across all sites; only one bat species, *Carollia perspicillata,* was consistently parasitized, and this was also the most abundant bat captured. The next two most captured bats, *Artibeus lituratus* and *Sturnira lilium,* were captured in all sites but not found to carry bat flies at several sites (see additional details in Chapter 3). Sampling completeness (SCw2) estimates of my ectoparasitic networks are >75%, which is above the threshold needed to avoid underestimating the more sensitive network metrics (Rivera-Hutinel et al., 2012) suggesting the sampling scheme achieved a good representation of the taxa present in these sites. Conversely, sampling completeness of my endosymbiont networks were more varied (20-72%). In contrast, Martinson et al., (2017) had reported to achieve 100% endosymbiont sampling completeness in *Drosophila* within the range of 2000-6000 sequences and 725 OTUs among 215 individuals. The lower sampling completeness despite the higher OTU count (1101) and sequencing depth may point to an extreme microbial diversity among the different bat fly genera. Given the variability in sampling completeness of the microbial networks, and that the SCw2 provides an estimate of unobserved interactions, I treat observations about the microbial network with some caution.

Bat fly species richness did not correlate with landscape metrics, but is correlated to host richness. (Hernández-Martínez et al., 2019b) reports similar findings that bat fly diversity mirrors their hosts which they speculate is driven by the obligate and specific nature of bat flies. Other studies have shown varying effects of host assemblage on parasite diversity. (Bordes et al., 2015a) found helminth diversity to be unaffected by fragmentation, pointing to the rat hosts being habitat generalists in their study, however both (Bush et al., 2013; Chasar et al., 2009) found blood parasite diversity to decrease with habitat area despite host persistence, suggesting a minimum habitat size for parasite preservation. I found that endosymbiont richness decreased as sites became smaller, more isolated, and less complex. Apart from the vertical transmission of maternally transmitted bacterial strains (ex: *Arsenophonus sp., Wolbachia sp.*), very little is known about the exact mode of bacterial recruitment in bat flies (Solon F Morse et al., 2012; David A Wilkinson et al., 2016). I observed that microbial diversity decreased in smaller, less complex fragments. Studies on field-captured tsetse flies (Griffith et al., 2018), mosquitoes (Zouache et al., 2011), mites (Dong et al., 2018), and bat flies (David A. Wilkinson et al., 2016b) have also reported an impact of environmental heterogeneity on the gut microbiota in parasitic arthropods, where it is speculated that bacterial recruitment occurs during free-living, host-seeking periods. Alternatively, the host bats themselves could also be a source of bacteria; blood meals have been shown to influence gut microbiota in ticks (Hawlena et al., 2013), and hosts can pick up bacteria from contact with their environment and in turn pass these microbes to their ectoparasites (Lawrence et al., 2015).

Choosing models to explain my data based solely on Δ AICc suggests that almost all network metrics for both layers are not sufficiently explained by the chosen landscape

variables alone, due to the null models in those sets consistently being within Δ AICc < 2, the exception being modularity for both layers having only a single model each within this range.

Using the Akaike weights (AICcWt) and evidence ratios in addition to Δ AICc allows more flexibility in discussing the merits of my models by using evidence ratios. For example, for models with Δ AICc < 2 in the connectance set for the ectoparasitic layer the evidence ratio shows that the top model (logArea) is 2.5x more likely to be the best approximating model than the null model. In this case I did not consider the second-to-top model (logArea +PCAhab2) for comparison because it is a nested model that has a lower weight, suggesting that the additional variable (PCAhab2) is uninformative in this set (Leroux, 2019).

(Burnham and Anderson, 2002) have also suggested creating a '95% confidence set' (a subset of candidate models beginning with the top model and moving downwards, whose cumulative weights tally up to 0.95) which implies that there is a 95% chance of the 'best model' being in this subset. The 95% threshold here is arbitrary and likely selected as a familiar frequentist confidence interval (Symonds and Moussalli, 2011). However, applying this approach onto my candidate model sets still included at least half of the initial candidate models in the subset, due to a combination of i) the small number of initial candidate models in each set, and ii) our top models not weighing highly enough to exclude the weaker models using this method at a 95% threshold.

For the ectoparasitic layer, I found some evidence that connectance is affected by area; larger sites appeared to have lower connectance. For the endosymbiont layer, area was also the most likely landscape variable to affect connectance, but this was

not well supported as the null model was weighted higher. Previous studies have reported similar trends for habitat loss decreasing connectance in plant-plant networks (Santos de Araujo, 2019) and pollination networks (Caron-Lormier et al., 2008), but there are exceptions even within some parasitic networks, particularly with complex parasites that require intermediary host species (Resasco et al., 2019). More species of bats were captured in larger fragments, which would affect the maximum number of potential links used to calculate global metrics like connectance. Despite an increase in 'hosts' for both ectoparasitic and microbial networks, the realised potential links may remain restricted by the specificity of the ectoparasites and microbes to their respective hosts. Other studies of parasitic networks point to phylogenetic constraints (Mouillot et al., 2008a, 2008b), or environmental barriers that inhibit connectance (Cardoso et al., 2018).

I found that the nestedness (weighted NODF) of ectoparasitic and endosymbiont networks had contrasting responses to different landscape metrics although both cases were only slightly better supported than the null models. Parasitic networks became more nested with increasing habitat complexity (PCAhab2), which may reflect increased ectoparasite-sharing amongst host species, however I approach this outcome with caution as this model was equally weighted with the null model. (Patterson et al., 2009) reported that the nested pattern of their bat fly parasitic networks diminished when they restricted their dataset to "primary" associations (flies on their typical host species), they posit that the nested structure is caused by both host specificity and transient accidental occurrences due to the mobile ectoparasites readily abandoning hosts when disturbed, and moving between individual bats in the same roost (Dick and Patterson, 2006). While no roosting information was collected for

the bats in my study, there are some studies linking habitat quality to bat roosting behaviours that could help explain my observations. Bats in felled and disturbed forests have been observed to have small colony sizes (Borkin et al., 2011), and were more prone to roost-switching (Willis and Brigham, 2004), both of which are thought to reduce the spread of parasites within bat populations (Kashima et al., 2013). This suggests that fragments with more complex vegetation facilitate the formation of larger bat roosting colonies, frequently containing multiple species. This could allow for more frequent movement of ectoparasite between hosts.

In contrast to the ectoparasitic networks, my endosymbiont networks became more nested in smaller and less isolated fragments. This model was 1.41x more likely than the null model. (Moeller et al., 2017) observed that gut microbiomes were not fully explained by the hosts' diet and phylogeny but were often restricted by the physical distance and co-occurrence of host species. In the context of my study, this might suggest that the nested pattern found could be related to the roosting behaviour of bats affecting the co-occurrence of bat flies. The effect of area on nestedness might also point to a change in the dispersal or diversity of microbes from the environment. (Peay et al., 2007) found that species richness of soil bacteria was positively correlated with habitat area and closeness to other fragments. In my case, this may point to generalist bacterial species being replenished through dispersal across fragments, and in more smaller habitats, the bacterial networks are less nested due to fewer specialists remaining.

Modularity of ectoparasitic networks decreased as fragment size was reduced. (McCann et al., 2005) identified that in a bipartite network, some species act as

module hubs, with a moderate to high number of links to species in the other layer. These hub forming species are frequently generalists or highly flexible species which resist environmental perturbations and maintain critical ecological services. In my study, *Carollia perspicillata* was present in every network and was infected by at least two bat fly species in each and was always included in the largest module. *Carollia* is a well-known environmental generalist and largely feeds on successional trees typical of disturbed habitat (Medellín et al., 2000). It is likely that *Carollia* acts as a main connecting node in all networks here because of these traits. In his study of seed dispersal using the same individual bats, Teixeira, (2019b) found that *C. perspicillata* was critically important to maintaining the core of ecological seed dispersal even in the smallest fragments in his study. I observe that this role also extends to the ectoparasitic level and thus *C. perspicillata's* abundance and ubiquitous presence across all studied fragments makes it critically important in maintaining multiple ecological services and network structure. The role of generalists, like *C. perspicillata*, as module hubs and connectors has been documented in plant networks (Dupont and Olesen, 2009), marine food-webs (Kortsch et al., 2015), and archipelagic birds (Carstensen and Olesen, 2009). Here I add the case for *C. perspicillata* acting to maintain ectoparasitic diversity in fragmented landscapes.

In my study, larger fragments hosted more bats and thus more batlfies but had reduced connectance due to a more modular structure. This suggests that as the environment becomes less rich, the network contracts and loses some of this dispersed nature as specialists drop out leaving only the generalist like *Carollia* to maintain the core community. Similarly, in (Teixeira, 2019b), three modules were identified in all networks regardless of fragment size which were associated with

foraging behaviour and were maintained primarily by the bat genera *Carollia*, *Artibeus*, and *Sturnira*. Even in the smallest most isolated fragment these three core modules remained.

In the microbial network modularity was most likely affected by site isolation, with lower modularity in more isolated fragments. Microbiome studies in other systems suggest that modularity decreases when host species that act as connectors are present (Lurgi et al., 2019). In my study, the core modules encompassed several bat flies and their bacteria. The more common bacteria such as *Arsenophonus sp., Wolbachia sp., and Bartonella sp.* acted as connector species. In modules with multiple bat flies, it was difficult to discern a primary hub species using our methods as many bat flies acted as their own hubs for specialist bacteria, however some consistent trends did emerge. The number of modules visualised for the microbial network would always be small (2-3) regardless of how many modules were identified in the corresponding ectoparasitic network, and in fragments where the bat fly *Basilia juquiensis* was collected, it tended to form a secondary module apart from other bat flies. *B. juquiensis* is the only nycteribid bat fly that was collected, and the tendency for its microbiome to form a separate modules from the streblids could have a phylogenetic basis as (Patterson et al., 2013a) found both families of bat flies to have independently acquired bacterial species on several occasions. Isolated habitats may exhibit lower network modularity due to the decrease of host species.

Modules in other microbiome networks have been suggested to form from co-occurrence of predator and prey (Kondoh, 2008), or the repeated interaction between plants and pollinators (Dupont and Olesen, 2009). Social groupings have also been

seen to affect module formation in other mammals (Perofsky et al., 2017). Given that in my networks the core bat species were retained in all fragments (*Carollia*, *Artibeus*, and *Sturnira*) it is unlikely that this effect is driven by the main bat species caught. A combination of phylogenetic history and maternally inherited microbiomes may be a primary reason why many of the bacteria are specialised, and why landscape gradients do not align with changes in modularity here.

It is important to consider the methods of bacterial acquisition and persistence in context of this study. Taking *Wolbachia* as an example, its role in sexual differentiation in arthropods and its transmission via infected eggs makes it easier to be confident that its presence in these bat flies is attributed to vertical transmission (Hong et al., 2002). A similar logic could be applied for *Bartonella*, in that its known potential as a pathogen of bats might suggest these bat flies have acquired the bacteria from feeding on infected hosts (Reeves et al., 2016). However (De Bruin et al., 2015) found that ked flies (Diptera: Hippoboscidae) could experience vertical transmission of *Bartonella* bacterium, while showing that other pathogens examined, *Anaplasma* and *Rickettsia* species were not likely to be transmitted in the same way. In addition, Heath et al., (1999) suggested that *Wolbachia* could have a natural method of horizontal transfer across insects via parasitoid wasp attack. Some phylogenetic evidence also suggests similar events have occurred for *Arsenophonus* in other taxa (Thao and Baumann, 2004). Although very little is known about parasitoid wasps interacting with bat flies, Shockley and Murray, (2006) observed braconidid wasp specimens from an unknown species of *Heterospilus* directly interacting with streblid *Paratrichobius dunni*, although no parasitoid egg was found within the bat fly, microscopic examination revealed a

wound in the abdomen. Other instances of parasitoid infection of neotropical bat flies have also been observed (E.Clare unpublished) but are not well documented.

Throughout this thesis I examined the bat fly gut bacteria at their genus levels based on the *V4* region of 16S rRNA. While the usage of regions of the 16S gene is common for screening bacteria (Kennedy et al., 2020; Li et al., 2017; Vences et al., 2005b), it has been suggested that 16S should be used alongside other markers when differentiating some families such as Enterobacteriaceae (Husník et al., 2011). (Jousselin et al., 2013) gave attention to genus *Arsenophonus* and suggested that a combination of horizontal transfer events between related insect hosts and recombination events can lead to emergence of new phenotypes and misinterpretation of phylogenies. An example where a revision in taxonomy could affect my data is found with *Enterobacteriaceae Candidatus Phlomobacter* which has been shown to be closely related to plant pathogens under genus *Arsenophonus* and renamed Cand. *Arsenophonus phytopathogenicus* (Bressan et al., 2012). I have chosen to keep the splitting of *Cand. Phlomobacter* from *Arsenophonus* rather than fuse them together as they co-occur (due to *Arsenophonus* being ubiquitious in my data) and so this single change is unlikely to heavily affect the analysis.

Due to the variable estimates, it is important to consider how the completeness and resolution of the endosymbiont networks may impact the results. The relative importance of sampling completeness, and the effect of undersampling for community-level networks is a much-discussed topic (Henriksen et al., 2018; Jordano, 2016; Macgregor et al., 2017; Meyer et al., 2015). Like other measures in community ecology, network metrics are sensitive to the number of interacting species, with some

properties like connectance found to be more affected by the species richness than others like nestedness, and modularity (Kuppler et al., 2017; Rivera-Hutinel et al., 2012) which are the focus here. Generally, the network metrics examined in this study are thought to be stable (Gibson et al., 2011; Nielsen and Bascompte, 2007), but susceptible to bias at very low sampling effort (Vizentin-Bugoni et al., 2016). (Falcão et al., 2016) found that it is more important to keep a consistent method and effort when sampling ecological data for networks. Undersampling also becomes less of an issue when considering ecosystem functioning, as studies have shown that the main contributions of ecosystem services tend to come from the abundant common species, and trait diversity rather than strict species richness (Gagic et al., 2015; Winfree et al., 2015), suggesting that missing rare species or rare interactions should not diminish the interpretation of the results and broader conclusions from discussing community processes despite influencing the structure of networks. Similarly, less information can be gathered from examining a network at the genus level as compared to the species or OTU level, and reconstructing the endosymbiont networks at a higher resolution could possibly affect the resulting network structure, however other studies have shown that coherent patterns can still be observed at genus (Lupatini et al., 2014), and even phylum levels (Banerjee et al., 2016). Consequently, I treat these observations with caution, but suggest my conclusions are likely robust.

The contrasting results obtained by excluding the continuous sites in the fragmented dataset (as seen in Table 4.5 and 7) shows how the 3 data points from the continuous sites influence the model estimates. Tables 6 and 7 demonstrate that including the REGUA values in the landscape variable *logArea* completely alters the coefficient for network modularity. Extreme values in data are sometimes interpreted as outliers and

removed (Seo and Gary M. Marsh, 2006). However, the extreme values associated with the REGUA sites are 'legitimate outliers' in my data as an unavoidable consequence of study design rather than sampling error, and its inclusion simply requires more caution in applying inference to my models (Osborne and Overbay, 2004). The lack of evidence for an effect of landscape on network structure in the fragmented dataset where an effect was found in the complete dataset (such as with modularity in both layers) point to my models being applicable when comparing pristine and disturbed habitats, as if associated with opposite sides of a threshold, where the turning point or range is not represented in our data. Bat species richness has been shown to respond to spatial and landscape variables in a combination of thresholds and gradients (Muylaert et al., 2016), and my results may hint at their networks reflecting this.

Studies showing an effect of landscape change on host-parasite networks has important implications for strategies for conservation and public health. The role played by arthropod vectors in the transmission and spread of pathogens from one host to another has been acknowledged to confound public health strategies in controlling emerging infectious diseases (Benelli and Duggan, 2018). Previous studies have used other network analysis concepts to link habitat changes to horizontal transmission of ectoparasites (Saldaña-Vázquez et al., 2019), and the robustness of host-parasite relations (Baumgartner, 2020).

My study highlights that landscape level metrics should be considered when examining ectoparasitic and endosymbiont networks. However, the explanatory power of my models (adj. R^2) suggests that additional variables should be examined alongside landscape. Environmental variables associated with climate such as temperature,

humidity, and rainfall have been shown to regulate pathogen transmission potential in some arthropod vectors (Ogden and Lindsay, 2016), and warrant some investigation into their effect in this system alongside landscape-level variables demonstrated here.

In summary, structures of both ectoparasitic and endosymbiont interactions do change when examined across different landscape metrics in a fragmented system, and while both trophic layers respond, this response was not always the same and likely caused by different mechanisms. Phylogenetic constraints appear to play a key role in shaping the networks, although the extent of this effect is difficult to establish, especially in endosymbiont networks where vertical transmission plays a major role in endosymbiont communities. By examining multiple trophic layers simultaneously, I have shown that not only do obligate symbiont communities have some capacity to respond to changes in the external environment, but they can respond independently from their respective hosts. However, an improved understanding of which properties of the external environment can influence these symbionts is still required for firmer conclusions to be made.

Chapter 5:
Elements of Metacommunity Structure in Symbiont Communities

Abstract

Habitat fragmentation alters natural landscapes and has implications for species assemblages within remnant habitat. Here I examine the metacommunity structure of two taxonomic groups (ectoparasitic bat flies, and their gut microbiota) across fragmented and continuous sites in the Atlantic forest. Both the bat fly and microbiome metacommunities were found to form Clementsian and quasi-Clementsian structures across sites based on gradients of habitat area and isolation, indicating that taxa exhibited coherent and clumped distribution patterns. For bat flies, I split the metacommunity to further examine a subset of fragmented sites and this subset exhibited a quasi-Gleasonean structure, which indicates that species show individualistic responses, in this case only to habitat area. My results suggest varying levels of species sorting shape the bat fly metacommunity in the forest fragments, where environmental limitations may exclude disturbance-intolerant bats and lead to fewer bat flies present in smaller forest fragments.

Introduction

Biodiversity and anthropogenic effects

Conserving biodiversity amidst increasing anthropogenic disturbance of ecosystems is an escalating challenge. One focal objective in this challenge is to understand the

mechanisms that shape communities (Svensson-Coelho and Ricklefs, 2011). Shelford's law of tolerance suggests that an organisms success is based on environmental conditions within a minimum, maximum, and optimal value for a given factor (Shelford, 1931). Habitat disturbances often changes the biotic and abiotic conditions affecting the abundance and probability of occurrence of species, as the latter is often a unimodal function of the former (ter Braak and Verdonschot, 1995). Habitat fragmentation and the usual resulting loss of habitat generally leads to smaller, more isolated habitat patches (Raphael K Didham, 2010), and has been known to limit the dispersal of species (Damschen et al., 2014), alter and reduce resource use (Martinson and Fagan, 2014), and put particular pressure on specialists (Krauss et al., 2003). The isolation of fragmented habitats can limit or prevent population and community replenishment through immigration from neighbouring patches (Magrach et al., 2011), although good colonisers and more vagile species are less hindered by this (Lynam and Billick, 1999). Habitat fragmentation has been implicated in species decline (Didham et al., 1998; Turner, 1996), local extinctions (Cushman, 2006; Michalski and Peres, 2005), and alterations in ecosystem structure and function (Höfer et al., 2001; Morris, 2010). However, species have varying responses to habitat fragmentation, with some species being more sensitive to perturbations while others thrive (Antongiovanni and Metzger, 2005). The underlying mechanisms that drive a species response can vary from abiotic factors such as changes in landscape layout (Didham et al., 1998), to biotic factors such as relative abundance of resources (Benstead and Pringle, 2004) and interactions between species (e.g. escape from predation (Roland, 1993b), and loss of obligate mutualists Bruna et al., 2005). This generates a complex dynamic where species may have individualised response to perturbations in habitat and resource distribution.

Parasite ecology

The study of symbiotic communities in modelling changes in ecological communities is vital because of the positive (Bruna et al., 2005), negative (Landmann, 2019), and synergistic (Jia et al., 2004) effects they have on host health, though they are frequently overlooked in any conservation planning scheme. The term 'symbiont' refers to organisms that must inhabit hosts and include all strategies across the parasite-mutualist continuum (EWALD, 1987), whereas parasites are defined as organisms that impose a negative, but non-lethal effect on their hosts (Esch and Poulin, 2006), resulting in reduced growth (Gorrell and Schulte-Hostedde, 2008), typically from stolen nutrients (Schwanz, 2006). These complex interactions can both exacerbate and moderate the effects of landscape level changes on host communities.

Studies of parasitism typically focus on a small number of species, or a single species, and document parasite growth (Macnab and Barber, 2012), host defence strategies (Field et al., 2007), or parasite burden (Brown et al., 1995) without often considering the interplay of host and environmental conditions. However, some studies have expanded the scope to include communities of parasite species and their hosts across geographical ranges (Guagan and Kennedy, 1993; Poulin and Morand, 1999). By examining multiple populations of parasites in parallel, environmental variables can be analysed for correlations with patterns expressed in parasite population dynamics. For example, Svensson-Coelho and Ricklefs, (2011) showed that the beta diversity of haemosporidian parasites in birds across the Lesser Antilles were not influenced by host species richness, but rather the geographical distribution and host genetic distance. Host and parasite communities tend to shape each other, for example

parasites often share traits when found on hosts that are geographically, phylogenetically, or ecologically similar (Locke et al., 2013), but the importance of any given factor may vary with ecological gradients. For example, phylogenetic relationships of cyprinid host species was a good predictor of metazoan parasite community similarity in some freshwater systems (Seifertová et al., 2008) but not others (Poulin, 2010). This system-dependent effect can also be seen in how parasites influence host-mediated trophic cascades, for example (Anaya-Rojas et al., 2019) found parasites reduced both body condition and population density of stickleback hosts, and found that stickleback populations with low parasite loads could initiate trophic cascades among zooplankton, but only in pelagic mesocosms not benthic ones. These studies highlight the importance of considering host, parasite, and environmental factors in conjunction when determining widespread effects and outcomes of infection.

Disease risks

Human infections by zoonotic diseases have often been related to changes in land-use increasing the interface between wildlife and human populations. This is often through land clearance for livestock in agricultural settlements (Jones et al., 2013), building human settlements in city outskirts to accommodate immigration (Nieto et al., 2012; Verdasquera Corcho et al., 2013), or forest fragmentation affecting roosting and feeding behaviour in vector species (Hahn et al., 2014b). Pathogen transmission dynamics can be attributed to complex interactions between vectors, hosts, and reservoirs. Understanding how the environment can influence symbiont community composition is an important contribution from the field of community ecology. There

are numerous examples of host health and pathogen transmission potential being affected by their symbiont communities. For example, mutualisms between bacteria in insects (Kaltenpoth, 2009) and fungi in plants (Faeth, 2002) contribute to host pathogen defence, and commensal viruses (Hall et al., 2016) and bacteria (Cirimotich et al., 2011) in insects reduce their hosts' vector potential. There has even been evidence of parasites and pathogens regulating each other for example, Johnson and Hoverman, (2012) found that natural populations with higher parasite richness and co-infection rates of helminths tended to lower overall infection success of even the most virulent species in their amphibian hosts.

Host diversity also has an impact in disease spread, where parasites require a minimum level of host diversity and population size to persist and so host and parasite diversity are often positively correlated (Lafferty, 2012). The loss of host diversity has been considered a driver for spreading infection. The dilution effect hypothesis suggests that communities with high species diversity inhibit disease spread by regulating susceptible host populations and interfering with parasite transmission and vice versa (Civitello et al., 2015; Keesing et al., 2006). Furthermore, both host diversity and parasite co-infection can interact to determine disease risk, for instance (Johnson et al., 2013) compared trematode *Ribeiroira ondatrae* infection in amphibian hosts on species-rich and species-poor assemblages, finding that co-infections by other parasites reduced *Ribeiroira* infections by 15%, and species-rich host communities had 40% fewer infections overall. Infection rates are also reduced through dilution effects by the buffering of abiotic stress. (Rottstock et al., 2014) demonstrated that host plant diversity affected fungal pathogen infection finding that while host and pathogen diversity were positively correlated, infection incidence and intensity decreased with

increasing host diversity, positing that complementarity and facilitation effects among host species reduced their susceptibility of infection due to nutritional stress.

The elements of metacommunity structure

Metacommunity ecology explores the roles of local and regional processes that shape species communities across space. A metacommunity is defined as a series of spatially distinct ecological communities, potentially linked by dispersal (Leibold and Mikkelson, 2002; Mihaljevic, 2012). The analysis of elements of metacommunity structure (EMS) was developed to determine how metacommunities of species are distributed (Leibold and Mikkelson, 2002). These elements (i.e., coherence, species turnover, and boundary clumping) of a metacommunity can be compared to several idealised metacommunity structures (Figure 5.1, for further elaboration on each element see Chapter 1) to identify a best fit structure and then subsequent ecological analysis can be used to identify drivers of these structures. There are significant limitations to this procedure, for example it cannot consider spatial factors in driving metacommunity structure, and so the influence of species dispersal is largely unrepresented in this analysis. However, EMS is convenient to use with data where a reliable abundance measure is difficult because the analysis only requires a presence and absence matrix.

EMS analysis has been used in a variety of contexts to determine community structure and possible environmental drivers of that structure. Some usages include snapshot studies (e.g. Paraguayan bats of different feeding guilds; Presley et al., 2009, stream organisms in Finnish basins; Heino et al., 2015b) and spatiotemporal studies that show how community structure changes with seasonality (e.g. bacterial communities in the South China Sea; Yeh et al., 2015, and small mammals in the Atlantic Forest; Delciellos

et al., 2018). Additionally, EMS has been useful in studying the impact of environmental disturbances by incorporating measured environmental gradients in multivariate regression analysis to test whether natural biotic and abiotic factors relate to the observed structure (e.g. land-use conversion and damselflies in the Amazon (Brasil et al., 2017), forest fragmentation and small mammals in the Atlantic Forest (de la Sancha et al., 2014). Based on the ecology of symbionts, the variables used in EMS to represent environmental gradients have been extended to include characteristics of their hosts (e.g. using body mass, longevity, and trophic status of hosts as environmental gradients; Dallas and Presley, 2014), in addition to using conventional abiotic gradients (e.g. predicting helminth metacommunity structure from degrees of human settlements; Costa-Neto et al., 2018). Here I apply the EMS approach to symbiont communities associated with bats in patches of Atlantic Forest recovering from fragmentation, with the goal of determining whether landscape features we associate with hosts, impact on the structure of their dependent symbiont communities which experience the landscape at a very different scale. The application of the EMS framework to host-parasite systems is a relatively recent endeavour, which highlights the importance of understanding the host-parasite relationship as reservoirs and vectors of disease (Daszak et al., 2000; Luis et al., 2013).

Symbiont metacommunities in the Atlantic forest

The Brazilian Atlantic Forest is considered a biodiversity hotspot, characterised by its high species richness (Bergallo et al., 2003), high levels of endemism (Costa et al., 2000), and heterogeneity (Martini et al., 2007). Originally spanning 150 million ha, the current natural environment in the Atlantic Forest has been greatly diminished due to

centuries of urbanisation and development, with forest fragments scattered throughout (Ribeiro et al., 2009). Models by (Liebsch et al., 2008) suggests it will take almost two thousand years after disturbance for existing fragments to match 40% of endemism found in the surrounding mature forests. Remaining fragments are of varying sizes with some as small as 100m from edge to edge, and this 500 year history of disturbance has attracted research into long term effects of habitat fragmentation (Lira et al., 2012b).

Bats (Chiroptera) represent a good model for studies on wide-spread ecological disturbance because of their diverse life histories. Bats are exceptionally mobile mammals (Shilton et al., 1999), but dispersal ability varies across species, allowing the effects of habitat fragmentation to vary accordingly (Christoph F.J. Meyer et al., 2009). Bats are hosts to specialised parasitic diptera called bat flies (Nycteribiidae and Streblidae), which are typically found as ectoparasites in their fur (Jobling, 1949). Faunal studies of bat flies have primarily focused on morphological and taxonomic descriptions (Wenzel, 1975), as well as records of occurrence (Dick and Gettinger, 2005; Patterson et al., 2008c). Ecological studies of bat flies have identified factors such as female-biased host preference (Patterson et al., 2008d), pupal deposition behaviours (Dittmar et al., 2009), and infection prevalence (de Vasconcelos et al., 2016; Patterson et al., 2007) shaping bat fly communities.

Investigations relating to the vector potential of bat flies were reviewed by (Szentiványi et al., 2019), finding that the literature recorded microparasites were mostly bacteria and fungi, followed by relatively marginal reports of protozoan blood parasites, viruses, and other arthropods. The obligate nature of bat flies and the

dispersal ability of their bat hosts may point to bat flies playing an important role in the maintenance and transmission of bat pathogens. There is particular attention given to bat flies for being potential reservoirs and vectors of Bartonella (Reeves et al., 2007), Rickettsia (David A. Wilkinson et al., 2016b), Ebola (Caron et al., 2018), and more recently COVID-19 (Poinar, 2020). For example, *Bartonella spp.* are bacteria that can infect several tissue types including red blood cells and endothelial cells (Billeter et al., 2008). Several zoonotic species are vectored by ticks (De Bruin et al., 2015), fleas (Reeves et al., 2007), and speculated to be vectored by biting flies (*Hippoboscidae*; Halos et al., 2004). Bat flies have been shown to harbour similar strains of *Bartonella spp.* bacteria to their infected host, suggesting they are able to pick up the microparasite from bats, however their ability to then transmit the pathogen has not been empirically demonstrated (Do Amaral et al., 2018; David A Wilkinson et al., 2016). However, Morse et al., (2012b), found evidence for early evolutionary association of *Bartonella* and subsequent radiation in distinct clades of bat flies and bats, further supporting the hypothesis of bat flies as a potential vector. By understanding the environmental factors that affect bat fly distributions, it may be possible to predict which regions have potentially higher risk of disease spread in host populations, and this has been demonstrated to be possible for other vectors (Brownstein et al., 2003; Diuk-Wasser et al., 2010).

Here I use EMS analysis to determine the metacommunity structures of bat flies and their bacterial symbionts and examine how properties of fragmented landscapes shape of these communities. While studies have documented the effects of seasonal change (Salinas-Ramos et al., 2018) and climate change (Pilosof et al., 2012b) on bat fly ecology, this is the first study to assess the impact of habitat ecology on bat flies and

their associated microbiome metacommunities simultaneously. I adopt a site-centric approach to analysing the metacommunities, using the spatially distinct habitat fragments to differentiate samples. Using this framework, I assess the structure of bat fly and microbe metacommunities in the fragmented Atlantic forest and determine which landscape gradients might influence this structure.

Methods

Data Collection and Preparation

The landscape and taxonomic data used here is described in chapter 3 and includes bat fly and microbiome data from all 13 sampling sites and the associated landscape metrics. For EMS analysis I generated community data matrices where rows represent taxa ranges corresponding to the sampling locations, columns represent taxa (bat fly species or microbial genera), and cell values represent the presence or absence of the taxon at that sampling site. This generated two data sets, one for all bat flies, and one for all the gut microbes found in those bat flies. For clarity, while 'symbiont' could encompass both focal bat flies and microbes in my study, I use 'Ectoparasite' and 'Endosymbiont' to refer to bat flies and their bacterial symbionts respectively.

Evaluating the 'Elements of Metacommunity Structure'

I evaluated the structure of my metacommunity matrices using the *metacom* package v1.5.2 (Dallas, 2014) in R v3.6.1 (R Core Team, 2019) which evaluates coherence, species turnover, and boundary clumping of metacommunity matrices following (Leibold and Mikkelson, 2002). I used the *Metacommunity* function on each matrix, this function first ordinates the matrix using reciprocal averaging before calculating the elements of metacommunity structure. I used default arguments to ordinate the

metacommunity matrix and simplify the species abundances into binary values. I generated graphical representations of these ordinated matrices using the *Imagine* function with the *fill* argument set to true, which artificially fills gaps in the matrices to visually maximise coherence (Figure 5.1). I then compared the coherent metacommunity matrix via z-tests (for coherence and species turnover) and chi-square tests of Morisita's Index (for boundary clumping) to 1000 ordinated null matrices, generated based on user-specified randomisation models. The structure of the metacommunity is determined based on the outcome of these tests, the current possible structures and requirements are summarized in Figure 5.2. I used two randomisation models, the ectoparasitic layer was randomised using a 'r1 model' which maintains species richness of sites while randomising species ranges based on their marginal probabilities, while the endosymbiotic layer was randomised using a 'r0 model' which still maintains species richness of sites, but otherwise completely randomises species ranges. The 'r0 model' was chosen for the endosymbiotic layer because the 'r1 model' is too computationally taxing for large matrices.

Canonical Correspondence Analysis to explain structures

For each matrix I examined both the primary and secondary axis of ordination. If a matrix had significant positive coherence suggesting a non-random structure, I applied canonical correspondence analysis (CCA) to measure the fit of species composition of the ordinated matrix and measured landscape variables, using the *cca* function available from the *vegan* package v2.5-5 (Oksanen et al., 2019). CCA functions similarly to multiple regression, where 'inertia' measures how well the explanatory variables align with the species composition, and the inertia scales similarly to the r^2 used for

regressions (ter Braak and Verdonschot, 1995). There are as many constrained canonical axes as there are explanatory variables included in a model (i.e. CCA1-CCAx, where x is the number of variables in the model), and any additional residual axes produced in the analysis hints towards important unmeasured variables (i.e. CA1-CAy, where y is the number of residual axes detected by CCA). I use the relative values of constrained and unconstrained inertia to evaluate the fit of the landscape variables in explaining the differences in metacommunity patterns, and I use the axis loadings calculated for each environmental variable to determine how well they can be used to discriminate between the sites and species. *Exploring Clementsian metacommunity structures*

For metacommunities exhibiting a Clementsian structure, I estimated the number of subgroups present and which sites belong to those subgroups. I calculate the beta diversity using the *multiplicativeBeta* function from package *velociraptr* (Zaffos, 2019). This iteration of beta diversity is based on a *true gamma diversity* formula as explained by (Tuomisto, 2010) where gamma diversity (total effective species in a dataset) is a product of alpha diversity (effective species per subgroup) and beta diversity (effective number of subgroups). I used a hierarchal clustering approach provided by the *recluster.cons* and *recluster.region* functions from the *recluster* package (Dapporto et al., 2013) to generate a consensus tree of sites sorted based on Sorensens-Dice coefficient (Dice, 1945) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sokal, 1958) to determine which sites belong to which subgroups in the metacommunity and better identify the point of range turnover suggested by the Clementsian structure. The Sorensens-Dice distance coefficient puts more weight on the similarity of species presence rather than

absences (Baselga, 2010), and is less sensitive to outliers like rare species (McCune et al., 2002). UPGMA is a hierarchical approach that forms clades from pairs of sites with the smallest distance coefficient, and is shown to successfully join replicate sites together, avoiding errors in overestimating matrix diversity (Cao et al., 1997).

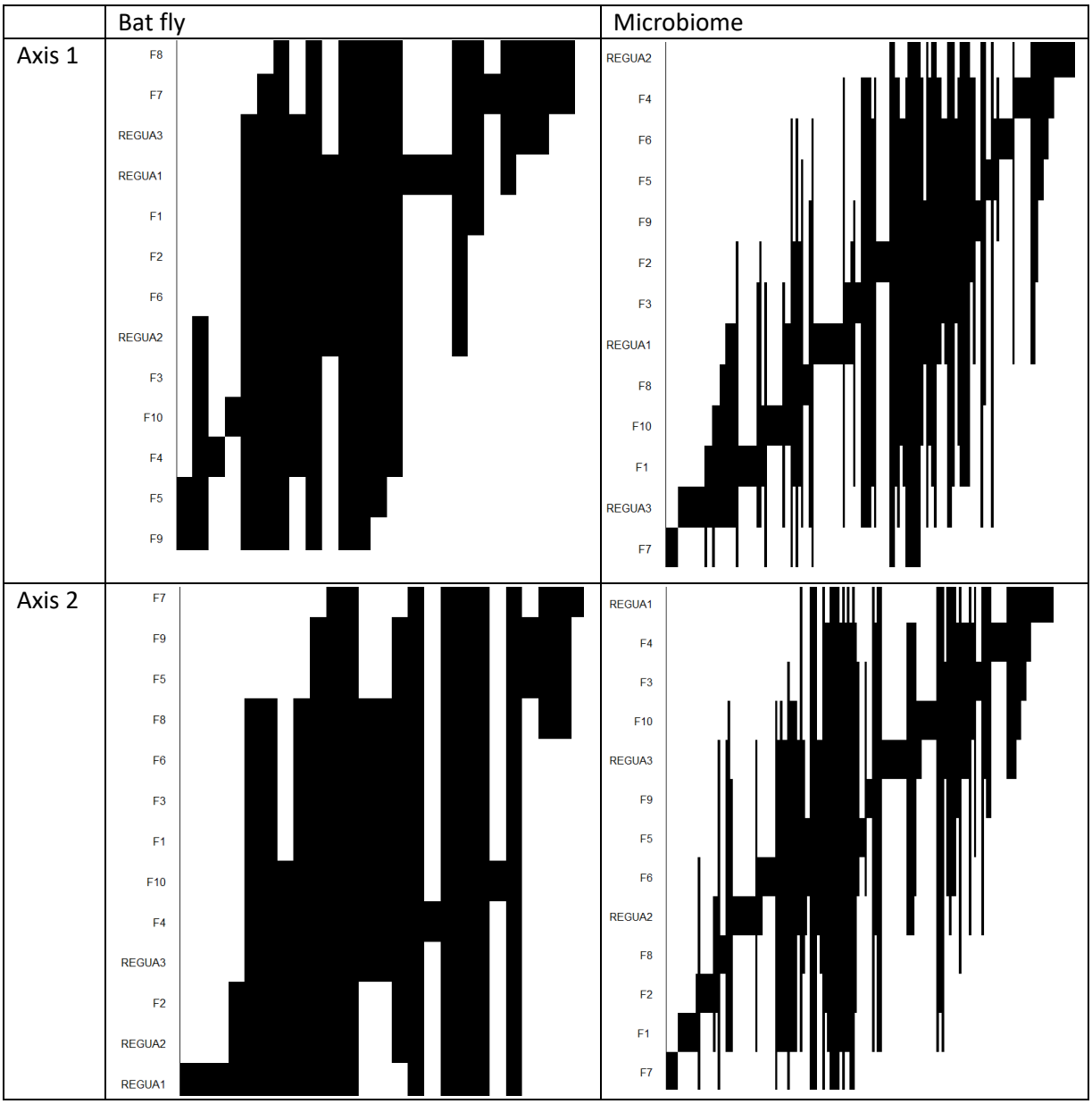
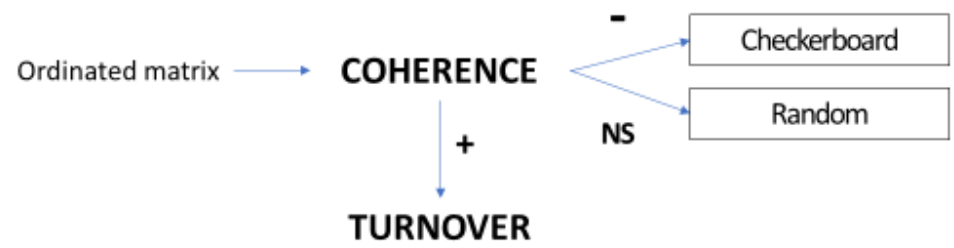


Figure 5.1: Ordinated species/genus distributions of each metacommunity for both axes of ordination. Structures exhibiting positive coherence were artificially filled to help visualise turnover and boundary clumping.



-	NS(-)	NS(+)	+	
Nested clumped species loss	Quasi-nested clumped species loss	Quasi-Clementsian	Clementsian	+
Nested stochastic species loss	Quasi-nested stochastic species loss	Quasi-Gleasonian	Gleasonian	NS
Nested hyperdispersed species loss	Quasi-nested hyperdispersed species loss	Quasi-evenly spaced	Evenly spaced	-

BOUNDARY CLUMPING

Figure 5.2: Flowchart and matrix showing the relationships between 14 idealised metacommunity structures based on the elements of metacommunity structure: coherence, turnover and boundary clumping. the '+' and '-' indicates the values of the element is significantly greater or lower than expected by chance, 'NS' indicates the value of the element is not significantly different than expected by chance.

Results

Metacommunity characteristics and structure

Population characteristics of the 13 sites are described in more detail in chapter 3. In summary, the ectoparasitic metacommunity is comprised of 16 bat fly species and was best described as a Clementsian structure on both axes of ordination, while the endosymbiont metacommunity had 50 bacterial genera and fit a quasi-Clementsian structure on both axes of ordination (for test statistics see Table 5.1). Both metacommunities had at least 1 taxon that was present in all sites (Figure 5.1). Estimates of beta diversity suggests the bat fly metacommunity had 2 subgroups (β = 2.66) and the microbiome metacommunity had 4 subgroups (β = 4.90). Subgroup membership for the bat fly metacommunity was an almost complete split between the REGUA and fragmented sites, however the same pattern was not seen in the microbiome metacommunity (Figure 5.3). Among the subgroups between the two metacommunities, all but one consisted of fewer than 4 sites, the exception was a bat fly subgroup (BSub) that consisted of 9 of the fragmented sites. BSub was found to have a quasi-Gleasonian structure on the primary axis but was quasi-Clementsian on the second axis (Table 5.1, Figure 5.4).

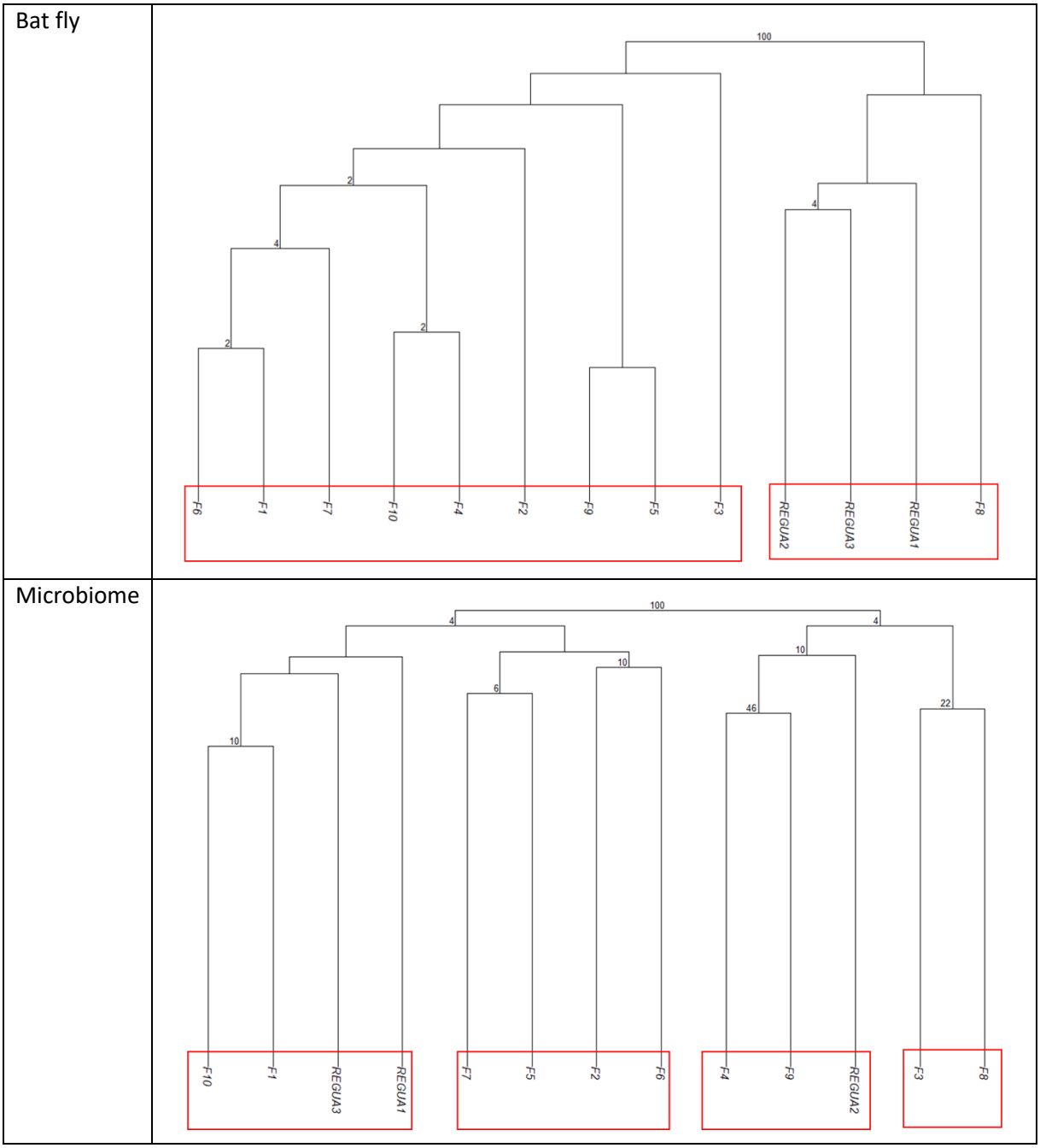


Figure 5.3: Dendograms illustrating similarities of sites based on Sorensen distances and UPGMA algorithm for both bat fly and microbiome metacommunities. Red boxes separate distinct subgroups as designated by recluster package when the number of subgroups is inferred from their respective values of β diversity.

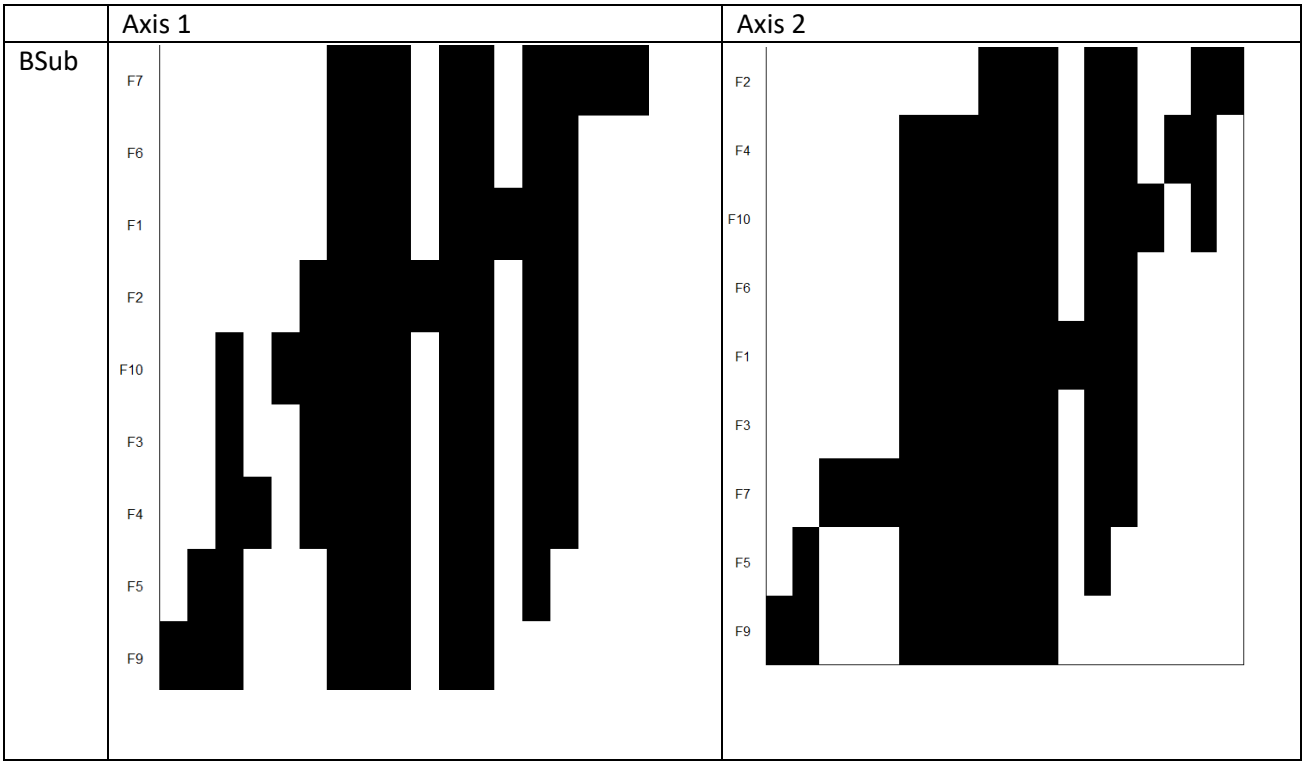


Figure 5.4: Ordinated species/genus distributions of the larger bat fly compartment for both axes of ordination. Axis 1 exhibits quasi-Gleasonian and axis 2 exhibits quasi-Clementsian structures.

Table 5.1: Coherence, species turnover, boundary clumping, and the resulting metacommunity structure across forest fragments for the bat fly metatcommunity, bat fly subgroup, and microbiome metacommunity. Rows represent different subsets of data as well as the axis of ordination (1: primary axis, 2: secondary axis). EmAbs = number of embedded absences, rep= number of replacements, I_m = Morisita's Index.

	COHERENCE				SPECIES TURNONVER				BOUNDARY CLUMPING		STRUCTURE
	EmAbs	z	*P*	Mean	rep	z	*P*	Mean	I_m	*P*	
Bat fly 1	68	-8.34	<0.001	157	854	1.97	0.04	643	1.49	0.008	Clementsian
Bat fly 2	100	-5.22	<0.001	155	754	2.76	0.005	463	1.67	0.01	Clementsian
Bat fly sub 1	33	-5.05	<0.001	64.7	142	1.19	0.23	113	1.07	0.32	Quasi-Gleasonian
Bat fly sub 2	25	-4.96	<0.001	61	147	1.01	0.31	115	1.47	0.02	Quasi-Clementsian
microbiome 1	868	-31.1	<0.001	1560	3.2E4	1.68	0.09	2.8E4	1.31	<0.001	Quasi-Clementsian
microbiome 2	903	-30.3	<0.001	1570	3.1E4	1.21	0.22	2.9E4	1.29	<0.001	Quasi-Clementsian

Table 5.2: Loadings from the first constrained and unconstrained axes from canonical correspondence analysis (CCA) for both entire metacommunities, and a subgroup from the bat fly metacommunity (BSub). Net EVs is (CCA1 EV – CA1 EV).

Codes		Bat fly Ax1	Bat fly Ax2	Microbe Ax1	Microbe Ax2	BSub1	BSub2
	Constrained Inertia	0.3494	0.422	0.3449	0.3264	0.60	0.60
	CCA1 EVs	0.2419	0.2335	0.3945	0.3855	0.2947	0.2771
	CCA1 % Inertia	0.1698	0.1638	0.1065	0.1041	0.25	0.2350
	CA1 EVs	0.2579	0.1981	0.4011	0.4053	0.2164	0.1979
	CA1 % Inertia	0.1810	0.1389	0.1082	0.1094	0.1835	0.1678
	Landscape variables						
LArea	Log Area	**0.9768**	**0.9542**	**0.9976**	**0.9868**	**0.9389**	**0.8609**
Iso	Isolation	**-0.693**	**-0.8235**	**-0.545**	**-0.6504**	-0.417	-0.1038
Hab2	PCA habitat 2	0.1747	0.2711	0.2562	0.3927	0.1941	0.115
Land2	PCA land 2	0.1348	0.1346	0.1388	0.1294	-0.0859	-0.2685

Environmental variables associated with CCA

Site area was highly associated with the structures of both metacommunities and BSub, while site isolation was only highly associated with the bat fly and microbe metacommunity, but not BSub (Table 5.2). Both PCA habitat and PCA land were not highly associated in any metacommunity. Overall, constrained inertia suggests the relationship between taxa, sites, and environmental variables was best explained by BSub on both axes of ordination (60%), followed by the bat fly metacommunity (34% for primary axis, 42% for secondary axis), and the microbiome metacommunity (34% for primary axis, 32% for secondary axis). Proportion of inertia explained by CCA1and CA1 of the bat fly metacommunity were both alike, accounting for around 15% of inertia. The microbiome metacommunity also followed this trend, with its CCA1 and CA1 both accounting for around 10% of inertia. The bat fly subgroup had a larger difference between % inertia explained across its CCA1 (around 25%) and CA1 (around 15%).

Discussion

Communities of species may respond to environmental gradients along a continuum from discrete groups with concordant boundaries to individual species responses (Presley et al., 2009). Species assemblages are affected by ecological disturbances, often towards reduced complexity (i.e. homogenization) and favouring disturbance-tolerant generalist species (Lôbo et al., 2011). Different groups within a metacommunity can have varying responses to ecological change and this has been

shown for groupings based on taxon (Presley et al., 2012), or functional traits (e.g. dispersal capacity, feeding mode; Tonkin et al., 2016). I found strong evidence for Clementsian structures across the bat fly metacommunity and quasi-Clementsian structure for the microbiome metacommunities. This suggests subcommunities which are independent within both trophic layers, although for the quasi-Clementsian microbiome metacommunity, the non-significant turnover signal suggests that the latent environmental gradient is only weakly responsible for its structure.

Factors which contribute to Clementsian structures of communities within the Atlantic Forest vary within and across taxa (da Silva and Rossa-Feres, 2017). For example, Clementsian structures for small mammal metacommunities have been attributed to patch size and habitat quality by (Delciellos et al., 2018), but (de la Sancha et al., 2014) found fragmentation to have no effect on structuring compartments, but instead represent pockets of endemism created by biogeographic regions. In the case here of bat flies and microbiome, compartments suggested by hierarchal partitioning did not align with the latent environmental gradients presented by reciprocal averaging. With the exception of fragmented site (F8) the clustering algorithm found a general split between fragmented and REGUA sites for the bat fly community. However, the microbiome metacommunity was split into 4 compartments without an obvious pattern. The CCA analysis suggests that among our landscape variables, habitat area had the strongest effect on both metacommunities, followed by habitat isolation but with a smaller effect. My results are concordant with other studies showing that landscape variables associated with habitat fragmentation do have an impact on ectoparasite interactions, including bat flies (Hernández-Martínez et al., 2019b; Pilosof

et al., 2012b), although none previously have considered EMS for bat flies communities.

(Teixeira, 2019a) found habitat area and isolation influenced the bat host metacommunities in this same system. (Presley, 2011) suggests that gradients in bat fly metacommunity reflect changes within their host assemblages (aggregation, co-occurrence) rather than the ectoparasites responding directly to forest disturbance. However, this obligate parasitic relationship does not negate the potential of other environmental variables directly affecting bat flies or the microbiome. The total constrained inertia provided by the 4 environmental variables account for less than half of the available inertia in both the bat fly and microbiome metacommunities, which suggests there are potentially important variables that shape these metacommunities outside of the scope of landscape. For example, climatic variables such as air temperatures and humidity have been shown to affect development in fleas (Krasnov et al., 2001), and it is not implausible that the development of some bat fly species would be hindered by these variables affecting their physiology, especially during pupal phase that occurs off their hosts.

There are many possible determinants of microbiome composition. Some bacterial symbionts are vertically transmitted. In bat flies, as in other insects, the genera *Arsenophonus* and *Wolbachia* are examples of vertically transmitted strains and are unlikely to be strongly influenced by the environment (Solon F Morse et al., 2012; Patterson et al., 2013b). The diet of arthropods has been shown to alter the composition of their microbiome (whole-prey; Kennedy et al., 2020; blood-meal Muturi et al., 2018; Swei and Kwan, 2017a), although in this study the bat flies are

unlikely to be feeding on anything other than bats, and it is not known whether the species of bat or their diet has an effect on the bat fly microbiome. It is more likely that dispersal ability and roosting behaviour of the bats and bat flies and contact between taxa is responsible for the variation in microbiome assemblages (Adair and Douglas, 2017), providing a link between the environment and the bat fly gut. However other studies have found evidence for genotype-dependent mechanisms curating the microbiome, limiting diversity through selection by the host species (Foster et al., 2017; Gaithuma et al., 2020).

The environmental gradients most associated with the microbiome metacommunity was the same as the bat fly metacommunity, habitat area and isolation. There have been examples of habitat degradation and human activity reducing the microbiome diversity in animals as a consequence of reduced diet variety (Barelli et al., 2015; Becker et al., 2010) or filtering by environmental conditions (Hughey et al., 2017; Kueneman et al., 2014), although the susceptibility of microbiomes to be affected by environmental disturbance appears to vary between host species even among closely related taxa (Mccord et al., 2014). (Ingala et al., 2019b) found the microbiota of blood-feeding vampire bats (*Desmodus rotundus*) varied across fragmentation gradients based on dietary breadth of the bats (i.e. proportion of livestock to wildlife), rather than site quality, although this variation was only found in terms of abundances and not composition of the microbial community. While our results could suggest the bat fly microbiome responds to habitat fragmentation, it is more likely a reflection of changes within the bat fly assemblage. Bacterial symbiont recruitment in bat flies is still largely untested (Solon F. Morse et al., 2012b; David A Wilkinson et al., 2016), but (Morse et al., 2013) suggests that a majority of the bat fly microbiome are vertically

transmitted obligates, and a similarly restrictive microbiome was found in the closely related tsetse fly (Gaithuma et al., 2020). The varying microbial assemblages shown in this study could then be a product of both microbes recruited from the external environment, genotype compatibility between bat fly and microbe, and species specificity between bat-bat fly and bat fly-microbe.

Exploring the compartments of Clementsian structures can provide insight into how these discrete communities differ (Presley and Willig, 2010). Unfortunately, most of the estimated compartments for both metacommunities examined had too few sites for meaningful analyses to follow. However, CCA analyses on the larger of the two compartments (BSub) in the bat fly metacommunity followed a quasi-Gleasonian structure and habitat isolation was no longer an important gradient. Notably, the 4 landscape variables now accounted for 60% of inertia (around double that of the total bat fly metacommunity). This provides some support for different emphasis in structuring mechanisms between communities in fragments and continuous forest, where landscape is less important in larger habitats, with other factors dominating (McKinney, 2008; Mokross et al., 2013).

This study demonstrates that examining metacommunity responses to environmental gradients can help us understand how these assemblages might be structured, and how our interpretation of the results can change depending on where ecological boundaries are drawn. Similar conclusions have been reached in other studies (Costa-Neto et al., 2018). Importantly, the effects of habitat disturbance can be seen in ectoparasites and their symbionts, through cascading effects from their respective hosts. However, it is also possible that a feedback loop exists for this system, as bat-bat

fly-microbiome interactions have immunological interfaces, and while not measured in this study, could be a confounding factor in studying this system (Woodhams et al., 2014), particularly when considering how environmental stresses effect immunocompetence (Acevedo-Whitehouse and Duffus, 2009). Diminishing species assemblages in forest fragments can have cascading consequences for ecological process. For example, animals such as birds and bats contribute immensely to seed dispersal in the Atlantic forest, the loss of these seed dispersers could lead to a habitat dominated by wind-dispersed trees instead (Silva and Tabarelli, 2001). Given the prevalence and pervasiveness of ecosystem damage and habitat fragmentation, this approach which considers multiple trophic levels simultaneously should allow researchers to consider the depth of environmental change and how more obscure or difficult to study organisms can be affected by environmental perturbation.

General discussion

In this thesis, I aimed to investigate the effects of habitat fragmentation on local interactions and metacommunity processes, focusing on two host-symbiont relationships: ectoparasitic bat flies found on bat hosts, and endosymbiotic bacteria inside bat flies. The data used in my habitat fragmentation studies were collected from 10 forest fragments and 3 sites in continuous forest within the Reserva Ecológica de Guapiaçú (REGUA) region of the Brazilian Atlantic Forest. Additionally, I conducted an *in-situ* DNA barcoding experiment in Lamanai, Belize using FTA® cards, miniPCR™ thermocyling, and MinION™ nanopore sequencing and compared its performance in the field to Sanger sequencing (chapter 2). A total of 25 species of bat flies were collected from the Atlantic Forest sites. I calculated and described the prevalence, intensity, and aggregation for each bat fly-bat association, and for the two most abundant pairings I compared their prevalence and intensities between forest fragments and continuous forest (chapter 3). For all 13 sites sampled, I used network theory to describe the associations present between bat flies and bats (ectoparasitic) as well as bacteria and bat flies (endosymbiotic) in terms of connectance, nestedness, and modularity and then used linear modelling to show how these network metrics varied with the landscape across the fragmented and continuous forest sites (chapter 4). For both the bat flies and endosymbionts I used the 'elements of metacommunity structure' (EMS; Leibold et al., 2004): coherence, species turnover, and boundary clumping; to determine which ecological processes best governs their distribution across all 13 sites (chapter 5).

Methods in DNA taxonomy

In chapters 2 and 3, a variety of DNA-based taxonomy (i.e. barcoding, metabarcoding, Sanger, and MiSeq) was utilized and accompanied by morphological methods to classify specimens and estimate their diversity, in addition to demonstrating alternative PCR and DNA extraction, and sequencing platforms available for molecular species identification in the field. I found FTA cards to be an effective method for collecting and extracting DNA from blood spots and to some degree was able to retrieve representative DNA barcodes from every bat species sampled. However, the quality of retrieved barcodes, and the taxonomic resolution was better (i.e. at species level) on Illumina MiSeq sequencer conducted in the laboratory, than the MinION sequencer experiment in the field. At the time of writing, the MinION is the only viable sequencer for *in situ* DNA sequencing (Seah et al., 2020) and the trade-off between sequence quality and on-demand analysis is well documented (Pomerantz et al., 2018; Tyler et al., 2018). I was able to mitigate this issue by focusing on a pre-selection of bats that were morphologically distinct enough to propose *a priori* species hypotheses, which complemented the *COI* barcode reference library prepared for this trial. The identification of bat flies was based on morphological keys and *COI* barcodes were only used to confirm divergent clades through clustering similar barcodes. Unlike for bats, a *COI* reference database for bat flies was not available for use making the a priori hypothesis method of Chapter 2 non-viable. (Virgilio et al., 2010) suggests that 98% of insect species still lack adequate representation in DNA barcode repositories (e.g. BOLD) and could cause false negative identifications thus the combined morphology and high-quality DNA approach was employed and was successful. Classification of bacterial symbionts was done through a combination of 16S rRNA

reference sequences and ASVs (i.e. OTUs clustered at the haplotype level). ASVs are said to provide a more comprehensive and accurate measure of sequence diversity when compared to using a close-reference OTU, as the former represents an exact sequence and the latter implies a sequence that is sufficiently similar to the associated reference (Callahan et al., 2017), a subtle but important difference. Molecular methods for taxonomy improves species discovery and description, and complements morphological methods (Hajibabaei et al., 2007), but as in the case of the bat flies does not replace it in less well known taxa. The coverage and reliability of available barcode libraries is an important factor in accurately identifying specimens (Ekrem et al., 2007), and facilitates research compilation based on a species ecological, physiological and morphological data (Padial et al., 2010). However, the lack of a reference database does not inhibit DNA-based identification specimens, as demonstrated by the variety of supplementary analyses utilised in this thesis.

Differences in site type

Using t-tests in chapter 3, linear modelling in chapter 4, and hierarchal clustering of metacommunities in chapter 5, I was able to detect differences in bat fly-bat interactions between forest fragment and continuous forest site types. I found that *Trichobius joblingi* infection on *Carollia perspicilata* was twice as prevalent in fragmented forest sites than in continuous forest sites, but no significant difference was found for the prevalence between site types for *Paratrichobius longicrus* and *Artibeus lituratus*. It is difficult to make generalisations from only 2 host-parasite pairs with contrasting outcomes, however there is precedence for bat fly-bat responses to habitat alteration to be highly host species-specific (Hiller et al., 2020). The differences seen between *C. perspicilata* and *A. lituratus* are possibly due to roosting differences,

as the former predominantly roosts in permanent structures where recruitment of bat flies is more consistent (e.g. caves, tree hollows) while the latter roosts in foliage and likely switch roosts often (Garbino and Tavares, 2018; Komeno and Linhares, 1999). Whereas the increased infection prevalence in the forest fragments may point to an increase in roost sharing or crowding among *C. perspicilata* within the smaller habitats (Hernández-Martínez et al., 2019c). Network structures of both ectoparasitic and endosymbiont trophic layers were not altered when compared across only fragmented landscapes but were both correlated with multiple landscape properties when the continuous forest sites were also considered. The symbionts in both trophic layers exhibit a high degree of specialization (Patterson et al., 2013b; Zarazúa-Carbajal et al., 2016), and their assemblages are likely dependent on composition of their respective hosts. It is possible that the contrast between the fragments and continuous sites reflects the loss of disturbance-sensitive bat species (and their associated symbionts) (Laurindo et al., 2019) and the remaining communities in forest fragments remain relatively stable to further landscape alterations. The metacommunity structure for bat flies was found to fit a Clemenstian structure, and hierarchal clustering suggested a split metacommunity where one group of sites contained 9 of the 10 fragmented forest sites (BSub), and the other group contained the 3 continuous forest sites and the remaining fragmented forest site. The splitting of the metacommunity in this suggests different processes governed metacommunity structure among the site types. However, this same splitting could not be observed by the bat or endosymbiont metacommunity, where bats has a random structure across the same 13 sites (Teixeira, 2019a), and endosymbionts also had a Clementsian structure but splitting formed 4 groups with no discernible pattern.

Landscape effects

When investigating how landscape properties affected the networks (chapter 4) and metacommunity structure (chapter 5) of both ectoparasitic and endosymbiont layers, I showed that all 3 metrics: habitat area, isolation, and habitat complexity were associated to changes in symbiont communities. Habitat area appeared most often as an explanatory variable in ectoparasitic networks, being negatively correlated to network connectance, and positively correlated to network modularity. Habitat area was also found to be the latent environmental gradient dictating the Gleasonean structure in the bat fly sub-community (BSub). Habitat isolation was only relevant in the endosymbiont community, being negatively correlated to network modularity. All three landscape properties were found to be correlated to richness of endosymbionts. These results point to networks becoming simpler (less modular, and more connected) in smaller and more isolated sites. The dependence of symbionts on their hosts makes it difficult to determine whether my results are a direct influence of landscape change on the symbiont organism, or a reflection of changes by their respective hosts in different environments, or a combination of both. The genetic basis for some host-symbiont relationships is established in bats, bat flies, and their microbiome (Barbier and Graciolli, 2016; Wilkinson et al., 2016), but it has also been recognised that the external environment can additionally modulate or filter symbiont community composition (Brinker et al., 2019).

Biodiversity-disease paradigm

Host health and susceptibility to disease can depend on local community composition and environmental variables (Guernier et al., 2004). In this thesis, I showed that ectoparasitic and endosymbiont communities and interactions are affected by changes in landscape. The mechanisms that control variation in disease risk can be attributed to spatial processes involving contact between susceptible hosts with an infectious agent (e.g. infected host, vector, or reservoir) (Killilea et al., 2008). Some pathogens are directly affected by biodiversity (e.g. zoonotic and vector-borne diseases), which involve multiple species in their transmission and can sometimes also infect humans (Plowright et al., 2017). The exact effect of biodiversity on disease risk can differ between host species, the pathogen, type of land use change, and geographic location (Yasuoka and Levins, 2007). Biodiversity can have a negative (dilution effect) or positive (amplified effect) relationship with disease risk (Johnson et al., 2015; Ostfeld, 2009). Dilution effects involve two hypothetical mechanisms involving disease: transmission interference and susceptible host regulation (Norman et al., 1999). As an example of transmission interference, in a multi-host system with a vector-borne disease, increasing the ratio of less competent host species to competent host species results in a more "wasted" bites from vectors on hosts that do not efficiently harbour the disease (Rosà et al., 2003). In contrast, susceptible host regulation occurs when competent host species population are kept low due to interactions with other species (e.g. predators, competitors). (Keesing et al., 2010) suggests that dilution effects occur more likely in systems with highly competent host (e.g. weak immune defences) that are tolerant to disturbance (e.g. human impact). However the dilution effect hypothesis relies on the assumption that high biodiversity does not instead favour

competent hosts over non-competent hosts, where then an amplifying effect on disease risk occurs (Wood et al., 2014). Examples where biodiversity loss decreases disease risk include the land clearing of the Brazilian Amazon correlating with a decline in malaria prevalence (Valle and Clark, 2013), and deforestation in East Africa also linked to declines in river blindness (Walsh et al., 1993), as both instances involves vectors associated with intact vegetation and high biodiversity.

Connectivity-disease trade-off

Loss of landscape connectivity is a key consequence of habitat fragmentation, whereby a species' capacity to disperse and forage for resources such as food, water and shelter is restricted (Benz et al., 2016; Raphael K. Didham, 2010). Anthropogenic landscape change reduces landscape connectivity when habitat is destroyed, and when infrastructure is built. Man-made infrastructure such as roads (Vos and Chardon, 1998), fences (Osipova et al., 2018), and river dams (Jansson et al., 2000) may not explicitly involve habitat destruction in their construction, but fundamentally they still present a barrier for species movement and a disruption to habitat. The segregation of natural habitat during anthropogenic land use change can be considered an unintended consequence rather than the primary objective (e.g. roads for vehicles, dams to regulate water pathways, land clearing for agriculture). While most fragmentation is tied to habitat loss, one important exception to this is fences which have been deliberately used to segregate wildlife from accessing areas across the barrier, and are a common method to mitigate wildlife-livestock and wildlife-human conflict by safeguarding species of interest, or managing sensitive habitats (Gortázar et al., n.d.; Osipova et al., 2018). Despite widespread use, fences are often unmapped

and receive less attention than more drastic forms of landscape change such as roads or forest clearing (Jakes et al., 2018); and because of their function to clearly delineate separated territory, they present an important example of fragmentation distinct from habitat loss or destruction.

The fragmentation of habitat can be deliberately deployed (e.g. using fences) as a means of restricting the spread of infectious diseases (Bozzuto et al., 2021). The relationship between connectivity and disease prevalence has been described as a trade-off by (Hess, 1996), whereby restricting connectivity between subpopulations may eliminates diseases that cannot persist in isolated population at the cost of increasing their vulnerability to stochastic extinctions. Mathematical models supporting this suggest positive relationships between subpopulation movement rate and epidemic duration (Jesse et al., 2008) as well as disease persistence (Gog et al., 2002). In practice, artificial fragmentation through cordon fences has the potential to limit wildlife contact and disease transmission to domestic animals (Batista Linhares et al., 2021; Jori and Etter, 2016). In South Africa, successful efforts to contain the spread of foot-and-mouth disease (FMD) included perimeter fencing around Kruger National Park to separate cattle from wild ungulates carrying the pathogen (Gortázar et al., 2020), as well as vaccinating the livestock from the disease (Brahmbhatt et al., 2012; Lazarus et al., 2017).

Artificial fragmentation by itself may not be sufficient to contain wildlife diseases, as exemplified from efforts to eradicate African swine fever virus (ASF) and chronic wasting disease (CWD) through fences in Europe (Mysterud and Edmunds, 2019; Mysterud and Rolandsen, 2019), partly because of the varied ways pathogens are

transmitted, as well as how different species respond to being contained by physical barriers. The European Food Safety Authority reported caveats to strategies in preventing the introduction and spread of ASF via hunting of wild boars in fenced areas, finding that certain hunting practices (e.g., artificial feeding) not only concentrated boar populations and thereby facilitated ASF spread, but intensive hunting led to dispersion of groups, making tracking difficult (EFSA, 2014). CWD fences meant to contain cervids were only effective at certain heights due some species jumping across (Vercauteren et al., 2010), in addition to the transport of farmed deer species, and wasting of contaminated meat and carcasses from hunting (Stegen et al., 2017) (Mysterud and Rolandsen, 2019) have contributed to the disease spilling over. Preventing disease emergence through fencing alone is also ineffective for pathogens circulated through water, soil or air (Stegen et al., 2017), as well as those involving flying hosts such as bats and birds (Gargas et al., 2009; James et al., 2011) that can bypass terrestrial barriers.

Following the connectivity-disease paradigm, the rehabilitation of fragmented habitat must carefully consider the consequences to both the reconnecting populations and diseases that could emerge (Bienen, 2002). Bioeconomic models tested by Horan et al. (2008) finds that it is more cost-effective to invest in increasing connectivity first, then focus on disease prevention and control in those highly connected habitats. This strategy particularly benefits endangered populations as the increased gene flow enabled through habitat corridors may promote disease resistance in hosts as population numbers grow (Horan et al., 2008; Jousimo et al., 2014). Disease resistance can be induced by administering vaccines against pathogens or their vectors, for example through injections for livestock (de la Fuente et al., 2020), or through wildlife

baits treated with orally acquired vaccines (Beasley et al., 2015). Another cost-effective method to tackle the connectivity-disease trade-off involves targeting only highly connected populations, such as those close to habitat corridors, as demonstrated by Haydon et al. (2006) deploying this low-coverage vaccination strategy on Ethiopian wolf metapopulations against rabies to avoid large outbreaks of the disease rather than its complete eradication from the region.

Implication for disease outbreaks

The recent COVID-19 pandemic has shown how little is still understood about the extent of the impacts wildlife mistreatment can cause (MacFarlane and Rocha, 2020b). Some authors believe the SARS-CoV-2 virus to have transmitted from bats to humans as they are natural reservoirs of similar viruses (e.g. SARS-CoV and MERS-CoV; Tiwari et al., 2020), while others hypothesize that illegal trapping of live wildlife such as pangolins alongside bats facilitated the virus's spread to humans when sold through wildlife markets (Andersen et al., 2020; Turcios-Casco and Cazzolla Gatti, 2020). (Platto et al., 2020) suggests three factors that influence the emergence of infectious diseases: (1) the frequency of human-wildlife interface, (2) the effects of environmental change on pathogen prevalence (Wolfe et al., 2005), and (3) the diversity of wildlife microbes (the zoonotic pool; Morse, 1993). Although measuring zoonotic transmission is beyond the scope of this thesis, the results from my studies can still provide insight on outbreak risks in this system. Aside from deliberate pursuit of animals for hunting, human-wildlife interactions occur as a result of competition between humans and animals for limited space (Pătru-Stupariu et al., 2020). Conflict arises due to wild animals encroaching upon man-made settlements in search for food and shelter (Lim et al., 2018; Tait et al., 2014), as well as domesticated animals expanding their range

into wild territories (Hanmer et al., 2017). The land clearing in the Atlantic Forest was primarily due to socioeconomic purposes (Izqulerdo et al., 2008). Non-forest matrix surrounding the sites in my Atlantic Forest studies consisted of agricultural land, farmland, and peri-urban settlements. Bats exhibit behaviours that increase the likelihood of human contact in these habitat matrices and therefore zoonotic transmission, for example by inhabiting human structures (Barros et al., 2015), feeding on fruit in plantations (Korine et al., 1999) and livestock on farms (Bobrowiec et al., 2015). The most abundant bat collected throughout my studies was *C. perspicilata*, and its primary bat fly *T. joblingi*. *C. perspicilata* is a disturbance-tolerant species (Laurindo et al., 2019), and in this study was found in every site. Comparing between forest fragments and continuous forests in chapter 3, there were 3 times as many *C. perspicilata* collected from forest fragments than continuous forest, and prevalence was twice as high in the former than the latter, suggesting a negative biodiversity-disease relationship. Based on networks in chapter 4, *C. perspicilata* was also highly connected to different network modules by being a generalist host, and this was more evident in networks of higher connectance in smaller forest fragments. Taken together, my results suggest that the smallest forest fragments are at higher risk of having pathogens spread farthest due to their high connectance, and persistence of generalist species. In terms of microbial diversity, I found that endosymbiont genus richness decreased in smaller, more homogenous sites. It is difficult to determine what this relationship means for disease risk without knowing the pathogenicity of the bacterial genus and species loss. However, known potentially pathogenic bacteria such as *Bartonella sp.* and *Rickettsia sp.* was prevalent in several species of bat fly and reduced microbiome diversity has been shown to increase susceptibility to pathogens

in other organisms (Schwarz et al., 2016; Swei and Kwan, 2017b). Overall, I hypothesise that the risk of diseases emerging from wildlife is higher in forest fragments that are smaller, more isolated, and have low biodiversity due to a combination of effects by bats, bat flies, and microbes from habitat disturbance that facilitates the infection of pathogens on susceptible hosts and its spread through host networks.

Future study design considerations

The generalisation of results from field ecological studies can be confounded by highly variable background environments, and community composition derived from an unknown history (Knapp et al., 2004). The results in this thesis consider 13 sites localised within the Reserva Ecológica de Guapiaçú (REGUA) region of the Brazilian Atlantic Forest. The proximity of the forest fragments to the continuous forest provides some confidence that the communities found at those sites would have been more homogenous historically (Chave, 2013), and changes in composition can be partially attributed to the change in land use. The landscape components of my analysis throughout this thesis were somewhat confounded by the stark contrast between fragments and control sites. For example, the dramatic size differences between fragments and control sites creates a large gap embedded within continuous variables which limits regression analyses. Missing data is a common occurrence for field work that can bias analyses (Bogoni et al., 2018; Horton and Kleinman, 2007). Some studies fill in incomplete data using methods called imputations and augmentation (Ellington et al., 2015; Horton and Kleinman, 2007), another way to close gaps in datasets is to conduct a meta-analysis and incorporate values from other similar studies (Ellington et al., 2015). Neither options were considered for this thesis,

as imputations have been heavily criticised (Nakagawa and Freckleton, 2011), and the scarcity of bat-bat fly-microbe network and accompanying landscape data available for use precludes meta-analyses. Rather than attempting to fill in the gaps for the continuous variables to create a smooth gradient between fragments and control sites, another way to analyse the data would be to treat the data as categorical (fragment vs continuous) as was done for chapter 3. In this case, the imbalanced sample design (10 fragments and 3 continuous sites) reduced statistical power for pair-wise comparisons such as T-tests (Hoover, 2002; Mendeş, 2005). In future analyses two options to improve analyses include sampling sufficient sites to equally populate the two categories of fragment and control data or to select fragments which create a more even distribution of size variables. One problem is that this may be too resource intensive, or not feasible due to the variability in natural systems but should be considered in future field sites. Using these data to conducting a power analysis prior to future field work may inform better sampling size and design (Fairweather, 1991).

Host health and disease risks were themes discussed at length in this thesis, but not directly measured. Discussions of bat flies as vectors for bacterial pathogens present in their microbiome would have benefited greatly from being paired with bat microbiome data, either through sampling blood or gut microbiota. The risks or costs to being parasitised instead were represented by prevalence and intensity measures, as a proxy to likelihood for disease transmission in the population. If similar bacterial species are found in both host and parasite, genome comparisons can be made to deduce whether an association between host-parasite microbiomes was present. For practicality, this focused look at microbiomes could be reserved for species of interest (e.g. connector species, threatened species).

Conclusions

In this thesis I show through multitrophic analysis of host-ectoparasite-endosymbiont communities, that the effects of landscape changes in fragmented habitats can be detected at fine level, and that symbionts can be affected by different environmental variables from their hosts to an extent. I also show how habitat disturbance affects symbiont interactions at different gradients and note how habitat area plays a large role in local and metacommunity processes. The rehabilitation of wildlife populations in small, isolated habitats should continue to be a priority to monitor disease risk in threatened species as well as prevent emerging infectious diseases. Evaluating disease risk of fragmented habitats can help identify which patches and which species present efficient use of resources to mitigate outbreaks when improving habitat connectivity.

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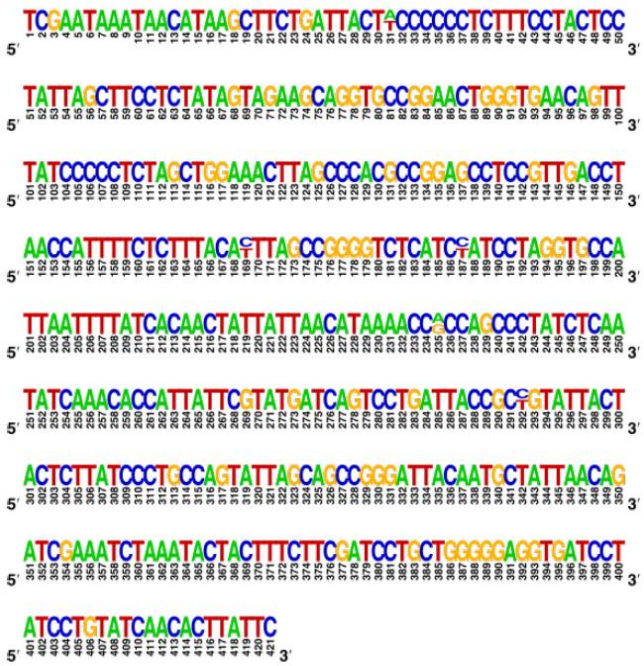
Appendix

Appendix 1.1: Sequence of Nanopore MIDs used

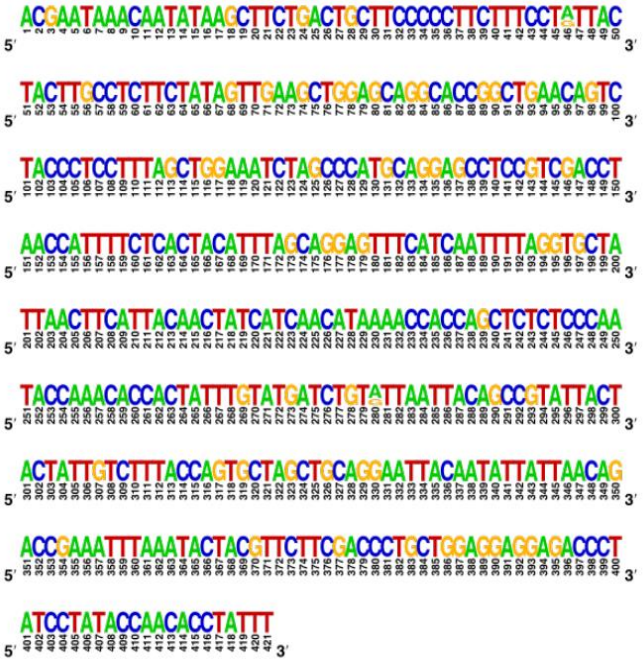
MID	Sequence	Assigned to
BC01	AAGAAAGTTGTCGGTGTCTTTGTG	*Uroderma bilobatum*
BC03	GAGTCTTGTGTCCCAGTTACCAGG	*Pteronotus mesoamericanus*
BC13	AGAACGACTTCCATACTCGTGTGA	*Sturnira parvidens*
BC25	GTAAGTTGGGTATGCAACGCAATG	*Saccopteryx bilineata*
BC38	ACCACAGGAGGACGATACAGAGAA	*Molossus rufus*
BC49	ACTGGTGCAGCTTTGAACATCTAG	*Desmodus rotundus*
BC73	AAGAAACAGGATGACAGAACCCTC	*Glossophaga soricina*
BC85	AACGGAGGAGTTAGTTGGATGATC	*Natalus mexicanus*

Appendix 1.2: Graphical representation of variation in 3 COI barcode sequences of 8 bat species

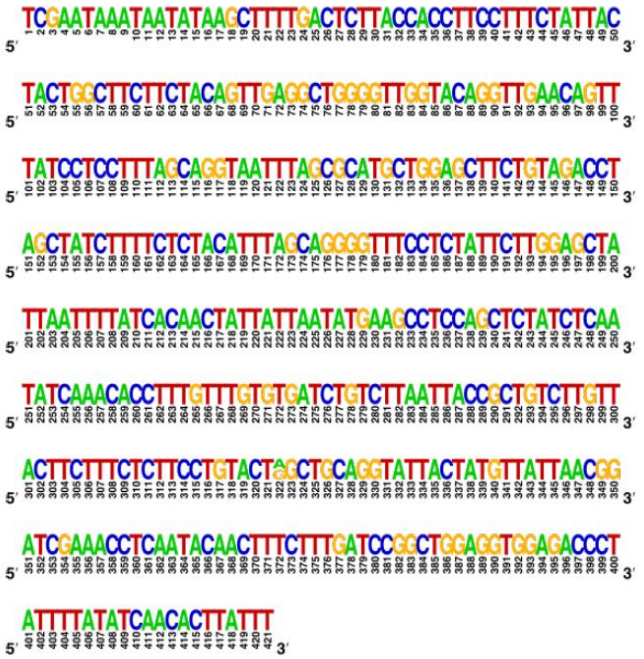
Natalus mexicanus



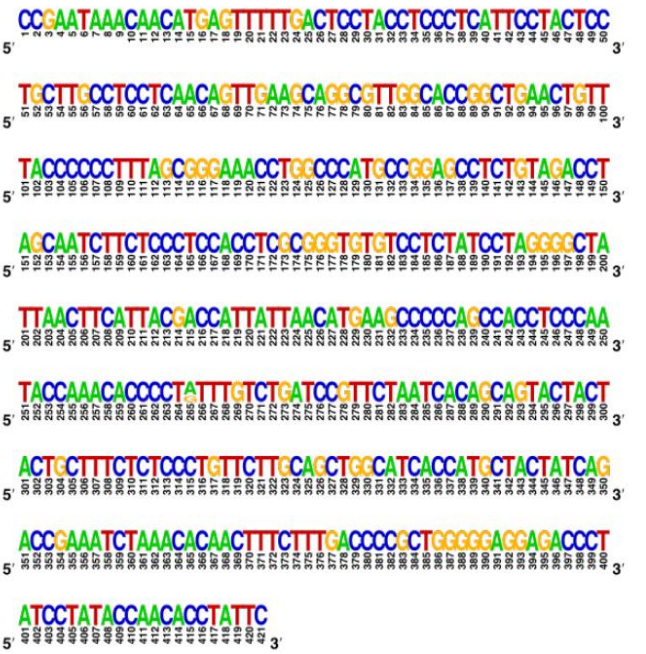
Molossus rufus



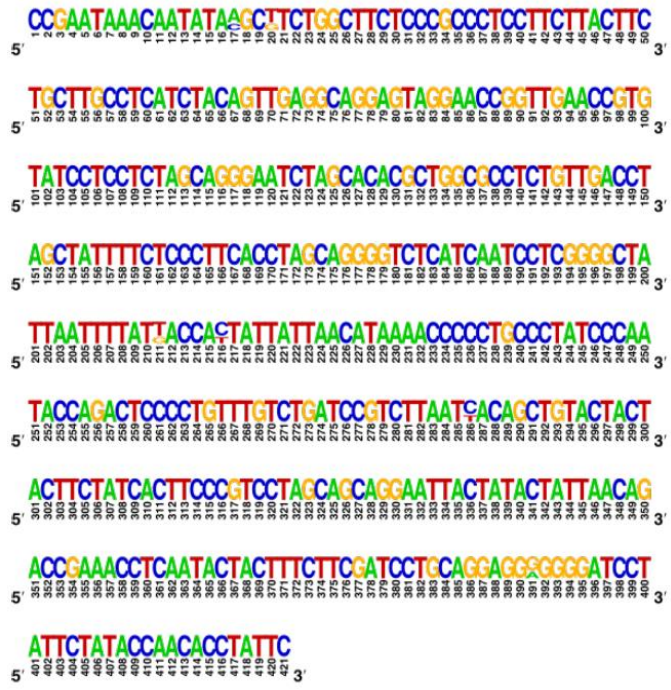
Glossophaga soricina



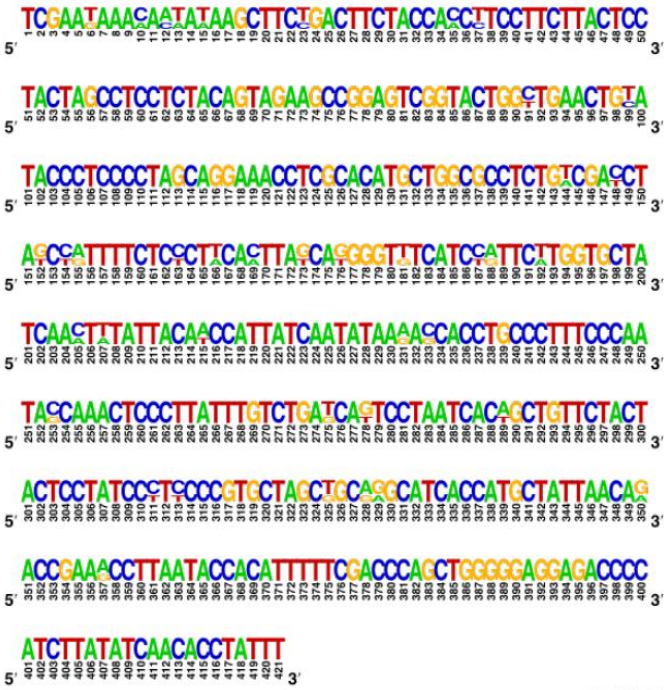
Desmodus rotundus



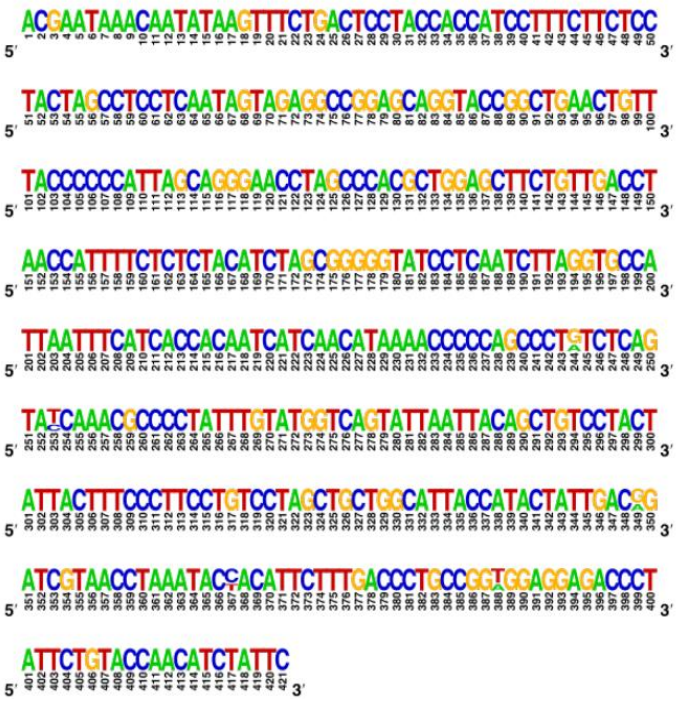
Uroderma bilobatum



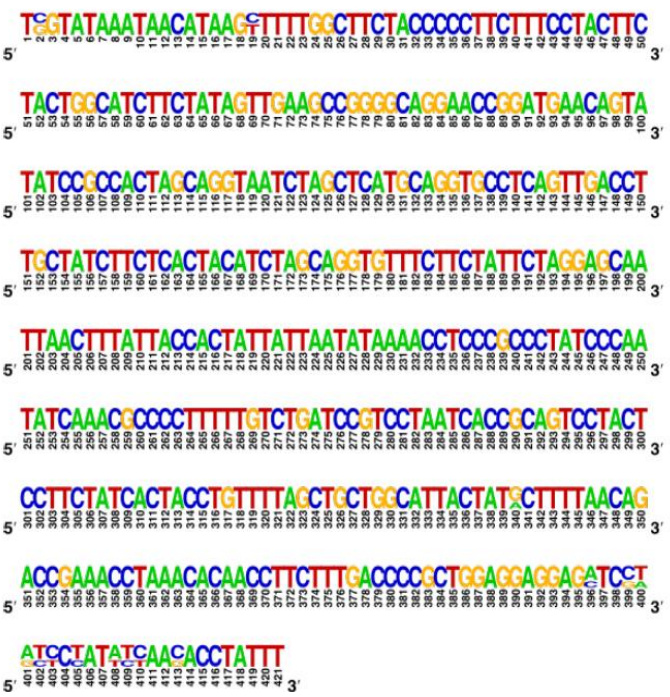
Sturnira parvidens

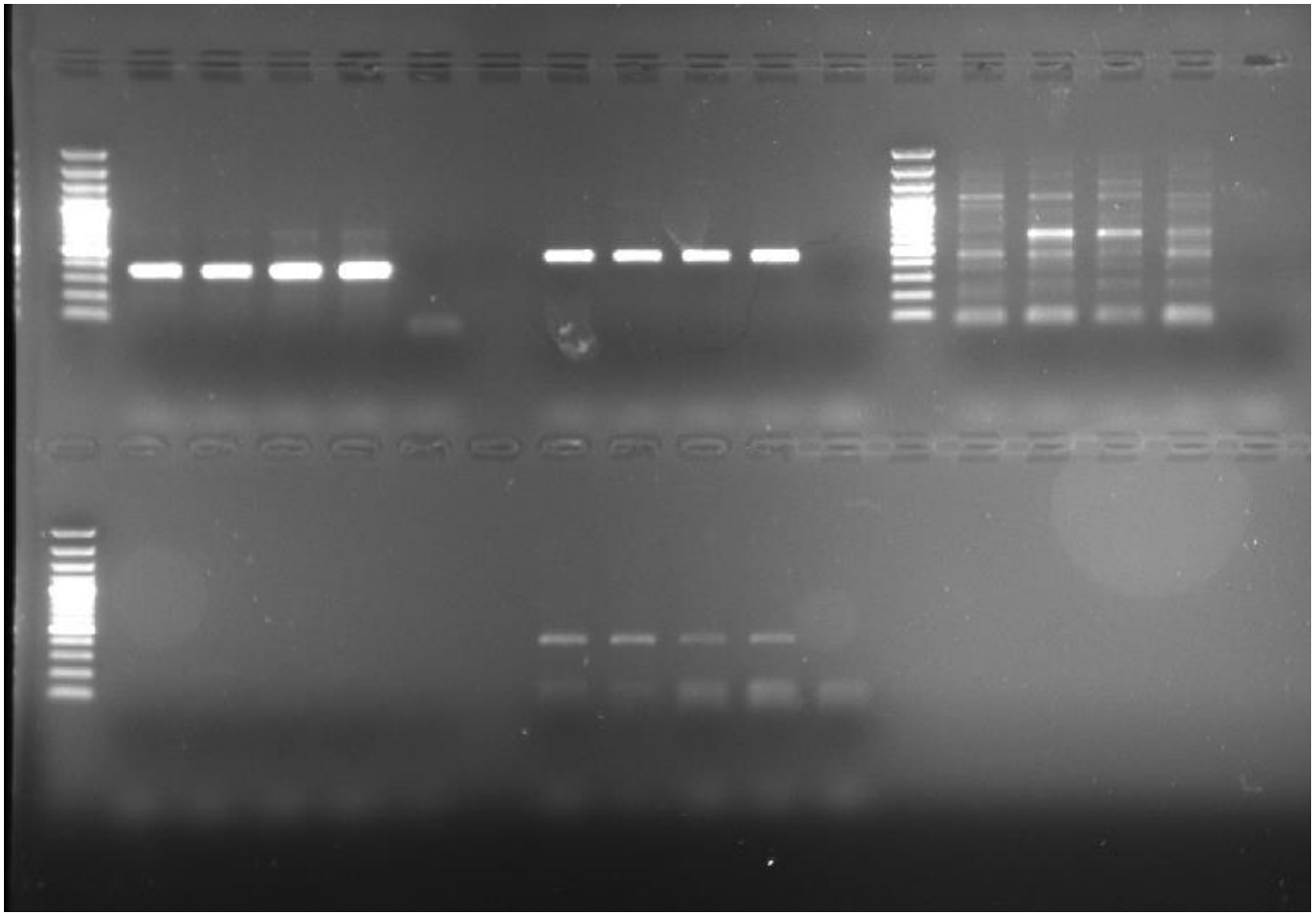


Pteronotus mesoamericanus

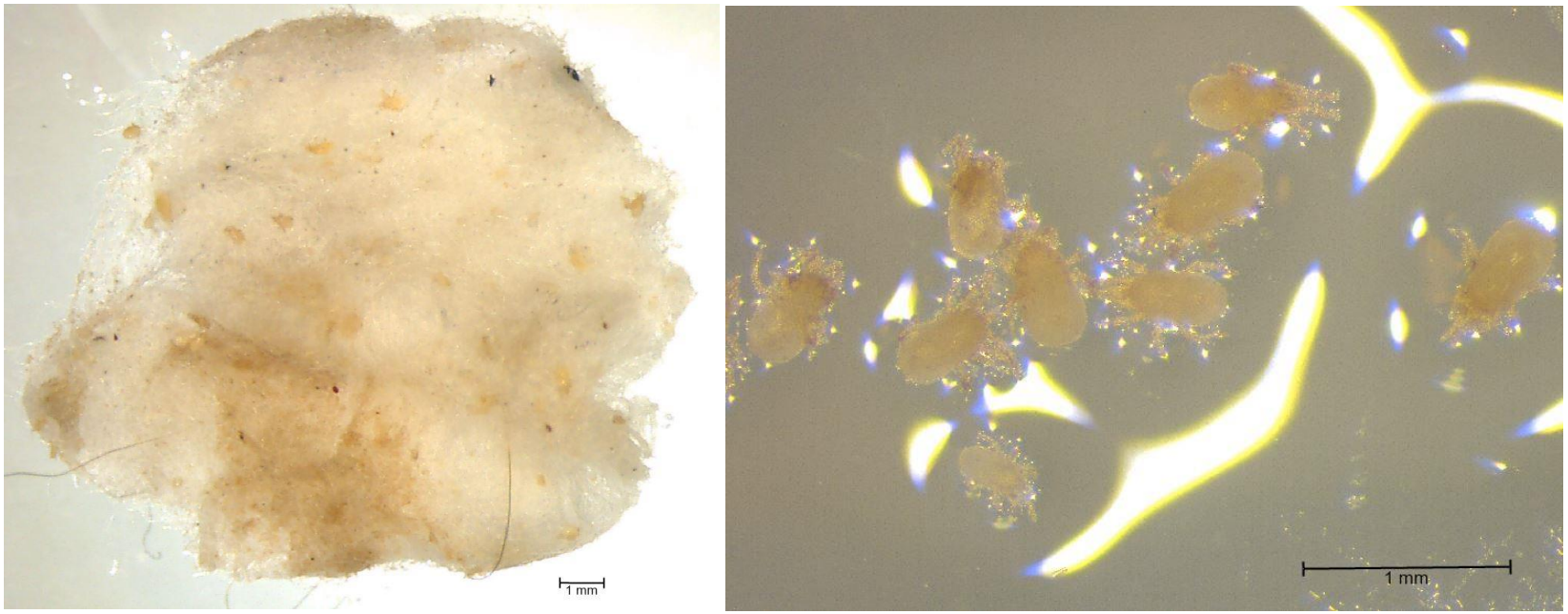


Saccopteryx bilineata

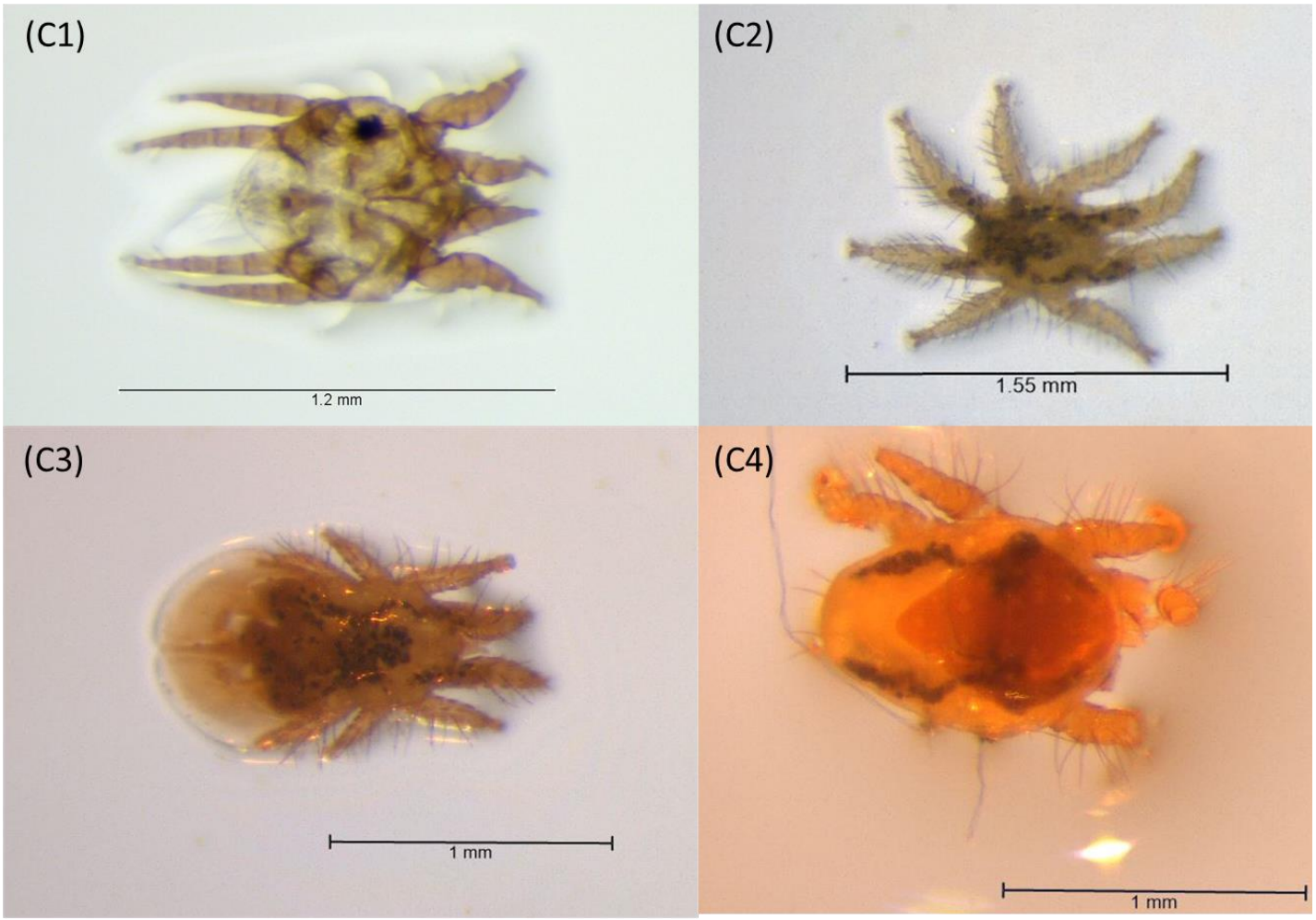




Appendix 2.1: four primer sets tested on cat fleas; (top left and top centre) MLepF1 and C_LepFolR, (top right) C_LepFolF and MLepR2, (bottom left) Uni-MinibarF1 and Uni-MinibarR1, and (bottom centre) ZBJ-ArtF1c and ZBJ-ArtR2c.



Appendix 2.2: Cotton swab with mites, suspected to be juveniles of Periglischrus mites



Appendix 2.3: Adult mites dorsal view, suspected to be (C1) female Spinturnix ,(C2) male Spinturnix, (C3) female Periglishrus,vargasi, (C4) female Spinturnix carloshoff; estimates based on diagrams in (Rudnick, 1960)