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Microbiomes in the context of insect communities

Ph.D. Thesis

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Annotation

The aim of this thesis was to investigate the deterministic patterns of insect-associated microbiome community composition and to investigate the role of symbiotic bacteria in insect model systems. I have shown that life stage is a key factor influencing microbiome composition, in both holometabolous and hemimetabolous insects, in addition to host species identity and local environment. I was able to find these deterministic patterns by controlling for diet in field studies of microbiomes, which is a well-known influential factor of microbiome communities. My thesis emphasises the importance of investigating the taxonomic and functional diversity of insect microbiomes and including symbiotic microbes in community level studies.

Declaration

I hereby declare that I am the author of this thesis and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 22.04.2021

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Joel J. Brown

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. Joel J. Brown, Joseph R. Mihaljevic, Lauren Des Marteaux, Jan Hrček. Metacommunity theory for transmission of heritable symbionts within insect communities. (2020). Ecology & Evolution 10 (3), 1703-1721. doi: 10.1002/ece3.5754. (IF = 2.39 (2019)). *JJB conceived the idea and wrote the manuscript (100%).*

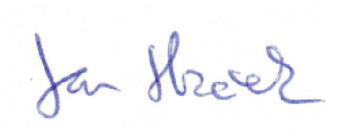
II. Joel J. Brown, Anna Jandová, Christopher T. Jeffs, Megan Higgie, Eva Nováková, Owen T. Lewis, Jan Hrček. Microbiome composition of a wild *Drosophila* community along tropical altitudinal gradients. (Manuscript). *JJB formulated hypotheses (80%), performed microbiome sequencing (80%), performed the statistical analyses (100%), and wrote the manuscript (95%).*

III. Joel J. Brown, Christopher T. Jeffs, Anna Jandová, Anna Macová, Megan Higgie, Michele Schiffer, Eleanor O'Brien, Jon Bridle, Eva Nováková, Owen T. Lewis, Jan Hrček. Long-term climatic shifts alter the structure of translocated insect-microbe metacommunities. (Manuscript). *JJB performed the field work and acquired the samples (50%), formulated hypotheses (50%), performed molecular work on insects and microbiomes (95%), performed the statistical analyses (95%), and wrote the manuscript (100%).*

IV. Joel J. Brown, Sonia M. Rodríguez-Ruano, Anbu Poosakkannu, Giampiero Batani, Justin O. Schmidt, Walter Roachell, Jan Zima Jr., Václav Hypša, Eva Nováková. Ontogeny, species identity, and environment dominate microbiome dynamics in wild populations of kissing bugs (Triatominae). (2020). Microbiome. 8 (1), 146. doi: 10.1186/s40168-020-00921-x. (IF = 11.607 (2020)). *JJB sampled insects (75%), formulated hypotheses (75%), performed some statistical analyses (60%), and led writing of the manuscript (90%).*

Co-author agreement:

Jan Hrček, the supervisor of Ph.D. thesis and co-author of 3 presented papers and manuscripts, fully acknowledges the major contribution of Joel J. Brown in all presented papers.



Mgr. Jan Hrček, Ph.D

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doc. RNDr. Eva Nováková, Ph.D.

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Chapter 1

Introduction

1.1 Scope of the thesis

The purpose of this thesis is to investigate the role of symbiotic bacteria in insect model systems and in community ecology. Chapter 2 advocates a method for achieving this goal by applying a metacommunity framework to insect-microbiome systems. Chapter 3 explores the elevational and lab-vs.-field differences in microbiomes of a novel *Drosophila* community from tropical Australia, whilst Chapter 4 explores the effects of whole-community translocation on *Drosophila* and their microbiomes. Chapter 5 explores the deterministic factors influencing the microbiome of a different insect system: kissing bugs (Triatominae), the vector of Chagas' disease (*Trypanosoma cruzi*). This chapter shows the microbiome dynamics of sylvatic bugs from multiple species native to the USA.

1.2 The symbiotic microbiome

In 1869, Simon Schwendener wrote the 'dual hypothesis of lichens'. He suggested that a lichen was a fungus and alga combined - a radical notion initially dismissed as absurd, yet eventually demonstrated to be true. The debate over lichens directly lead to Albert Frank coining the term 'symbiosis' in 1877. It was quickly modified by Heinrich Anton de Bary to refer to the spectrum of interactions that encompasses parasitism at one end and mutualism at the other. The lichen discussion, combined with new terms & definitions, spawned more detailed investigation into other organisms. Biologists quickly realised that symbiosis was everywhere: viruses were found in bacteria, algae were found inside corals and sponges, and zoochlorellae and xoothanellae in protists (Honegger 2000). Throughout the 20th century, nobody championed the importance of symbiosis more than Lynn Margulis, who proposed a then-controversial theory suggesting that symbiosis was crucial to the evolution of early life (Sagan 1967). Specifically, she proposed that a mitochondrion was originally an independent bacterium that was engulfed by a primitive archaea, making what

we would now call a eukaryotic cell (Margulis 1971, 1996). Margulis would eventually be proven (mostly) correct, and our understanding of symbiosis has grown to the point where we now recognise whole communities of symbiotic microbes associated with nearly every organism on the planet.

Symbiosis with whole communities of microbes leads us to the term 'microbiome'. It was first defined as "a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physico-chemical properties", with the authors making clear to note "the term thus not only refers to the microorganisms involved but also encompasses their theatres of activity" (Whipps *et al.* 1988). The standard definition includes bacteria, archaea, microscopic eukaryotes (predominantly protists, fungi, and unicellular eukaryotes), and their respective genomes (Marchesi & Ravel 2015; Berg *et al.* 2020). These symbiotic organisms - henceforth symbionts - include mutualists, commensals, and parasites (the term 'symbiont' is colloquially used interchangeably with 'mutualist', which is admittedly very confusing. I am opting for the definition that doesn't provide any bearings on the type of relationship with the host). A microbiome can be referred to at multiple levels of biological organisation (e.g. - an organ, individual, population, or entire species) or by focusing on a specific symbiont taxa within a microbiome, and then classifying the rest as the 'remainder microbiome' (Brinker *et al.* 2019). The mass-exploration of microbiomes has led to two more important terms of classification: 'holobiont'— which comprises the host and all associated symbionts (terminology further explored in (Gilbert *et al.* 2012; Rosenberg & Zilber-Rosenberg 2014, 2018; Bordenstein & Theis 2015; Carthey *et al.* 2019) and 'hologenome'— the host's genome plus the genomes of all its associated symbionts.

The majority of microbiome studies have focused on bacteria and archaea. Partly because hosts and their associated bacteria likely evolved synergistically, but also

because the timeline of microbiome research shows that the 16S rRNA marker gene was refined for selective amplification relatively early on (Martínez-Murcia *et al.* 1995; Tajima *et al.* 1999), enabling researchers to employ culture-independent techniques and study prokaryotes with unprecedented ease. Further molecular advances (High Throughput Sequencing; meta- and pan-genomics; meta-transcriptomics) coupled with major initiatives like the Human Microbiome Project (Turnbaugh *et al.* 2007) and Earth Microbiome Project (Gilbert *et al.* 2014) have revolutionised our understanding of the diversity and functioning of host-associated microbiomes, presenting unique challenges and new questions for studying organismal biology.

A seminal moment for recognising the importance of host-associated bacteria came when human obesity was shown to be reflected in differences in the gut microbiome (Ley *et al.* 2005, 2006). This ground-breaking research was reinforced when the gut microbiome of lean and obese people were introduced into germ-free mice, and the microbiome-associated phenotypes manifested in the receiving mice (Turnbaugh *et al.* 2008). This result had enormous connotations for the medical community regarding the perception of diet, health, and treatment (Turnbaugh *et al.* 2009). From that point onwards, the "microbiome revolution" has grown exponentially, with human-focused research leading to a number of microbiome studies on related primates to resolve phylogenetic relationships and determine the physiological relationship with the gut microbiome (Muegge *et al.* 2011; Sanders *et al.* 2014, 2017; Gomez *et al.* 2015; Aivelo & Norberg 2017; Amato *et al.* 2018), as well as spawning new funding initiatives and breakthroughs in health and disease treatment (Kong *et al.* 2012; Gevers *et al.* 2014; Rooks *et al.* 2014; Dheilly *et al.* 2017; Singh *et al.* 2017; Woodhams *et al.* 2019). For example, amphibian populations have been decimated by fungal pathogens (*Batrachochytrium dendrobatis* and *B. salamandrensis*; collectively called chytrid fungi) in recent decades, leading to a large increase in

amphibian extinction rate and the number of at-risk species (McCallum 2007; Bower *et al.* 2017; Scheele *et al.* 2019). Research into these fungal pathogens determined that they induce symptoms (and eventually, death) by preventing amphibian respiration through the skin (Jani & Briggs 2014) but further study has determined that bacteria on their skin (the skin microbiome) can potentially save amphibians from the lethal effects of chytrid fungus (Harris *et al.* 2009; Bletz *et al.* 2017). Thus research efforts are being tailored towards probiotic treatments of amphibians to help them ward off the fungus (Bletz *et al.* 2013; Kueneman *et al.* 2016; McKenzie *et al.* 2018; Woodhams *et al.* 2019), providing a key example of the benefits of studying microbiomes and disease in complex organisms.

Beyond vertebrate-centric symbioses, other taxa shown to have fundamentally vital relationships with microbes are coral (Bourne *et al.* 2016). Coral-microbe symbioses are incredibly extensive, including bacteria, archaea, photosynthetic dinoflagellates, viruses, protists, and fungi. The symbiotic relationship with photosynthetic microbes has been an area of particular focus (Bourne *et al.* 2013) due to coral's propensity to reject them under heat stress, a phenomenon called coral bleaching. The first investigation into coral symbiotic bacteria discovered a suite of rare bacteria that are important functional components of the coral microbiome (Ainsworth *et al.* 2015). Such an extensive array of symbioses presents a wealth of horizontal gene transfer (HGT) opportunities (McDaniel *et al.* 2010; Kelly *et al.* 2014). HGT events within the coral holobiont can have a substantial effect facilitating the adaptation of corals to new or changing habitats (e.g. warming and acidifying oceans; Shinzato *et al.* 2011), indicating the significant importance of the symbionts for the wellbeing of the coral host.

Despite numerous examples of close association between host and microbe, it should be noted that not *all* organisms are so dependent. Hammer *et al.* (2019) raised

compelling points about an expectation of beneficial symbioses with bacteria based on a paradigm shift that has resulted from the explosion in microbiome studies. They provide numerous cases where wild invertebrates do not have a resident microbiome, yet their fitness does not appear to suffer. This inconsistency across the animal kingdom adds layers of complexity to our understanding of how and why microbiomes form, and their diversity of purpose. We have undeniably begun to make inroads in understanding the composition, diversity, and function of the microbiome within a select handful of species (Yatsunenko *et al.* 2012), but we have also opened up a "Pandora's Box" of new mechanistic questions with much remaining to be discovered about microbial symbiosis throughout the natural world (see Figure 2 in Woodhams *et al.* 2020 for the small proportion of microbiome studies on non-mammalian systems). As with many other aspects of biology, a large proportion of our understanding of the microbiome has come from using insects as model systems.

1.3 The bacterial microbiome and insect hosts

Insects are model systems in many areas of biological research, including microbial symbioses. Honeybees, *Drosophila melanogaster*, and aphids are model organisms for microbiome research, partly due to their success as laboratory-reared species (Gómez-Valero *et al.* 2004; Chandler *et al.* 2011; Shin *et al.* 2011; Moran *et al.* 2012; Hansen & Moran 2014; Chaplinska *et al.* 2016; Guo *et al.* 2017; Adair *et al.* 2018, 2020; Hrček *et al.* 2018; McLean *et al.* 2018; Leonard *et al.* 2020). Additional focal organisms for insect-microbiome research are vectors of major diseases, including: *Anopheles gambiae* (malaria)*, Aedes aegypti* (Dengue fever), and tsetse flies (trypanosomiasis). Insect-microbiome work has taken on extra significance with advocated schemes of microbe manipulation as a form of biocontrol, reducing vector- and pest-species population numbers in order to limit parasite spread and agricultural damage (Alphey *et al.* 2007; Bourtzis *et al.* 2014; Ross *et al.* 2017; Turelli *et al.* 2018; Vorburger 2018; Gao *et al.* 2020).

An important aspect of insect-associated symbiotic bacteria, in particular, is the widespread nature of endosymbioses (Douglas 2016). Endosymbionts are micro-organisms that have successfully invaded host cells. They are often well adapted to their host as a result of extensive host-symbiont co-evolution that subsequently reduces the bacterium's ability to persist in other environments (and often characterised by a reduced genome and A-T bias; Degnan *et al.* 2009; Fisher *et al.* 2017; Chong & Moran 2018). Endosymbionts are divided into obligate (primary) symbionts, that are essential for host survival, and facultative (secondary) symbionts, that are non-essential but sometimes valuable for host survival (Table 1). In contrast, categorising non-endosymbiotic bacteria is more convoluted. For instance, when all the symbiotic bacteria within the guts of *D. melanogaster* larvae were removed (i.e. the flies were made axenic) the larvae still successfully developed to adulthood (Broderick & Lemaitre 2012). This adds weight to the argument presented by Hammer *et al.* (2019) that I outlined above. Without a doubt, the symbiotic bacteria that comprise the gut microbiome can perform valuable (if not essential) roles within their host, and most individuals possess a set of key bacteria in their guts that persist for long periods (Faith *et al.* 2013). Yet the ability of some organisms to survive in the complete absence of a gut microbiome undermines the argument that they are of universal, vital importance (Hammer *et al.* 2017, 2019; Ravenscraft *et al.* 2019).

Table 1: Characteristics of bacterial symbionts in insects. Adapted from (Douglas 2015).

Obligate symbionts	Facultative symbionts
Restricted to cells containing bacteria (bacteriocytes)	May be found in bacteriocytes or haemolymph.

Present in all individuals	Intermediate, fluctuating prevalence
Vertical transmission	Vertical and horizontal transmission
Necessary for host survival	Supplementary, but non-essential, for host survival

Another important aspect of insect-microbiome associations is the distinction between 'open' and 'closed' associations. Open associations are subject to invasion by external microorganisms, such as the gut or cuticle, whereas closed associations are isolated from invading microbes - like the aforementioned intracellular endosymbioses (because cells are not routinely invaded; Douglas 2015). The gut microbiome is an open association, routinely undergoing rapid and dramatic changes in community composition (e.g. when host diet changes, Chandler *et al.* 2011; Muegge *et al.* 2011; Hammer & Bowers 2015; Muturi *et al.* 2016; Adair *et al.* 2018). When focusing on gut-associated microbes, it is important to contextualise the microbiome community with the host insect's developmental pathway, i.e. whether it is holometabolous or hemimetabolous. Holometabolous insects undergo complete metamorphosis, which includes shedding the gut lining (and associated microbes), thus undergoing a complete microbiome community shift (Hammer & Moran 2019). Hemimetabolous insects, comparatively, have a less dramatic change in their guts when they moult from one life stage to the next, thus their gut microbiomes are comparatively less disturbed.

A crucial aspect of some insect bacterial symbionts is their heritable nature (i.e. vertical transmission from parent to offspring; Bennett & Moran 2015; Corbin *et al.* 2017; Mao & Bennett 2020). Obligate symbiotic bacteria have co-evolved with their host to become an essential part of the host's life. However, many facultative symbionts transition between being beneficial and detrimental to their host, depending on the host's biotic and abiotic environment and including interactions

with other symbionts (Oliver *et al.* 2008; Simon *et al.* 2011; Vorburger & Gouskov 2011; McLean *et al.* 2018; Monnin *et al.* 2020; Weldon *et al.* 2020). In the wrong conditions, there can be significant ecological and evolutionary costs to a host carrying particular symbiotic bacteria (Vorburger *et al.* 2013; Polin *et al.* 2014), resulting in loss of the bacteria in question, or host population death. Thus, variation in the frequency of facultative symbiont transmission has made generalisable conclusions elusive. Symbionts are often described as remaining at intermediate levels of abundance, usually because of seasonal fluctuations averaged out over time or from balancing selection, where positive and negative evolutionary forces act to keep symbiont abundance somewhere in the middle (Oliver *et al.* 2014).

Further complicating transmission of symbionts, and perception of their importance, are cases where a particular bacterium is demonstrably important yet is not transmitted directly from parent to offspring (Engel & Moran 2013). Kikuchi *et al.* (2007) found that *Riptortus clavatus* must reacquire their symbiont (*Burkholderia)* from soil in each new generation, rather than directly from the parent, despite its importance to the host. Multiple 'indirect vertical transmission' strategies have been found in other heteropteran true bugs, like egg smearing (Hosokawa et al., 2013) or trophallaxis (Kaltenpoth, 2009). The likely explanation for these transmission patterns are a trade-off between maintaining a beneficial symbiont and needing to maintain an internal environment hostile to parasites. One clear situation with opportunities to circumvent this trade-off is when multiple ontogenetic stages share an environment or substrate, enabling indirect transmission of microbes outside a host insect. *Drosophila* are an ideal example of this because they defecate, regurgitate, and oviposit into a resource that is utilised by larvae and adults alike (Martinson *et al.* 2017a, b; Hammer & Moran 2019). Other insects acquire their microbiome from the walls of brood cells (e.g. Sphecidae, *Philanthus spp.*). Eusocial insects routinely opt for direct transmission of gut microbes, through oral exchanges,

individual interactions, and sharing resources (Martinson *et al.* 2012; Powell *et al.* 2014; Lanan *et al.* 2016; Zhukova *et al.* 2017).

A notable feature of some insect endosymbionts (e.g. *Wolbachia* and *Spiroplasma*) is their ability to manipulate host sex ratios. *Wolbachia* can cause cytoplasmic incompatibility by modifying the sperm of infected males during spermatogenesis. This results in paternal chromosomes condensing when an egg is fertilised, thus killing the embyro (Jiggins 2016). *Spiroplasma* can kill male embryos selectively or blindly kill embryos of both sexes (Masson *et al.* 2020). The outcome in either case is skewed sex ratios with potentially damaging consequences for host populations. Another common feature of well-studied, heritable endosymbionts is their ability to facilitate defence against natural enemies. Symbiotic bacteria can play a crucial role in facilitating the ecological and evolutionary dynamics of host-parasite/parasitoid interactions. *Wolbachia* and *Spiroplasma*, as well as *Hamiltonella, Rickettsia, Serratia,* and *Regiella* can significantly influence the chance of host survival from parasitoid or pathogen attack. *Hamiltonella defensa* was first described from whiteflies (Clark *et al.* 1992) and has since been documented in aphids, where it is known to protect its host from parasitoids by making the internal environment untenable for the development of a parasitoid after oviposition (Oliver *et al.* 2003; Asplen *et al.* 2014; Hrček *et al.* 2016; Rothacher *et al.* 2016; Zytynska & Weisser 2016). Similarly, a strain of *Regiella insecticola* was found to protect aphids against parasitoids (Vorburger *et al.* 2010) even though it was initially found to not provide protection. The complexities of context-specific effects are magnified in these scenarios, when the influence of a single strain of symbiont is drastically different to other strains of the exact same species (McLean & Godfray 2015; Smee *et al.* 2021). These concepts are explored further in Chapter 2.

1.4 Microbiome of hematophagous insects

The microbiome revolution has been particularly influential on our understanding of hematophagous (blood-feeding) insects, including an estimated 14,000 species of sandflies, black flies, bat flies, bed bugs, and lice, in addition to the previously mentioned mosquitoes, triatomines, and tsetse flies (Adams 1999; Weiss & Aksoy 2011; Budachetri *et al.* 2014; Rio *et al.* 2016; Husnik 2018; Duron & Gottlieb 2020). Hematophagous organisms have adapted to feeding on a low nutrient food source, somewhat comparable to herbivores breaking down cellulose. Blood lacks vitamins, is heavily biased towards proteins, and has high salt content (Ribeiro & Arcà 2009). This often requires some highly specialised adaptations to aid the breakdown of blood compounds and eliminate the potentially toxic by-products (like haem and urea; Mesquita *et al.* 2015). Haemolytic activity is an important characteristic of bacteria that colonise the midgut of hematophagous insects. The consensus dominant bacteria associated with *Aedes aegypti*, *Enterobacter* and *Serratia,* both consistently present strong haemolytic activity (Gusmão *et al.* 2010; Gaio *et al.* 2011). Additionally, some obligate symbionts (e.g. *Wigglesworthia, Rhodococcus, Coxiella*) synthesise B vitamins within their arthropod hosts (Rio *et al.* 2016), a necessity for host survival based on the aforementioned depauperate nature of blood meals (Douglas 2017). Within hematophages, there is still noticeable variation in the dependence on different symbiotic microbes (e.g. within blood-feeding vertebrates, Song *et al.* 2019) but the unique nature of blood as a source of nutrition results in an inevitable degree of functional convergence in symbiotic microbes.

One notable route of microbiome colonisation in hematophagous invertebrates is through their blood meal (Husnik 2018). This can include accidental uptake of pathogens that then colonise the host's internal environment (e.g. salivary glands; Strand 2018) and microbes from the skin of the host. Prominent hematophagous insects have many different life-history strategies that influence their microbiome

composition. For example, mosquitoes lay their eggs in water, and phlebotomine sandflies lay theirs in humid soil, which means the larval microbiomes of both taxa are heavily influenced by the specific microenvironment they develop in. The common bacterial genera in major mosquito vectors are *Asaia, Acinetobacter, Aeromonas, Pantoea, Pseudomonas,* and *Serratia* (Wilke & Marrelli 2015; Gao *et al.* 2020). These bacteria are also common in other insects that acquire their gut microbiota from the environment (Yun *et al.* 2014). In contrast, the Hippoboscoidea (includes the tsetse flies, bat flies, and keds) reproduce by adenotrophic viviparity, which means the larvae hatch *in utero* and then pupate almost immediately after "birth" (Geiger *et al.* 2018). Thus there is ample opportunity for direct microbe transmission between mother and offspring (Gaithuma *et al.* 2020).

Hematophagous insect microbiomes are an area of high development for biocontrol strategies. As mentioned in section 1.3, manipulating the microbiome has become a favourable method for reducing the abundance of targeted insect species. Bacteria have been isolated from the guts of important insect vectors (e.g. *Rhodnius prolixus, Anopheles albimanus, Anopheles funestus*) that have demonstrable effects on vector competence and survival (Azambuja *et al.* 2005). Similarly, there is potential to introduce a bacterium with anti-parasitic effects (e.g. promoting host immune defence or actively producing anti-parasitic molecules like hemolysins or malloproteases; Geiger *et al.* 2018; Oliveira *et al.* 2018) into a vector's microbiome. The most developed microbiome approach to vector control is in *Aedes aegypti* where *Wolbachia* limits the host's ability to carry pathogens (Moreira *et al.* 2009), specifically shown with Dengue fever (Frentiu *et al.* 2014) and Chikungunya virus (Aliota *et al.* 2016). However, as I alluded to in section 1.3, there are complex and context-dependent interactions to consider with host-microbiome studies. Hancock *et al.* (2016) showed that *Wolbachia* doesn't spread as well as expected in *A. aegypti* populations, due to host density-dependent population dynamics. Similarly, different

Wolbachia strains differed dramatically in their response to heat stress (Ross *et al.* 2017) demonstrating that microbiome manipulation of vectors needs to be extensively tested before it can be used with widespread success.

1.5 Microbiomes as communities & metacommunities

All communities of organisms are created by a combination of assembly processes: selection, dispersal, drift, and diversification (Vellend 2010; Costello *et al.* 2012; Kohl 2020). Host-associated microbiomes are no different. From the community ecology perspective, the study of microbiomes can be compared to the study of eukaryotic macroparasites, which have a much richer history in the ecological literature (Poulin 2007; Johnson *et al.* 2015). These parasites are also symbionts, by definition - they live within a particular host but have been treated as separate entities and not considered within the context of a host organism's whole microbiome, until recently (Clements *et al.* 2020). Thus, the parasite ecology literature is awash with community-level host-parasite studies but relatively little data exists on hosts and the 'remainder' microbiome together.

The microbiome of an individual can be considered a local community that is colonised from a regional species pool - which is the pool of possible species that can colonise a local community based on historical, biogeographic, and environmental filters (Cornell & Harrison 2014; Karger *et al.* 2016). When a bacterial species arrives inside a new host (i.e. colonising the host's microbiome community), there are two basic outcomes: it establishes in the host microbiome community, or it does not. Hypothetical bacterial species 'X' may be excluded because the already-established bacterial species 'Y' is occupying a shared niche, or has sufficiently altered the niche in a manner that makes it unsuitable for species 'X' (Fukami 2015). This outcome could be because species 'Y' is a superior competitor or is due to dispersal, where species 'Y' becomes established in the microbiome simply because it colonised first.

Alternatively, species 'X' could fail to establish because the hosts' internal environment is not suitable for its development (environmental filtering), for example because the temperature or pH are too far from the optimum for species 'X'. Another option is drift, otherwise described as stochasticity, randomness, or neutral dynamics. Stochastic events include microbiome disturbance (e.g. through illness, unenforced diet change) and, by nature, have a random probability distribution and cannot be precisely predicted (Zaneveld *et al.* 2017). Similar to macroorganism community ecology, there is divided opinion on the importance of stochastic events for community composition. Some studies suggest that microbiome community structure matches neutral dynamics well (Koskella *et al.* 2017; Zeng & Rodrigo 2018; Sieber *et al.* 2019; Heys *et al.* 2020), others suggest that stochastic events are of minimal influence (O'Dwyer *et al.* 2015; Li & Ma 2016), and some suggest both depending on specific contextual requirements (Burns *et al.* 2016). The importance of different deterministic factors depend on individual studies, yet evidence sufficiently accumulates to show that, in many cases, host-associated microbiome communities are readily determined by multiple interacting processes.

Our understanding of microorganism communities is primarily limited (compared to macroorganism communities) by our ability to perceive microbial interactions. Constituting what defines an interaction, and how to quantify them, are old and familiar philosophical issues in community ecology. Genetic studies of microbial communities do not provide data on whether taxa are alive or dead, nor whether one taxon is interacting directly with another. Interactions are often inferred by analysing species-species covariation within the community. Subsequently looking at changes in relative abundance (which are simplex values and thus must sum to 1) tells us that an increase of taxon 'A' must be compensated by a reduction in taxon 'B', suggesting that 'A' has a negative effect on 'B' but without telling us the ecological basis behind

the observed change (whether the outcome is a product of direct or indirect processes) and thereby whether deterministic or stochastic factors play a more prominent role. Improvements in co-culturing methods and longitudinal sampling of microbiome communities (in order to parameterise time-series models) are required to improve our understanding of interactions within microecological communities.

A metacommunity is, simply, a community of communities. It's a community-level extension of the metapopulation framework popularised by Ilkka Hanski (Hanski & Gilpin 1991). The metacommunity concept is essentially a hybrid between biogeography and community-level study. For example, in a chain of 10 islands each island is a habitat patch that contains a community. Each of these communities is linked together by the dispersal potential between each of the islands, thus creating a metacommunity (Holyoak *et al.* 2005; Urban *et al.* 2008; Leibold & Chase 2017; Toju *et al.* 2017; Thompson *et al.* 2020). The key purpose of a metacommunity is simultaneously analysing local *and* regional processes, and how these both influence communities across time and space. For studying host-associated microbiomes with the metacommunity paradigm, we first take a community of hosts (e.g. insects). Each individual insect becomes a discrete habitat patch which contains a local community of symbionts. Each insect patch is then linked together by the dispersal of symbionts between hosts (Mihaljevic 2012; Miller *et al.* 2018; Brown *et al.* 2020a). The combination of the metacommunity framework and insect-microbiome research are the basis for Chapter 2. This chapter is based on concepts from parasite ecology, a logical comparison if we consider microbiome studies as a logical branching from the stem of parasite ecology. Mihaljevic (2012) first presented the idea of host-symbiont metacommunities, with the term 'symbiont' including both microbiome bacteria and parasites, by definition. Yet because the ecological study of parasites has a much richer history, community ecology has influenced the study of parasites (Johnson *et al.* 2015) much more than microbiomes. Tad Dallas and Joe Mihaljevic have

attempted to advance broad-scale studies on symbionts (Mihaljevic *et al.* 2015, 2017; Dallas *et al.* 2017, 2019a, b, c; Ranjeva *et al.* 2019), mostly on parasites, with mutualistic bacterial microbiome remaining unconsidered in broad community-level studies.

1.6 Our model insect community

A substantial portion of this thesis is based on a new model system of *Drosophila* and parasitoids from tropical Australia. One of the tasks throughout my doctoral research was to help establish this novel system in the Hrček lab, collecting live flies and wasps for transport to the Czech Republic, thereby bridging the field and lab components of study. Many of the parasitoids are yet to be taxonomically described (Lue *et al.* 2021). Not all species survived in lab conditions, but most are listed in Jeffs *et al.* (2021). This host-parasitoid community survey formed the basis of all the projects undertaken within the lab group and was the starting point of Chapters 3 and 4.

Work by the Hoffman group at the University of Melbourne determined that *D. pseudotakahashii, D. bipectinata,* and *D. pandora* all host different strains of *Wolbachia* (Richardson *et al.* 2016, 2018; A. Hoffman, *pers. comms.*). We performed PCRs on collected flies using *wsp* primers (Braig *et al.* 1998; Zhou *et al.* 1998) and indeed found *Wolbachia* in isofemale lines of these three species. In addition, we found that *D. pseudoananassae* also contains *Wolbachia* (Chapter 3). One isofemale line of *D. pallidifrons* had a positive result for *Spiroplasma*, but this well-described endosymbiont was otherwise absent in our flies.

1.7 Summary of datasets

The chapters presented in this thesis are comprised of data and studies from around the world (Table 2). The majority of my PhD research was conducted along two

altitudinal gradients in the Wet Tropics World Heritage Area (WTWHA), Queensland, Australia (Fig. 1).

Table 2: Original sources of data for the chapters included in this thesis

Chapter	Dataset	Source of origin	Molecular methods used	Year
3	Host-parasitoid community survey	Australia: Paluma & Kirrama altitudinal transects, QLD (Fig. 1)	COI + 16S rRNA metabarcoding; Sanger seq	2016
4	Host-parasitoid translocation experiment	Australia: Paluma & Kirrama altitudinal transects, QLD (Fig. 1)	COI + 16S rRNA metabarcoding; Sanger seq; multiplex PCR	2017
5	Field collection of triatomines	USA: Tucson, AZ; Bisbee, AZ; San Antonio, TX; Riverside, CA	16S rRNA metabarcoding; Sanger seq;	2017-18

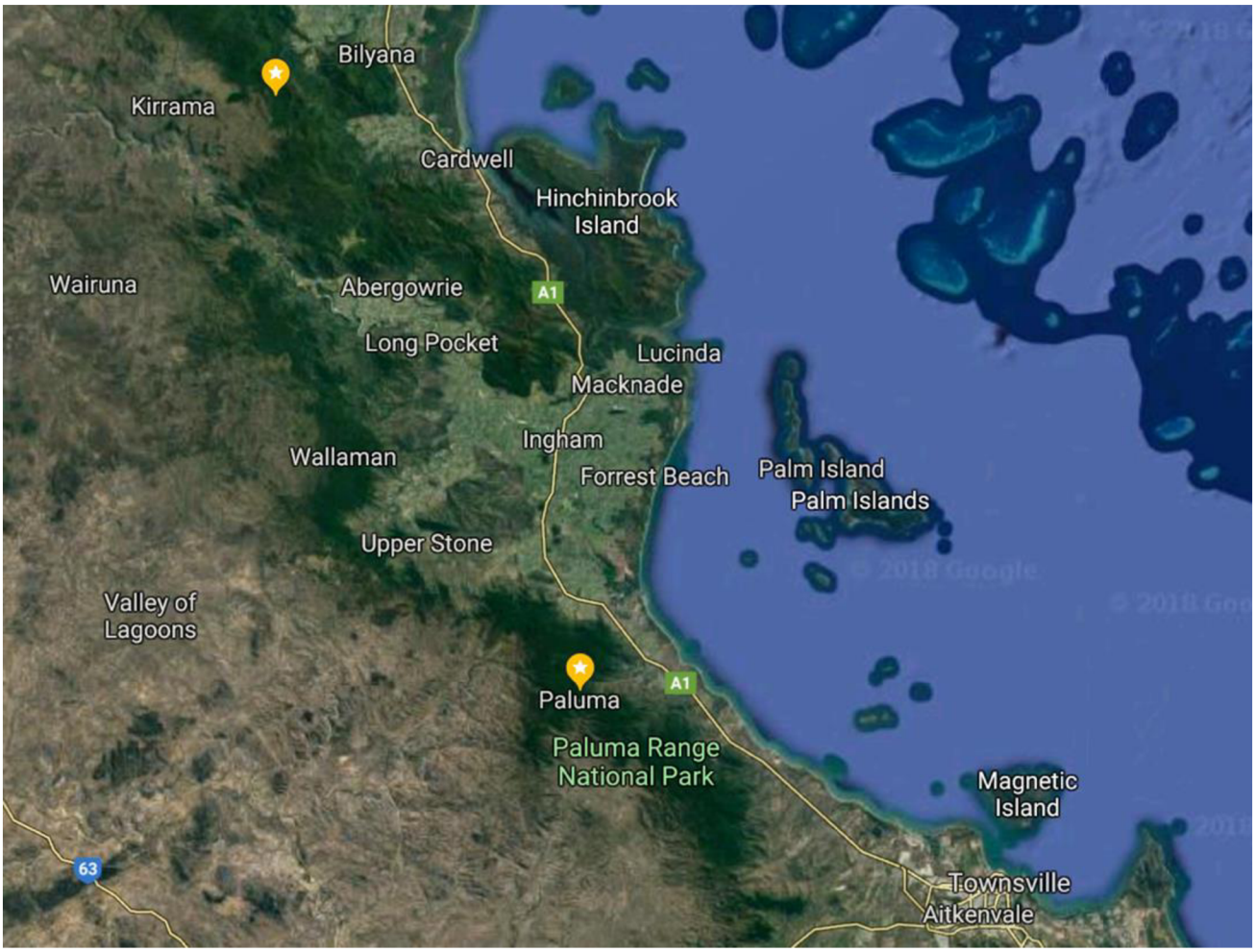

Figure 1: Location of altitudinal gradients (starred) in Queensland, Australia used for the field components of Chapters 3 and 4. From Google Maps (accessed on 04/04/2021).

1.8 Molecular methods

The chapters in this thesis used a suite of molecular techniques for sample analysis, which are described in Table 3. I wanted to provide additional description and justification of the methods themselves. The focal tool of sample analysis in these studies is Illumina MiSeq metabarcoding (Shokralla *et al.* 2015). Metabarcoding is a unified product of two biological tools: high-throughput DNA sequencing and DNA-based taxonomy (Cristescu 2014; Hrček & Godfray 2015; Aylagas *et al.* 2016; Miller *et al.* 2016). The 'barcode' part is a short sequence of DNA that possesses an intermediate amount of variability: enough to resolve recent taxonomy, but also

changes at a relatively stable rate through evolutionary time. Developing these standardised barcodes is more challenging for metabarcoding, because the primers have to be versatile enough to equally amplify many different targeted groups (Cristescu 2014). Thus the amplified fragments must have good taxonomic resolution, ideally to species level. For animals, the most commonly used locus is a section of the mitochondrial cytochrome oxidase subunit I (COI) gene (Ji *et al.* 2013). For fungi; the ITS2 gene (Blaalid *et al.* 2013). For bacteria, the most commonly used is the 16S rRNA gene which is exceptionally well-conserved, evolving at ~1-2% per 100 million years (Kuo & Ochman 2009) making it a near-ideal candidate locus for identifying bacteria. Techniques have been developed to the point where DNA barcodes now exist for a huge variety of life forms (Zimmerman *et al.* 2014), which can be used *en-masse* on large volumes of libraries in a metabarcoding format.

For sequencing microbiome bacteria from my samples, I used a 16S rRNA gene dual-barcode strategy complete with a customised 18S rRNA gene blocking primer, adapted from the Earth Microbiome Protocol (EMP; Brown *et al.* 2020). The custom 18S rRNA gene blocking primer is the unique element of our 16S rRNA gene sequencing process. The Earth Microbiome Protocol-proposed modified 16S rRNA gene primers can have low sequence specificity, resulting in a large proportion of 18S rRNA gene sequences from the host instead. To avoid this, the blocking primer was intentionally employed at 10x normal concentration to avoid amplification of 18S rRNA gene sequences during the PCR process. Supplement 1 of Chapter 5 shows the significant increase in the proportion of 16S sequence reads when the blocking primer is used. The process is described in detail within the Methods of Chapters 3-5.

1.9 Aims of the thesis

The central aims of my thesis are to explore symbiotic microbes in insects and the role of microbiomes in community ecology. In particular, I aimed to advance the concepts exploring microbiota using the metacommunity framework (Chapter 2) and identify the microbial and environmental factors determining microbiome community composition and species interactions over time (Chapter 3, Chapter 4, and Chapter 5). In addition to this, I explored whether there is convergence in microbiomes of an insect vector when sampled from the same primary host species (Chapter 5).

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Chapter 2

Metacommunity theory for transmission of heritable symbionts within insect communities

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REVIEW

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Metacommunity theory for transmission of heritable symbionts within insect communities

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Abstract

Microbial organisms are ubiquitous in nature and often form communities closely associated with their host, referred to as the microbiome. The microbiome has strong influence on species interactions, but microbiome studies rarely take interactions between hosts into account, and network interaction studies rarely consider microbiomes. Here, we propose to use metacommunity theory as a framework to unify research on microbiomes and host communities by considering host insects and their microbes as discretely defined "communities of communities" linked by dispersal (transmission) through biotic interactions. We provide an overview of the effects of heritable symbiotic bacteria on their insect hosts and how those effects subsequently influence host interactions, thereby altering the host community. We suggest multiple scenarios for integrating the microbiome into metacommunity ecology and demonstrate ways in which to employ and parameterize models of symbiont transmission to quantitatively assess metacommunity processes in host-associated microbial systems. Successfully incorporating microbiota into community-level studies is a crucial step for understanding the importance of the microbiome to host species and their interactions.

KEYWORDS

bacteria, dispersal, heritable, insect, metacommunity, microbiome, species interactions, symbiont, transmission

1 | INTRODUCTION

Microbial organisms readily live in symbiosis with their host, often forming communities referred to as a microbiome. The microbiome is a broad term that defines the microscopic, symbiotic organisms associated with a particular host, and which can provide essential services for their host (e.g., aiding in immunity and digestion), thus providing insight into the health of the host organism (Fierer et al., 2012). The microbiome can have strong influence on the ecological niche occupied by the host species (Henry, Maiden, Ferrari, & Godfray, 2015; Hoffmann, Ross, & Rašić, 2015), and these symbiont-induced changes to host ecology have increasingly clear impacts on the identity, strength, and outcome of interactions between hosts within communities (Berry & Widder, 2014; Cusumano et al., 2018; Frago, Dicke, & Godfray, 2012; Frago et al., 2017; Hrček, McLean, & Godfray, 2016; McLean, Parker, Hrček, Henry, & Godfray, 2016; Oliver, Smith, & Russell, 2014; Xie, Vilchez, & Mateos, 2010; Zhu et al., 2018). Understanding the spatiotemporal distribution and function of symbiont communities therefore has implications for basic and applied ecological theory.

A promising framework under which symbiont community dynamics can be explored is the metacommunity. An ecological

community is an assemblage of multiple species living in a specified place and time with the potential to engage in ecological interactions (Agrawal et al., 2007; Vellend, 2010). A metacommunity scales up from this definition, linking multiple communities together via dispersal of multiple potentially interacting species (reviewed in Leibold et al., 2004). The crucial element of metacommunity theory, and where it differs from standard community ecology, is the exploration of how local and regional processes interact to influence patterns of community composition across space and time (Leibold & Chase, 2017). The metacommunity framework has been most frequently applied to natural communities defined by discrete habitat patches (such as alpine meadows and aquatic pools; Leibold & Chase, 2017; Logue, Mouquet, Peter, & Hillebrand, 2011; Mihaljevic, 2012). The relevance of studying organisms in a community context applies at both microbe and host levels, with the metacommunity concept allowing us to consider both levels simultaneously. Logue et al. (2011) found that empirical metacommunity studies lacked data on trophic interactions, in addition to lacking experimental work from terrestrial systems. We believe that symbiont–host metacommunities are ripe to fill these research gaps and provide further insight into currently unanswered questions in symbiosis research and community ecology.

Specifically, we believe that the metacommunity concept will help us explore (a) symbiont vertical and horizontal transmission (dispersal), and (b) the influence of symbiont–symbiont interactions on their transmission and phenotype. The study of symbiont dispersal must take into account how local processes, such as interactions between multiple symbionts, shape symbiont populations sizes and density-dependent dispersal (transmission). From the host community perspective, we must account for the effects of symbionts present in the local community and the dispersal processes that facilitate symbiont migration into a host. The importance of symbiotic bacteria to a wide variety of insect hosts (Box 1) suggests that symbiont communities and the processes that structure them are crucial for understanding the biology of the host insects, both as single entities and in the context of the wider insect community (Ferrari & Vavre, 2011; Hrček et al., 2016; McLean et al., 2016). The metacommunity concept provides us with a necessarily broad approach that includes local and regional processes. In this review, our use of the term "symbiont" refers broadly to commensal, mutualistic, or parasitic bacteria that exist in close physical association with their host. We focus on insect–bacteria associations because insects are often a model system for both community ecology and symbiosis studies, and bacteria are common members of microbiomes that have a well-documented history of affecting insect host ecology (Bourtzis et al., 2014; Corbin, Heyworth, Ferrari, & Hurst, 2017; Crotti et al., 2012; Ross et al., 2017) and are relatively easy to identify with modern molecular methods. More specifically, we focus on the heritable bacteria that contextually transition between being beneficial and detrimental for their host. This includes both facultative endosymbionts (those found within host cells and hemolymph) and the symbiotic bacteria associated with the gut (commonly referred to as the "gut microbiome").

Recently, several studies have advocated for the application of metacommunity theory to understand the dynamics of symbiotic and/or pathogenic organism communities within and among their hosts (Borer, Laine, & Seabloom, 2016; Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; Fierer et al., 2012; Johnson, Roode, & Fenton, 2015; Mihaljevic, 2012; Miller, Svanbäck, & Bohannan, 2018; Seabloom et al., 2015). However, most have proposed conceptual models without sufficient advice on how to empirically or quantitatively assess such dynamics. Furthermore, most of the empirical approaches that have been suggested are in the realm of inferring processes from static patterns of community composition. More powerful approaches involve an integration of longitudinal data and dynamical models to infer the dominant, mechanistic processes that influence community composition over space and time. Here, we extend the metacommunity concept to heritable symbionts, specifically considering their transfer (i.e., dispersal). The concepts discussed here will apply to other symbioses (e.g., plants and endophytic fungi, vertebrates and their organ microbiomes, or insect–virus–plant systems), but for the sake of clarity we focus on insect–bacteria associations. We believe that using a metacommunity approach will facilitate a deeper understanding of insect–symbiont systems, by focusing on the local and regional ecological processes that influence symbiont community assembly, the process of symbiont dispersal via horizontal and vertical transmission, and the consequences for the host organisms.

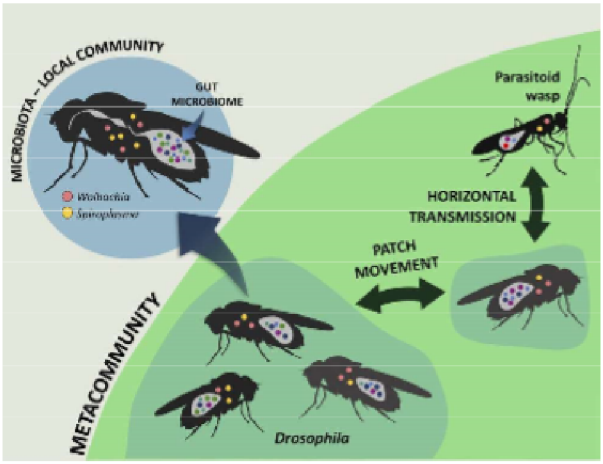

FIGURE 1 Applying the metacommunity concept to microbial communities of insects, in this case a community of hosts (*Drosophila*) and parasitoids. Each individual insect is a "patch" that harbors a local community of endosymbiotic bacteria. The green area represents the regional metacommunity of hosts. Bacteria can be present both within the gut and inside host cells and hemolymph (with *Wolbachia* and *Spiroplasma* as specific examples of the latter category). Differently colored circles within an insect each represent a different bacterial genus. Arrows indicate horizontal transmission (dispersal) of bacteria among local communities (host microbiomes). This diagram represents one of multiple ways to apply metacommunity theory to host–symbiont systems; see Table 1 scenarios B-E for alternative approaches

1.1 | Objectives

In this review, we explore how symbiosis research can be fruitfully integrated with metacommunity theory to advance both fields. First, we provide an overview of the influence of microbial communities on the biology and interactions of their insect hosts (Box 1, see also McLean et al., 2016 and Corbin et al., 2017 for recent reviews on symbiotic bacteria in insect communities). This is followed by an examination of microbial transmission and its importance for host communities. We then propose how and why the metacommunity concept should be considered for advancing our understanding of symbiont transmission within insect–microbe networks, and highlight the future directions these studies could take (Figure 1, Boxes 2 and 3, Table 1). Specifically, we introduce a mathematical modeling framework and give concrete examples of how to conduct experiments with insect study systems to parameterize these models and better understand the roles of metacommunity processes in structuring symbiont communities. Our aim is to stimulate ideas for combining research on the microbiome and host community ecology. We present the metacommunity framework as a possible method to achieve this, but recognize that other macroecological approaches could be complementary. As we will outline in this paper, the importance of the microbiome to host biology suggests that microbiomes should be considered when studying communities of host organisms.

2 | INSECT-ASSOCIATED SYMBIOTIC BACTERIA

For the purpose of this paper, we focus on both endosymbiotic and symbiotic gut bacteria within insect hosts. Endosymbionts (bacteria living within the host's cells or hemolymph) can be obligate (primary) symbionts and thus necessary for host survival, or facultative (secondary) symbionts which are often helpful but not required for host survival. Obligate symbiont transmission is predictable because it is inextricably linked to host reproduction, whereas transmission of facultative symbionts is much more variable, leading to fluctuation in their abundance and diversity (explained further in "Microbiome transmission," below). Pea aphids (*Acyrthosiphon pisum*) have the best known endosymbiont community to date, with a total of seven (up to four can be present in one individual). *Drosophila* species have a maximum of two known endosymbionts while spiders, another well-studied invertebrate group, have a total of five (Goodacre, 2011). Gut symbionts are often collectively referred to as the gut microbiome. Insects have highly variable gut symbiont species richness (Christian, Whitaker, & Clay, 2015) which is largely dependent on the diet and lifestyle of the host species (Blum, Fischer, Miles, & Handelsman, 2013; Kaltenpoth, Winter, & Kleinhammer, 2009; Martinson, Douglas, & Jaenike, 2017; Nováková et al., 2017). For example, saproxylic beetles and termites have demonstrably large and diverse gut microbiomes based on their consumption of decaying wood (i.e., cellulose; Ohkuma, 2008), whereas some caterpillars have relatively depauperate gut microbiomes because they only feed on a single host-plant species (Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017).

Symbiont dispersal (their transmission between hosts, see "Microbial transmission" below) is an important determinant of microbiome diversity within the host (Henry et al., 2013). The profile of symbiotic bacteria within a particular host can in turn influence various aspects of host biology, including feeding behavior, sex ratios, resistance to parasitism, and thermal tolerance (Figure 2; Box 1; see also Feldhaar, 2011; Ferrari & Vavre, 2011; Ottman et al., 2012; McLean et al., 2016; Martino, Ma, & Leulier, 2017). This interaction between the host and symbiont community therefore ultimately shapes the spatial distributions of insects and their inter- or intraspecific interactions, with cascading effects on community and broader ecosystem processes (Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Frago et al., 2012, 2017; Hrček et al., 2016).

2.1 | Interactions within microbial communities

Interactions between the microbial species in an individual host impact both the host and the function of the microbiome itself. Foster and Bell (2012) reported that the majority of interactions between microbial species were competitive, and thus classified as negative. Competition between gut microbiome species is also associated with a reduction in cooperation, which results in a decrease in community productivity (i.e., an inability to digest as efficiently; Oliveira, Niehus, & Foster, 2014). Ecological modeling by Coyte, Schluter, and Foster (2015) showed that competition between microbes facilitated stability within microbial communities, to the extent that the stabilizing effects were sufficient to counteract any destabilizing effects caused by increased cooperation or diversity. Based on this evidence, species interactions (such as competition) within a microbial community have both positive and negative effects and are therefore crucial factors to consider when analyzing animal–microbe symbioses. When viewed from a metacommunity perspective, there is strong potential for interactions between symbionts to affect their distribution among insect hosts, and consequently the biology and interactions of their hosts as well.

Microbes can also facilitate the establishment of other microbial species within the microbiome community. Some symbiont species are more likely to occur in coinfections; for example, *Fukatsui symbiotica* (Manzano-Marín, Szabó, Simon, Horn, & Latorre, 2017) is a facultative symbiont that is almost always found in coinfection with *Hamiltonella defensa* in aphids feeding on *Medicago sativa* in Europe and North America. McLean et al. (2018) found stable coinfections to be possible between multiple combinations of different aphid symbionts and even between multiple strains of the same symbiont, *H. defensa*. Similarly, in a long-term study of aphid symbiont communities, Rock et al. (2017) found that the bacteria *Serratia symbiotica* and *Rickettsiella viridis* co-occurred more often than expected, a phenomenon that was explained by their ability to promote each other's

transmission to the next host generation. *Wolbachia* is also positively associated with *Spiroplasma* within *Drosophila neotestacea* (Fromont, Adair, & Douglas, 2019).

3 | MICROBIAL TRANSMISSION

In the context of metacommunity theory, the dispersal of organisms among habitat patches can influence local interactions and ultimately affect the community composition across space. For symbionts, dispersal can occur across host generations, between individuals of a single species, and across multiple species and trophic levels. Symbiont dispersal depends on two main factors: the ability to transmit from one host to the next and the ability to successfully establish within the new host. Symbionts can be transmitted vertically (parent to offspring) but also horizontally (between individuals or via the environment; Caspi-Fluger et al., 2012; Haselkorn, Markow, & Moran, 2009; Hosokawa et al., 2016; Jaenike, 2009; Li et al., 2017).

3.1 | Vertical transmission

Vertical transmission is typically the dominant form of symbiont dispersal (especially among endosymbionts) and occurs primarily from mother to offspring, although rare cases of paternal transmission have been documented (Moran & Dunbar, 2006). Gut microbes are generally not considered to be heritable, but are often transmitted from parent to offspring either directly or through the environment (Estes et al., 2013; Shukla, Vogel, Heckel, Vilcinskas, & Kaltenpoth, 2018). Some insects, especially true bugs, even display specialized behaviors that transmit their bacteria to offspring (e.g., via parental postoviposition secretions; Kaltenpoth et al., 2009). This "indirect inheritance" of gut microbes can be crucial to the well-being and functioning of the new generation, and therefore influences how individuals of the new generation interact in their communities.

3.2 | Horizontal transmission

Horizontal transmission of a symbiont includes transmission via host-to-host contact (either inter- or intraspecific) as well as acquisition from the environment. The precise mechanisms are poorly known, but it is widely presumed that horizontal transmission is a key mode of symbiont dispersal (Henry et al., 2013). Evidence for this presumption is provided by broad analyses of endosymbiont distribution. For example, strains of *Wolbachia* (the most common endosymbiotic bacteria in insects) are not distributed throughout insect clades in a phylogenetically or geographically clustered way, suggesting multiple horizontal transfer events in which the endosymbiont jumped from one species to another of distant relation (Smith et al., 2012). In the case of *Wolbachia*, multiple acquisitions from the environment are unlikely because the symbiont cannot survive outside hosts. A similar lack of phylogenetic clustering has been shown for incidences of symbiont infection within aphids (Henry et al., 2015). On an intraspecific level, dispersal of symbionts can be viewed as a pool of adaptations available for selection when they are advantageous to their host (Henry et al., 2013). The mechanism of horizontal transmission supported by the most evidence is that of "the dirty needle effect," whereby an uninfected parasitoid picks up a bacterium when parasitizing an infected host and then transmits the bacterium to a new uninfected host in a second parasitism event (Ahmed et al., 2015; Gehrer & Vorburger, 2012). Gehrer and Vorburger (2012) demonstrated this phenomenon by allowing parasitoids to attack an aphid clonal line possessing *H. defensa* and then attacked aphids of a "recipient" clonal line, allowing any survivors of attempted parasitism to mature and reproduce. In a number of cases, the offspring of these "recipient" aphids tested positive for *H. defensa*. Ahmed et al. (2015) showed that the parasitoids of *Bermisia tabaci* whiteflies picked up *Wolbachia* from infected hosts on their mouthparts and ovipositors, and could then effectively transmit *Wolbachia* to new hosts for 2 days.

3.3 | Establishment

Successful establishment of a symbiont within a novel host is an important component of symbiont transmission. A symbiotic bacterium could survive for a short period of time in a novel host but may ultimately fail to reproduce or survive in the long term. Therefore, an important biological distinction must be made between the occurrence of a horizontal transmission event and successful symbiont establishment. Establishment success is an important filter for interspecific transmission, and as a result, the establishment rate of symbionts is highly variable. Gehrer and Vorburger (2012) reported an estimated 8.6% rate of establishment for *H. defensa* that was transmitted via parasitoids (the dirty needle effect), whereas Ahmed et al. (2015) found a 93.8% transmission rate of *Wolbachia* via parasitoids during their experiment. In another example, Łukasik et al. (2015) found that *H. defensa* established more easily when it was transferred from an individual of the same species as the recipient host. Similarly, establishment was most successful when the introduced symbiont strain was more closely related to the pre-existing symbiont strain in the host (also shown by Tinsley & Majerus, 2007). In some cases (and perhaps more often than not), introduction of a symbiont into a novel host species can severely reduce host viability (Hutchence, Fischer, Paterson, & Hurst, 2011; Nakayama et al., 2015). The mechanisms underlying these harmful introductions have yet to be fully explored, but the consensus hypothesis is that novel symbiont failure is not simply a product of host responses to infection. Obadia et al. (2017) determined that stochastic factors were the main drivers of gut microbiome establishment, based on alternative stable states of colonization and high between-individual variability in composition. Therefore, gut microbiome establishment is an inherently difficult process to predict.

Box 1 Insect–microbe interactions

Below, we detail key areas in which symbionts can affect host phenotype, and thus the host's ability to interact with its environment and its community (Cagnolo, Salvo, & Valladares, 2011; Ferrari & Vavre, 2011; McLean et al., 2016).

Herbivory

The microbiome affects host-plant use, as acquisition of novel endosymbionts, or gut microbes, can potentially facilitate species interactions with different plants (Hansen & Moran, 2014; Figure 2a) and the acquisition of novel resources (Hammer & Bowers, 2015). New food sources can change population and community dynamics due to rapid expansion of host populations following sudden resource availability (Frago et al., 2012; Hulcr & Dunn, 2011). Symbionts are also capable of mediating interactions with plants. Frago et al. (2017) found that several endosymbionts reduced parasitoid wasp recruitment by attenuating the release of volatiles from a plant under attack by aphids, further indicating the wide-reaching role played by host-associated microbes (also see Cusumano et al., 2018; for viral symbionts).

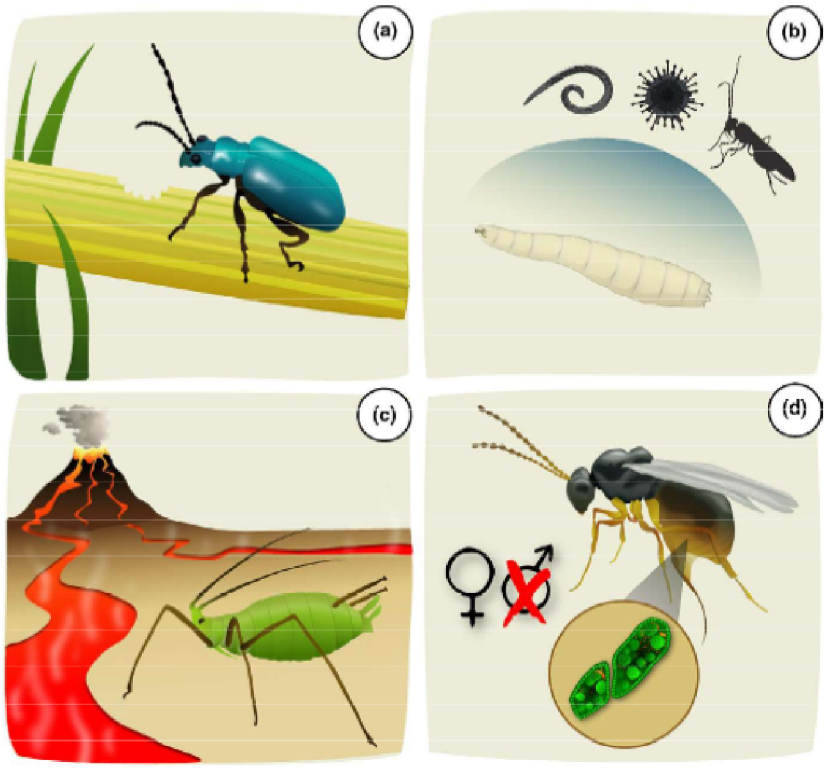

FIGURE 2 Representative examples of how microbial symbionts influence insect host ecology, physiology, and health. (a) novel symbioses can facilitate host insect feeding on a new food source; (b) the presence of specific microbes can protect a host against natural enemies such as parasitoids, fungi, and nematodes; (c) symbionts can modify host thermal tolerance in both positive and negative ways; and (d) some symbionts, like *Wolbachia* and *Spiroplasma*, manipulate host sex ratios by male-killing, genetic feminization, and by inducing cytoplasmic incompatibility

Protective symbiosis

Microbiota have been shown to alter host defense against natural enemies (Imler, 2014; Parker, Spragg, Altincicek, & Gerardo, 2013; Rothacher, Ferrer-Suay, & Vorburger, 2016; Figure 2b). One of the best studied endosymbionts with regard to parasitoids is the bacterium *Hamiltonella defensa*, which has been demonstrated to provide aphids with protection against parasitoids in the laboratory (Oliver, Russell, Moran, & Hunter, 2003) and in the field (Hrček et al., 2016; Rothacher et al., 2016) by providing phage-encoded

Box 1 **(Continued)**

toxins that kill developing parasitoids (Oliver, Degnan, Hunter, & Moran, 2009). Other endosymbionts, including *Regiella insecticola*, *Wolbachia*, *Spiroplasma*, and *Rickettsia*, also provide their hosts with protection against parasitoids (Fytrou, Schofield, Kraaijeveld, & Hubbard, 2006; Hamilton & Perlman, 2013; Vorburger et al., 2010; Xie, Butler, Sanchez, & Mateos, 2014; Xie et al., 2010), fungi (Łukasik, Guo, Asch, Ferrari, & Godfray, 2013; Parker et al., 2013), nematodes (Haselkorn & Jaenike, 2015; Jaenike, Unckless, Cockburn, Boelio, & Perlman, 2010), and RNA viruses (Cattel, Martinez, Jiggins, Mouton, & Gibert, 2016; Hedges, Brownlie, O'Neill, & Johnson, 2008). Additionally, bacteria from the gut microbiome have been shown to regulate insect immunity (Koropatnick et al., 2004; Round & Mazmanian, 2009), with changes in gut microbiome community composition resulting in demonstrable changes to immunity and host resistance to parasitoids (Chaplinska et al., 2016; Ferguson et al., 2018).

Thermal tolerance

Symbionts can both increase and decrease thermal tolerance in a variety of hosts (Bensadia, Boudreault, Guay, Michaud, & Cloutier, 2006; Lazzaro, Flores, Lorigan, & Yourth, 2008; Figure 2c). Heat-shock tolerance in the whitefly *B. tabaci* increases with reduction in *Rickettsia* numbers and the symbiont-led expression of genes associated with stress response (Brumin, Kontsedalov, & Ghanim, 2011). Conversely, in *A. pisum*, *Rickettsia* increases heat tolerance by allowing the aphid to retain a higher percentage of bacteriocytes (Montllor, Maxmen, & Purcell, 2002). Disruption of specific regions of the microbiome (e.g., the gut) can have negative consequences for host thermal tolerance because the gut microbiome has positive influence on induction of thermal tolerance proteins within cells (Henry & Colinet, 2018; Liu, Dicksved, Lundh, & Lindberg, 2014). Heat shock can further affect bacterial density in their hosts, which may lead to increased variation in vertical transmission rates (Hurst, Johnson, Schulenburg, & v d & Fuyama, Y., 2000; McLean et al., 2016; Watts, Haselkorn, Moran, & Markow, 2009). In some cases, insects have lost their endosymbionts completely following sufficiently strong heat-shock events (Thomas & Blanford, 2003). The sensitivity of bacterial symbionts to temperature suggests that the benefits and costs provided to hosts could be substantially altered in scenarios of significant environmental (Ross et al., 2017) and seasonal (Ferguson et al., 2018) change. These responses require further investigation, especially in the context of changing temperatures predicted to cause increased abiotic stress (Corbin et al., 2017).

Reproductive manipulation

Some facultative symbionts (***Wolbachia*** and ***Spiroplasma***) are known for impacting host reproduction through male-killing, genetic feminization, and inducing cytoplasmic incompatibility (Harcombe & Hoffmann, 2004; Haselkorn & Jaenike, 2015; Mateos et al., 2006; Montenegro, Solferini, Klaczko, & Hurst, 2005; Werren, Baldo, & Clark, 2008; Xie et al., 2014; Figure 2d). This leads to altered sex ratios in the host population, reducing mating opportunities, and overall population growth rates. ***Wolbachia*** infection in some insect species has been documented at >90% prevalence, with extreme evolutionary and behavioral consequences (Jiggins, Hurst, & Majerus, 2000). For instance, one study commonly observed ***Wolbachia*** infections in parasitoid wasps (Vavre, Fleury, Lepetit, Fouillet, & Boulétreau, 1999), and in one species (***Leptopilina heterotoma***), fecundity, adult survival, and locomotor performance were all affected by ***Wolbachia*** (Fleury, Vavre, Ris, Fouillet, & Boulétreau, 2000). The mechanisms behind ***Wolbachia*** are still poorly understood (see Jiggins, 2016).

3.4 | Transmission of function

In cases where a symbiont successfully transfers and establishes in a novel host, it is still not guaranteed that it will provide the same function(s) in the new host. A symbiont that confers a protective phenotype for one host genotype may (Parker, Hrček, McLean, & Godfray, 2017) or may not (Chrostek et al., 2013) provide the same benefit in other host genotypes or species (Veneti et al., 2012). Transmission of symbiont function (or phenotype) is an important reason to integrate the microbiome with host community ecology. Particularly in cases where symbionts facilitate host defense (see Box 1), transmission of symbiont function can have drastic effects on host survival and interactions with other species (e.g., *Regiella insecticola* protects aphids against parasitoids; Vorburger, Gehrer, & Rodriguez, 2010). In the case of the dirty needle effect described in the "horizontal transmission" paragraph above, *B. tabaci* whiteflies that received *Wolbachia* from a wasp had subsequently increased survival and reduced development times, a tangible benefit for the host that received the symbiont (Ahmed et al., 2015). Parker et al. (2017) demonstrated that the strength of protective phenotypes conferred by transfer of *Regiella* varied with host genotype, providing further evidence for the complexities of context dependency in host–symbiont interactions. Similarly, Veneti et al. (2012) showed that a male-killing *Wolbachia* strain did not transfer that phenotype when introduced to novel hosts, despite the novel hosts being sister species of the original host. Variation in phenotype transfer is likely a product of host and symbiont genotypes, and how they have evolved

together. The function of each symbiont is therefore important to consider when discussing the possibilities of phenotype transfer to novel hosts. For example, symbiont-induced male-killing can transfer more readily (Ahmed et al., 2015) than defense against parasitoids (Gehrer & Vorburger, 2012).

Transmission of function is a more intricate and difficult process to consider when the particular function in question is a direct result of community complexity. For example, immunity or digestion can be improved with a more complex microbiome (Chaplinska, Gerritsma, Dini-Andreote, Salles, & Wertheim, 2016). Loss of microbiome complexity and species abundance, often referred to as dysbiosis, is shown to have negative health effects in insects, corals, and humans (Bajaj et al., 2014; Hamdi et al., 2011; Petersen & Round, 2014; Raymann, Shaffer, & Moran, 2017; Sansone et al., 2017), among others. Currently, it is unclear whether keystone species (i.e., those required for healthy gut function in the host) occur within microbiome communities. Experimental species removal (or insertion) from the microbiome could be one approach to determine whether particular species play disproportionately important roles for host function.

Many facultative symbionts exist at intermediate abundance within host populations as a result of balancing selection and seasonal fluctuation (Oliver et al., 2014). In certain scenarios, hosts experience ecological and evolutionary costs from carrying symbionts. These costs can be subtle, yet significant, for host survival (Polin, Simon, & Outreman, 2014; Vorburger, Ganesanandamoorthy, & Kwiatkowski, 2013). Fitness costs also have important implications for the transmission of symbionts. The line separating a beneficial symbiont from one that is detrimental to its host is often blurred and context-dependent. For example, a facultative symbiont that protects against a parasitoid can also reduce the host's competitive ability in the absence of said parasitoid or in different abiotic environments (Oliver, Campos, Moran, & Hunter, 2008), subsequently reducing host longevity (Vorburger & Gouskov, 2011) and fecundity (Simon et al., 2011). This variable selection pressure means that facultative symbionts will not always be transmitted, vertically or horizontally.

The effect of symbionts on their hosts (Box 1) demonstrates the importance of microbiota in insect community dynamics. On an ecological timescale, symbionts influence the way in which their hosts feed, reproduce, compete, and defend themselves against natural enemies (McLean et al., 2016). Over evolutionary time, these influences may facilitate host species' coexistence, cause localized deterministic extinctions, or impact species coevolutionary dynamics (Frago et al., 2012; McLean et al., 2016). To connect insects, microbiota, and the environment into a wider context, and to consider the importance of horizontal transmission in particular, we advocate a macroecological viewpoint with the dispersal-led concept of metacommunity theory.

4 | INTEGRATING METACOMMUNITY THEORY AND INSECT–SYMBIONT STUDIES

Considering interactions and diversity at multiple scales through the prism of metacommunity theory raises new possibilities for the study of insects and their associated microbes. In these networks, each individual host insect harbors its own community of symbionts and gut bacteria. The interactions between bacteria within a host (intrahost) are joined to other hosts (interhost) at larger spatial scales by transmission (i.e., dispersal) of these symbionts, linking individual insects into a metacommunity (Figure 1, Table 1, Box 2; Mihaljevic, 2012). Metacommunity theory will also enable us to account for patch creation, movement, and destruction, as new host insects are born, move, and die (e.g., Box 2). As we discussed above, microbes play vital roles in host biology and mediate interactions throughout the whole community. These same microbes thus alter metacommunity-level processes through their own vertical and horizontal transmission. The impacts of microbes on their hosts, and their own transmission, can then be modeled as feedback loops to account for biotic changes (Miller et al., 2018). Organizing these systems into a metacommunity framework provides opportunities for us to explore host interactions at a community scale while simultaneously considering the associated symbionts. This will have subsequent benefits for our broader understanding of how symbionts influence host health (Imler, 2014; Parker et al., 2013; Rothacher et al., 2016), how symbionts become contextually detrimental to their hosts, and the circumstances under which hosts eject their symbionts completely (Polin et al., 2014; Vorburger et al., 2013).

One of the most productive ways to implement the metacommunity framework for studying insect–symbiont systems is to use a dual approach that is both mechanism-based and model-based, to best explain observable patterns of community assembly, diversity, and abundance. From a modeling perspective, one method for incorporating hosts and symbionts into metacommunities is by adapting models developed to explain the spread of infectious diseases. Seabloom et al. (2015) introduced a flexible mathematical framework to describe pathogen metacommunity dynamics. The model tracks the spread of two infectious agents among host individuals in a population, where hosts can be infected with one or both pathogens, following the standard susceptible-infectious-removed (SIR) framework (Anderson & May, 1979; Keeling & Rohani, 2008). While this framework has broad applicability to the study of symbiont metacommunity dynamics, there have been no attempts to guide researchers with regard to integrating these types of models with empirical data. For instance, how do we estimate the key parameters of these models, and how do we test whether our models accurately represent symbiotic systems? In Box 2, we show simple SIR-type models to explain the vertical and horizontal transmission of symbionts among hosts and assess which processes are most important for explaining patterns of symbiont community composition over space and time. In Box 3, we highlight how conducting experiments with insect model systems will allow us to parameterize these models, and we offer suggestions for how to use data-model integration to explicitly test metacommunity theory.

One of the issues with studying natural communities (and applying metacommunity theory to natural habitats) is that they

Box 2 A metacommunity model of vertically transmitted symbionts

Here, we build upon epidemiological models (Anderson & May, 1979; Keeling & Rohani, 2008; Seabloom et al., 2015) to explain the horizontal and vertical transmission of symbionts among insect hosts, and the movement of hosts among habitat patches. Thus, the models capture the dynamics of a simple insect metacommunity, where the dynamics of the symbionts are summarized at the level of a host population, i, and host dispersal links all J populations in the host metapopulation. We begin with a generalized model framework of two symbionts and one host species:

$$S_i' = \overbrace{D_s(\mathbf{V},\theta_D) + T_s(\mathbf{V},\theta_T)}^{\text{Within-patch dynamics}} + \overbrace{M_{s_i}(\mathbf{S},\theta_M)}^{\text{Among-patch dynamics}}$$

$$I_{A_i}' = D_{I_A}(\mathbf{V},\theta_D) + T_{I_A}(\mathbf{V},\theta_T) + M_{I_{A_i}}(\mathbf{I_A},\theta_M)$$

$$I_{B_i}' = D_{I_B}(\mathbf{V},\theta_D) + T_{I_B}(\mathbf{V},\theta_T) + M_{I_{B_i}}(\mathbf{I_B},\theta_M)$$

$$X_i' = D_X(\mathbf{V},\theta_D) + T_X(\mathbf{V},\theta_T) + M_{X_i}(\mathbf{X},\theta_M)$$

In this set of differential equations, hosts are susceptible (S), infected with a single symbiont (I_A or I_B), or coinfected with both symbionts (X). The D, T, and M functions represent the dynamics of host demography and vertical symbiont transmission (D), horizontal symbiont transmission (T), and host migration (M). These are functions of the model variables, captured by the vector $\mathbf{V} = (S_i, I_{A_i}, I_{B_i}, X)$, as well as vectors of the respective parameters, stored in θ. Migration is a function of all other subpopulations in the host metapopulation, such that, for example, vector $\mathbf{S} = (S_1, S_2, \ldots, S_J)$. This set of differential equations therefore allows for flexibility in defining the specifications of each of the D, T, and M functions. We will use the following expansion of the above equations to suggest a more concrete model of the system.

$$S_i' = \overbrace{\nu_b\left[\phi\left(X_i + I_{A_i} + I_{B_i}\right) + S_i\right] - \nu_d S_i}^{\text{Host Demography and Vertical Symbiont Transmission}} - \overbrace{\frac{\beta_A\left(I_A + qX\right)S}{N_{i(t)}} - \frac{\beta_B\left(I_B + qX\right)S}{N_{i(t)}}}^{\text{Horizontal Symbiont Transmission}} - \overbrace{mS_i + \sum_{I\neq i}^{J}\rho_{i,I}mS_I}^{\text{Host migration}}$$

$$I_{A_i}' = \nu_b\left[\left(1-\phi\right)\left(c_A X_i + I_{A_i}\right)\right] - \nu_d I_{A_i} + \frac{\beta_A\left(I_A + qX\right)S}{N_{i(t)}} - \frac{\psi\beta_B\left(I_B + qX\right)I_A}{N_{i(t)}} - mI_{A_i} + \sum_{I\neq i}^{J}\rho_{i,I}mI_{A_I}$$

$$I_{B_i}' = \nu_b\left[\left(1-\phi\right)\left(c_B X_i + I_{B_i}\right)\right] - \nu_d I_{B_i} + \frac{\beta_B\left(I_B + qX\right)S}{N_{i(t)}} - \frac{\psi\beta_A\left(I_A + qX\right)I_B}{N_{i(t)}} - mI_{B_i} + \sum_{I\neq i}^{J}\rho_{i,I}mI_{B_I}$$

$$X_i' = \nu_b\left[\left(1-\phi\right)\left(1-\left(c_A + c_B\right)\right)X_i\right] - \nu_d X_i + \frac{\psi\beta_B\left(I_B + qX\right)I_A}{N_{i(t)}} + \frac{\psi\beta_A\left(I_A + qX\right)I_B}{N_{i(t)}} - mX_i + \sum_{I\neq i}^{J}\rho_{i,I}mX_I$$

The model tracks host demography via reproduction and death rates, ν_b and ν_d, and we assume that infection with the symbionts does not affect these rates. The model also incorporates vertical transmission of the symbionts. The parameter ϕ is the fraction of births that result in fully symbiont-free, susceptible hosts, while $1-\phi$ is the likelihood of vertical transmission occurring. Parameters c_A and c_B are the conditional likelihoods of coinfected hosts reproducing and leading to singly infected offspring, assuming they produce

Box 2 (Continued)

offspring with any infection. The term $1-(c_A+c_B)$ is therefore the probability of producing coinfected offspring, again conditional on producing offspring with any infection, $(1-\phi)$.

We assume horizontal transmission occurs in a frequency-dependent manner via contact between susceptible and infectious hosts (sensu Seabloom et al., 2015), such that the transmission rates for each symbiont, β_A and β_B, are divided by the habitat patch- and time-specific population size $N_{i(t)}$. Population sizes within a host habitat patch may fluctuate over time due to within-patch demography and among-patch migration. The likelihood of singly infected hosts becoming coinfected is mediated by the infected hosts' susceptibility to a secondary infection, ψ. Susceptible hosts can be infected by single- or coinfected hosts, and the transmissibility of symbionts from coinfected hosts is modulated by q, but we assume coinfection occurs sequentially (i.e., a host first becomes infected with one symbiont, then the other).

Host migration occurs when hosts emigrate from the patch, at a per-capita rate m, or when hosts immigrate to patch i from other patches. The probability of migration from patch l to patch i, $\rho_{i,l}$, can then be a function of the distance between patches $d_{i,l}$. And, importantly, the sum $\sum_{l\neq i}^{J}\rho_{l,i}=1$ so that all individuals emigrating from a patch eventually end up in some other patch.

Addressing metacommunity questions with the model

Although this model seems complex, it could be quite useful for both theoretical explorations and empirical tests of metacommunity theory (e.g., Box 3). For instance, analytic and numerical model analysis could reveal how the likelihoods of vertical and horizontal transmission affect local and regional coexistence of symbionts in the context of host migration between habitat patches. Additionally, the roles of trade-offs in symbiont coexistence could be analyzed, such as trade-offs in the host traits (e.g., demography and migration) compared to trade-offs in the symbiont traits (e.g., rates of vertical and horizontal transmission). Furthermore, in Box 3 we demonstrate how this model could be parameterized with empirical studies of insect–symbiont systems. The parameterized models can then be used to determine how well model predictions match observed patterns of symbiont community composition across space. Thus, insect–symbiont systems could be used to rigorously test the role of metacommunity dynamics in structuring symbiont communities.

rarely have defined boundaries (Leibold et al., 2004). The confinement of microbiota within an insect host is thus advantageous for defining community boundaries in a spatially explicit manner, as the microbiota of an individual represents a single local community (Gucht et al., 2007) and the whole host insect population represents the regional part of the metacommunity (Figure 1 and Table 1, Scenario A). This is significant because the specific definition of "region" strongly influences how patch processes affect metacommunities (Leibold & Chase, 2017; Logue et al., 2011; Moritz et al., 2013). The reduced ambiguity over defined scale (because the local community is the host's microbiota) makes it more straightforward to apply spatially explicit models to these systems. Even with this framework, we can still include the surrounding environment as the metacommunity matrix, thus enabling us to include environment-sourced horizontal transfer events. One caveat is that, in this proposed insect–microbiome metacommunity, the "patch" (host) is not static in space, so dispersal rates of microbes partly depend on the dispersal of the host. However, spatial frameworks similar to metacommunities (e.g., metapopulation and epidemiological models; Keeling, Bjørnstad, & Grenfell, 2004, and island biogeography; Reperant, 2009) have been successfully applied to systems with mobile hosts. Similarly, the metacommunity framework has been applied to systems without clearly definable patches (Marrec, Pontbriand-Paré, Legault, & James, 2018). Therefore, it is still possible to match spatial assumptions under these circumstances. Box 2 shows how we can add implicit spatial dynamics into an SIR-type modeling framework, and how we can start to parameterize these models as well. Other modeling approaches, including probabilistic, event-driven approaches (e.g., Gillespie's Direct Algorithm, Gillespie, 2007), could also be simulated, and custom model-fitting code could be generated to fit these stochastic models to experimental or observational time-series data. This approach could be particularly appropriate for more complex models, where model parameters may have hidden correlations (Kennedy, Dukic, & Dwyer, 2015).

One of the benefits of using metacommunity ecology to study insect–symbiont systems is the flexible use of definitions. As we outline in Table 1, there are multiple scenarios where metacommunity theory can be applied to these systems. The local community scale, especially, can be designated at the discretion of the investigator. We outlined above, and in Figure 1 and scenario A of Table 1, the possibility of treating each individual insect as a local community of bacteria. Below (and in other scenarios of Table 1), we suggest future applications of metacommunity ecology to insect–symbiont systems, including scenarios where symbionts are being actively manipulated as a form of vector control.

Box 3 Integrating theory and empirical data to understand metacommunity dynamics

There have been few attempts to guide researchers with advice for integrating metacommunity models with empirical data. This process is critically important to test whether metacommunity theory can explain patterns of symbiont community composition across space and time, and more specifically to explore which local and regional processes are most important for explaining these patterns. Parameterized models can also be used to make forecasts which can be useful, for instance, in the microbial control of insect populations. Here, we briefly highlight methods of model parameter estimation using laboratory experiments and offer suggestions for how to use data-model integration to test metacommunity theory with insect model organisms. Our goal is to emphasize the utility of insect–symbiont systems for understanding the applicability of metacommunity theory to communities of host-associated microorganisms. Supplemental code for model fitting is provided. We note that our methods rely on longitudinal sampling of host populations, but other methods of estimating transmission do not rely on taking multiple samples through time (Dwyer, Elkinton, & Buonaccorsi, 1997), but are perhaps less generalizable.

Introduction to model fitting for parameter estimation

To begin parameterizing the equations in Box 2, we deal with horizontal transmission, which is arguably the most complex dynamic. We must first measure the transmission rates of each symbiont. One approach to estimate transmission rate is to conduct a simple laboratory experiment in which the researcher releases infected hosts into a population of susceptible (uninfected) hosts and documents the change in prevalence over time (Table 1, Figure 3). Then, the researcher can fit a simplified SIR model to these data to estimate transmission rates. We assume the dynamics of the experimental system can be represented by the simple equation:

$$I' = \beta I\,(N-I)\,/N.$$

In this differential equation model, we assume that a host population of constant size N is made of susceptible hosts (S) and infected hosts (I), such that $N=S+I$. The rate of change in the infected class is mediated by the transmission rate β and contact between susceptible ($N-I$) and infectious hosts. If we experimentally expose a known number of susceptible hosts to a known number of infectious hosts, we can track the proportion of hosts that become infected over time. We can then fit this simple dynamical system to the experimental data. Specifically, we compare the fraction of the experimental host population infected at any given time point to the fraction infected in our model, and we can assume the likelihood of the data $P\,(D|\beta)$ follows a binomial probability distribution (Figure 3). This can be done in a Bayesian framework, for instance, by fitting the differential equation model to the data in *Stan*, an open-source statistical programming language (Carpenter et al., 2017). This same model-fitting routine can be used for more complex SIR-type models (e.g., below).

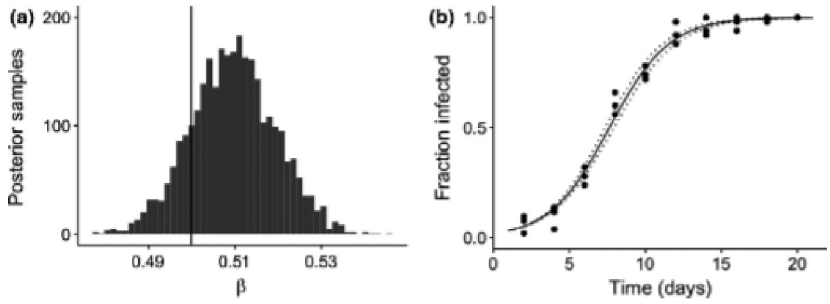

FIGURE 3 Graphs represent fitting a simple susceptible-infected (SI) model to hypothetical experimental data. In this experiment, a single-infected host was released in a population of 49 susceptible hosts, and this was replicated across three host populations. Symbiont transmission occurs horizontally, from infected individuals to susceptible individuals. We simulated the data based on the SI model, adding observation error, and setting the transmission rate to 0.50 day^{-1} $host^{-1}$. The model was then fit to the synthetic data with *Stan* using 3 Hamiltonian Monte Carlo chains, with a 2,000 iteration warm-up period, and 5,000 total iterations, thinning by 3. A vague prior ($N(0, 5)$) was used for the transmission rate. (a) Marginal posterior estimate of transmission rate, with vertical line delineating the true parameter value (0.50). (b) Fit of the model (median and 95% credible interval) to time-series data of the fraction of the population infected, where the three populations were sampled every 2 days of the experiment

Box 3 **(Continued)**

Multisymbiont model and experiments

To continue parameterizing the equations in Box 2, we must understand how multiple symbionts interact in the system. We can simplify the model to only include horizontal transmission to encompass the dynamics of an experiment that occurs on a timescale with no host demography, and in which migration is not allowed.

$$S' = -\frac{\beta_A (I_A + qX) S}{N} - \frac{\beta_B (I_B + qX) S}{N}$$

$$I_A' = \frac{\beta_A (I_A + qX) S}{N} - \frac{\psi \beta_B (I_B + qX) I_A}{N}$$

$$I_B' = \frac{\beta_B (I_B + qX) S}{N} - \frac{\psi \beta_A (I_A + qX) I_B}{N}$$

$$X' = \frac{\psi \beta_B (I_B + qX) I_A}{N} + \frac{\psi \beta_A (I_A + qX) I_B}{N}$$

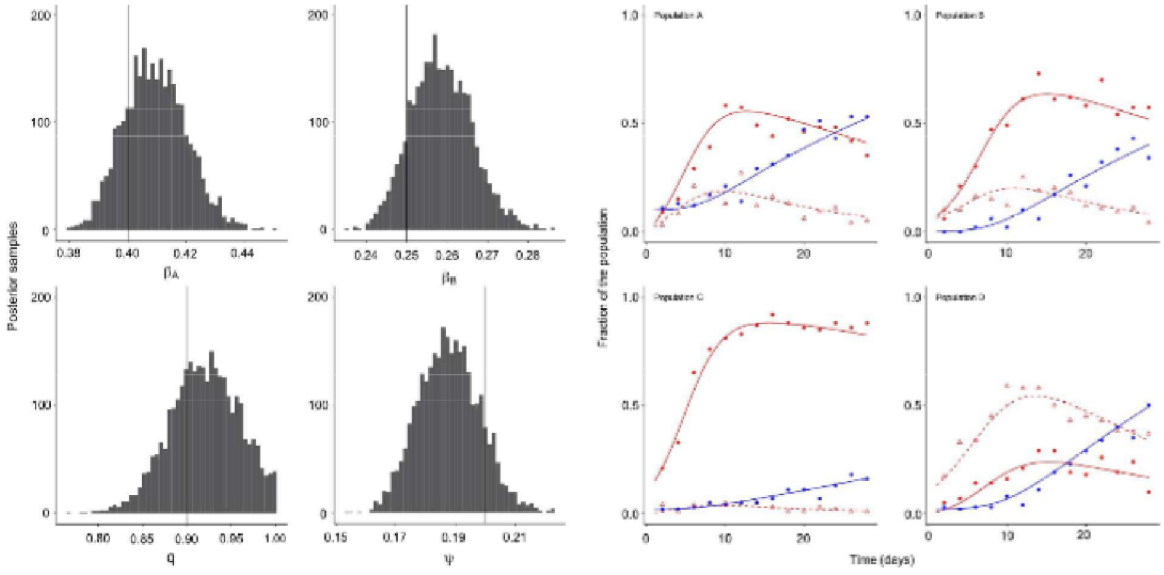

FIGURE 4 Fitting the two symbionts—one host species SI model to synthetic data, from Box 2 "Multisymbiont model and experiment" section. Four populations of 100 hosts were exposed to variable initial numbers of hosts infected with symbiont A (closed red circles, red line), symbiont B (open red triangles, dashed red line), or coinfected with both symbionts (closed blue circles, blue line). Experimentally manipulating the initial conditions enables us to estimate the parameters with more power, because we observe more variable dynamics in the system. Specifically, the initial conditions for each simulated population ($S(0), I_A(0), I_B(0), X(0)$) are as follows: (a) 90, 0, 0, 10; (b) 90, 5, 5, 0; (c) 88, 10, 0, 2; (d) 88, 0, 10, 2. We chose these values to demonstrate that the transient dynamics of the model are influenced by subtle changes to initial conditions, and we should see these dynamics reflected in the experimental data. Again, the model was fit to the synthetic data with *Stan* using vague priors for each of the four parameters, and 5,000 total sampling iterations. Graphs in the left-hand panel show the marginal posterior samples for each parameter, with the vertical line delineating the true parameter value. To reiterate, the parameters are as follows: β_A and β_B are the transmission rates of the two symbionts, respectively; q modulates the likelihood that susceptible hosts become infected through contact with coinfected hosts (i.e., $q = 1$ would mean that there was an equal likelihood of susceptible hosts being infected by single- or coinfected hosts); and ψ modulates the likelihood that single-infected hosts will become coinfected by a secondary symbiont. Graphs in the right-hand panel depict the simulated, synthetic data, where the fraction of hosts infected with one or both pathogens changes over time. The lines represent the median model predictions. Only median posterior model predictions are shown, for clarity

Box 3 **(Continued)**

Although this model seems complex, there are only four parameters, two of which (the transmission rate of symbiont A, β_A, and the transmission rate of symbiont B, β_B) can be estimated with the experiment outlined above. Therefore, we can conduct another experiment to estimate the remaining two parameters. And when we use Bayesian inference, we can use prior probability distributions for β_A and β_B derived from the single-symbiont experiments.

In a multisymbiont experiment, we can create experimental populations of hosts, and we can expose these populations to varying numbers of single- or coinfected hosts. We again track how the fractions of single- and coinfected hosts change over time, as the symbionts spread. We construct a likelihood function that compares the model's predicted number (or fraction) of hosts in each class to the experimentally derived numbers. By altering the starting conditions (i.e., the initial numbers of susceptible, singly and coinfected hosts), we gain more power to estimate the parameters, allowing for estimation of all four parameters from a small number of experimental populations (Figure 4).

Host demography, vertical transmission, and spatial processes

We do not spend much time on measuring the parameters of host demography or vertical transmission in the equations in Box 2. First, empirically estimating the rates of host demography in ecological models has been covered in great detail (McCallum, 2008). In addition, the parameters of vertical transmission could be easily measured by determining the probability of singly and coinfected hosts producing singly or coinfected offspring, or fully susceptible offspring. Measuring the rates of host migration can admittedly be complex, but will likely be simpler for insect model organisms (Table 1). Mark–recapture studies, for example, have been used to estimate mosquito dispersal rates for decades (e.g., Reisen et al., 1991). Therefore, emigration rates and quantitative dispersal kernels could be parameterized by determining the probabilities of short-range and long-range movements in the laboratory and/or in the field.

Model comparisons to test metacommunity theory

The examples above assume that the mathematical model presented in Box 2 is an appropriate representation of the system's dynamics. However, this is not necessarily true. In other words, the applicability of metacommunity theory to a particular system is a testable hypothesis. We can construct different versions of our mathematical models, including or excluding specific assumptions and processes, and then fit these models to our time-series data. We can then use formal model-comparison approaches (Hooten & Hobbs, 2014; Vehtari, Gelman, & Gabry, 2016) to determine which models best explain observational data. For instance, we can collect data from the field on how the composition of the symbiont community changes through time in a host metapopulation. By comparing how different metacommunity models fit to these data, we can therefore test which local and regional mechanisms are most important.

In summary, integrating time-series data and model-fitting approaches can expand our understanding of metacommunity dynamics. Furthermore, insect–symbiont communities are unique and experimentally tractable model systems for exploring the applicability of metacommunity theory to host-associated microbial communities (Table 1).

5 | DIRECT APPLICATION OF INSECT–MICROBIOTA METACOMMUNITIES

A direct way to study dispersal in an insect–microbiome metacommunity could be to focus on horizontal transmission of facultative symbionts throughout a host–parasitoid community, as horizontal acquisition of symbionts can be key for host survival against natural enemies (Haselkorn et al., 2009; Jaenike, 2009; Moran & Dunbar, 2006). One way to investigate this experimentally would be to use hosts that are axenic (devoid of all bacteria) or gnotobiotic (possessing select microbiota only) before initiating colonization with a community of bacteria, then allowing dispersal across the host community to occur (Table 1, Scenario A) by introducing parasitoids to facilitate the spread of bacteria, for instance (the "dirty needle effect"; see section on "Horizontal transmission"). This could be expanded upon by measuring symbiont dispersal in conjunction with other effects. For example, symbiont dispersal under different temperature regimes will provide information on how host–symbiont metacommunities might respond to a changing climate, and thus, how they would be expected to affect host performance (Corbin et al., 2017; Feldhaar, 2011). A similar experimental approach for insect–microbiota metacommunities is to determine the effects of disturbance on stability and interactions within the metacommunity by feeding hosts with antibiotics.

TABLE 1 Suggested scenarios for the application of metacommunity theory to insect–symbiont systems, taking into consideration community definitions, the possible questions that could be addressed with each system, and outlining a potential experiment to test address the question

Scenario	Local community	Regional community	Question(s) addressed	Experimental outline	Metacommunity response variable
A (see also Figure 1)	Individual insect	Host insect community	• How much horizontal transmission of bacteria between individual insects occurs over a single host generation? • How do abiotic factors or variable parasitoid pressure influence horizontal transmission?	Introduce a target bacterium to a metacommunity of axenic insects, and sample them at the end of one host generation to see how much the target bacterium has spread via horizontal transmission	Individual insect microbiome (local community) diversity
B	One insect host species	Multiple insect host species	• What barriers exist between species preventing horizontal transmission of symbionts? (e.g., Is coevolution of host and symbiont a predominant barrier preventing horizontal transmission from one host species to another?)	Experimentally, again with axenic hosts, one could introduce a symbiont in different 'doses' to determine the point where dispersal is sufficient to overcome natural dynamics	Microbiome (local community) diversity
C	One individual plant	Multiple plants of single or multiple species, with their insect pests and symbionts included	• How does a spatially structured metacommunity change the dynamics of herbivore–symbiont dispersal? • Metacommunity structured by the location of plants, with parameters changed relative to previous scenarios by plants not moving and having much longer life spans	Comparison of different plant spatial configurations with measures of herbivore density, the number of symbionts, and the dispersal of symbionts, as a result of the distance between plant-associated communities	Diversity of insects and associated symbionts on a particular plant
D	All insects associated with one plant individual	All insects associated with multiple plant individuals	• How much does pest dispersal facilitate symbiont movement between plants? • This scenario is a combination of **scenarios B and C**, based on the coevolved barriers between insect species and their impacts on symbiont dispersal, and the plant-focused spatially structured metacommunity	Dispersal measured as the movement of insect herbivores (e.g., aphids) between plants, and the subsequent impacts on symbiont dispersal within the metacommunity (see Brady et al., 2014; Frago et al., 2017 for the associations between symbiont, insect, and plant)	Diversity of insects and associated symbionts on one particular plant
E	One local site of a focal symbiont-infected host species, and close relative species of the host	Multiple sites of the focal insect host, its symbiont, and closely related species	• Which insect species does a biocontrol symbiont spread to within a wild community? • Will other species in the microbiome of wild hosts facilitate establishment of *Wolbachia*? This is a specific application toward biocontrol efforts. The example presented is the attempt to use male-killing strains of *Wolbachia* to reduce populations of the dengue fever mosquito (*Aedes aegypti*)	In this scenario, dispersal is a combination of the mosquito's movement, transmission of the symbiont, and establishment of the symbiont, measured over time and space by capturing individuals of *A. aegypti* (and closely related species) and measuring them for the used *Wolbachia* strain. This enables us to quantify dispersal distance over time, and simultaneously consider spillover events into other insects in the natural community	Insect microbiome diversity

These synthetic metacommunities will also reveal the effects that changes in microbiome (local community) diversity have on the local community structure (Adair & Douglas, 2017) and regional host community structure, with subsequent possibilities for relating structure to metacommunity stability through these local manipulations (Leibold et al., 2004; Loreau, 2010).

Theoretical metacommunity models, like those shown in Boxes 2 and 3, have the potential to identify the most important factors in insect–microbiome metacommunity assembly by fitting alternative models to experimental data. Modeling metacommunities can also deepen our understanding patterns of diversity of host-associated microbiomes. Previous work on microbiomes has suggested that stochasticity plays a significant role in community assembly, and that the process is inherently hard to predict (see Adair, Wilson, Bost, & Douglas, 2018; Obadia et al., 2017; Sieber et al., 2019; Vega & Gore, 2017), based on findings that are consistent with the neutral theory of biodiversity (Hubbell, 2001). Recent models for metacommunity diversity (e.g., O'Sullivan, Knell, & Rossberg, 2019) can be utilized to answer questions about ecological structural stability influencing microbiome diversity, and whether the microbiome adheres to broad ecological patterns of diversity. For instance, testing whether symbiont communities fit the species-abundance distribution (SAD) or species-area relation (SAR). The aforementioned studies indicating that stochasticity plays a prominent role in microbiome composition would appear to infer that diversity patterns in microbiomes differ from those observed elsewhere in ecology. Thus, a pressing question in microbial ecology is to determine whether patterns of microbial community composition are driven by the same mechanisms that drive patterns of free-living community composition. More work is required to unravel microbiome diversity, and metacommunity modeling is a potential avenue to further explore this aspect of microbiomes.

Another potential application for metacommunity theory and insect–symbiont systems is to improve understanding of symbiont dynamics in scenarios where symbionts are being utilized for human benefit (Table 1, Scenario E). A prominent example is the use of *Wolbachia* to manipulate host sex ratios as a form of biocontrol against undesirable species (Hoffmann et al., 2015), particularly disease-spreading mosquitoes such as *Aedes aegypti* (Frentiu et al., 2014; Ross et al., 2017). One of the most important aspects for releasing *Wolbachia*-infected mosquitoes is knowing how they will disperse, both in terms of how the infected hosts will move and how the wild symbiotic communities will respond to *Wolbachia* introduction. The structure of their dispersal routes is crucial for infected mosquitoes to access wild insect communities and for *Wolbachia* to disperse. An equally important aspect of *Wolbachia* dispersal is understanding how *Wolbachia* will interact with other endosymbionts and the gut microbiome (see subsection "Interactions within microbial communities"). One possibility could be to aid *Wolbachia* dispersal via facilitation from another symbiont. In addition, we also need to understand symbiont dynamics for scenarios where a host becomes a pest species due to protective symbiosis (McLean et al., 2016). To counteract pests with biocontrol, we need to know the best potential control option, and therefore must know which enemies can be countered with protective symbionts and how these symbionts disperse throughout the host population (e.g., if applying a parasitoid for biocontrol of a pest risks facilitating defensive symbiont dispersal via the dirty needle effect). Using the metacommunity framework to explicitly measure symbiont dispersal within a community-wide context could provide new insights into currently unexplained patterns, such as the lack of phylogenetic clustering exhibited by *Wolbachia* and other symbionts in their host species (Henry et al., 2015; Smith et al., 2012).

6 | CONCLUSION

Strong evidence that host-associated microbiota influence interactions among their hosts warrants greater consideration of the mechanisms that drive symbiont diversity in large-scale studies, and we propose metacommunity theory as a framework to achieve this. We recommend that insect–microbiota model systems be used to investigate the role of symbionts in shaping host interactions within metacommunities, the importance of phenotype transfer as a result of symbiont dispersal, and the ecological consequences of symbiont transmission. Through the microbial prism, we are likely to achieve greater understanding of the mechanisms that influence metacommunities and the dynamic processes within them.

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CONFLICT OF INTEREST

The authors declare no known conflict of interest regarding the publication of this manuscript.

AUTHOR CONTRIBUTIONS

JJB, JRM, LDM, and JH contributed to development of the ideas and the writing of the manuscript.

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DATA AVAILABILITY STATEMENT

Model code is included as Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Chapter 3

Microbiome composition of a wild *Drosophila* community along tropical altitudinal gradients and in comparison to laboratory lines

Microbiome composition of a wild *Drosophila* community along tropical altitudinal gradients and in comparison to laboratory lines

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Abstract

Understanding host-associated microbiome communities is important because they can provide their host with invaluable benefits, including natural enemy protection, essential nutrients, and improved thermal tolerance. Two major confounding factors in many microbiome studies are i) how comparable laboratory animals' microbiotas are to their wild counterparts and ii) how much does the broader environment structure the microbiota in free-ranging animals. Model insects, like *Drosophila spp.*, provide a tractable system to explore these factors because they are naturally pervasive and survive well in lab conditions. In this study, we analysed microbiomes from both field-caught and laboratory-reared pupae and adults of 4 *Drosophila* species. We controlled for diet to help elucidate other deterministic patterns of microbiome composition. We show that microbiome community composition differs radically between lab and field flies. We also found some notable taxa-specific differences in *Drosophila* microbiomes at different altitudes, and between different species. We suggest these differences are the products of environments with different bacterial species pools. We caution against determining microbiome composition from lab-only specimens and recommend that future field studies are designed to control for deterministic factors of microbiome composition.

Introduction

Patterns of diversity over environmental gradients (e.g. latitude, elevation, environmental degradation) have long been of interest in community ecology (Chown and Gaston, 2000; Fierer et al., 2011; Wang et al., 2011; Pärtel et al., 2016; Roslin et al., 2017; Thompson et al., 2017), and are of renewed interest as an angle to study the potential consequences of climate change. Many studies have focused on animals and plants to investigate these patterns, but now bacterial communities are getting increased attention. Some studies suggest environmental bacteria do not follow the

same broad biogeographic patterns as plants and animals (Fierer and Jackson, 2006; Lauber et al., 2009; Meyer et al., 2018). For example, Fierer et al., (2011) showed that soil bacteria had no significant change in diversity when sampled across an elevational gradient. Other studies have found inconsistent patterns in bacterial communities sampled from streams and soils across elevational gradients, with differences usually being attributed to changes in pH and C:N ratio (Wang et al., 2011; Shen et al., 2015; Siles and Margesin, 2016; Meyer et al., 2018). Yet in contrast to environmental microbial communities, the effect of elevational change on insect-associated microbiome communities has yet to be investigated in-depth. The most conspicuous aspect of a change in elevation is a difference in mean temperature, creating different abiotic environments that can be used as a proxy for climate change scenarios (Wadgymar et al., 2018; Nottingham et al., 2019). We would expect to see differences in microbiome composition because both insects (Economos and Lints, 1984; James et al., 1997; Kinjo et al., 2014; Tochen et al., 2014; Brankatschk et al., 2018) and bacteria (Ratkowsky et al., 1982; Pettersson and Bååth, 2003; Tsuji et al., 2017) develop in temperature-dependent manners. Thus, at different elevations and in climate change scenarios, insect-associated microbiomes could develop differently.

Many insects maintain intimate communities of symbiotic microbes (their 'microbiome'). Host insects and their microbes influence each other in many ways. Insect microbiomes can play important roles in host health, digestion, thermal regulation, and protection against natural enemies (reviewed in McLean et al., 2016; Corbin et al., 2017; Brown et al., 2020a). In turn, many factors can influence insect microbiome composition, some biotic (e.g. diet, insect species identity, ontogeny, and parent-to-offspring transmission), others abiotic (e.g. local environment and temperature) (Colman et al., 2012; Yun et al., 2014; Xie et al., 2015; Martinson et al., 2017; Nováková et al., 2017; Bing et al., 2018; Park et al., 2019; Brown et al., 2020b).

Symbioses between insects and bacteria have been particularly well investigated (Douglas, 2016), notably because their microbiome communities tend to be less complex than those of vertebrates (Woodhams et al., 2020).

Many microbiome studies have been performed on lab-reared organisms, and many studies have sampled organisms from the field, but few have done both. Those that have compared both environments have found conflicting results (Morrow et al., 2015; Bost et al., 2018a; Hegde et al., 2018; Dada et al., 2020). Some studies suggest that microbiome richness is not appreciably different between the lab and the field, whilst others find greater differences between captive and wild animals. Model organisms such as mice, *Drosophila melanogaster,* and mosquitoes have had their microbiomes sequenced as part of laboratory studies. The predominating conclusion from most of these studies is that microbiomes are very different in lab-reared individuals, for a variety of reasons, but primarily because the colonising bacterial species pool is heavily reduced in laboratory housing (Amato et al., 2013; Nelson et al., 2013; Kohl et al., 2014; Clayton et al., 2016; Adair et al., 2020; Dada et al., 2020). Additionally, many microbiome studies have sampled from animals in captivity (e.g. zoos). However, few studies directly compare the microbiotas of lab- and field-reared specimens in the same study. For many free-ranging animal species, such an experimental design is not feasible due to the difficulty of bringing wild animals into the laboratory, but insects offer a tractable model system to directly compare lab and field microbiotas from the same host species.

One consideration when selecting insects for such a study is host life history. All insects undergo metamorphic ontogenetic development, either holometabolous (complete metamorphosis) or hemimetabolous (incomplete metamorphosis). These different strategies have contrasting but important consequences for their

microbiomes and gut physiology. Holometabolous insects routinely have life stages that are radically different in form and function from one another (Truman, 2019). This has two important implications for microbiome composition. Firstly, complete metamorphosis means that the gut is completely restructured from one ontogenetic stage to another, so many symbionts have to relocate and adjust to novel habitats or risk being purged from the insect's body (Hammer and Moran, 2019). Also, many holometabolous insects induce bactericidal activity in their guts at the onset of pupation (Johnston et al., 2019). Secondly, the distinctiveness of each life stage can result in adaptive decoupling, which results in further ecological specialisation (e.g. Lepidoptera larvae focus on growth, adults focus on reproduction). This, in turn, results in further specialisation in the microbiome, making it more distinctive between life stages. In contrast, in hemimetabolous insects, there is more opportunity for a symbiont to pass between one nymphal stage to another because their body plan is more stable across development. However, certain hemimetabolous species still have highly complex ontogenetic development with radical changes in microbiome composition (Rodríguez-Ruano et al., 2018; Brown et al., 2020b).

Drosophila spp. are well-established models for studying holometabolous insect-associated microbiomes (Chandler et al., 2011; Blum et al., 2013; Wong et al., 2013; Chaplinska et al., 2016; Adair et al., 2018, 2020) because they are naturally abundant and easy to maintain in laboratory cultures. *Drosophila*-associated microbiomes have important functional impacts on many aspects of their ecology, mainly development (Elgart et al., 2016), ability to recognise kin (Lizé et al., 2014), thermal tolerance (Henry and Colinet, 2018), and immunity (Sansone et al., 2015; Chaplinska et al., 2016). Additionally, some *Drosophila* species possess intracellular endosymbionts (*Wolbachia* and *Spiroplasma*) that demonstrably influence host immunity and protect

against natural enemies, including pathogenic fungi, nematodes, and parasitoids (Chrostek et al., 2013; Hamilton and Perlman, 2013; Haselkorn et al., 2013; Xie et al., 2014; Yadav et al., 2018). *Drosophila* bacterial microbiomes are of moderate-to-poor diversity, making their microbiome communities relatively simple to characterise. The well-studied nature of *Drosophila* makes them ideal candidates for investigating insect-associated microbiomes over elevational gradients, and in a field vs laboratory setting.

Here we present one of the first analyses examining the effects of altitude-induced temperature change on insect microbiome composition. This study was specifically designed to find the key deterministic factors shaping microbiome composition and establish if there was any consistency in deterministic patterns in the microbiomes in lab-bred individuals of the focal species. We chose to study four species of frugivorous *Drosophila* from two mountain gradients in tropical Australia - *Drosophila rubida, D. pseudoananassae, D. pallidifrons,* and *D. sulfurigaster*. These species occur throughout north Queensland, including along multiple altitudinal gradients in the Wet Tropics. We specifically opted for these four species because they occur in sympatry across the full elevational gradient at our chosen study sites (Jeffs et al., 2021). We hypothesised that we would see a difference in microbiome composition between high and low elevation populations as a result of the differences in temperature at these respective sites. To reinforce our investigation, we sampled microbiomes from lab-reared flies of the same species which were collected from the same field sites, to see if fly microbiomes retained any species- and site-specific differences. To control for diet we exclusively sampled pupae from banana-baited bottle traps (Jeffs et al., 2021), thus guaranteeing that each individual sample originated from an egg laid in our bottle traps and therefore that it fed solely on yeasted banana as a larva. We expected *a priori* to find high among-individual

variation and hypothesised that species identity, elevation, and environment (i.e. lab vs field) would be the primary causes of difference in host microbiome community composition (Chandler et al., 2011; Staubach et al., 2013; Adair et al., 2018, 2020).

2. Material & Methods

2.1 Study Sites

The Australian Wet Tropics World Heritage Area (WTWHA) is a 450 km long, narrow section of rainforest along Queensland's northeast coast between Cooktown and Townsville (15-19'S, 145-146.30'E). Samples were collected from two altitudinal gradients: Paluma Range Road (within Paluma Range National Park 19°00'S, 146°14'E) and Kirrama Range Road (within Girramay National Park 18°12'S, 145°50'E). The Paluma gradient ranges from 59 m to 916 m above sea level (a.s.l.) and the Kirrama gradient ranges from 92 m to 770 m a.s.l (Jeffs et al., 2021). We chose sites at high, middle, and low elevations (Paluma: 880m, 350m, 70m; Kirrama: 730m, 390m, 70m) to capture a ~5°C temperature range (mean temperatures 21°C at high elevation, 26°C at low elevation; Jeffs et al., 2021).

2.2 Field Samples

Samples of *Drosophila* pupae were collected from banana-baited bottle traps placed at low, middle, and high elevation sites along the Kirrama and Paluma altitudinal gradients. Bottle traps were exposed for either 11-12, 14-15, or 24 days, to capture the natural variation in community colonisation and variation in ontogenetic development in different *Drosophila* species (Jeffs et al., 2021). Each bottle trap had a piece of cardboard to assist *Drosophila* larvae in pupation. On the day of sampling, these cards were removed and sealed in ziplock bags. From what we know of frugivorous *Drosophila* life-history, we can guarantee that the samples we collected

had only fed on banana bait for their entire lives. Pupae from each card were sampled by placing the card on a plate and adding distilled water, with all pupae being removed with a small paintbrush. Each individual pupa was placed into an individual well in 96-well PCR plates and preserved in 100% ethanol. Adult flies were collected from bottle traps (using an aspirator) 2 days after provision of fresh banana bait and placed into individual vials in 100% ethanol.

2.3 Sample Selection & Samples from Lab Lines

Based on the results of Jeffs et al., (2021) which identified the *Drosophila*-parasitoid food web to species level with COI metabarcoding and Multiplex PCR methods, we selected a stratified subset of 214 field samples focused on the four most common species that occurred at all elevations along both altitudinal transects: *D. rubida, D. pseudoananassae, D. pallidifrons,* and *D. sulfurigaster*. Eight samples of *D. rubida* were parasitised, enabling us to examine if there are any changes in richness or unique microbial taxa associated with a developing parasitoid. We also sampled 70 pupae and 70 adults from isofemale laboratory lines (2-4 per species) of these four elevationally ubiquitous species (20 pupae and 20 adults from *D. sulfurigaster, D. rubida,* and *D. pseudoananassae*, and 10 pupae and 10 adults from *D. pallidifrons*) to investigate if suspected natural patterns (site- and species-specific influence) were retained in lab-reared flies. These isofemale lines were established from the same populations sampled in the field (i.e. they were collected at the same sites and shipped live to the lab in Czech Republic, one year after the field samples used in this experiment). Isofemale lines were kept in the lab for between 18-30 months by the time of sampling. We also took 10 samples of the food source used in keeping lab-reared *Drosophila* and 20 samples of the banana bait we used in our field sampling Complete sample breakdown is shown in Table 1.

Table 1: Breakdown of the sample set used in this study. PL = Paluma Low, PH = Paluma High, KL = Kirrama Low, KH = Kirrama High, JCU = James Cook University campus.

Species	Stage	Origin	Sites	Number of samples
D. rubida	pupae	field	PL, PH, KL, KH	79
D. rubida	adult	field	JCU	14
D. pseudoananassae	pupae	field	PL, PH, KL, KH	48
D. pallidifrons	pupae	field	PL, PH, KL, KH	39
D. sulfurigaster	pupae	field	PL, PH, KL, KH	10
D. rubida	pupae	lab	PL, PH, KL, KH	20
D. rubida	adult	lab	PL, PH, KL, KH	20
D. pseudoananassae	pupae	lab	PL, KL, KH	20
D. pseudoananassae	adult	lab	PL, KL, KH	20
D. pallidifrons	pupae	lab	PH, KH	10
D. pallidifrons	adult	lab	PH, KH	10
D. sulfurigaster	pupae	lab	PL, PH, KL, KH	20
D. sulfurigaster	adult	lab	PL, PH, KL, KH	20
banana bait	na	field	PL, PH, JCU	20
lab fly food	na	lab	na	10

2.4 Library Preparation & Sequencing

Field sample DNA was extracted using GeneAid Blood and Tissue kits for host-parasitoid identification (published in Jeffs et al., 2021). Lab and bait samples were extracted using the same single column method according to manufacturer instructions, with one extraction negative control accompanying every 29 samples. All samples were subsequently moved to 96-well plates in a randomised order. DNA templates were stored at -75ºC. These templates were used for amplification of ~400 bp of the V4/V5 hypervariable region of the 16S rRNA gene according to Earth Microbiome Project standards (EMP; http://www.earthmicrobiome.org/protocols-and-standards/16s/). Sample multiplexing was based on the EMP-proposed double barcoding strategy using the recommended modifications (12 bp Golay barcodes included on the forward primer 515F, and additional 5 bp barcodes on the reverse primer 926R; Supplementary Information after Chapter 5). We also added a custom 18S rRNA gene blocking primer (named 926X) to counteract the low specificity of EMP primers towards the 16S rRNA gene (Brown et al., 2020b). PCR amplification was confirmed with gel electrophoresis. PCR products were purified with AMPure XP (Beckman Coulter) magnetic beads, pooled to equimolar concentration (based on DNA concentration measured using a Synergy H1 (BioTek) spectrophotometer), then cleaned again using Pippin Prep (Sage Science) to eliminate all fragments outside the 300-1100 bp range. To confirm barcoding success, we included four negative controls from the extraction procedure (ENC), eight negative controls from the PCR process (NC), and eight positive controls (PC) of mock microbiome communities. PCs were supplied commercially and comprised 4 samples of gDNA templates with equal abundance of 10 bacterial species (ATCC® MSA-1000™) and 4 samples with staggered abundance for the same bacteria (ATCC® MSA-1001™). We sequenced four plates of samples. In each sequencing plate, we ensured that there was one ENC, two NCs, and two PCs - one of the even mock community and one of the staggered mock

community. The four purified libraries were sequenced by a single run of the Illumina MiSeq platform using v3 chemistry with 2 x 300 bp output (Norwegian High Throughput Sequencing Centre, Department of Medical Genetics, Oslo University Hospital).

2.5 Data Processing and Statistical Analyses

The sequencing process returned 15,893,914 reads. These raw reads were quality checked (FastQC; Andrews, 2010) and trimmed using USEARCH v9.2.64 (Edgar, 2013), to keep the quality score above Q20. We trimmed the primers then demultiplexed and merged the reads which resulted in a final amplicon length of 357 bp. We clustered the reads at 100% identity for a representative set of sequences and used the USEARCH global alignment option at both 99% and 97% identity (Edgar, 2013) for *de novo* OTU assignment. We subsequently used the BLAST algorithm (Camacho et al., 2009) on the representative sequences, matching them against the SILVA 132 database (Quast et al., 2013) for taxonomic identification, producing a dataset of 1108 OTUs at 97% identity and 1118 at 99% identity. We used the 97% identity OTU table as the primary dataset and used the 99% identity table as a supplemental dataset to confirm that the patterns we found were not a product of bioinformatic decision making.

Any chloroplast, mitochondrial or eukaryotic OTUs were identified in the OTU table and excluded. Potential bacterial contaminants were systematically identified by examining the prevalence of reads found in negative controls using the R package 'decontam' (V1.5.0; Davis et al., 2018). Specifically, OTUs with a higher proportion of reads in negative controls than in actual samples were labelled as contaminants and excluded (Fig. S2). 43 OTUs were eliminated from the dataset via this process. Singletons were also excluded. We set the minimum threshold to 2000 reads (because

all the negative controls had a total number of reads beneath this number), which excluded 38 individual samples. We then subsampled to a fixed minimum depth of 2000 reads without replacement across samples and agglomerated the OTUs at the Genus level. These procedures resulted in a dataset of 117 OTUs and 343 samples. We used Shannon index and Bray-Curtis dissimilarity as quantitative measures of community diversity and calculated ordination analyses (non-metric multidimensional scaling; NMDS) with PERMANOVA tests to determine significant community differences, using the packages 'vegan' (Oksanen et al., 2019) and 'phyloseq' (McMurdie and Holmes, 2013) in R (R Core Team, 2019). In each NMDS, we included trap identity as a random factor.

With negative controls remove from the data, we had a mean average of 16,898 reads per sample and a median of 14,751 reads. From our positive controls, we recovered microbiome profiles that matched the expected community composition in each of the 'staggered' and 'even' mock communities. In the staggered mocks, there were two species present at 0.04% and our sequencing detected reads of those species in all four staggered mock samples. In the even mocks, there was consistent overrepresentation of *Clostridium beijerinckii* and *Escherichia coli* (1.4x - 4.7x expected), leading to subsequent reductions in other taxa. Overall, the positive controls in this sequencing run matched our previous results (Rodríguez-Ruano et al., 2018; Brown et al., 2020b).

3. Results

3.1 Microbiomes across altitude

Altitudinal gradient had a small but significant effect (NMDS ordination, mean stress ≈ 0.15, PERMANOVA, $R^2 = 0.035$, $p \leq 0.001$) when comparing pupal samples from the

different field sites, suggesting that the differences in temperature and geographic location (as a result of altitude) and gradient location have a minor effect on *Drosophila* microbiome composition.

3.2 Microbiome and environment of origin

The dominant trend in our results was a significant reduction in microbiome richness in lab-reared flies of all species, compared to those from the field, based on ANOVA tests between taxon richness and Shannon index values (Fig. 1). We found this significant trend in both pupae and adult *Drosophila*. In multivariate analyses (NMDS), environment of origin was the dominant explanatory factor for microbiome community composition, with consistent significant differences between pupae sampled from the lab and the field (Fig. 2; Fig. S4). These differences were observed for all species, but were particularly obvious for *D. rubida,* our most sampled species (Fig. 2, mean stress ≈ 0.15; PERMANOVA $R^2 = 0.299$, $p \leq 0.001$, with significant Beta-dispersion $F = 242.71$, $p \leq 0.001$ on 999 permutations). In the more diverse field samples the dominant genera were *Acinetobacter, Klebsiella*, and *Providencia.* The dominant bacteria genera in lab-sampled microbiomes were *Acetobacter, Gluconobacter,* and *Lactobacillus,* with *D. pseudoananassae* maintaining the endosymbiont *Wolbachia*.

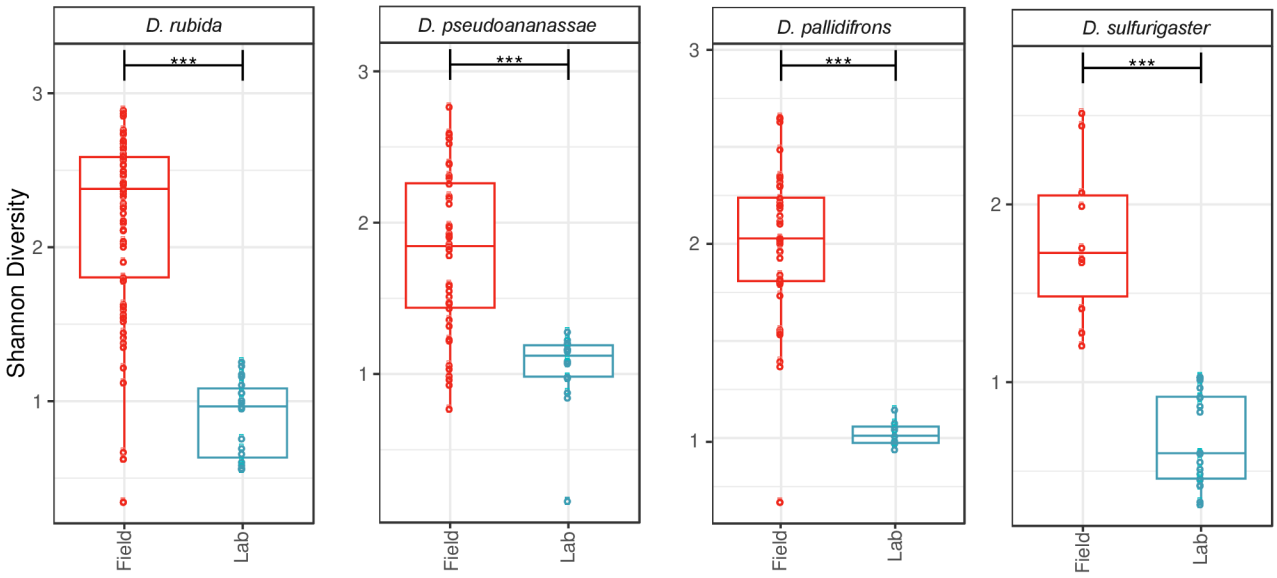

Figure 1: Comparison of richness and Shannon index values for pupal samples from each species of *Drosophila* in the lab and the field. A) = *D. rubida*, B) = *D. pseudoananassae,* C) = *D. pallidifrons,* D) = *D. sulfurigaster.* Field samples are shown in red; lab samples are shown in blue.

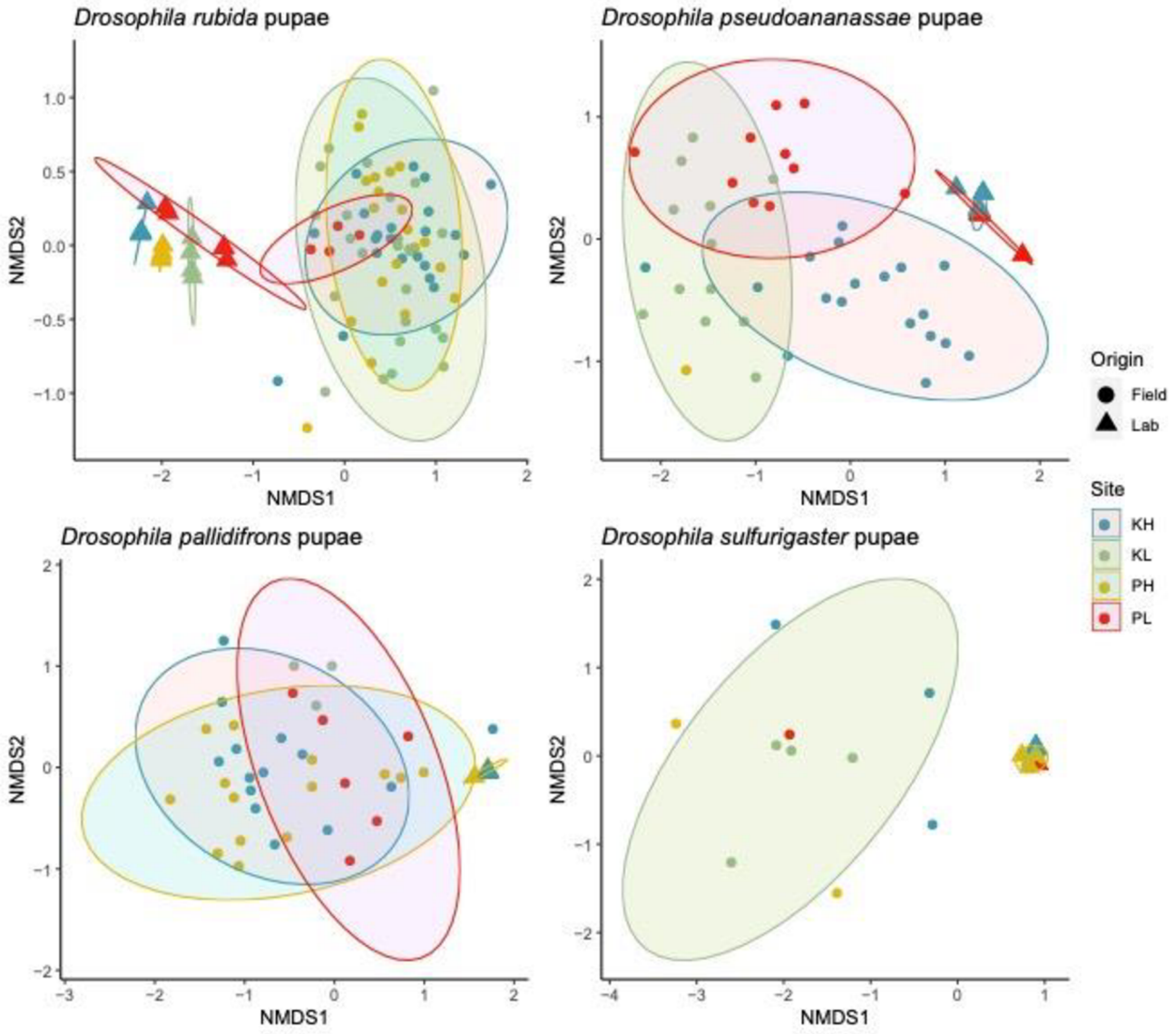

Figure 2: NMDS analysis of microbiome communities from pupae samples of *Drosophila rubida*, *D. pseudoananassae*, *D. pallidifrons*, and *D. sulfurigaster* in the lab (triangles) and the field (circles). Ellipses are significant at 0.05 confidence interval. Colours represent each field site, so for lab samples represent site of origin.

3.3 Species-specificity

We found some evidence of species-specific differences amongst pupae from the field (PERMANOVA R^2 = 0.077, $p \leq 0.001$). These minor differences in community composition can be recognised on sample microbiome profiles (Fig. 3), for example, *D. rubida* did not contain any *Acetobacter* whereas the other three species did.

Microbiome communities are mostly similar between species, with *D. rubida, D. pseudoananassae, D. sulfurigaster,* and *D. pallidifrons* all primarily composed of *Acinetobacter, Klebsiella, Providencia,* and *Pseudomonas* (four of the five most abundant bacterial genera). *D. rubida* microbiomes had a much greater relative abundance of *Providencia* than in any of the other 3 species. The other dominant bacterial genera were evenly distributed throughout all four *Drosophila* species sampled here, including *Acinetobacter,* which was the most dominant genera overall. Fig. 3 shows that *D. rubida* microbiomes contain a greater proportion of 'other' taxa, i.e. bacterial genera not in the top 20 for relative abundance, suggesting greater intraspecific variation in microbiome composition. There was no detectable difference in microbiome diversity of parasitised pupae of *D. rubida,* compared to unparasitized pupae. Examination of microbiome composition indicated that there were no unique bacterial genera in parasitised samples.

In contrast to the field, there was much stronger species-specificity in the lab-reared samples (PERMANOVA $R^2 = 0.292$, $p \leq 0.001$). *D. rubida* contained a much higher proportion of *Corynebacterium* and *Providencia* in their microbiomes, compared to any of the other species. *D. pseudoananassae* was the only species to contain *Wolbachia*, which made up a significant proportion of the reads in many individuals. Additionally, *D. pallidifrons* was the only species to contain *Weissella* (Fig. S1).

3.4. Microbiome and other factors

In the field we exposed baits for different lengths of time to ensure we captured a full picture of the insect community. The field sample results suggest that this had minimal influence on microbiome composition, in comparison to the dominant patterns we identified (3% variation explained vs 7% or more). In the lab, the number of generations a fly line had been in the lab was a significant explanatory variable in

NMDS analysis, but it only explained 1.5% variation and there was no discernible difference in bacterial genera (Fig. S1). There was no significant difference in microbiome composition of flies fed on lab food or the banana bait that we used in the field. Both are completely dominated by *Acetobacter* and *Lactobacillus,* with some lab food samples containing *Gluconobacter*. In lab-reared flies, these 3 genera dominated the microbiomes of pupae and adults. In the field, however, *Acetobacter* and *Lactobacillus* were not the most dominant genera. There was still some congruence because these taxa were still present in high relative abundance, but field-caught fly microbiomes were much richer, so the relative abundance of *Acetobacter* and *Lactobacillus* was proportionally lower.

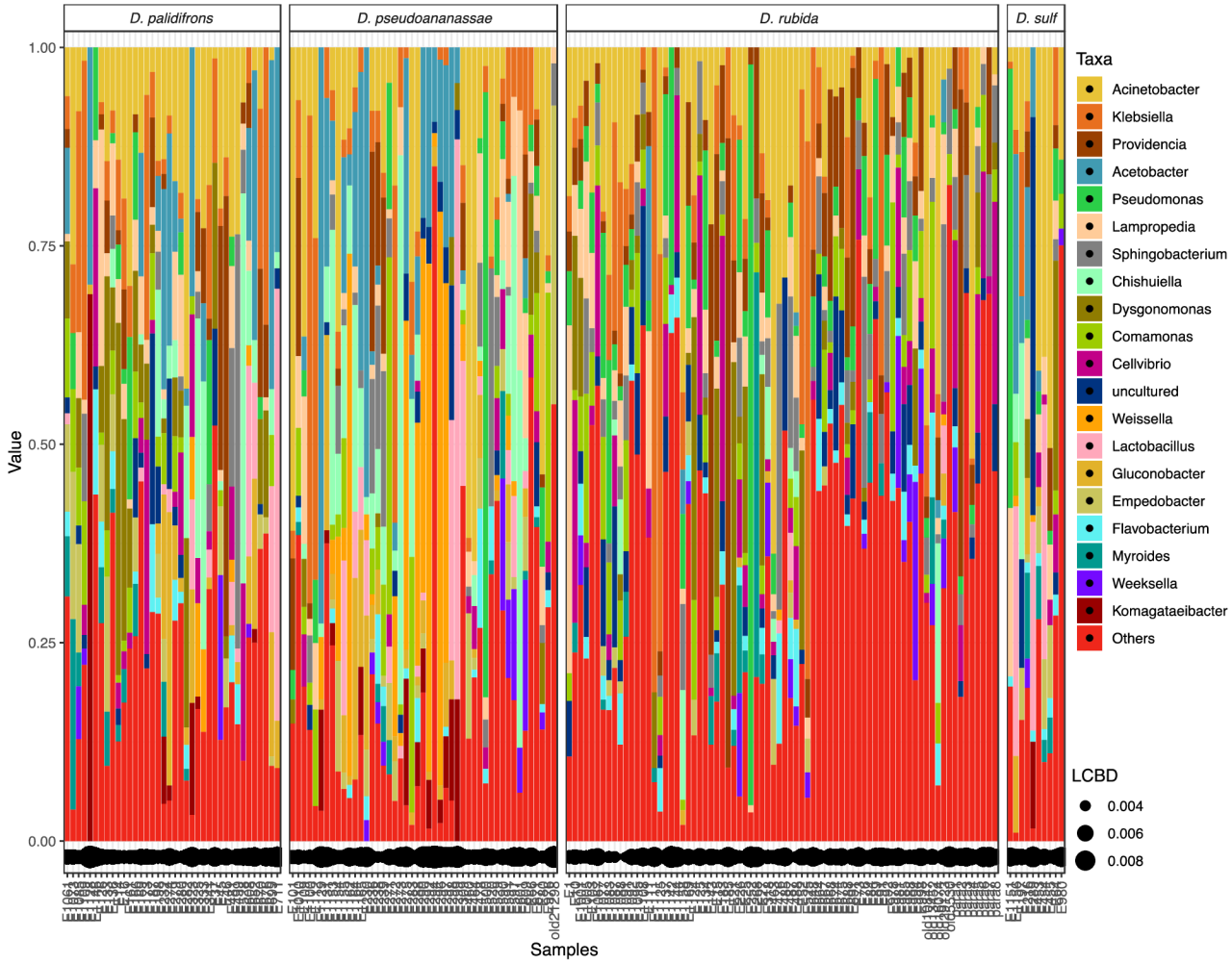

Figure 3: The top 20 bacterial genera for field-reared pupae samples of all four *Drosophila* species. Each individual column represents an individual sample. LCBD = Local Contribution to Beta Diversity.

Discussion

We examined *Drosophila* microbiome community-level patterns across multiple elevations, species, environments, and life stages. We specifically focused on elevation as a potential factor influencing microbiome composition, due to the lack of prior investigation and the natural variation in temperature that elevation gradients provide. Our results show significant differences in community dissimilarity between high and low elevation across both gradients, but these results are small compared to variation between sites. This finding is likely a result of the species

sampled here being ubiquitous across elevation and not forming sufficiently distinct populations at high and low sites, and because the ~5°C temperature shift between our sites is not strong enough to drastically alter microbiome composition. This result was unexpected, because there is well-documented evidence of both insects and bacteria developing differently according to differences in temperature (Pettersson and Bååth, 2003; Kinjo et al., 2014; Tochen et al., 2014; Tsuji et al., 2017; Brankatschk et al., 2018; Cooper et al., 2021). Despite previous studies demonstrating a lack of change in bacterial diversity across elevation (Fierer et al., 2011; Wang et al., 2011), we expected to find a difference in *Drosophila*-associated microbiomes because an insect's internal environment is very different from soil or streams. Naturally, diet might differ along an elevational gradient based on the fruits that develop in different environments. By standardising diet, we showed that minor changes can be expected in different *Drosophila* species due to non-dietary forces. Furthermore, the disparity in microbiome composition between bait samples and pupae in the field setting suggests that diet is not always the most important variable structuring *Drosophila* microbiomes either. Both natural microbiome community variation and similarity from their homogenous diet used for sampling likely played a role in this result. From a broad perspective, changing global temperatures may not result in large changes of insect-associated microbiomes, at least in insects like *Drosophila*.

The most pronounced differences in microbiome composition were between individuals raised in the lab and those raised in the field. Multiple factors coalesce to explain this distinction. Firstly, lab and field individuals were exposed to agar-yeast fly food medium and banana, respectively, thus their dietary sources were different, but the food sources themselves have very similar microbiome profiles. The bacterial community from lab food matches well with the microbiomes found within pupae and adult flies. This would be expected, because it shows a well-established pathway

of insect microbiome colonisation - they ingest food and acquire the bacteria associated with that food source. Yet in the field, the fly microbiomes do not correspond well with the bacterial community found on the banana bait samples. We can therefore infer that the exhibited differences are mostly due to significant differences in microbiome colonisation from environmental bacterial species pools (Cornell and Harrison, 2014; Kohl, 2020). The disparity in banana bait microbiome and pupae microbiome could be because of variety in age. Some of our pupae were collected from traps with 24-day-old banana bait, and our bait samples were 2-12 days old, showing little variation between them - thereby suggesting that the bacteria on the food doesn't change radically day-to-day. The flies sampled from the lab come from a highly regulated environment, with a specific and consistent food source provided into heat-sterilised glass vials, so the only 'available' bacteria for colonising their microbiomes comes from the food and vertically inherited endosymbionts (e.g., *Wolbachia* in *D. pseudoananassae*). In contrast, the bacterial species pool in Australian tropical rainforest comprises much greater diversity and abundance of different bacteria, creating a greater variety of possible microbiome communities within *Drosophila* hosts. This diversity of taxa creates more room for ecological drift, dispersal, and selection to act on microbiome communities, in turn creating greater among-individual and between-species variation in wild flies. The selective forces acting on wild *Drosophila* microbiomes are unlikely to be negative because we see consistent diversity - suggesting that bacteria are not being selectively removed from communities and that low-biomass microbiomes are predominantly colonised by diet-induced transients (i.e. microbes that come directly from a food source and are lost from the microbiome after a dietary switch; Hammer et al., 2019) or from the wider environment. The traps were visited by other organisms, which could have functioned as a source of bacteria indirectly transmitted to the *Drosophila* sampled in this study.

Furthermore, there was high congruence between the microbiome communities in lab-reared pupae and lab-reared adults, suggesting that low diversity within pupae is an accurate representation of lab-reared microbiomes. This ontogenetic congruences implies that other life stages (eggs, larvae) would likely have similar microbiomes too. The result was surprising because we anticipated some stage-specific microbiome community patterns, given that *Drosophila* are holometabolous insects and thus undergo substantial gut remodelling during complete metamorphosis (Hammer and Moran, 2019). The consistency across life stages from lab-reared individuals provides further evidence for the simplicity of the lab environment. In contrast to the lab, the field-caught adults of *D. rubida* lacked congruence with the field-caught pupae. The parsimonious explanation is that the adults were caught from a different site to the other field samples but results from the focal field sites show that there is not much geographic variation in microbiome composition, so this likely doesn't fully explain the discrepancy. With adult flies we can't rule out that they might have fed on a substance other than our yeasted banana bait. Given the influence of diet in *Drosophila* microbiome composition, it's clear that different food sources could explain the microbiome incongruence. The substantial differences in microbiomes between lab and field specimens suggests that future studies should be cautious in interpreting microbiome community composition from lab-kept specimens, as these are highly unlikely to be representative of natural microbiomes (Fig. S1) (Bost et al., 2018b; Dada et al., 2020).

Previous studies on *Drosophila* have demonstrated high intra- and interspecific variation in microbiome community composition from wild-caught and lab-reared flies (Adair et al., 2018, 2020; Bost et al., 2018b, 2018a; Solomon et al., 2019). We also found species-specific differences in microbiome composition amongst wild flies.

Controlling for diet, in the field and especially in the lab, allowed us to recognise this species-specificity more accurately by eliminating dietary variation. In lab flies, there was significantly reduced microbiome richness and every species maintained the same few bacterial genera. We found a significant effect of species identity, but it did not explain as much variation as the results from Adair et al., (2020). This small discrepancy could be a product of the species themselves (i.e. in this study we used a different set of species) or the number of species studied (we studied four species here; Adair et al., (2020) studied eighteen), but the evidence from both studies suggests that species-specificity is maintained in the lab. It should be noted that other insect species have experienced dramatic microbiome shifts after being introduced to the lab (Dada et al., 2020). Since the four species we sampled are all frugivorous, sympatric *Drosophila* species, it is unlikely that *Drosophila* diversification played much of a role in generating the microbiome differences we found here. This study is a snapshot of the communities involved and not multi-generational, so it is difficult to tell.

The discrepancy in microbiome diversity found between lab and field flies suggests that *Drosophila* are not heavily reliant on their bacterial microbiomes, because a core group of bacterial taxa has not been consistently maintained between species or between environments. Hammer et al., (2019) raised compelling points about bacterial microbiome functionality and demonstrated multiple invertebrate species that appear to have no resident gut microbiome. In other insect species, host transmission of extracellular symbionts (like those in the gut) have been hypothesised to result in long-term associations between insect and microbe (Sanders et al., 2014; Kwong et al., 2017; Sinotte et al., 2020). The long-term survival of these four *Drosophila* species in the lab with near-completely different microbiomes than in the

field suggest that they do not fit the hypothesis of long-term association between host and microbe.

A crucial factor in this study was ensuring each field sample received the same food source, in order to control for diet as a factor influencing microbiome composition. By sampling pupae from fermented banana baits in bottle traps hanging from branches we can guarantee that the pupae we sampled spent their whole life cycle within the baited trap, thus we can ensure that our field-reared pupae only consumed the substances within their bottle trap, in turn providing some control over diet as a factor influencing their microbiomes. We believe this element of our study was crucial for recognising other deterministic factors of *Drosophila* microbiome community composition. Controlling for diet (a known influential factor on microbiome composition) in a study involving wild insects provides a new option for investigating microbiome community assembly processes.

Overall, we found significant differences in the microbiomes of lab-reared and field-caught *Drosophila*, which were consistent across species and life stage. Species identity was also a significant variable in explaining microbiome community variation, in flies from the lab and the wild. We hypothesise that these differences are the products of environments with markedly different bacterial species pools. To elucidate functional conclusions from insect-microbiome analyses, more in-depth molecular analysis (e.g. metagenomics, transcriptomics) is required. We recommend that microbiome studies focus on wild-caught individuals and caution against determining microbiome composition from lab-only specimens. We advocate that future field studies are designed in a manner that controls for deterministic factors of microbiome composition.

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Supplemental Figures

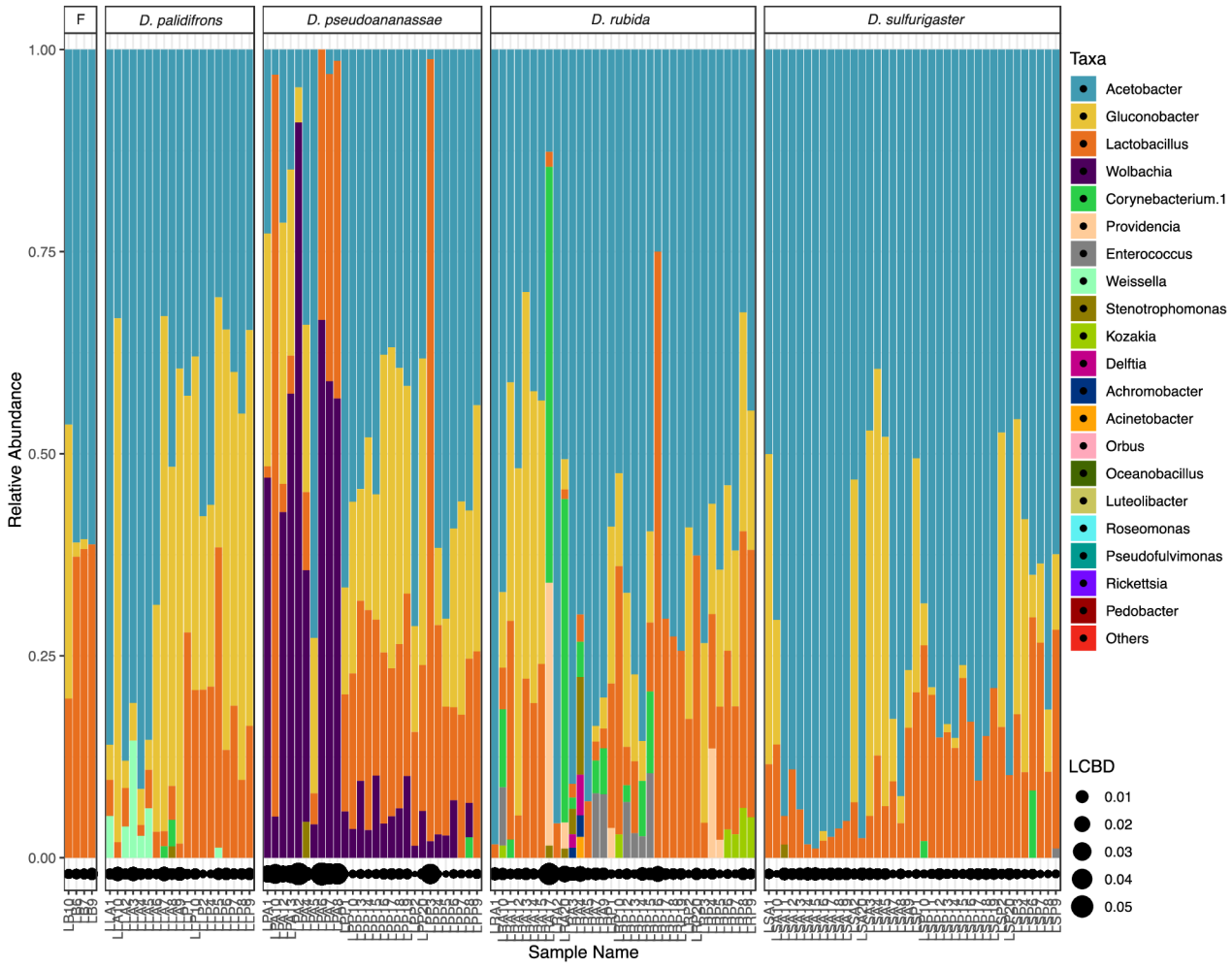

Figure S1: The top 20 bacterial genera for lab-reared pupae samples of all four *Drosophila* species. Each individual column represents an individual sample. LCBD = Local Contribution to Beta Diversity.

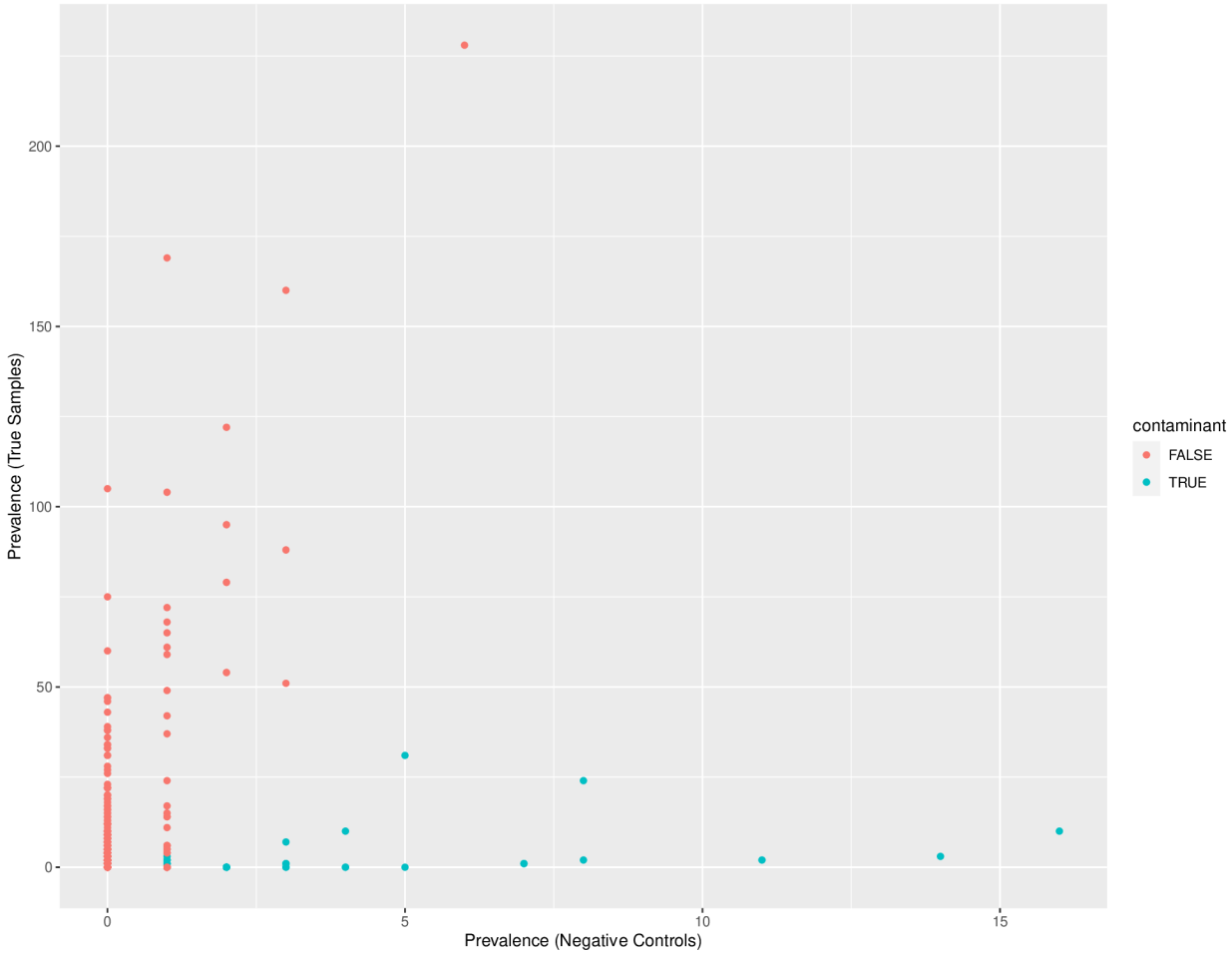

Figure S2: Plot of contaminant OTUs identified by 'prevalence' function in the R package 'decontam'.

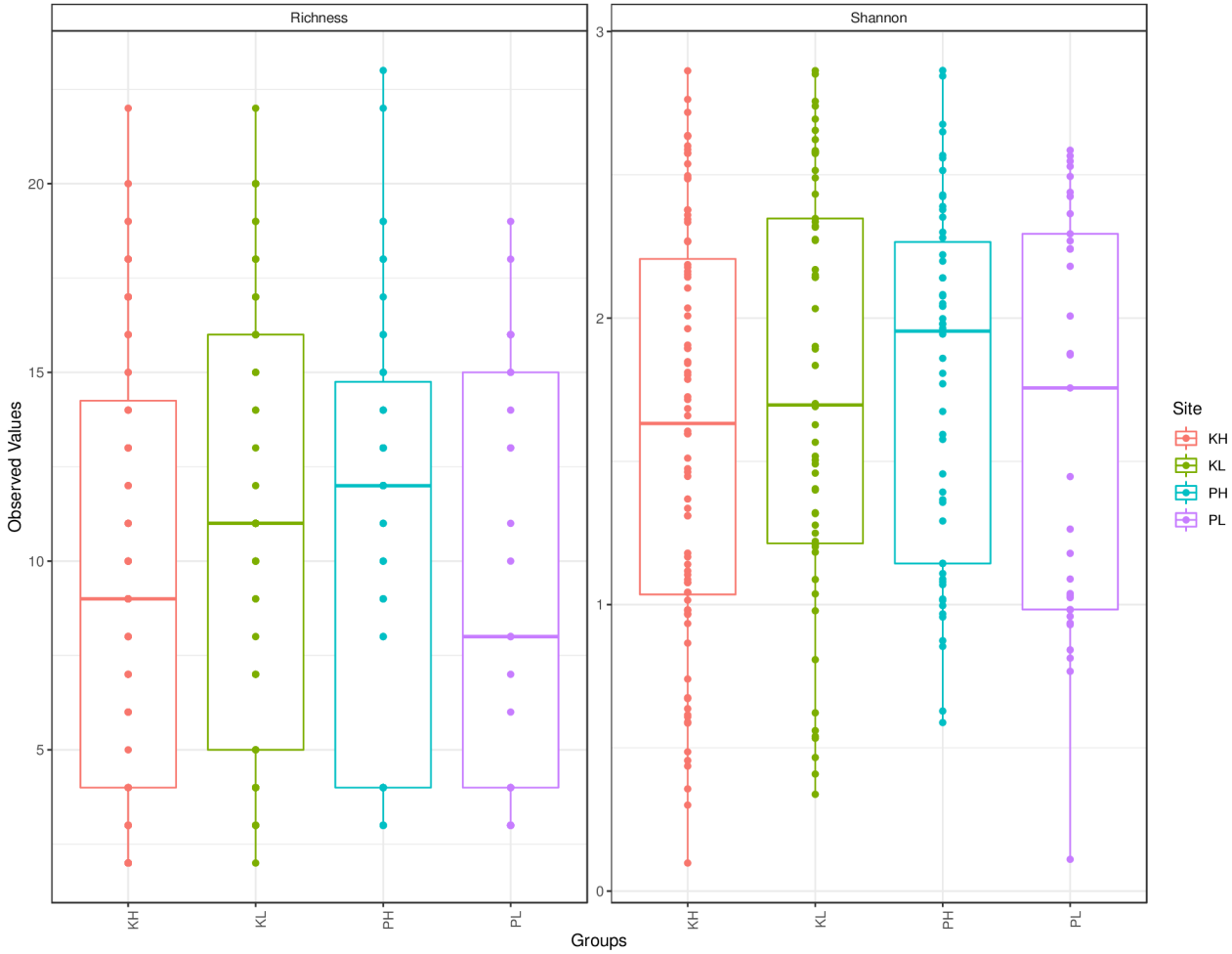

Figure S3: Pupae of all four *Drosophila* species organised by site of origin.

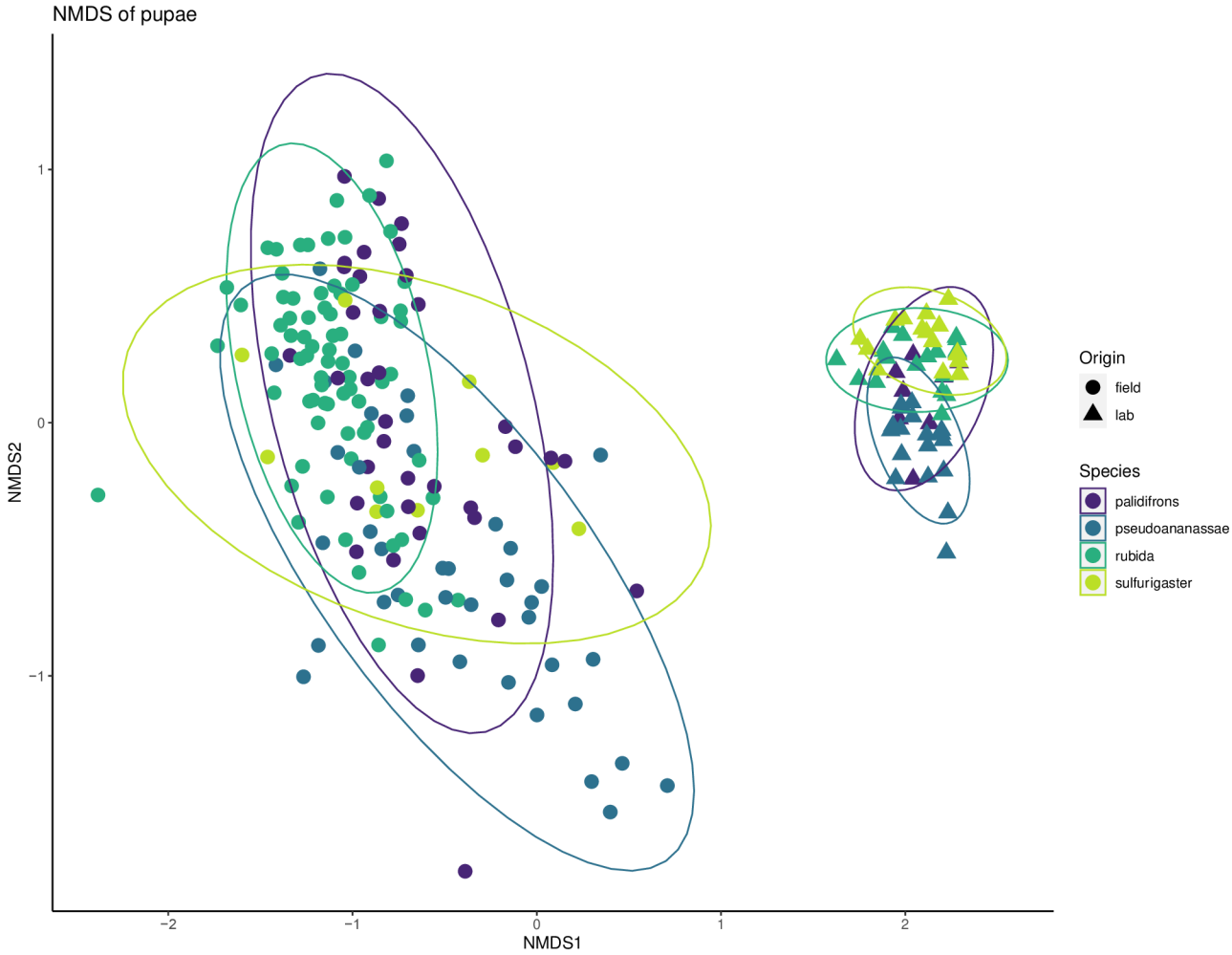

Figure S4: NMDS of microbiome communities from all pupae in this study. Symbols represent site of origin and different colours represent different species. This result was generated from the dataset BLASTed at 99% sequence identity.

Chapter 4

Long-term experimental translocations suggest tropical insect-microbiome communities are more resilient to climate change than expected

Long-term experimental translocations suggest tropical insect-microbiome communities are more resilient to climate change than expected

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Statement of authorship: JJB, CJ, MH, EOB, JB, OL, JH designed the research. JJB, CJ, JH executed the fieldwork. MS identified *Drosophila* specimens. JJB, AJ, AM, EN performed the molecular work. JJB, EN, JH analysed the data. JJB wrote the manuscript. All authors contributed to the improvement of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

Keywords: climate change; community; *Drosophila;* elevation gradient; insect; interactions; microbiome; parasitism; symbiosis; translocation

Abstract

How communities will respond to climate change is one of the most pressing issues ecologists are facing. To unravel the potential effects of long-term temperature change on biotic interactions, we simulated climate change by translocating tropical insects over elevation transects. We moved high-elevation *Drosophila*-parasitoid-microbiome communities to middle and low elevation sites, thus exposing them to prolonged warming for multiple generations. We found that tropical insect communities have surprising resilience to warming temperatures and *Drosophila* in warmer environments managed to consistently maintain their microbiome communities, albeit with reduced richness. Our results imply that ecological drift was a stronger factor in structuring these communities than response to temperature.

1. Introduction

Climate change has substantially affected global weather patterns, directly impacting species abiotic environments (IPCC 2014). These changes have resulted in shifts in species ranges by elevation and latitude, changes in species' phenology and life history, and rewiring of ecological networks (Colwell *et al.* 2008; Chen *et al.* 2011; Kortsch *et al.* 2015; Macgregor *et al.* 2019; Birrell *et al.* 2020; Mamantov *et al.* 2021). Populations of species do not respond to temperature change in isolation, because they naturally interact with other species (Gilman *et al.* 2010; Sheldon *et al.* 2011; Nadeau & Urban 2019). These biotic interactions strongly modify species' response to their abiotic environment (Blois *et al.* 2013; Gårdmark & Huss 2020), and biotic interactions themselves respond to temperature changes in their own diverse and complex ways (Tylianakis *et al.* 2008; Barton *et al.* 2009; Barton & Schmitz 2009; Petchey *et al.* 2010; Frances & McCauley 2018; Bartley *et al.* 2019). Therefore to understand the full effects of climate change it is important to take community-level interactions into account (Ockendon *et al.* 2014).

One valuable method for understanding community response to temperature variation is to utilise elevational gradients (O'Brien *et al.* 2017; Pellissier *et al.* 2017; Tylianakis & Morris 2017; Jeffs *et al.* 2021). Temperature can change rapidly with elevation, thus creating transects that can approximate climate change scenarios. Translocation of communities from cooler environments into warmer ones provides a simulation of climate change, by subjecting the focal community to warmer temperatures. This provides the benefit of a controlled experiment in a natural setting and avoids the limitations of laboratory-based studies (Wadgymar *et al.* 2018; Nottingham *et al.* 2019).

In the tropics many species are operating close to their critical thermal maximum (CT_{max}) and are therefore highly sensitive to abiotic changes, as a result of long-term climatic stability and lack of temperature seasonality (Deutsch *et al.* 2008; Angilletta 2009; Laurance *et al.* 2011; Kellermann *et al.* 2012; Shah *et al.* 2017b). Janzen's 'seasonality hypothesis' predicts that reduced seasonality in the tropics results in species with narrower thermal niches (Janzen 1967), thus limiting where they can exist. Ectothermic species, like insects, are incapable of regulating their own temperature and are especially sensitive to changes in environmental temperature (García-Robledo *et al.* 2016). In ectotherms, temperature strongly predicts important physiological functions like growth and reproduction (Frazier *et al.* 2006; Laughton *et al.* 2017; Burger *et al.* 2019; Huey & Kingsolver 2019; May *et al.* 2019), with substantial consequences at the population, community, and species level (Chen *et al.* 2011). Thus, we may predict that tropical insect communities are more at risk due to climate change.

Insect-associated microbiomes are important for considering how insects might respond to climate change. Symbiotic microbiomes are communities of bacteria, archaea, viruses, and unicellular eukaryotes inhabiting a host. Many insects benefit

from symbiont-mediated protection against natural enemies (Xie *et al.* 2010, 2014; Brandt *et al.* 2017; Jamin & Vorburger 2019; Smee *et al.* 2021), or from microbiome-facilitated nutrient provision (Gaio *et al.* 2011; Hansen & Moran 2014; Jing *et al.* 2020). But these beneficial symbioses can be affected by thermal stress. For example, aphids carrying *Fukatsuia* that suffered a heat shock were more susceptible to parasitoid attack than those without the symbiont, even though *Fukatsuia* is normally protective (Heyworth & Ferrari 2016). A recent study found that the unique ability of bees to thermoregulate is important for establishing and maintaining symbionts (Hammer *et al.* 2021). If ectothermic insects are exposed to increased temperatures, then their inability to self-regulate temperature could result in thermal stress on their microbiomes, potentially altering important symbiotic interactions that damage the host further (reviewed in Corbin *et al.* 2017). Thus, climate change could create important feedback loops between insects and their microbiome with negative consequences for both.

To test the effects of climate change on tropical insects and their microbiota, we focused on a *Drosophila*-parasitoid-microbiome community from North Queensland, Australia, which has been characterised previously (Jeffs *et al.* 2021). The different components of this system allow us to include competitive (conspecific *Drosophila*), trophic (parasitoids), and symbiotic (microbiome) interactions, thus capturing a diverse range of biotic interaction types to better understand how climate change might affect communities. We simulated the impact of climate change on our focal community by translocating entire communities from high elevation to middle and low elevations on two tropical mountain gradients and allowing them to develop there for 74-76 days. We predicted that: 1) communities exposed to elevation shifts will show changes in species' abundances, parasitism rates, and structure, due to species ability (or inability) to survive at different temperatures. 2) Communities exposed to more extreme climate regime shifts will experience greater changes in

composition, with more local extinctions in high elevation-adapted species like *Drosophila pseudotakahashii*, and 3) *Drosophila* species exposed to temperature increase will suffer greater loss of microbiome diversity compared to conspecific populations at cooler temperatures.

2. Methods

2.1 Study Sites

The Australian Wet Tropics World Heritage area (WTWHA) is a 450 km long, narrow section of rainforest along Queensland's northeast area between Cooktown and Townsville (15-19'S, 145-146.30'E, Wilson, Trueman, Williams, & Yeates, 2007). We established study sites along two rainforest elevation gradients within this area, with permission for site use from the relevant governing bodies: Paluma Range Road (Paluma Range National Park 19°00'S, 146°14'E) and Kirrama Range Road (within Girramay National Park 18°12'S, 145°50'E) and span altitudes from 59 – 916 m above sea level (a.s.l.) (Fig. 1). We selected sites within enclosed rainforest at three elevations, high, medium, and low, along each gradient. Sites were established in the same locations as our previous *Drosophila*-parasitoid community survey quantification for this study system (Jeffs *et al.* 2021). The temperature gradients across our elevation transects reflect current predictions of climate warming (1 - 6ºC for Australia by 2100; Wilson, Trueman, Williams, & Yeates, 2007; further detail on temperature data below).

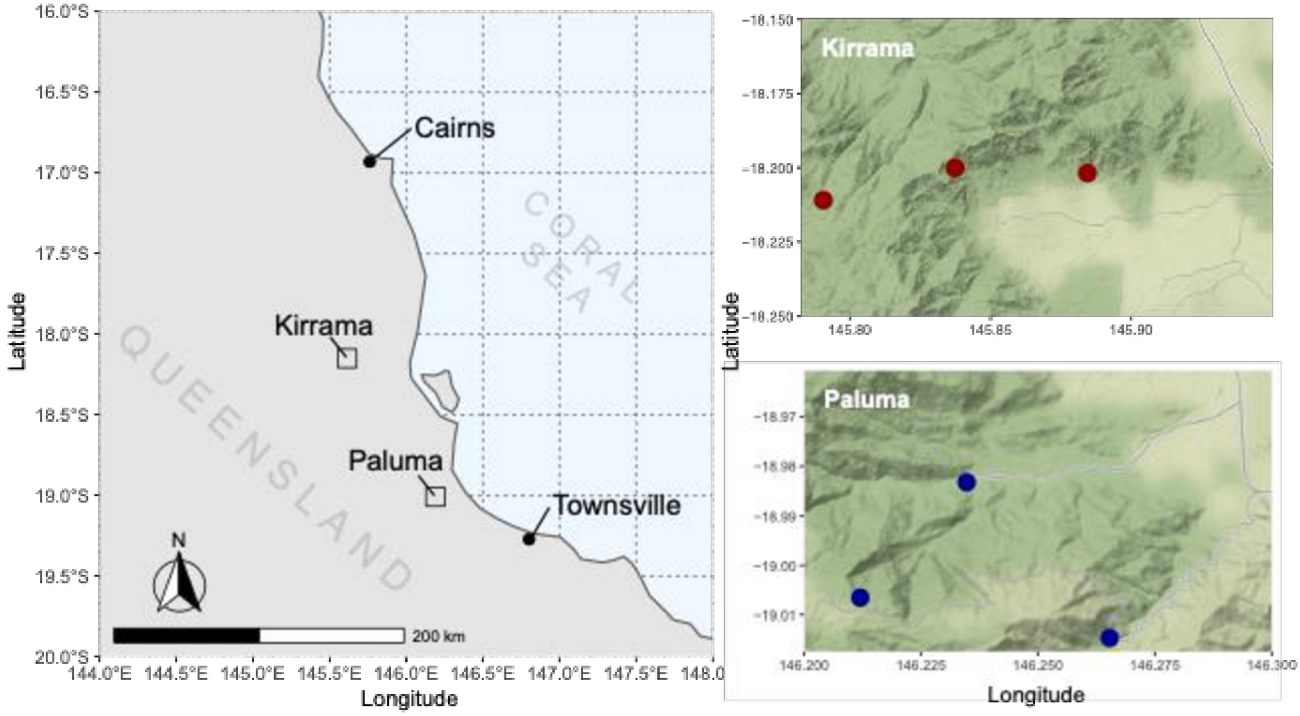

Figure 1: Location of sites used for this field translocation experiment in Queensland, Australia. Top right inset is the Kirrama gradient, bottom right inset is the Paluma gradient.

2.2 Insect community colonisation

Our focal community was forest-dwelling, frugivorous *Drosophila*, their hymenopteran parasitoids (that we described in Jeffs et al., (2021) and Lue et al., (2021)), and their endogenous microbiomes (Chapter 3). We obtained source flies and wasps using glass vials (2.5 cm diameter x 9 cm high; LabTek) 1/4 filled with yeasted mashed banana placed in the field to attract flies. Bananas were mashed 24 hours prior to field placement, with a 1/4 tsp of bakers' yeast. We included a range of banana consistencies in each vial to attract different *Drosophila* species that utilise bait of different decomposition stages and added a 2.5 cm x 7.5 cm strip of 280gsm folded coaster board into each vial to provide larvae with a pupation site.

We placed vials inside 5L (230 mm x 240 mm x 230 mm) plastic buckets (henceforth 'colonisation buckets') hung from branches by twine between 1 - 2 m above the

ground. We covered colonisation buckets with an aluminium foil tray (30 x 60 cm) to prevent flooding, and a 1 m x 1 m black plastic sheet tented across the branch from which the colonisation bucket was hanging, to prevent from flooding and direct sunlight. We placed ten colonisation buckets at each high elevation site; Paluma High (PH; ~880 m) and Kirrama High (KH; ~730 m). Colonisation buckets were separated by a minimum of 50 m along total transect lengths of 1 km and were placed a minimum of 5 m inside the closed forest bordering mountain roads or walking tracks.

We placed 32 bait vials in colonisation buckets at Paluma on days 1, 6, and 11 of the experiment, and vials at Kirrama on days 3, 8, and 13 (Fig. 2). Batches of vials were colonised for ten days each before being transferred to sealed 'experimental cages' (Fig. 2). This ten-day colonisation period maximises the diversity of colonising fly and parasitoid species that may utilise different baits/hosts without allowing any pupal emergence for that generation. Staggering vial colonisation increases overlap of generations within and between species to ensure an asynchronous mixed community at the end of the experiment when food-web structure is being assessed (see below).

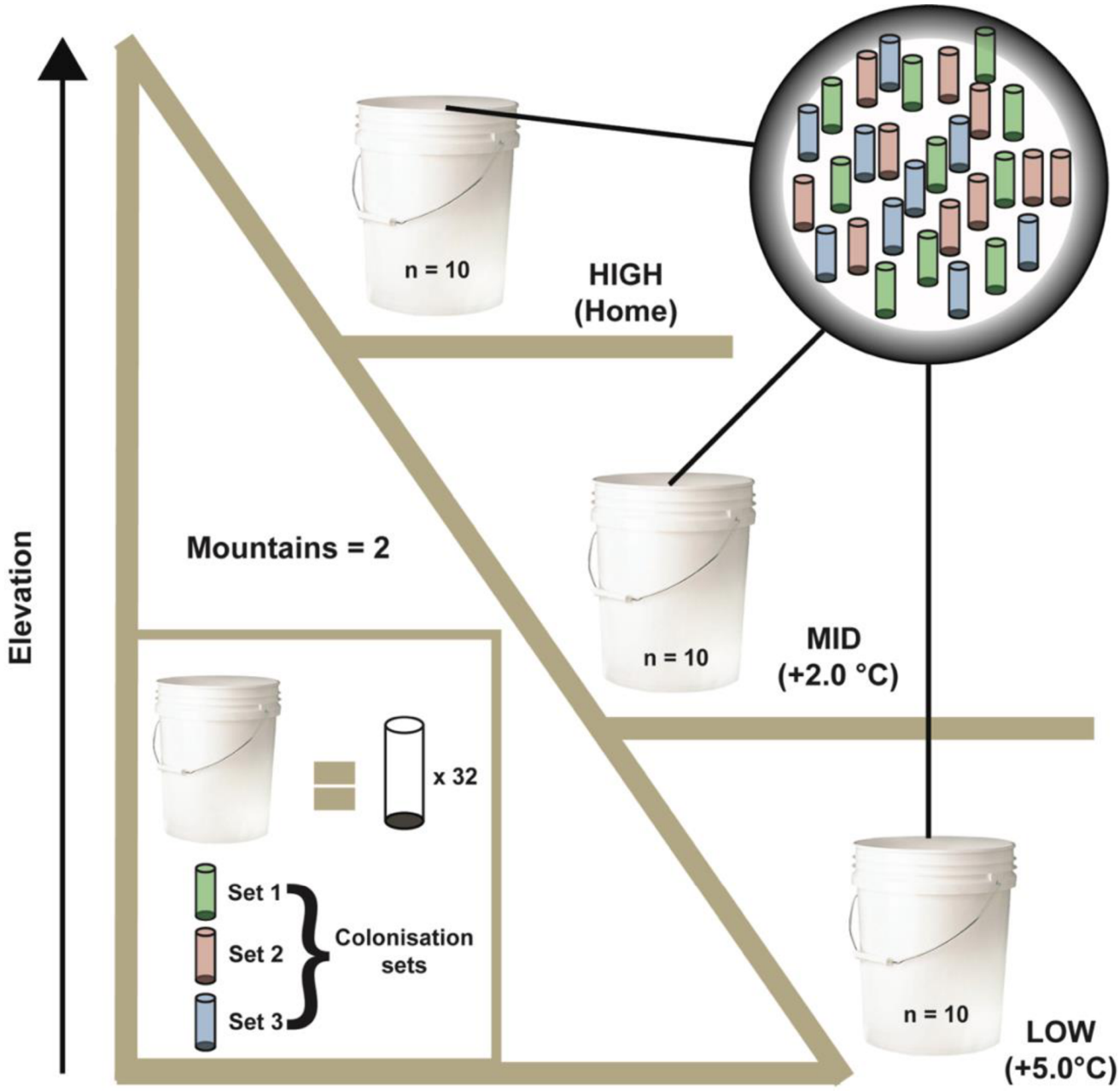

Figure 2: The experimental set up along both of our elevational gradients. After our community colonisation process each enclosed experimental community had the same number of vials from 3 colonisation sets, providing a homogeneous and diverse community to begin the translocation experiment with.

2.3 Insect community rearing

Experimental cages (henceforth just 'cages') consisted of a 5L plastic bucket sealed with 15 denier nylon mesh to keep experimental insects inside and prevent additional entrants, ensuring a closed community. To prevent interference/damage from larger

animals we fastened a dome of wire mesh around cages, which were then hung 1 - 2 m below branches with twine. Cages also had aluminium foil dishes attached above the wire mesh and were hung 30 cm under 1 m x 1 m plastic sheets. We accessed vials within the cage through a tied-off extension of nylon mesh once removed from the wire mesh dome. We added Tanglefoot insect glue around the circumference of the bucket and reapplied it every week to prevent insects (primarily ants) crawling onto and chewing through the nylon mesh, thus opening our closed community. We set up ten experimental cages at a high, middle, and low elevation site along both transects, totalling 60 experimental cages at six experimental sites. We put temperate and relative humidity dataloggers (EasyLog USB Data Logger, Lascar Electronics) on the outside of seven randomly selected cages per site, and a logger on the inside of three of the seven selected cages with a reading taken every hour or half-hour for the duration of sampling (based on estimated battery longevity) (Figs. S1 & S2).

At the end of the ten-day colonisation period for each staggered batch of vials, we split the 32 vials per colonisation bucket between 30 experimental cages (one vial in each of the 30 cages across all elevations per transect) with the two remaining vials frozen for sorting (see Fig. 2). Thus, after the third translocation of colonisation vials, each experimental cage contained 30 vials from the same colonisation source. Once all colonisation vials were in experimental cages, we allowed the community to develop for 70 days within the sealed cage until sampling at the end of the experiment (see below). We periodically replaced older vials with fresh banana bait vials to provide new substrate for community development.

Thirty-five days after vials were sealed in cages, we replaced five vials from each batch of 10 colonisation vials with vials of mashed banana and pupation card (prepared as described above in section *2.2 Insect community colonisation*). The bait and pupation card from the removed colonisation vials was added to the remaining colonisation

vials within the cage, to make sure that we did not accidentally remove slowly developing parasitoids. On experiment days 26 & 27 we temporarily removed all experimental cages from Kirrama and Paluma respectively due to predictions that Severe Tropical Cyclone Debbie would pass through our study sites. All cages were placed within a 22°C 16:8 light:dark controlled temperature room at James Cook University, Townsville (JCU). We added four new banana bait vials inside each cage on Day 28 and returned all cages to their exact original positions in Kirrama and Paluma on Day 32 and Day 33 respectively. We removed the remaining 5 colonisation vials 45 days after their initial placement into experimental cages and replaced them with five new vials of mashed banana with pupation card. These new vials were removed 10 days later, sealed with tissue paper-covered foam stoppers and reared in the JCU temperature-controlled room, to see for the presence of parasitoids. A complete schedule of the experiment is available in the Supplementary Information for clarity (Table S1).

2.4 Temperature data

In addition to data loggers on experimental buckets (as described above in *2.3 Community rearing*), we also have long-term data from February 2016 - April 2018. These loggers were placed at all six sites used in this study (PH, PM, PL, KH, KM, KL), and collected readings every 12 hours for a 2-year period.

2.5 Pupae and larvae collection

At the end of the experiment, we selected seven experimental cages from each site for sampling. For each cage, five vials were collected in two staggers 8 days apart, with three vials randomly selected for comprehensive sampling of pupae and larvae.

Immediately after collection from the field, we removed the original pupation card within each vial and froze it at -15°C in separately labelled vials. We subsequently

added a replacement pupation card into the vial, then removed and froze it three days later. We repeated this process two more times resulting in a total of four pupation cards per vial that spanned a nine-day period post-removal from the cage. The staggered collection of multiple pupation cards allowed us to sample species with different development rates until all individuals were collected from each vial. We checked vials twice daily to remove low numbers of emerging adults and prevent oviposition.

We chose two vials from each cluster of five for each stagger from each cage for sorting. To sort them, we put each frozen pupation card on a plastic plate with 0.5 cm of water and used a fine-tip paintbrush to break up the card and store pupae and larvae within individual wells of 96-well PCR plates, then added 2 ml of 96% ethanol to preserve the samples. Plates were sealed and frozen at -15°C until shipping at ambient temperature between JCU and the Biology Centre of the Czech Academy of Sciences, Ceske Budejovice, Czech Republic. Upon arrival, all samples were immediately frozen at -20°C to minimise DNA degradation. We then selected the three experimental buckets with highest abundance at each site, and randomly selected samples from these buckets for extraction.

2.6 DNA isolation and host sequencing

Samples were extracted using single column GeneAid Blood and Tissue kits, according to manufacturer instructions. Each set of 29 extracted samples was accompanied by an 'extraction negative control' (ENC). We used custom-developed multiplex PCR primers based on COI and/or ITS2 genes for identification of 11 *Drosophila* species previously detected in the studied community (Jeffs *et al.* 2021). In cases where the result of multiplex PCR identification was ambiguous, we sequenced the diagnostic locus. All samples were screened for parasitic wasps using custom PCR detection primers based on 28S D2 gene region.

2.7 *Microbiome sample selection and sequencing*

For microbiome sequencing, we selected a stratified subset of 360 field samples from the three most common species from our sample identifications: *Drosophila rubida, D. pseudoananassae,* and *D. sulfurigaster*. We selected these species because they are naturally ubiquitous across the elevational gradient and because species identity had a major effect in our previous analyses (Chapter 3), so we wanted to be able to understand microbiome changes beyond simple host species turnover. All samples were subsequently moved to 96-well plates in a randomised order. For each sequencing plate, we also included one negative control from the extraction procedure (ENC), two negative controls from the PCR process (NCs), a blank well, and two positive controls (PC) of mock microbiome communities, totalling 384 samples. The PCs were supplied commercially and comprised 4 samples of gDNA templates with equal abundance of 10 bacterial species (ATCC® MSA-1000™) and 4 samples with staggered abundance for the same bacteria (ATCC® MSA-1001™). Each plate contained a 'staggered' and an 'equal' mock community.

DNA templates were used for 16S rRNA gene amplification according to Earth Microbiome Project standards (EMP; http://www.earthmicrobiome.org/protocols-and-standards/16s/). We used the EMP-proposed double barcoding strategy with their recommended modifications (12 bp Golay barcodes included on the forward primer 515F, and additional 5 bp barcodes on the reverse primer 926R) for sample multiplexing. We also used our custom 18S rRNA gene blocking primer (named 926X) to counteract the low specificity of EMP primers towards the 16S rRNA gene (details in Brown et al., 2020). We amplified a ~400 bp portion of the 16S rRNA V4/V5 hypervariable region. Triplicate PCR amplification was confirmed with gel electrophoresis. We used AMPure XP (Beckman Coulter) magnetic beads to purify PCR products, which were subsequently pooled to equimolar concentration (based

on concentration measurements with a Synergy H1 (BioTek) spectrophotometer). Then we used Pippin Prep (Sage Science) to eliminate all fragments outside of 300-1100 bp range. The purified libraries were sequenced on a single run of the Illumina MiSeq platform using v3 chemistry with 2 x 300 bp output (Norwegian High Throughput Sequencing Centre, Department of Medical Genetics, Oslo University Hospital).

2.8 Sequence processing

The sequencing process returned 16,522,311 reads. These raw reads were quality checked (FastQC; Andrews 2010) and trimmed using USEARCH v9.2.64 (Edgar 2013), keeping the quality score above Q20. Then we trimmed the primers, demultiplexed the reads, and merged them, which resulted in a final amplicon length of 357 bp. We then clustered the reads at 100% identity for a representative set of sequences. We used the USEARCH global alignment option at 97% identity for *de novo* OTU assignment (Edgar 2013). We used the BLAST algorithm (Camacho *et al.* 2009) on the representative sequences, matching them against the SILVA 138 database for taxonomic identification. Finally, we removed chloroplast sequences and mitochondrial OTUs using QIIME 1.9 to produce a dataset at 97% identity. To show that our bioinformatic processing had minimal influence on the results, we analysed the final OTU table as two independent datasets. The 'regular' dataset was made by following the steps described above. We followed the same steps to make the 'ultraclean' dataset, and then employed more stringent filtering. We kept OTUs that matched the following criteria: representing more than 1% of reads in a sample and being found in more than one sample.

We used the R package 'decontam' (V1.5.0; Davis *et al.* 2018) to identify potential contaminant sequences from our negative controls. Examining sequence abundance in our NCs indicated 9 contaminant OTUs, which were excluded. We also excluded

any singletons or OTUs with less than 5% abundance in a single sample. We set the minimum threshold to 5000 reads based on the maximum number of non-contaminant reads in negative controls, which excluded 24 samples. Then we normalised the reads to get proportional relative abundance of OTUs across each sample. The final dataset encompassed 77 different bacterial OTUs for 350 samples.

2.9 Statistical analyses

For insect community analysis, we used raw species abundance data and quantified Bray-Curtis community dissimilarity at each site and tested differences with Mantel tests, using the package 'vegan' (Oksanen *et al.* 2019). In each ordination analysis, we treated 'site' as a proxy for temperature because each site is a combination of gradient and elevation. For microbiome analysis, we used Shannon index, and Bray-Curtis dissimilarity as community quantitative measures and calculated ordination analyses (non-metric multidimensional scaling; NMDS) accompanied by PERMANOVA tests to determine significant community difference, using 'vegan' and 'phyloseq' (McMurdie & Holmes 2013) in R (R Core Team 2019).

We found a mean average of 26,586 reads per sample and a median of 23,574 reads (not including NCs). The OTUs in our positive controls matched the expected community composition in each of the 'staggered' and 'even' mock communities. In the even mock communities, there was slight overrepresentation of *Clostridium beijerinckii* and *Escherichia coli* (1.4x - 2.0x expected), leading to small reductions in *Rhodobacter sphaeroides* and *Enterococcus faecalis*. The other taxa were consistently present at ~10% relative abundance. In the staggered mock communities, there were two species present at 0.04% and our sequencing detected reads of those species in all four staggered mock samples. Our positive controls were consistent with our previous sequencing results (Rodríguez-Ruano *et al.* 2018; Brown *et al.* 2020; Chapter 3).

3. Results

3.1 Insect community analysis

The dominant species at all sites by the end of the experiment was *D. pseudoananassae* (Fig. 3). *D. sulfurigaster*, *D. rubida,* and *D. pallidifrons* were the only other species found in communities at every site on both transects (Fig. 3). There was no significant difference in community Shannon index across elevation along either gradient (PERMANOVA; F = 1.268, R^2 = 0.241, p = 0.353). Site explained 24% variation, but it was non-significant.

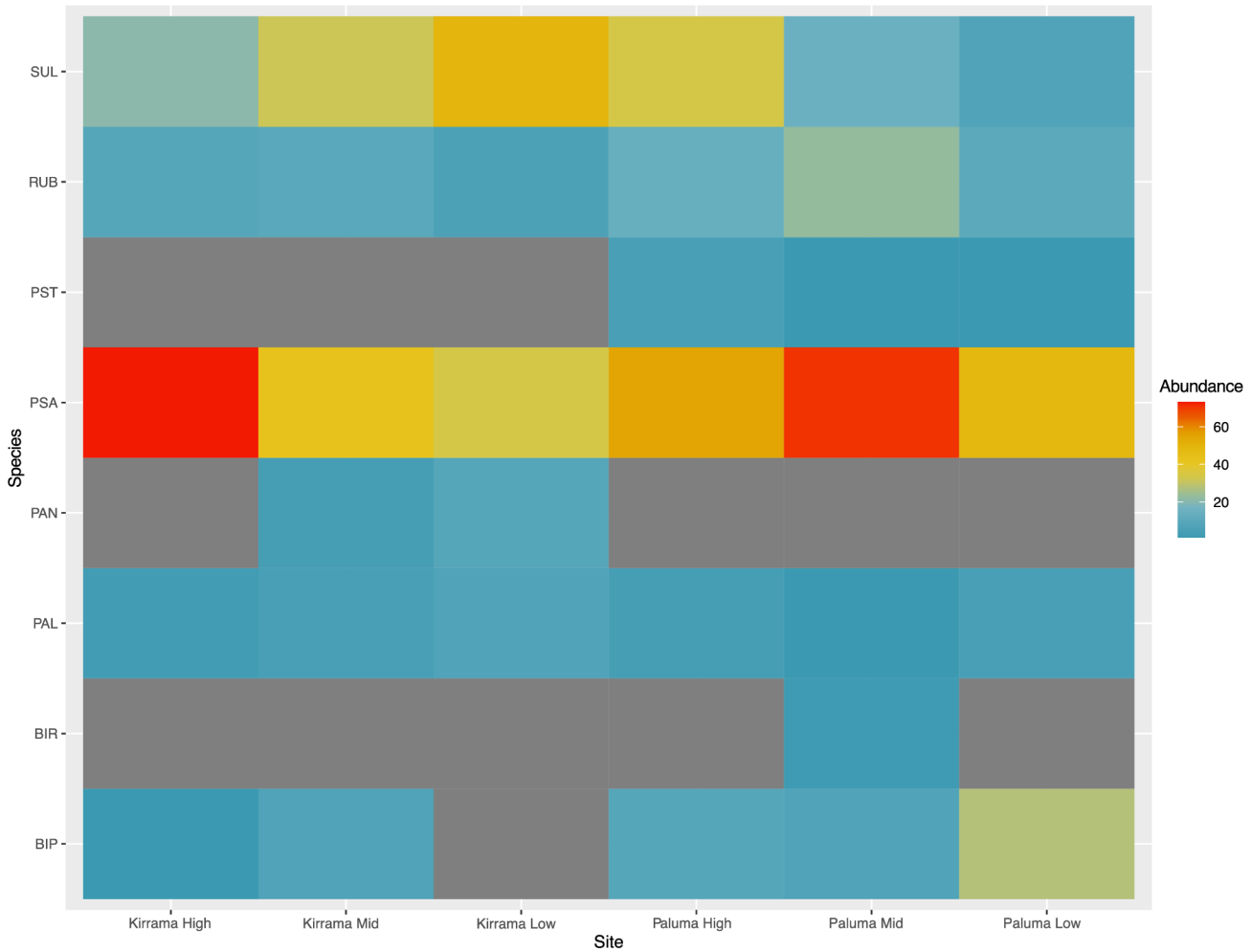

Figure 3: Heatmap of *Drosophila* species abundance by site. Grey tiles have zero abundance. SUL = *D. sulfurigaster,* RUB = *D. rubida,* PST = *D. pseudotakahashii*, PSA

= *D. pseudoananassae,* PAN = *D. pandora,* PAL = *D. pallidifrons,* BIR = *D. birchii,* BIP = *D. bipectinata.*

We found a small number of parasitised samples, with no discernible effect of site (Table 1). Parasitism rate varied between <1% and 7.5%.

Table 1: Parasitised samples by site

Site	**Total parasitised samples**	**Parasitism %**
Paluma High	**2**	**2**
Paluma Mid	**9**	**7.5**
Paluma Low	**3**	**3**
Kirrama High	**1**	**0.9**
Kirrama Mid	**4**	**4**
Kirrama Low	**1**	**0.9**

3.2 Microbiome community analysis

There were minimal differences in microbiome alpha diversity between species and life stage at each site. However, beta diversity metrics show that life stage was the most influential factor in determining microbiome composition (NMDS, mean stress ≈ 0.24; PERMANOVA R^2 = 0.234, $p \leq 0.001$, with non-significant Beta-dispersion F = 0.654, p = 0.414 on 999 permutations; Fig. 4). Site and host species identity explained little variation in the NMDS (7% and 4%, respectively, compared to 23% variation explained by life stage). There were also minimal differences in microbiome diversity according to experimental cage of origin (Shannon index values; Fig. S3), suggesting that there was not much variation in microbiome composition caused by separate cages.

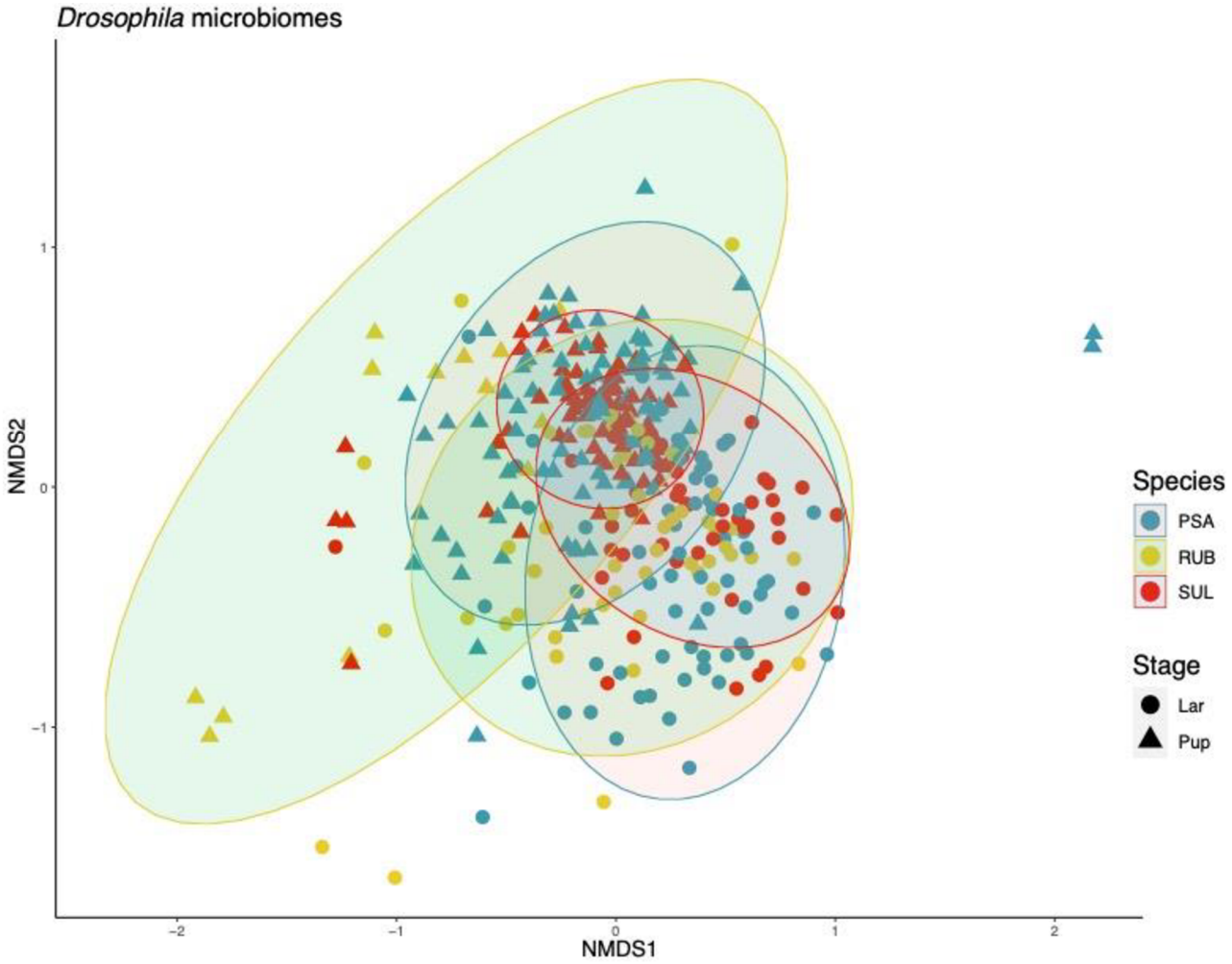

Figure 4: NMDS of microbiome Bray-Curtis dissimilarity values from all individual samples of three species and two life stages.

The dominant bacterial genus in *Drosophila* larvae was an uncultured genus in the Orbaceae family, whereas in pupae the dominant genera were *Komagataeibacter* and *Acetobacter* (Fig. 5). Combined, these 3 genera regularly comprised over 50% of the microbiome. *D. pseudonananassae* retained *Wolbachia* throughout the duration of the experiment, and none of the other *Drosophila* species obtained *Wolbachia*, suggesting there was no horizontal transmission induced by our experimental conditions.

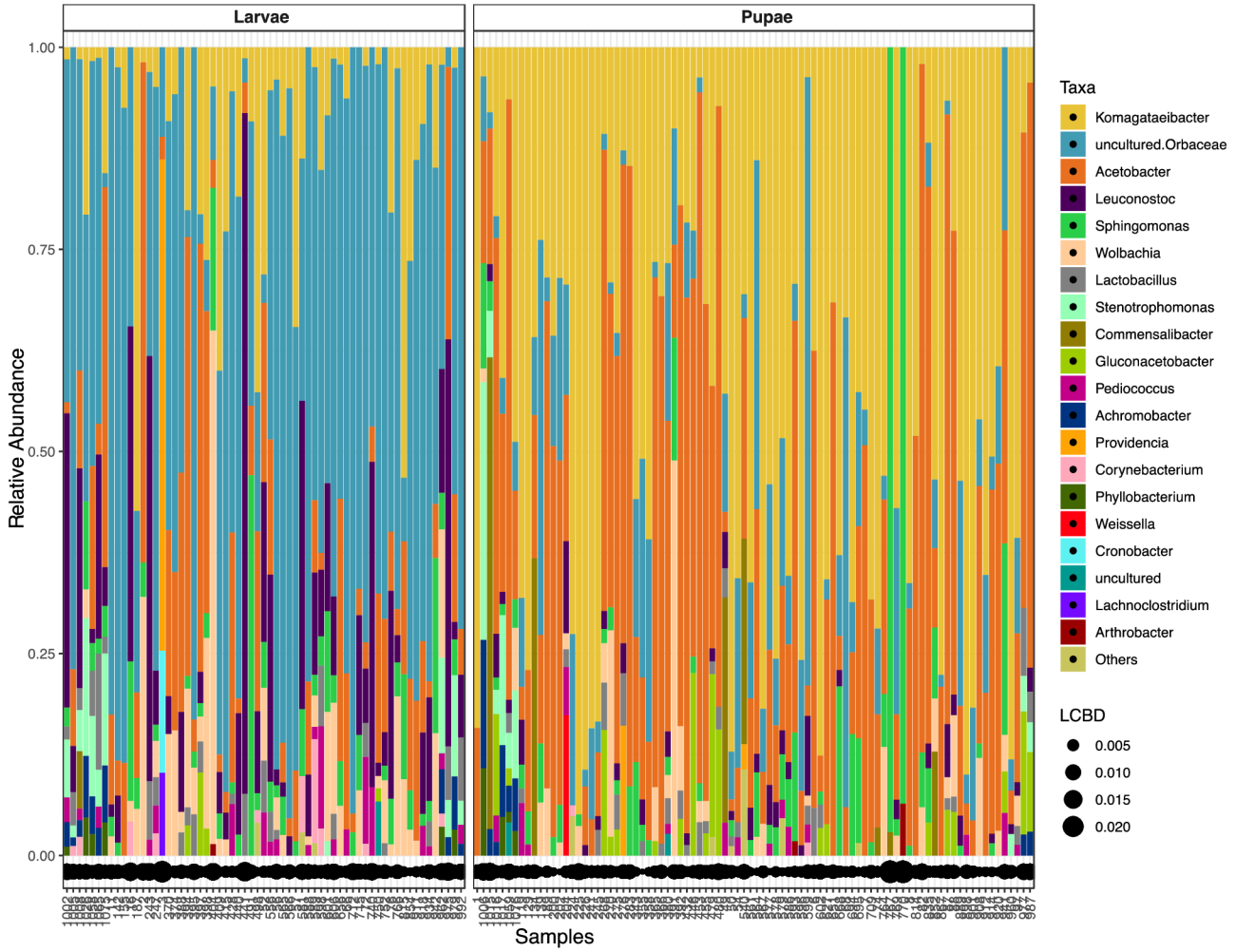

Figure 5: Dominant bacterial genera in microbiomes of *D. pseudoananassae* larvae and pupae. LCBD = Local Contribution to Beta Diversity.

4. Discussion

Our results provide revealing insights into the effects of climate change on a tropical insect community. We expected that the slowest developing species and high elevation specialists would be most likely to go locally extinct in communities at warmer temperatures, based on existing knowledge of tropical insect temperature sensitivity (Deutsch *et al.* 2008; Shah *et al.* 2017b, a; Montejo-Kovacevich *et al.* 2020). However, we found that *D. pseudotakahashii* - a high elevation specialist - did not go extinct at lower elevations during the duration of this experiment (although n = 1 at Paluma middle and low elevations, so it could be functionally extinct). Possibly if the experiment was continued for more time *D. pseudotakahashii* might have been lost

from the community completely. Similarly, *D. rubida*, the largest-bodied and slowest developing species - traits considered unfavourable in warming conditions - was not lost from communities at any site and was actually the third most abundant species. Thus the species predicted to be most vulnerable to climate change performed surprisingly well in this study. *D. pseudoananassae* achieved ubiquitous dominance at each site, not just in warmer environments. This is notable because *D. pseudoananassae* does not dominate natural communities to the extent shown in this experiment (see Fig. 3 in Jeffs *et al.* (2021)) suggesting that it might have had a competitive advantage in our experimental setup. *D. pseudoananassae* is one of the smallest species in this community, and one of the fastest developing (generation time can be as fast as 8 days in high temperatures; Thierry *et al.* 2021). Thus it has life history traits favourable for success in artificially enclosed environment.

Since abiotic factors did not strongly influence community composition, biotic factors or ecological drift may have played a more influential role. All communities are created by the same interacting processes: selection, dispersal, drift, and diversification (Vellend 2010). Our experimental setup prevented dispersal, and the timescale of our experiment was not sufficient to result in diversification. It is therefore more likely that factors structuring this community could have been drift and selection through biotic interactions. Yet given the apparent lack of successful parasitism, apparent stability of microbiome communities, and fairly consistent communities across buckets, it appears that biotic interactions were not a strong structuring factor either. This has added importance when the experimental setup could have created artificially high competition and parasitism, but apparently did not (see below). One possible explanation is that our results match the hypotheses presented by Saito *et al.* (2021) who suggest that higher temperatures result in a greater proportion of individual deaths from metabolic processes (McCoy & Gillooly 2008), leading to reduced competitive differences between species. Their hypothesis

proposes that populations (and by extension: communities) are under weaker control of niche-based processes leading to added importance of stochasticity, which matches our findings. Thus we conclude that, at this experimental scale, ecological drift was an important component in structuring and maintaining these insect communities (Siqueira *et al.* 2020).

Interestingly, the translocation seemed to have minimal impact on community structure, suggesting that abiotic factors (e.g. temperature) did not have strong influence on our communities. We know from lab experiments on this system (Terry *et al.* 2021; Thierry *et al.* 2021; N. Pardikes & M. Gonzalez, *unpublished data*) that there are limits to species co-existence and thermal tolerance, and trophic interactions change with temperature (Barton 2010; Gilbert *et al.* 2014; Frances & McCauley 2018; Bartley *et al.* 2019), yet in this field experiment temperature did not have a strong effect. This could be a product of daily temperature cycles providing some respite (Paluma typical daily variation ~5°C, highest = 18°C; Kirrama typical daily variation ~4°C, highest = 20°C), whereas lab experiments are routinely performed at constant temperatures, so study organisms are under consistent thermal stress. However, whilst the *Drosophila* community seemed to handle warmer environments well, temperature increases could have been more detrimental to host-parasitoid trophic interactions. Jeffs & Lewis (2013) identified three primary responses of parasitoids to warming: i) changing distributions to cooler environments, ii) phenological shifts, and iii) persistence through phenotypic plasticity or adaptation. In this experiment the first option was not an available response, so the experimental parasitoids could only rely on phenological shifts or their ability to adapt to local conditions. A lab heatwave experiment on species in this community found that exposure to 34°C for 4 hours was enough heat shock to fundamentally change parasitoid survival, so we anticipate parasitoids in our experimental cages could not overcome exposure to extreme heat either (N. Pardikes, *unpublished data*).

In *Drosophila* microbiome communities, there were minimal effects of temperature on community composition. Instead, life stage was the most important factor for explaining variation in community composition. On one hand, this is not a surprising result because holometabolous insects undergo complete metamorphosis, which has well-documented effects on gut physiology and microbiome composition (Hammer & Moran 2019). On the other hand, it was surprising to see such strong, consistent differences between larvae and pupae, specifically because a pupa is in the process of development. In other words, the pupae we sampled had not completed their transition to adult and therefore had not yet completely turned over their gut physiology (and associated microbiome community). We also know that the sampled pupae were developing from larvae in the exact same micro-environment (experimental cage) with the same diet (banana bait). So whilst we *a priori* acknowledged the documented differences between larvae and pupae, we did not expect the differences in our results to be as substantial as they were, especially compared to other factors like temperature or species.

Furthermore, we know the individuals we sampled were in their experimental enclosures for anywhere between 6-11 generations (depending on species and temperature combination) and thus had not been properly exposed to the full environmental bacteria species pool for multiple generations, which will naturally reduce microbiome richness (Chapter 3). Between these results and those in Chapter 3, we have a reasonably complete picture of the microbiomes from common *Drosophila* species in this community. In Chapter 3 we found significant differences in microbiome composition between lab-reared and field-caught pupae. In this field experiment we kept wild-caught insects in quasi-captivity (enclosed in experimental cages), so their microbiomes are richer than those reared in the lab, but not as rich as those of 'truly wild' pupae. This is likely because the cage environment and controlled

diet restricted the size of the regional species pool, so there were less bacterial taxa available to colonise *Drosophila* microbiomes. Additionally, the experimental cages likely caused increased interactions between individual *Drosophila*, resulting in more microbial horizontal transmission and thereby making microbiome communities more homogeneous overall.

The dominant bacterial taxa in microbiomes from this experiment was an 'uncultured Orbaceae', which we did not find in our previous sequencing run (Chapter 3). The Family (and Order Orbales) was created to accommodate novel taxa sequenced from the guts of bees (Kwong & Moran 2013), and has since been found in high relative abundance in other plant-feeding insects (e.g. Lepidoptera, Hammer *et al.* 2020). One possibility is that our *Drosophila* have a potentially new gut bacterium. This would not be unprecedented, because cactophilic *Drosophila* in Mexico have *Orbus* in their microbiomes (Martinson *et al.* 2017). Given that many bacteria in Orbaceae come from plants, it seems likely that our 'uncultured Orbaceae' came from the banana bait. We note that bananas are not insect pollinated, so it is unlikely that this result is due to transmission of taxa from another insect into *Drosophila*, via banana bait. Nonetheless, it would be interesting to use more advanced molecular techniques to establish precisely what this taxon is.

Our results provide some encouragement for how insects and their microbiomes might respond to long-term temperature change. However, one component that we did not intentionally consider in this study but no less pertinent to climate change-related effects is extreme weather events, like heatwaves (Perkins-Kirkpatrick & Lewis 2020). Lab experiments suggest that the species involved in this study have differing tolerance to short-term extreme heat shock events (N. Pardikes, *unpublished data*), with starkly different consequences depending on the life stage affected. Our experimental data loggers show that our communities were subject to strong heat

shock events and occasionally extreme day-to-day temperature variation. At Kirrama, the highest temperature experienced was 46°C for 2 hours and at Paluma the highest temperature was 38°C for 3 hours. Whilst we can't know precise differences in species abundances (because we didn't sample the communities immediately afterwards) these heat shock events clearly did not eliminate the community completely. Thus it appears that the natural *Drosophila* community is quite resilient to temperature change, both long-term and short-term. A logical future step would be to do an experiment combining long-term temperature changes with short-term heatwave events, to get a realistic picture of how species (and communities) respond to both aspects of climate change simultaneously.

We believe that our experimental set-up did not drastically influence the experiment for multiple reasons. Firstly, when comparing our end-of-experiment communities with the natural communities described in Jeffs et al., (2021), we can assert that our experimental enclosure functioned as intended and kept out non-target species. Secondly, we determined from our sorting procedures that vials from the end of the experiment had similar densities to colonisation vials from the beginning of the experiment colonisation vials (both frequently yielded 200+ larvae per vial). Thus we do not believe our cage environment lead to artificially high densities across the whole community, but it might have inflated the overall abundance of *D. pseudoananassae* (as outlined above). On a related note, the general abundance of flies at the end of the experiment suggests that there was minimal experimental disruption caused by the cyclone and associated safety measures. Thirdly, deliberately staggering the addition of fresh bait ensured that uncolonized bait was available to different fly and wasp species with different development times, enabling us to obtain a full picture of the natural community. Fourthly, we believe our starting experiment communities were homogeneous because splitting colonised vials evenly across all replicate experimental cages retains natural variation in starting

communities (due to random sampling effects) but reduces greater variation in starting community composition (due to random factors associated with the microhabitat of each colonisation bucket site). Finally, we note that humidity was not controlled by the experimental design, but our loggers show that it was 100% (or close to it) for the duration of the experiment. Thus any abiotic changes were much more likely to be a product of temperature.

Currently the most limiting factor in elucidating stronger conclusions from our data is sample size, which may be improved with further molecular analysis. We have not found a strong effect of translocation site on insect community dissimilarity, but trends might appear with a greater sample set. Similarly, we have low numbers of parasitized samples in our dataset. Some parasitoids were clearly able to survive until the end of the experiment because the number of parasitized larvae plus pupae is above zero, but we anticipated greater parasitoid abundance. This suggests that we did not artificially inflate parasitism rate with our experimental setup, and also that parasitoids might have suffered more during heat shock events (as discussed above).

Overall, our study shows that tropical insect communities have surprising resilience to warming temperatures based on our translocation-led simulation of climate change. This resilience is further represented by consistent maintenance of their microbiome communities. Community translocations over elevational gradients represent a valuable experimental tool to help us elucidate the effects of climate change. In future, we suggest that experiments combine long-term temperature change with short-term heat shock events, because these more extreme occurrences might be having a greater effect on community interactions.

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Supplementary Information

TABLE S1: Full experimental schedule from initial colonisation to final sampling

Day	Experiment Note
1	Paluma D1 Group 1
2	
3	Kirrama D1 Group 1
4	
5	
6	Paluma D6 Group 2
7	
8	Kirrama D6 Group 2
9	
10	
11	Paluma D11 Group 3/ SHIFT Group 1
12	
13	Kirrama D11 Group 3/ SHIFT Group 1
14	
15	
16	SHIFT Group 2
17	
18	SHIFT Group 2
19	
20	
21	SHIFT Group 3
22	
23	SHIFT Group 3
24	
25	
26	**Collected in Kirrama cages**
27	**Collected in Paluma cages**
28	**Added 4 fresh banana vials per cage**
29	JCU CT room

30	JCU CT room
31	JCU CT room
32	**Put back Kirrama cages**
33	**Put back Paluma cages**
34	PUT IN MORE BAIT VIALS = pre-cyclone ones stay as sources, the later ones collected in as parasitism assessments.
35	
36	
37	
38	
39	
40	
41	
42	
43	
44	
45	
46	Paluma 1st batch replacement vials out; replace Group 1
47	
48	Kirrama 1st batch replacement vials out; replace Group 1
49	
50	
51	Paluma 2nd batch replacement vials out; replace Group 2
52	
53	Kirrama 2nd batch replacement vials out; replace Group 2
54	
55	
56	Paluma 3rd batch replacement vials out; replace Group 3
57	
58	Kirrama 3rd batch replacement vials out; replace Group 3
59	
60	
61	
62	

63	
64	
65	
66	Paluma 1st batch final sampling vials out
67	
68	Kirrama 1st batch final sampling vials out
69	
70	
71	
72	
73	
74	Paluma 2nd batch final sampling vials out/ 1st batch in
75	
76	Kirrama 2nd batch final sampling vials out/ 1st batch in
77	
78	
79	
80	
81	
82	2nd Paluma batch in
83	
84	2nd Kirrama batch in

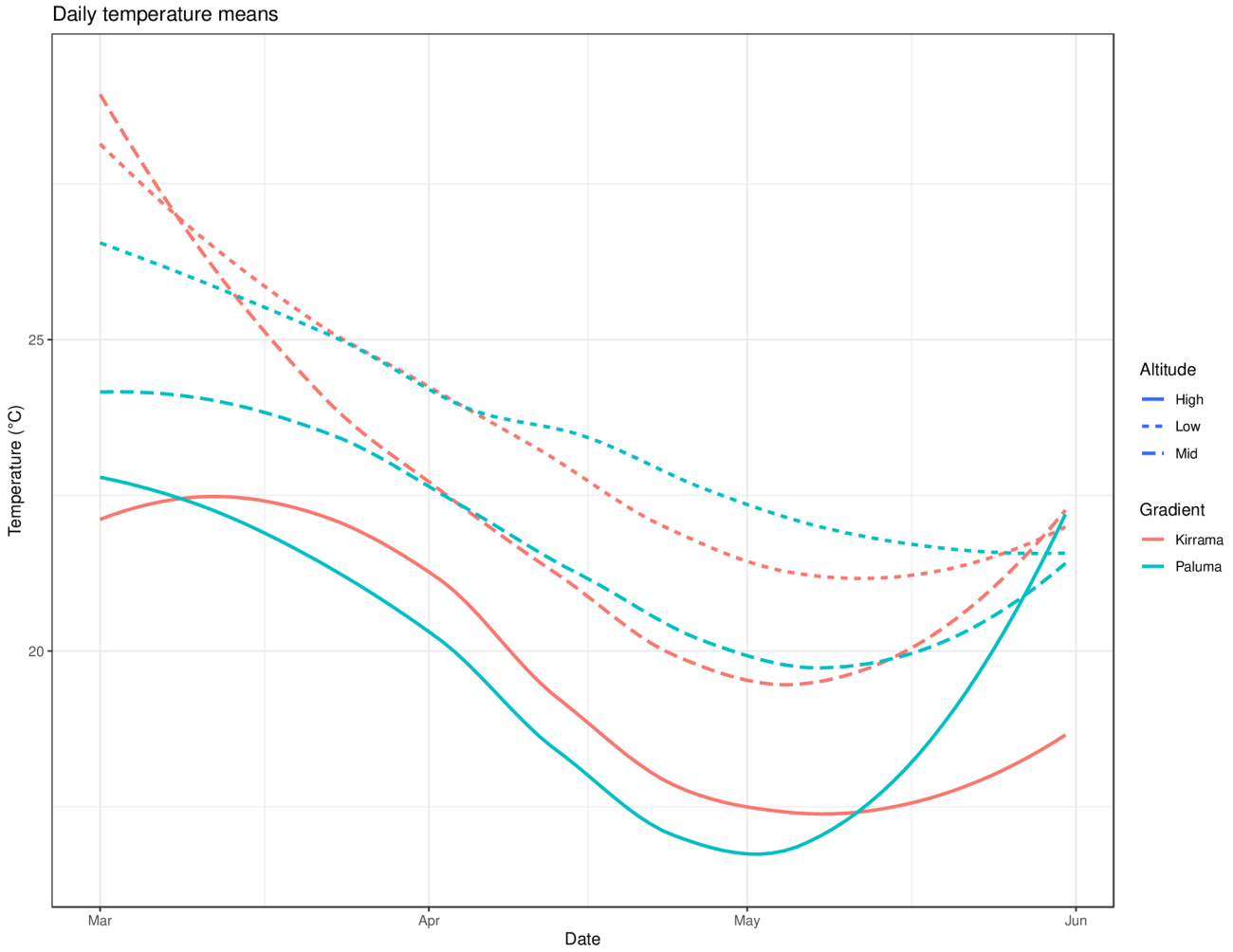

Figure S1: Mean temperature at each site for each day of the experiment. Different line types represent different elevations, Kirrama is shown in Red and Paluma is shown in blue.

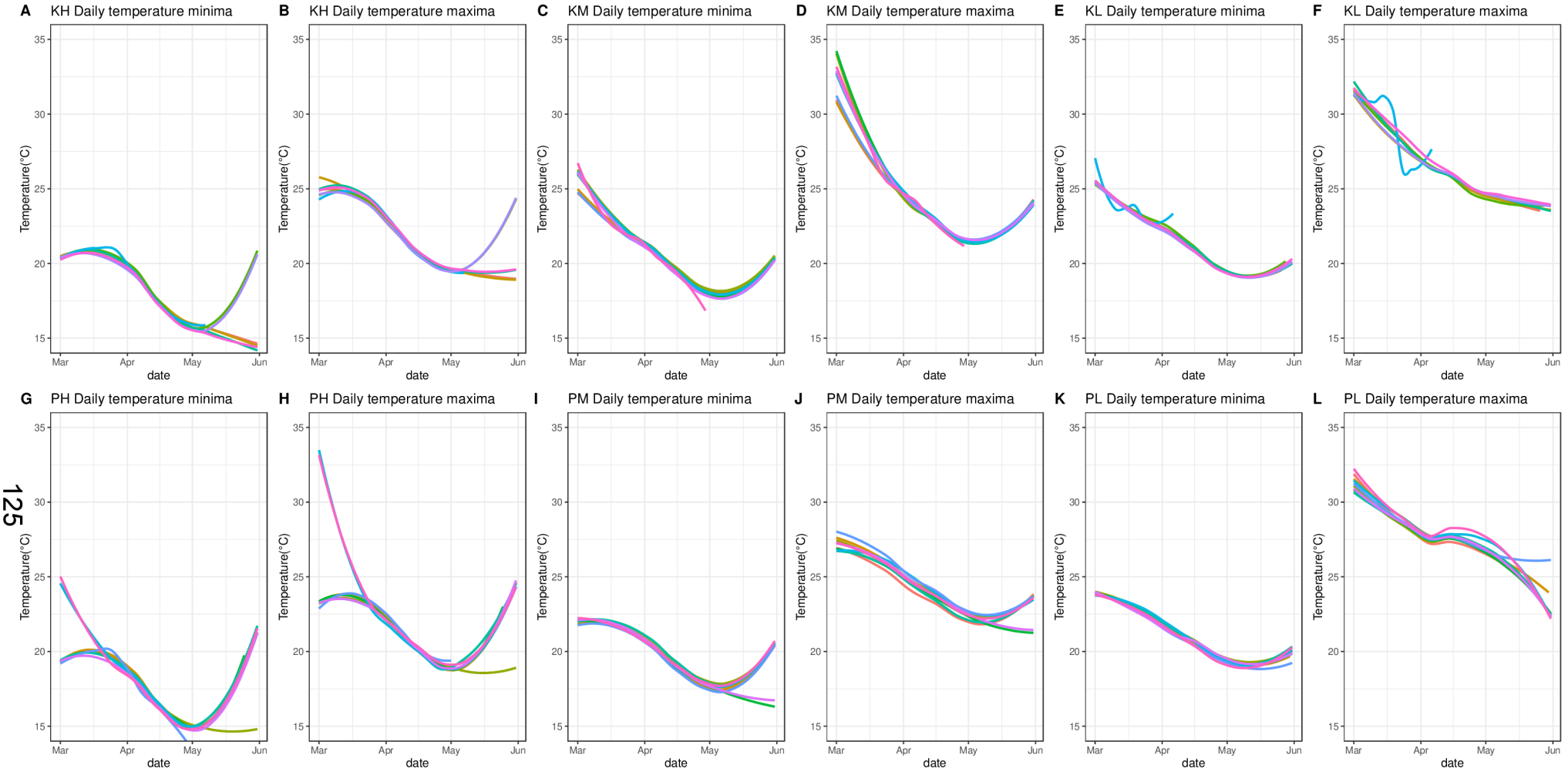
A KH Daily temperature minima
B KH Daily temperature maxima
C KM Daily temperature minima
D KM Daily temperature maxima
E KL Daily temperature minima
F KL Daily temperature maxima
G PH Daily temperature minima
H PH Daily temperature maxima
I PM Daily temperature minima
J PM Daily temperature maxima
K PL Daily temperature minima
L PL Daily temperature maxima
Temperature(°C)
35
30
25
20
15
Mar
Apr
May
Jun
date

Figure S2: Daily minimum and maximum temperatures at each site used in our experiment.

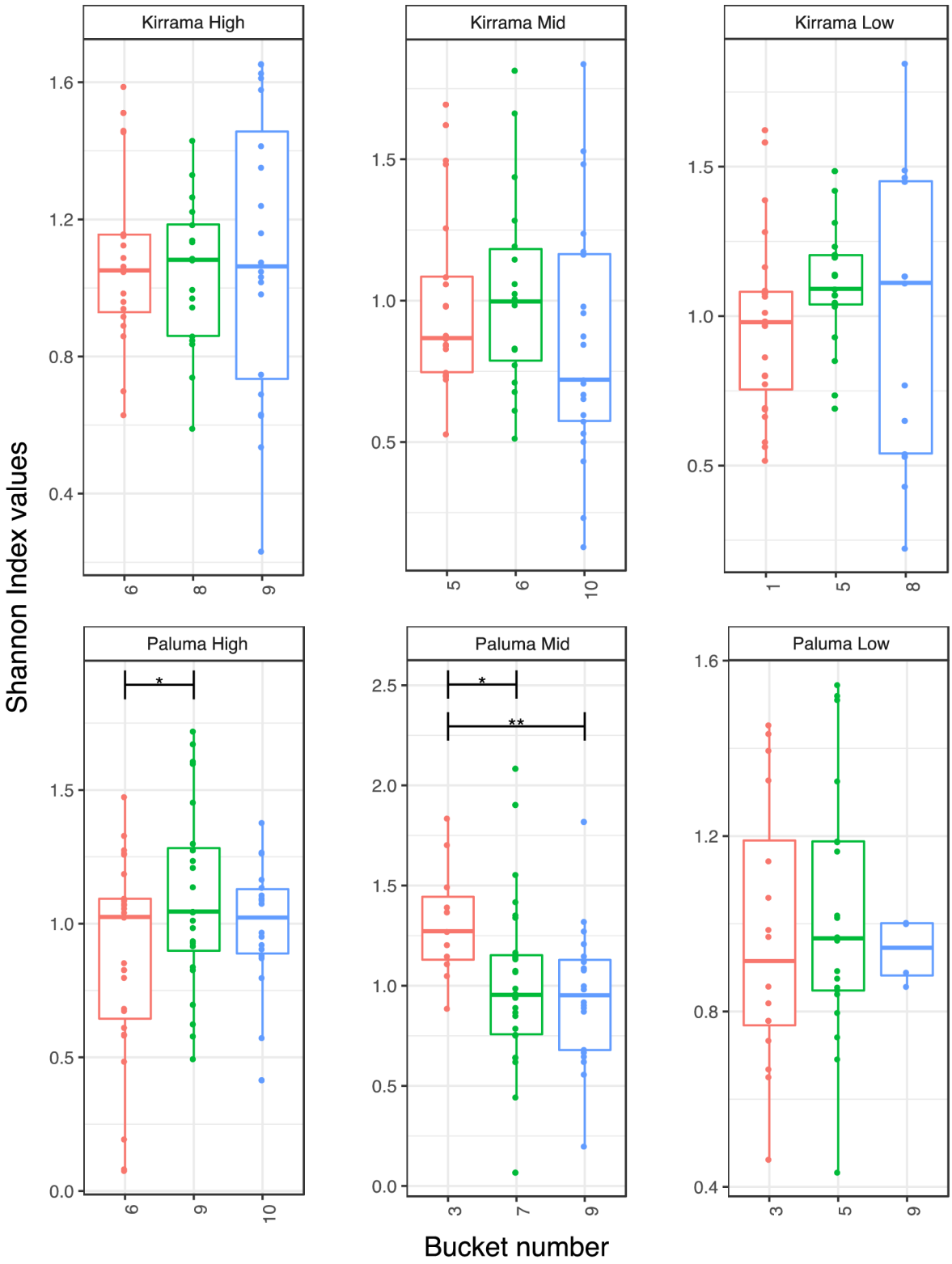

Figure S3: Shannon index values from each experimental cage at each site.

Chapter 5

Ontogeny, species identity, and environment dominate microbiome dynamics in wild populations of kissing bugs (Triatominae)

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RESEARCH Open Access

Ontogeny, species identity, and environment dominate microbiome dynamics in wild populations of kissing bugs (Triatominae)

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Abstract

Background: Kissing bugs (Triatominae) are blood-feeding insects best known as the vectors of *Trypanosoma cruzi*, the causative agent of Chagas' disease. Considering the high epidemiological relevance of these vectors, their biology and bacterial symbiosis remains surprisingly understudied. While previous investigations revealed generally low individual complexity but high among-individual variability of the triatomine microbiomes, any consistent microbiome determinants have not yet been identified across multiple Triatominae species.

Methods: To obtain a more comprehensive view of triatomine microbiomes, we investigated the host-microbiome relationship of five *Triatoma* species sampled from white-throated woodrat (*Neotoma albigula*) nests in multiple locations across the USA. We applied optimised 16S rRNA gene metabarcoding with a novel 18S rRNA gene blocking primer to a set of 170 *T. cruzi*-negative individuals across all six instars.

Results: Triatomine gut microbiome composition is strongly influenced by three principal factors: ontogeny, species identity, and the environment. The microbiomes are characterised by significant loss in bacterial diversity throughout ontogenetic development. First instars possess the highest bacterial diversity while adult microbiomes are routinely dominated by a single taxon. Primarily, the bacterial genus *Dietzia* dominates late-stage nymphs and adults of *T. rubida*, *T. protracta*, and *T. lecticularia* but is not present in the phylogenetically more distant *T. gerstaeckeri* and *T. sanguisuga*. Species-specific microbiome composition, particularly pronounced in early instars, is further modulated by locality-specific effects. In addition, pathogenic bacteria of the genus *Bartonella*, acquired from the vertebrate hosts, are an abundant component of *Triatoma* microbiomes.

(Continued on next page)

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(Continued from previous page)
Conclusion: Our study is the first to demonstrate deterministic patterns in microbiome composition among all life stages and multiple *Triatoma* species. We hypothesise that triatomine microbiome assemblages are produced by species- and life stage-dependent uptake of environmental bacteria and multiple indirect transmission strategies that promote bacterial transfer between individuals. Altogether, our study highlights the complexity of Triatominae symbiosis with bacteria and warrant further investigation to understand microbiome function in these important vectors.

Keywords: Bacteria, Blood, Hematophagous, Insect, Microbiome, Ontogeny, Pathogen, Triatominae, Vector

Background

Kissing bugs (Hemiptera: Reduviidae: Triatominae) are hemimetabolous blood-feeding insects predominantly found across the Americas. They are the vectors of Chagas' disease (CD; caused by the trypanosomatid parasite *Trypanosoma cruzi*) which the majority of ~ 150 known species can transmit to a wide range of mammalian hosts, including humans [1, 2]. There are 11 endemic North American species, whose epidemiological relevance has been overlooked compared to their neotropical relatives. However, multiple recent studies have recorded high prevalence of *T. cruzi* in kissing bugs and reservoir mammals, like packrats, racoons, and opossums [3–5], and others have confirmed cases of autochthonous human CD in the USA [6–10]. Thus, *T. cruzi* transmission by native US vectors has become a current health concern, emphasising the need for in-depth understanding of triatomine vector biology.

Hematophagous (blood-feeding) organisms are broadly affected by their associated microbial communities (referred to as the "microbiome"). Microbiome diversity, composition, and function directly influence various fundamental aspects of host biology, such as immunity, thermal tolerance, and digestion [11–13]. Nutritionally, blood is rich in proteins and salt, lacks vitamins, and its breakdown releases toxic amounts of haem and urea [14, 15]. Many symbiotic bacteria facilitate blood meal digestion and synthesise essential vitamins, making them important mutualists for their hosts [16–23]. Furthermore, the gut bacteria of hematophagous vectors interact with parasites (like *T. cruzi*) occupying the same niche [2, 24, 25]. The microbiome can potentially impede parasite transmission through direct (competition for resources) and indirect (promoting immune response) interactions [26–32]. The most comprehensive background on hematophagous microbiomes has been derived from mosquitos, ticks, and tsetse flies (reviewed in [33]), whereas triatomine-bacteria associations remain neglected.

To establish a basic background for studying the dynamics and potential function of Triatominae microbiomes, we need to elucidate the main factors determining their composition. In other systems, microbiomes usually display species specificity, i.e., they differ even among closely related host taxa (e.g., [34]). In some cases, the differences reflect the phylogenetic relationships of the hosts (phylosymbiosis, e.g., [35]). On the other hand, since environment is a natural source of at least some portion of the microbiomes, the habitat and geographic location of the host may significantly affect microbiome composition (e.g., [36–39]). In triatomines, thirteen high throughput sequencing studies published since 2015 have indicated a wide range of factors that potentially influence their microbiomes. However, it remains difficult to derive any consistent cross-species patterns since the studies utilised a wide variety of DNA templates, including pooled or individual bodies, entire abdomens, the distal part of the abdomen, whole guts, midguts, faeces, and cultured bacterial colonies [40–52], and were often further complicated by other variables (e.g., sex, locality, instar, *T. cruzi* infection status). Apart from these methodological differences, the disparity among studies could also reflect true biological characteristics of Triatominae. These include lengthy development times through five nymphal instars [48–52], complex physiology of the alimentary tract [48], and accessory feeding strategies, like haemolymphagy (feeding on arthropod haemolymph), kleptohematophagy (stealing a blood meal from another triatomine), and conspecific coprophagy (feeding on faeces) known to be employed by some triatomine species [2, 53–56].

To date, only a single study [52] has sampled microbiomes from multiple wild populations of any triatomine species. Others have mostly targeted South American vectors in domestic environments or laboratory-reared specimens [44–49], with little consideration for non-urban systems. In this study, we thus focus on wild populations of 5 *Triatoma* species in southern Texas and Arizona. Sampling triatomines within the nests of a favoured host, the white-throated woodrat (*Neotoma albigula*), substantially increases the probability of an identical blood source (a factor known to influence microbiome composition [57–59]) and allows us to collect all five instars and adults. Furthermore, centring this study on *T. cruzi*-negative individuals eliminates another variable known to influence microbiome composition.

Our controlled design thus provides the opportunity to (i) evaluate microbiome development across the full ontogenetic spectrum (first to fifth instars plus adults) in natural populations, (ii) determine any relationship between triatomine genetic background and microbiome diversity, (iii) examine an environmental effect on microbiome composition in species from multiple distinct geographic areas, (iv) determine microbiome specificity among *Triatoma* species from the same microhabitat, and (v) identify pathogens acquired through feeding on the vertebrate host.

Methods

Study sites and sample set

Samples were collected from 3 sites in southern Texas in July 2017 (Chaparral Wildlife Management Area, Camp Bullis and Lackland Air Force base in San Antonio) and 3 sites in southern Arizona in July 2018 (Las Cienegas National Conservation Area, University of Arizona Desert Station in Tucson, and San Pedro Riparian National Conservation Area). Sites were accessed with full written permission from the relevant governing bodies (see the "Acknowledgements" section). Larval instars and adults, molecularly determined as *Triatoma rubida* (N = 128), *T. lecticularia* (N = 20), *T. sanguisuga* (N = 25), *T. gerstaeckeri* (N = 42), and *T. protracta* (N = 34; see below), were collected from the nests of white-throated woodrats (*Neotoma albigula*). We recorded nest coordinates, developmental stage, morphospecies, engorgement, and sex (adults only) for every individual. All samples were preserved in individual vials with 100% ethanol. Additional samples of adult *T. protracta* and *T. rubida* were provided from two houses neighbouring the University of Arizona Desert Station. Since these were adult individuals attracted by black light and not a permanent domestic infestation, we included them in the study.

DNA extraction and basic molecular analyses

The entire abdomen (comprising the whole gut length) of each individual sample was used as a template for DNA isolation with DNeasy Blood and Tissue kits (Qiagen) according to manufacturer instructions. DNA templates were stored at −75 °C prior to molecular analyses, which included host molecular taxonomy, *T. cruzi* infection status, and 16S rRNA gene amplicon library preparation. To determine *Triatoma* species identity and phylogenetic distance we used the primers 7432F (5′-GGACGWGG WATTTATTATGGATC-3′) and 7433R (5′-GCWC CAATTCARGTTARTAA-3′) to amplify a 663 bp fragment of the *cytB* gene [60]. However, the primer pair 7432F and 7433R failed to amplify a PCR product in samples morphologically identified as *T. protracta*, for which some difficulties with *cytB* sequencing have previously been reported [61, 62]. We therefore designed alternative primers, Tpr_F (5′-CCTACTATCCGCGGTTCCTT-3′) and Tpr_R (5′-GGGATGGATCGGAGAATTGCG-3′) using three available *T. protracta cytB* sequences and seven sequences of different *Triatoma* species from GenBank. Under the same conditions published by Monteiro et al. [60] the amplification resulted in 380-bp long sequences. The PCR products were cleaned from primers using Exonuclease I and FastAP (Thermo Scientific) enzymes and Sanger sequenced. Phylogenetic background of the *Triatoma spp.* sample set was reconstructed from aligned sequences using maximum likelihood with the best fitting model determined by a corrected Akaike information criterion in jModelTest2 [63]. Representative sequences for each species are available in GenBank under the following accession numbers MT239320–MT239329.

To eliminate infection status as a variable affecting the host microbiome, all samples were screened for the presence of *T. cruzi* in three PCR reactions (as described in Rodríguez-Ruano et al. [51]). In brief, *T. cruzi* presence/absence was confirmed with the universal primer pair TCZ1/TCZ2, targeting any discrete typing unit (DTU) as described by Moser et al. [64]. Additionally, two primer sets (ME/TC1 or TC2) were used to distinguish different discrete typing units of *T. cruzi* [40, 65]. The representative PCR products of all three primer pairs were Sanger sequenced (as described above) and their identity evaluated based on BLASTn searches to confirm the specificity of the screening process. All bands of the expected size from the PCR products were identified as *T. cruzi* DTUs. 57 *T. cruzi*-positive samples were subsequently excluded from the analyses. The complete metadata for the samples used in this study are provided in Additional File 1.

Amplicon library preparation

Extracted DNA templates were used for 16S rRNA gene amplification according to Earth Microbiome Project standards (EMP; http://www.earthmicrobiome.org/protocols-and-standards/16s/). Sample multiplexing was based on a double barcoding strategy with EMP-proposed 12-bp Golay barcodes included in the forward primer 515F [66], and additional 5-bp barcodes (designed in our laboratory) within the reverse primer 926R [66, 67]. Barcodes and primers are available in Additional File 1. The resultant amplicons were approximately 500 bp long, including adapters, barcodes, primer sequences, and approximately 400 bp of the 16S rRNA gene V4/V5 hypervariable region.

Novel 18S rRNA gene blocking primer

Since our previous sequencing of insect-associated microbiomes with the 515F/926R primer pair [66, 67] revealed low specificity towards the 16S rRNA gene (resulting in high numbers of host 18S rRNA gene amplicons; unpublished data) we implemented a custom 18S rRNA gene blocking primer (see [68] for pertinent application of a blocking primer). The 8 bp at the 5′ end of the blocking primer 926X (5′ GTGCCCTT**CCGTCA ATTCCT**-C3 3′) were designed to specifically match the 18S rRNA gene sequence (conserved in representatives of 23 Insecta orders, a human, and a mouse; Additional File 1), while the last 12 bp partially overlaps with the 926R (5′ **CCGYCAATTYMT**TTRAGTTT 3′) annealing site. The C3 spacer modification at the 3′ end of 926X was introduced to prevent any prolongation of the blocker. In addition, the blocking primer was used at 10× higher concentration compared to that of the amplification primers [68, 69]. This concentration disparity results in the 926R primers being outcompeted by the blocker 926X during any possible annealing to the 18S rRNA gene, thus increasing the 16S rRNA gene amplification efficiency (detailed information on blocking primer design and validation are provided in Additional File 2).

Library controls and amplicon sequencing

In order to confirm the barcoding output and evaluate any effect of our blocking primer on 16S rRNA gene amplification (e.g., possible amplification bias towards some bacterial taxa), the library contained two types of commercially available microbiome mock communities and three microbiome samples of colony-reared *Rhodnius prolixus* sequenced in our previous projects [51]. The mock communities comprised three samples of gDNA templates with an equal composition of 10 bacterial species (ATCC® MSA-1000™) and three samples with staggered composition of the same 10 bacteria (ATCC® MSA-1001™). Altogether seven negative controls (NC) were used to control for the extraction procedure (2 NC), PCR library preparation (2 NC), and well-to-well contamination (3 NC: PCR water template). The PCR amplicons were cleaned using AMPure XP (Beckman Coulter) magnetic beads and equimolarly pooled based on DNA concentration measured with a Synergy H1 (BioTek) spectrophotometer. Since the bead purification did not completely remove the high concentrations of the blocking primer, the final pooled library was purified using Pippin Prep (Sage science) in order to remove all DNA fragments shorter than 300 bp and longer than 1100 bp. The purified library was sequenced in a single run of Illumina MiSeq using v3 chemistry with 2 × 300 bp output (Norwegian High Throughput Sequencing Centre, Department of Medical Genetics, Oslo University Hospital).

Sequence processing

The raw reads were quality checked (FastQC) and trimmed (necessary for reverse reads due to the reduced end-of-read quality) using USEARCH v9.2.64 [70]. The reads were processed according to the following workflow, implementing suitable scripts from USEARCH v9.2.64 [70]. Pair-end reads were demultiplexed and merged. Primers were trimmed and sequences were quality filtered, resulting in a final amplicon length of 369 bp. The dataset was clustered at 100% identity to get a representative set of sequences for de novo OTU picking, using the USEARCH global alignment option at 97% identity match [70]. Taxonomy was assigned to the representative sequences using the BLAST algorithm [71] against the SILVA 132 database trimmed for the SSU rRNA gene [72]. Chloroplast sequences, mitochondrial OTUs, and singletons were removed from the final OTU table using QIIME 1.9 [73].

We analysed the final OTU table as three independent datasets, to make sure that our bioinformatic approach did not influence the results. We made the "*basic*" dataset (567 OTUs) by filtering extremely low abundant OTUs (as recommended by Bokulich et al. [74]). We generated the "*decontam*" dataset (5553 OTUs) from the final OTU table by filtering potential contaminants, using the R package "decontam" (V1.5.0) [75] to systematically identify and discard a total of 118 OTUs (complete list in Additional File 1) with a frequency-based approach combined with the post-PCR concentration of each sample. Three of the computationally identified contaminant OTUs (one assigned to the genus *Sphingomonas* and two to *Geobacillus*) were present in all of our negative controls, comprising 223 ± 195 mean total bacterial reads. We generated the "*ultraclean*" dataset (183 OTUs) from the "*decontam*" dataset by employing stringent filtering steps to reduce data complexity, based on our previous experience with insect microbiomes. We retained the OTUs that met the following conditions: first, representing more than 1% of reads in any individual sample, and second, being found repeatedly, i.e. in at least two samples across the dataset.

Statistical analyses

We carried out all downstream analyses on the three normalised datasets using rarefaction at 1000 sequences per sample for "*basic*" and "*decontam*", and 500 sequences per sample for "*ultraclean*". We used the "vegan" [76] and "phyloseq" [77] packages in R [78] to calculate community quantitative measures (Richness and Shannon index) and ordination analyses (non-metric multidimensional scaling, NMDS; based on Bray-Curtis dissimilarities). We supported the ordination analyses using PERMANOVA tests with beta-dispersion to

determine the significance of the tested factors on shaping the microbiome composition.

To test the effect of ontogeny, we analysed the microbiome communities across host developmental stages in *T. rubida*, *T. protracta*, *T. lecticularia*, and *T. gerstaeckeri*. To test for differentiation in microbiome communities with a distinct geographic background, we analysed *T. rubida* samples from two different locations in Arizona. We assessed possible species-specific differentiation by comparing individuals from all 5 species, and by comparing *T. gerstaeckeri* and *T. lecticularia* collected from the same nest in southern Texas (thus eliminating the geographic variable). We evaluated the possible effect of host phylogeny on species-specific microbiome patterns. Using Mantel test (implemented in the R package "ecodist" [79]), we tested correlations between microbiome Bray-Curtis dissimilarities and host genetic distance (obtained using neighbour-joining analysis with Tamura-Nei model for *cytB* alignment with discarded gap and ambiguous positions). The same approach was used to evaluate the effect of geographic distance among sampling sites calculated in QGIS [80].

Results

Molecular data

The Illumina MiSeq run generated 11,991,455 reads. With negative controls removed, we retrieved a mean average of 13,883 reads per sample and a median average of 9398 reads. In our positive controls, we retrieved consistent profiles of expected diversity from the commercially produced mock communities (Additional File 1). Two of the staggered mocks lacked one taxon with 0.04% abundance. The composition of equal mocks was consistently biassed towards an overrepresentation of *Clostridium* (2.8 times the expected value of 10%), which led to 0.5–8.6% decreases in other taxa. Within the staggered communities, we retrieved most taxa in the expected proportions (from 0.04% to 44.78%). Three of the low abundant taxa (*Clostridium*, *Lactobacillus*, and *Streptococcus*) were overrepresented. The most underrepresented component was *Rhodobacter* (see Additional File 1). All three *Rhodnius prolixus* positive controls showed consistent profiles: *Enterococcus* (mean(SD) = 86(2)%), *Bacillus* (mean(SD) = 10(1)%), and *Arsenophonus* (mean(SD) = 4(1)%), which matched the results of our previous sequencing runs [51].

The results focus on 170 *T. cruzi*-negative samples from the "*ultraclean*" dataset (see section 2.6; metadata available in Additional File 1). The corresponding results of ordination analyses from the "*basic*" and "*decontam*" datasets are available in Additional File 3. Phylogenetic clustering based on maximum likelihood (Additional File 4) unequivocally determined the 170 samples from "*ultraclean*" to be *T. rubida* (N = 81), *T. lecticularia* (N = 13), *T. sanguisuga* (N = 15), *T. gerstaeckeri* (N = 27), and *T. protracta* (N = 34).

Microbiome dynamics and host ontogeny

Host ontogeny is a major factor influencing triatomine microbiomes (Fig. 1a–c). *T. rubida* (the most abundant species in our data) shows a pronounced pattern of diversity loss and *Dietzia* accumulation throughout ontogenetic development. *Dietzia* is absent in our "*ultraclean*" data from the earliest instars, progresses into some L3s, and then clearly increases in L4 nymphs. In most adults it completely dominates the microbiome, comprising 100% of the reads in some individuals (Fig. 1a). The same ontogenetic pattern exists in *T. gerstaeckeri*, *T. protracta*, and *T. lecticularia* but was not analysed with statistical support due to smaller sample sizes and some instar unavailability (see Additional File 5). Furthermore, there were significant differences in *T. rubida* microbiome diversity between early and late life stages (6 pairwise comparisons retrieved significant differences at the 0.001 confidence interval, Fig. 1b). First instars had the highest Shannon index value (L1 median average = 2.75) and adults had the lowest (L6 median average = 0.01). In a non-metric multidimensional scaling analysis (NMDS), *T. rubida* microbiomes clustered into significantly distinct groups reflecting their ontogenetic development (Fig. 1c; mean stress ≈ 0.16; PERMANOVA R^2 = 0.288, $p \leq 0.001$, with significant beta-dispersion F = 3.252, p = 0.014 on 999 permutations). A single *T. rubida* L3 outlier with 100% reads from *Streptobacillus* (Additional File 1) has been removed from our analyses.

The results presented in Fig. 2 are for two ontogenetic subsets: early (L1–L3) and late (L4–L6) stages, based on their significantly different variance (Additional File 6). There was high among-individual variation in microbiome diversity for all species and instars (Fig. 1a and Additional File 5) but all harboured bacteria from two classes, Actinobacteria and Gammaproteobacteria (Fig. 2). We found species-specific patterns in microbiome composition: only *T. sanguisuga* and *T. gerstaeckeri* contained Acidobacteria and TM6 class bacteria, and only *T. protracta* possessed Bacteroidia in high abundance (particularly the genus *Proteiniphilum*, which dominated some adults; Additional File 5). At the genus level, *Dietzia* dominated the later developmental stages of *T. protracta*, *T. lecticularia*, and *T. rubida*, yet was completely absent from *T. sanguisuga* and *T. gerstaeckeri* (Fig. 2). Specifically, *Dietzia* comprised 2 OTUs (62% prevalence; abundance median [min–max] = 31.6% [0–100%]) in *T. protracta*, 2 OTUs (77% prevalence; abundance median [min–max] = 79.0% [0–100%]) in *T. lecticularia*, and 3 OTUs (29% prevalence; abundance median [min–max] = 0.0% [0–100%]) in *T. rubida*. In contrast, *T. sanguisuga*

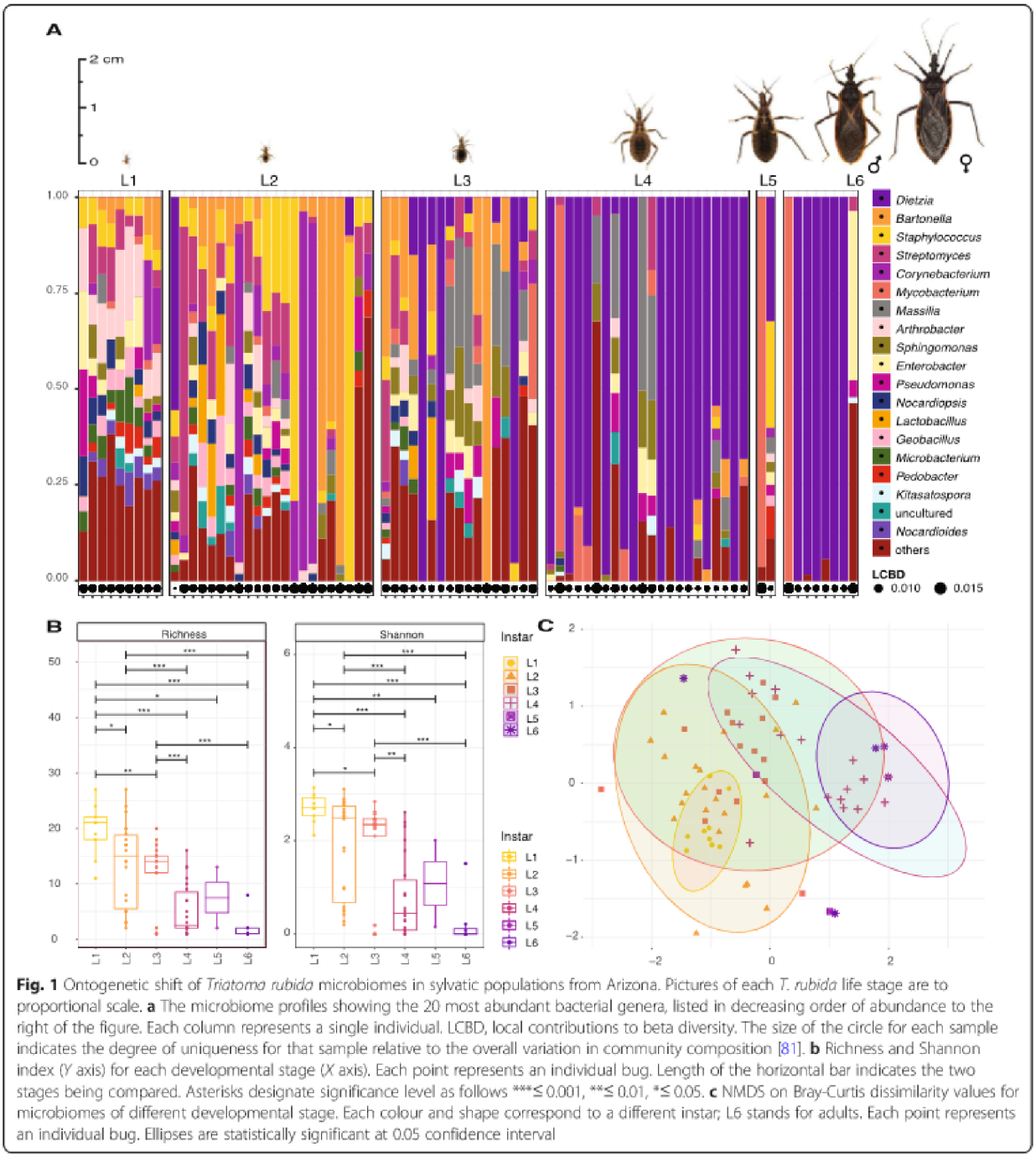

Fig. 1 Ontogenetic shift of *Triatoma rubida* microbiomes in sylvatic populations from Arizona. Pictures of each *T. rubida* life stage are to proportional scale. **a** The microbiome profiles showing the 20 most abundant bacterial genera, listed in decreasing order of abundance to the right of the figure. Each column represents a single individual. LCBD, local contributions to beta diversity. The size of the circle for each sample indicates the degree of uniqueness for that sample relative to the overall variation in community composition [81]. **b** Richness and Shannon index (*Y* axis) for each developmental stage (*X* axis). Each point represents an individual bug. Length of the horizontal bar indicates the two stages being compared. Asterisks designate significance level as follows ***≤ 0.001, **≤ 0.01, *≤ 0.05. **c** NMDS on Bray-Curtis dissimilarity values for microbiomes of different developmental stage. Each colour and shape correspond to a different instar; L6 stands for adults. Each point represents an individual bug. Ellipses are statistically significant at 0.05 confidence interval

was dominated by the genus *Streptomyces* (5 OTUs; 75% prevalence; abundance median [min–max] = 19.1% [0–90%]), as was *T. gerstaeckeri* (6 OTUs; 93% prevalence; abundance median [min–max] = 23.6% [0–90%]).

Microbiome and host genetic background

We found species-specific differences between the microbiome communities of all five *Triatoma* species (Fig. 3). Evaluation of early instar microbiomes confirmed significant differences among clusters reflecting host species identity (NMDS ordination, mean stress ≈ 0.02; PERMANOVA, R^2 = 0.18, $p \leq 0.001$, beta-dispersion on 999 permutations: p = 0.034, Fig. 3). To further test host species specificity in microbiome composition, we specifically compared *T. gerstaeckeri* (17 individuals) and *T. lecticularia* (13 individuals)

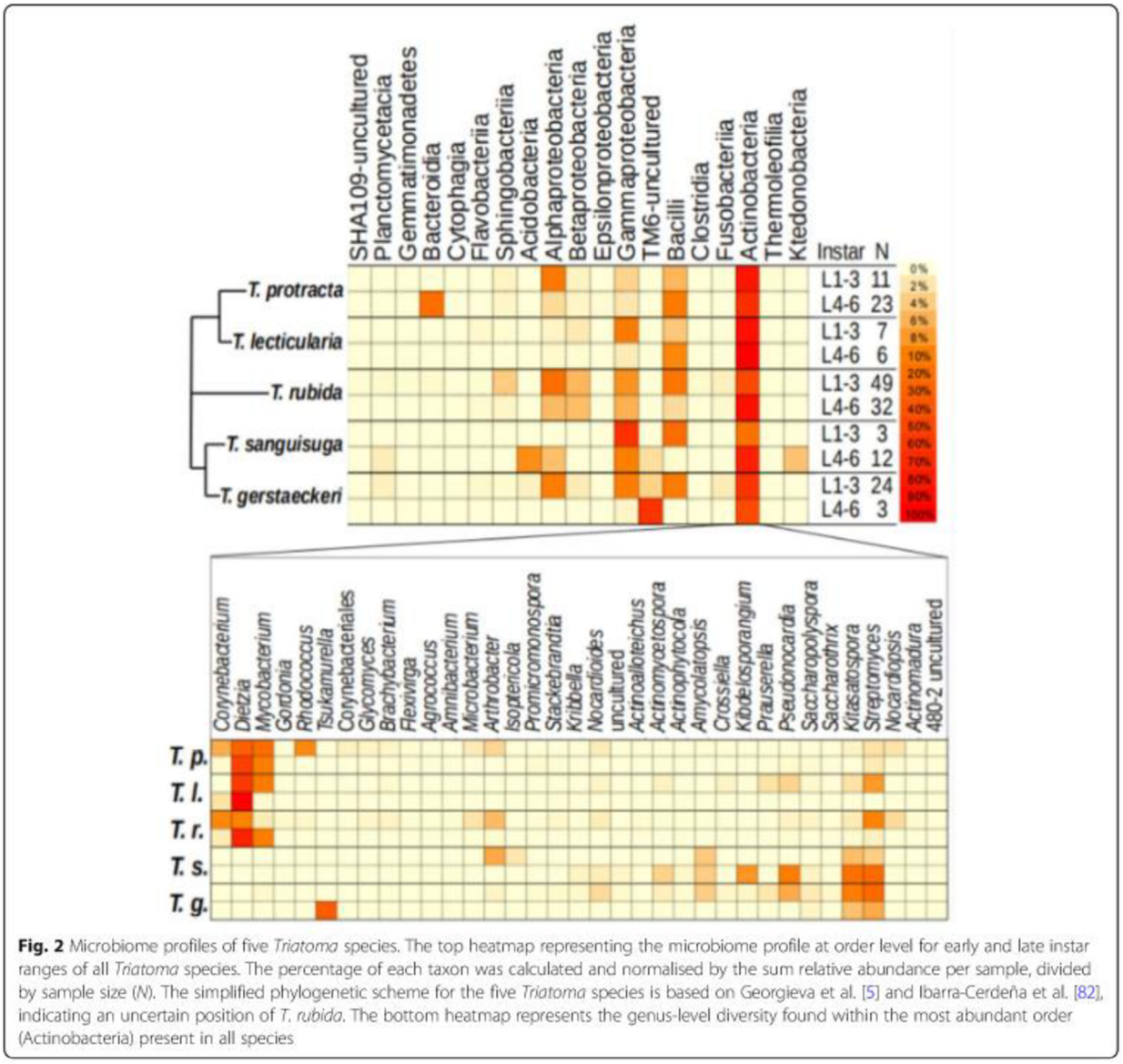

Fig. 2 Microbiome profiles of five *Triatoma* species. The top heatmap representing the microbiome profile at order level for early and late instar ranges of all *Triatoma* species. The percentage of each taxon was calculated and normalised by the sum relative abundance per sample, divided by sample size (*N*). The simplified phylogenetic scheme for the five *Triatoma* species is based on Georgieva et al. [5] and Ibarra-Cerdeña et al. [82], indicating an uncertain position of *T. rubida*. The bottom heatmap represents the genus-level diversity found within the most abundant order (Actinobacteria) present in all species

sampled simultaneously from the same *N. albigula* nest in Chaparral, Texas. These species formed distinct clusters with all individuals included in the analysis (NMDS with mean stress ≈ 0.09; PERMANOVA R^2 = 0.268, p = 0.002, beta-dispersion on 999 permutations: p = 0.022; Fig. 4). When we analysed the 23 early instar (L1-L3) individuals, we found the same distinct clusters (NMDS with mean stress ≈ 0.11; PERMANOVA R^2 = 0.150, $p \leq 0.001$, beta-dispersion on 999 permutations: p = 0.522). They notably differed in microbiome taxonomic composition, with *Dietzia* conspicuously absent from *T. gerstaeckeri* but highly abundant in *T. lecticularia* (Fig. 4).

In addition, we performed two-sided Mantel tests on 93 early instars (L1-L3) to determine if species-specific microbiome differences were a product of host phylogenetic constraint. We identified positive correlations between microbiome dissimilarities and respective host phylogenetic distances (Spearman's rank correlation: r = 0.29, $p \leq 0.001$). We illustrated this result with a neighbour-joining tree of Bray-Curtis microbiome dissimilarities that specifically included triatomine species identity and geographic origin (Fig. 5). *T. sanguisuga* and *T. gerstaeckeri* microbiomes were arranged in a single cluster which reflects the host's close phylogenetic relationship. Microbiomes of *T. lecticularia* predominantly clustered according to host phylogeny

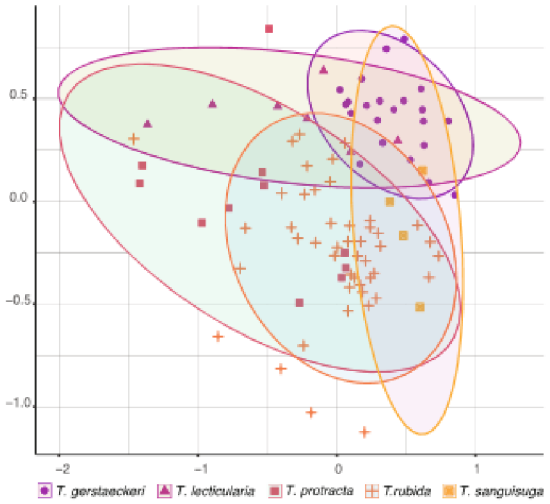

Fig. 3 *Triatoma* microbiome species specificity. NMDS ordination for early instars (L1–L3) of all five *Triatoma* species (*T. rubida*, *T. protracta*, *T. gerstaeckeri*, *T. sanguisuga*, and *T. lecticularia*) based on Bray-Curtis dissimilarities of their microbiome communities. Each point is an individual bug. Each species is represented by a distinct colour and shape shown beneath the *x* axis

(close to *T. protracta* samples), despite the common geographic origin of *T. sanguisuga*, *T. gerstaeckeri*, and *T. lecticularia*. *T. protracta* branching was affected by both phylogeny and geographic origin. Overall, both phylogeny and geographic origin partially explain microbiome composition. Analysis of late instars (74 individuals L4–L6) resulted in a notably lower degree of correlation (Spearman's rank correlation: $r = 0.18$, $p \leq 0.001$).

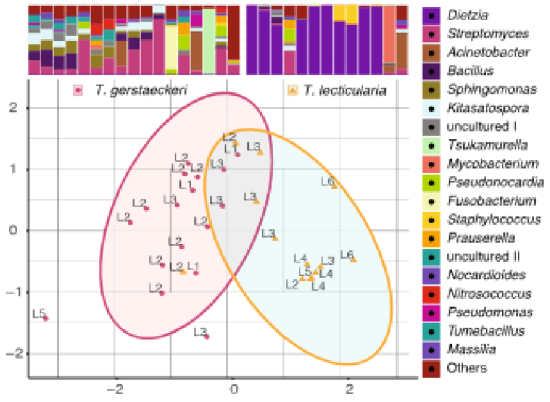

Fig. 4 Microbiome species-specific differences in a single habitat. NMDS ordination calculated with microbiome Bray-Curtis dissimilarity values from *T. gerstaeckeri* and *T. lecticularia* residing in the same nest in Chaparral, TX. Each point represents an individual bug, with the life stage listed next to it. Statistical ellipses were calculated using a 0.05 confidence interval. Microbiome profiles for both species are provided above the plot, with the 20 top bacterial genera colour-coded and listed in decreasing order of abundance to the right of the plot. "uncultured I" stands for bacteria from the order Bacillales, and "uncultured II" for Sphingomonadales.

Microbiome and host geographic origin

Geographic location was a small but significant factor explaining microbiome variation at the intra-species level. We demonstrated this by comparing *T. rubida* from nests in two Arizona locations (Las Cienegas National Conservation Area (LCNCA) and University of Arizona Desert Station (UADS)). We grouped them into early and late instar ranges, to account for ontogenetic changes in microbiome composition, and found their microbiomes significantly differed based on locality. NMDS (mean stress ≈ 0.17) showed statistically significant clusters (PERMANOVA, $R^2 = 0.08$, $p \leq 0.001$, with non-significant beta-dispersion on 999 permutations, $F = 0.393$, $p = 0.537$; Fig 6).

Furthermore, we analysed the microbiomes of 21 *T. rubida* from 6 different nests within UADS to see if *N. albigula* nests function as natural isolated microhabitats, potentially structuring the population variability among *T. rubida* microbiomes. Our results show that microbiome variability reflected the nest origin among early instars (L1–L3), supported with statistically significant clusters in the NMDS analysis (PERMANOVA, $R^2 = 0.45$, $p \leq 0.001$; Additional File 7) and a modest correlation with geographic distance between the nests (Mantel test, Spearman's rank correlation: $r = 0.164$, $p = 0.019$, at 95% confidence).

Inherited taxa and bacterial pathogens

Sampling across the ontogenetic spectrum allowed us to examine bacterial taxa shared between adult triatomines and their presumed progeny. For this analysis, we only considered adults and early instar individuals originating from the same nest and sharing 100% identity among their *coxB* gene sequences. Cross-referencing our "*decontam*" unrarefied data (to deliberately include low abundance taxa that were excluded in the "*ultraclean*" dataset) with early and late developmental stages of *T. rubida* (comparison between three L2s and one adult from nest number 4 in LCNCA, AZ) indicated 17 shared OTUs with abundance > 0.05% per sample, associated with all individuals. 11 OTUs from *Actinobacteria* represented the genera *Dietzia*, *Mycobacterium*, *Corynebacterium*, *Brachybacterium*, *Amycolatopsis*, *Kitasatospora*, *Nocardiopsis*, and *Streptomyces* (4 OTUs); 1 OTU of an uncultured bacterium from *Bacteriodetes*; and 5 OTUs from *Firmicutes* (*Geobacillus*, *Staphylococcus*, *Lactobacillus* (2 OTUs), and *Ruminococcus*). For *T. lecticularia* (comparison between three L2 individuals and two adults from nest number 2 in Chaparral, TX), we found 4 shared OTUs with abundance > 0.05% per sample

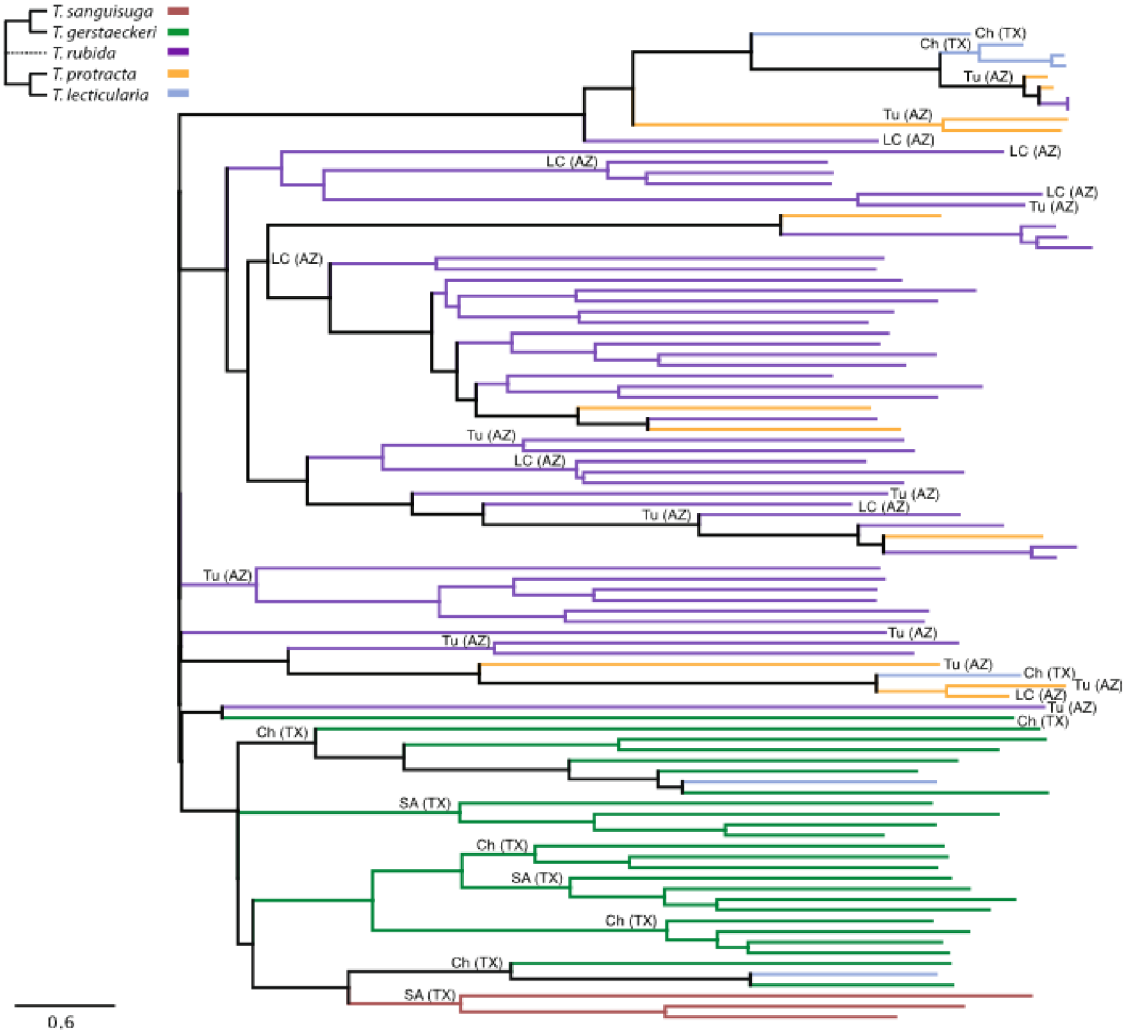

Fig. 5 The neighbour-joining tree of microbiome Bray-Curtis dissimilarities for 93 *Triatoma* early instar individuals, showing microbiome species specificity is determined by host phylogeny and geographic origin. The nodes are coloured according to host phylogeny. The simplified phylogenetic scheme for the five *Triatoma* species is based on Georgieva et al. [5] and Ibarra-Cerdeña et al. [82]. The dashed line reflects the uncertain position of *T. rubida*. Sample geographic origin is designated as follows: SA (TX) San Antonio, Texas; Ch (TX) Chaparral, Texas; Tu (AZ) University of Arizona Desert Station, Tucson, Arizona; LC (AZ) Las Cienegas National Conservation Area, Arizona

associated with all five individuals. These represented the genera *Dietzia* and *Kitasatospora* (*Actinobacteria*), *Bacillus* (*Firmicutes*), and *Enterobacter* (*Proteobacteria*).

Since microbiomes of hematophagous insects often contain acquired bacterial pathogens [83, 84], we examined our data for known pathogenic genera. Two OTUs assigned to the genus *Bartonella* were found in the "*basic*" and "*decontam*" datasets, one of which was retained in the "*ultraclean*" data. This *Bartonella* OTU was highly prevalent (51%) among all *T. protracta* and *T. rubida* individuals. Using *Bartonella gltA* gene-specific primers [85], we retrieved 272-bp sequences from *T. rubida* individuals sampled from LCNCA and UADS. We found 99-100% pairwise similarity between the sequences from UADS and *Bartonella vinsonii* isolates from *N. albigula* blood (available in GenBank; KJ719286-7). *Bartonella gltA* sequences retrieved from LCNCA samples were equally similar with *Bartonella vinsonii* isolates from unspecified rodents (AF148491, AF148493, AF148481), suggesting that *T. rubida* acquired pathogenic *Bartonella* from its' vertebrate hosts (Additional File 8). The representative sequences for *B. vinsonii* found here are available in GenBank under the following accession numbers MT112947–MT112949.

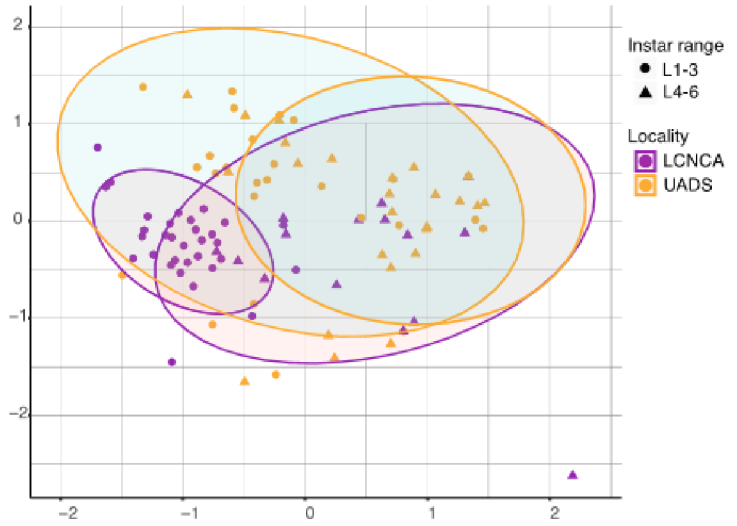

Fig. 6 NMDS visualisation of the microbiome differences in *T. rubida* from *N. albigula* nests, sampled in two different locations in southern Arizona (LCNCA and UADS; represented by different colours). The results are also grouped into early- and late-instar groups (represented by different shapes). The statistical ellipses were assigned using a 0.05 confidence interval

Discussion

A wide range of factors have been suggested to determine the microbiomes of triatomines. Species identity, ontogeny, sex, blood meal source, geographical origin, physiological state, and *T. cruzi* infection have all been implicated from the current literature [24, 43, 49, 50]. However, the actual importance of these factors remains inconclusive or controversial. To address this issue within a broader phylogenetic and geographic context allowing for more general conclusions, we designed the first large-scale investigation of triatomine microbiomes, sampling wild populations of five *Triatoma* species. Our data shows that microbiomes of wild triatomines are determined by three main factors: ontogeny, species specificity, and geographic origin, and are also influenced by pathogen uptake from their vertebrate hosts.

Losing diversity: the main ontogenetic shift in microbiome composition

Wild *Triatoma* microbiomes display dramatic compositional shifts from high diversity in first instars towards low diversity dominated by a single bacterium in adults. This general pattern, previously shown only from laboratory-reared *Rhodnius prolixus* [51], is well documented in *T. rubida* (our most sampled species) and indicated in three other species (*T. gerstaeckeri*, *T. protracta*, and *T. lecticularia*). Statistically, ontogeny is the most important explanatory factor of the microbiome dissimilarities found among *T. rubida* individuals. However, there are two caveats to this general pattern of microbiome diversity. Firstly, there is high among-individual variation in richness reduction. For example, some L2s retained highly diverse microbiomes; others showed large reductions in richness reflected by single taxon dominance. The trend towards single taxon dominance increases in later developmental stages (L4 to L6). Among-individual variability was independent of engorgement status (scores are recorded in metadata), suggesting that it is not a product of host physiological state. Furthermore, it suggests that individual triatomines can maintain various different microbiome arrangements (a rich microbiome vs. one dominated by a single taxon). Secondly, *Dietzia* is clearly the dominant bacteria in most late-stage individuals of *T. rubida*, *T. lecticularia*, and *T. protracta*, but some of their microbiomes are dominated by other genera (*Mycobacterium*, *Proteiniphilum*). High single taxon prevalence in late ontogenetic stages likely reflects a real biological process rather than a methodological artefact (e.g., artificial over-amplification). We base this assertion on three major points: (i) our positive control profiles did not indicate any major preferential amplification in the data; (ii) the non-random occurrence of this pattern, i.e., *Dietzia* dominates the late ontogenetic stages of three *Triatoma* species; and (iii) concordant results of Mann et al. [50] showing 65% of *T. sanguisuga* and *T. gerstaeckeri* adult microbiomes are dominated by a single bacterial taxon, often *Bacillus* or an unspecified *Enterobacteriaceae*.

Developmental stage has been recognised as a key determinant of microbiome composition in other arthropod vectors (e.g., ticks [86–88]). In *Triatoma*, we can only hypothesise about the mechanisms underlying the ontogenetic shift from taxon-rich microbiomes in early instars to single taxon-dominated microbiomes in adults.

Ontogenetic reduction of microbiome diversity may be random or induced by a specific physiological state, e.g., the moulting process. Insects typically shed the foregut and hindgut linings during moulting, causing loss or suppression of symbiotic bacteria in the process [89–91]. The rich microbial community of *Triatoma* first instars (possibly acquired from their eggs [92, 93]) may be periodically shed from the gut with each moulting. After five moulting events, adults thus possess a microbiome with significantly reduced richness. However, the relationship between ontogeny and microbiome composition has not been investigated in other hemimetabolous blood-feeding insects (bed bugs or lice [21];), making generalisable conclusions between biologically similar taxa elusive. Similar examples of decreasing microbiome diversity can be found throughout the lifecycle of some holometabolous insects, e.g., dung beetles [94] and cabbage flies [95]. For holometabolous insects, ontogenetic changes in gut bacterial communities are generally explained by two main factors: the substantial remodelling of the gastrointestinal tract during metamorphosis [89] and different dietary needs between larvae and adults [94]. Although hemimetabolous Triatominae depend on a blood-based diet throughout their entire development, their preferences for accessory feeding strategies, especially haemolymphagy and coprophagy, may decrease over time and thus limit opportunities for any microbiome enrichment. Since gut microbiome analysis of natural populations requires killing the specimen, we cannot record microbiome shifts throughout ontogenetic development of a single individual, and thus cannot currently determine whether diversity loss is a permanent change to triatomine microbiomes.

Origin of the microbiome bacteria: inheritance vs, environment

The results shown in Figs. 2, 3, 4, 5, and 6 demonstrate that triatomine microbiome composition (especially in early instars) is shaped by host species and locality. For both variables, there was partial overlap in microbiome composition, likely due to the notable degree of among-individual variability (discussed above). Species-specific differences were present even when multiple triatomine species were sampled in the same nest, showing that they are not caused by different environmental sources of bacteria. In theory, they could be explained by multiple different mechanisms, like specific maternally inherited bacteria or differential uptake and retention of environmental bacteria. Due to microbiome ontogenetic changes, most of the early stage diversity is lost in the later stages and these bacteria are therefore unlikely to be transmitted vertically. Thus, only the bacteria present in both early stages and adults remain possible candidates for maternal inheritance. To address this issue, we cross-referenced bacterial OTUs from late-stage (L6) and early-stage (L1/L2) triatomines captured from the same nest. One nest from Arizona contained early- and late-stage *T. rubida*, as did one nest in Texas for *T. lecticularia*. In both instances there were common genera within all individuals (13 in *T. rubida*, 4 in *T. lecticularia*). We hypothesise that these shared taxa are the most likely candidates for vertical transmission. Moreover, the majority of the candidate bacteria fall within Actinobacteria, shown to be maternally inherited in other true bugs and wasps [93, 96–98]. However, we cannot exclude other potential causes for the patterns found, such as host immune selective pressure favouring these bacterial taxa.

While the significance of maternal inheritance is unclear, the effect of environmental bacteria is more evident. A prominent component of environmental microbe acquisition is potential vertebrate pathogens in the blood meal. In some hematophagous arthropods, vertebrate pathogens have evolved into symbionts (e.g., *Francisella* in the Gulf Coast tick [84]). Others, like sheep keds (*Melophagus ovinus* [99]) and a single kissing bug species (*Eratyrus mucronatus* [100]), were found to carry *Bartonella* species of an unknown phenotype. In our data, *Bartonella* was the second most abundant taxa found in every life stage of *T. rubida*. Molecular analysis showed that the bacterium is a pathogen acquired from *N. albigula*. A possible phenomenon for future consideration is whether *Bartonella* is a transcriptionally active component of *Triatoma* microbiomes, or a transient taxon reacquired with each feeding. *Mycobacterium*, the sixth most abundant OTU within *T. rubida*, provides another potential example of a pathogen [101, 102] acquired from the environment. Strict vertical inheritance and environmental uptake (horizontal transmission) are biologically distinct modes of acquiring bacterial symbionts. Triatomines engage in accessory feeding behaviours that potentially interconnect these two sources, such as coprophagy and kleptohematophagy [2, 55]. For instance, coprophagy is employed by first instar *Rhodnius prolixus* to acquire *Rhodococcus rhodnii* from parental faeces [103]. This form of symbiont acquisition is not strict maternal inheritance, because offspring do not acquire *Rhodococcus* in utero or from the mother's ovaries. Instead, coprophagous symbiont acquisition represents both "indirect" vertical transmission and environmental acquisition. Currently, we cannot determine whether *Triatoma* microbiome species specificity is due to transmission of maternally provided bacteria or genetically determined uptake of environmental bacteria (e.g., the lack of *Dietzia* in *T. gerstaeckeri* and *T. sanguisuga* could be linked to the host's close phylogenetic relationship), or a combination of both.

Dominant taxa and endosymbiosis

The consensus for many arthropod vectors is strong reliance on obligate bacterial endosymbionts (e.g., *Wigglesworthia* in *Glossina*, *Riesia* in *Pediculus* lice, *Coxiella*-like symbiont in ticks [16–20]) that facilitate essential functions like vitamin synthesis and participate in blood meal breakdown. Triatomines appear to establish less intimate symbioses with extracellular bacteria in their gut lumen, instead of possessing obligate intracellular symbionts [49, 51, 104]. Some studies indicated that *Rhodococcus* (an extracellular symbiont) was important for successful development and reproduction of *Rhodnius prolixus* [105–107]. However, later molecular studies showed *Rhodococcus* is not omnipresent throughout Triatominae, and not even among other *Rhodnius* species [47, 108]. In our results, *Dietzia*, a bacterium closely related to *Rhodococcus* [109], is the dominant bacterium in late instars of *T. rubida*, *T. protracta*, and *T. lecticularia*. *Dietzia* has been described from other triatomine species [40, 41, 43, 46, 47, 51] and other hematophagous insects (*Aedes albopictus* [110], *Glossina pallidipes* [111]), suggesting it may be an important mutualist. However, unlike typical primary symbionts, *Dietzia* does not seem to be transmitted vertically. In contrast to its obvious dominance in later instars, the presence of *Dietzia* in L1 is questionable. The "*decontam*" dataset showed that five *T. rubida* L1s had 1–3 reads of *Dietzia* (from a median average of 1864 reads). Such low read numbers cannot be discriminated from marginal well-to-well contamination and do not provide evidence of *Dietzia* presence in first instars. Further studies with quantitative and in situ approaches are required to unequivocally determine the transmission mechanism and presence of *Dietzia* in first instar triatomines. One hypothesis is that individual bugs acquire *Dietzia* from other triatomines via "indirect" vertical transmission (analogous to the *R. prolixus* and *Rhodococcus* example described above [103]) or from the environment, strategies that have been found in other true bug (Heteroptera) species, including trophallaxis, egg smearing, and endosymbiont reacquisition from soil [93, 112–117]. To further investigate transmission and function of triatomine microbiomes, we will require tissue specific whole genome sequencing and functional transcriptomic studies.

Consistency of the patterns: biology vs. methodology

Previous studies on triatomine microbiomes have suggested various factors, including ontogeny [48, 51], species identity [41, 47, 50, 51, 108], sex [44, 50], blood meal source [44], and *T. cruzi* infection [24, 43, 45, 49, 50], as determinants of microbiome composition, while another study claimed triatomine microbiomes have no determining factor [40]. Since many were based on limited sample size (e.g., $N = 4$ in [40], $N = 14$ in [44], $N = 20$ in [42], $N = 9$ in [41], $N = 29$ in [51]) and largely fragmented by host taxonomy, ontogeny, geographic origin, *T. cruzi* infection status, or were restricted to colony-reared bugs, it is difficult to draw comparative conclusions. We thus paid particular attention to our sampling design and molecular approach, ensuring that our study enabled multiple comparisons at different scales (i.e., different species from the same locality, different species from the same microhabitat, and different localities for the same species) across all ontogenetic stages. Furthermore, by introducing a novel method with a blocking primer (see the "Novel 18S rRNA gene blocking primer" section), we achieved greater sequencing depth of 16S rRNA amplicons in our mixed templates. As a result, this study presents multiple deterministic patterns consistent across several triatomine species for the first time.

Some of our findings contradict the patterns reported by other authors. The most conspicuous example is the ontogenetic decrease of microbiome diversity in North American species, which is supported by Mann et al. [50], but contrasts two studies on South American species [47, 48]. Oliveira et al. [48] reported an increase in microbiome diversity throughout ontogenetic development in *T. sordida*, and Waltmann et al. [47] found no ontogenetic effect in *T. infestans*. There are two possible reasons for these differences. One is biological, because the other studies worked with South American species and species-specific differences are a clear component of microbiome dissimilarity, as our results and the results of others show [41, 51, 108]. The other is methodological, since the design of Oliveira et al.'s [48] study involved pooled samples and therefore does not allow evaluation of individual microbiome composition in different ontogenetic stages. Considering the among-individual compositional variability we observed, it is clear that pooling samples may have significantly distorted the profiles. A similar methodological artefact has been shown in mosquitoes [118]. The lack of ontogenetic differences in Waltmann et al. [47] could reflect the sample source (faeces) and incompleteness of the ontogenetic spectrum (L3 to adults only) rather than a real biological pattern in natural populations.

The importance of sampling the complete ontogenetic spectrum is well demonstrated by comparing our results with the recent survey of Mann et al. [50], which focused on *T. sanguisuga* and *T. gerstaeckeri*. By profiling microbiomes of 74 specimens, they also revealed a high degree of among-individual variability. However, since their study was based solely on adults, they reported weak species specificity, whereas we found that species-specific microbiome patterns were more pronounced in early instars. In addition, Mann et al. [50] found support

for locality-based effects which corroborates our findings for *T. rubida* from multiple locations in southern Arizona. These examples show that there is some consistency across triatomine microbiome studies, at least regarding US species. However, the current paucity of data does not allow for broader cross-study comparisons. To reach more generalisable conclusions for all Triatominae, we require added breadth (more studies) and depth (metagenomics and transcriptomics) of molecular data.

Conclusion

This study has contributed key information on triatomine microbiomes, which constitutes a crucial component of their biology. We identified ontogenetic shift, species identity, and the environment as the major factors determining microbiome composition in natural populations of *T. rubida*, *T. protracta*, *T. lecticularia*, *T. sanguisuga*, and *T. gerstaeckeri*, thus observing consistent deterministic patterns across multiple triatomine species for the first time. We hypothesise that the high among-individual variability of Triatominae microbiome assemblages is produced by inconsistent uptake of environmental bacteria, including vertebrate pathogens, and multiple indirect bacterial transmission strategies. The epidemiological relevance of Triatominae and their microbiome communities both warrant more in-depth exploration for successful implementation of microbiome-based vector control strategies. To achieve this, we advocate that future studies are designed to allow comparison of detected patterns across different triatomine populations, species, biogeographic areas, and environments.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40168-020-00921-x.

Additional File 1: Sample metadata including list of discarded contaminants.

Additional File 2: Methodological Supplement for 18S rRNA gene blocking primer design and validation

Additional File 3: Results of ordination analyses based on '*basic*' and '*decontam*' datasets.

Additional File 4: Sample set phylogenetic background inferred from *coxB* sequences.

Additional File 5: Microbiome ontogenetic shift in other *Triatoma sp.*

Additional File 6: Significant difference in beta dispersion of the instar range groups (L1-L3 and L4-L6) calculated from the *ultraclean* dataset.

Additional File 7: NMDS analyses of *T. rubida* microbiomes from early instar (L1-L3) individuals found in different *N. albigula* nests at UADS.

Additional File 8: *Bartonella* phylogenetic analysis of gltA sequences retrieved from *T. rubida*.

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Authors' contributions

EN designed the research. JJB, EN, SRR, and WR collected the field samples. JOS provided supplementary samples. EN and JZ designed the novel 18S rRNA gene blocking primers. JZ, SRR, and EN performed the DNA isolation and library preparation. AP performed *T. cruzi* screening. GB performed *Bartonella* sequencing. JJB, EN, SRR, AP, and VH analysed the data and interpreted results. JJB, EN, SRR, and VH wrote the manuscript. All authors contributed to improving the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article and are included with the article and its additional files. A representative subsample of *cytB* sequences for all *Triatoma* species in this study were uploaded to NCBI (accession number MT239320–MT239329). Representative sequences for *Bartonella vinsonii* are available under the following accession numbers MT112947–MT112949. Raw, demultiplexed microbiome data for this study and 926X blocking primer validation were uploaded to ENA (project numbers: PRJEB36515 and PRJNA657483). Complete R code is available at https://github.com/apoosakkannu/MS-Triatominae.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Supplemental Information

Information on the blocking primer 926X used in Chapters 3-5

Ontogeny, species identity and environment dominate microbiome dynamics in wild populations of kissing bugs (Triatominae)

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Additional File 2: Methodological Supplement for 18S rRNA gene blocking primer design and validation

Background

Since 2010, two general primer pairs 515F/806R [1] and 515F/926R [2] that were introduced by the Earth Microbiome Project (EMP [3]) have been broadly used in numerous studies (cumulative number of citations according to Web of Science equals 3334 as of August 14, 2020). Both primer combinations have highly comprehensive coverage that spans sequences of small ribosomal subunits of Bacteria, Archaea but also Eukaryota; i.e. 16S rRNA and 18S rRNA gene amplicons. The number of 18S rRNA reads amplified with 515F/806R was previously evaluated from mosquito samples, averaging 4% for single individuals and pooled templates (calculated from the average read numbers [4]). For the 515F/926R primer pair [2] the authors note even lower specificity towards the bacterial 16S rRNA gene. On average, they retrieved 17% of 18S rRNA gene amplicons from plankton samples. In principle, there are two main reasons why the general amplification properties and potential biases in amplicon analyses with the EMP primer pairs have been broadly overlooked. The first is solely methodological: the data are processed with various analytical pipelines that remove non-overlapping paired-end reads. 18S rRNA reads are approximately 200bp longer and thus discarded and not analysed further. The second reason originates in biological properties of analysed samples, i.e. different proportions of bacterial and eukaryotic DNA. Thus, while some studies can benefit from the comprehensive coverage of EMP primer pairs (e.g. using 18S rRNA reads for the host molecular taxonomy [4]), these might pose a major drawback for microbial analyses of templates with a low proportion of targeted bacterial DNA. Our initial trial for microbiome analyses of hematophagous kissing bugs (Triatominae) with 515F/926R primers failed due to preferential amplification of 18S rRNA sequences, reaching up to 100% of retrieved reads in some samples (as illustrated here in Figure 3C). We assumed that such a bias stems from a high eukaryotic content of our samples, consisting of Triatominae gDNA, prey gDNA from the blood meal, and gDNA of eukaryotic parasites associated with kissing bugs (e.g. *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Hepatozoon* sp.). This may be overcome by using an 18S rRNA blocking primer.

Methods

Design of 18S rRNA gene blocking primer and initial PCR evaluation

While we have primarily designed the blocking primer (designated here as 926X) to lower the numbers of 18S rRNA amplicons retrieved for various *Triatoma* species, its annealing site is conserved in representatives of 23 Insecta orders, a human and a mouse (a single nucleotide mismatch was found for Thysanoptera, Psocoptera and Strepsiptera; Figure 1 and Additional File 1). The eight bp at the 5' end of the blocking primer 926X (5' GTGCCCTT**CCGTCAATTCCT**-C3 3') specifically match the 18S rRNA gene sequences, while the last 12 bp partially overlap with the 926R (5' **CCGYCAATTYMT**TTRAGTTT 3') annealing site. We used a 3' C3 spacer CPG modification (available from most suppliers of custom oligos) that prevents elongation during PCR and does influence annealing properties [5].

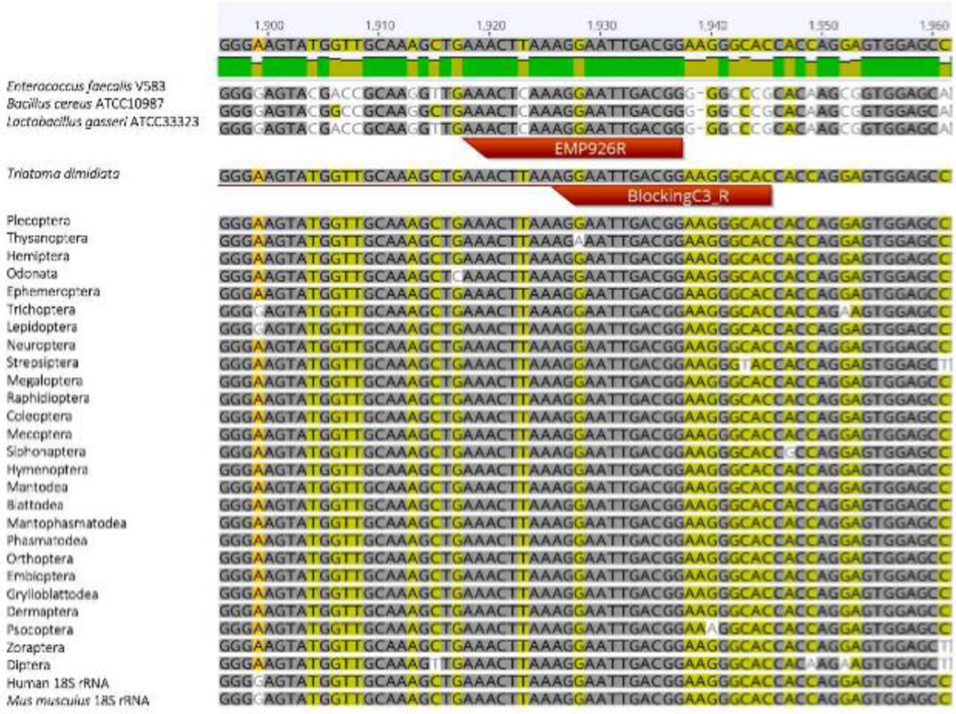

Figure 1. Illustration of 18S rRNA sequences alignment composed of 26 insect orders along with human, mouse, and *Triatoma dimidiata* sequences showing the conservative annealing site of the 926X blocking primer. Three 16S rRNA sequences were included to illustrate the mutual position of 926R amplification and 926X blocking primers.

The performance of the 926X blocking primer was initially evaluated by a simple PCR assay and gel electrophoresis using four DNA templates (A and B not producing a detectable 16S rRNA PCR product with 515F/926R primers and C and D producing a faint band (Figure 2). The blocking primer was added to 50 µL reaction with Q5 High-Fidelity 2X Master Mix in tenfold higher concentration compared to that of 515F/926R primers (final concentrations of 5 µM and 0.5 µM, respectively). PCR conditions as recommended by the EMP 16S Illumina Amplicon Protocol (https://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/) were followed.

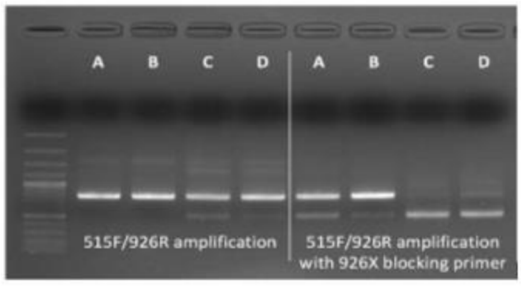

Figure 2. Gel electrophoresis of PCR products amplified from four different Triatominae DNA templates (A-D) with 515F/926R primers (rows 2-5) and with 515F/926R in combination with the novel 926X blocking primer (rows 6-9). Upper bands represent 18S rRNA products (app. 740 bp), lower bands are 16S rRNA products (app. 470 bp). The ladder used is Gene Ruler 100 bp Plus (Thermofisher Scientific).

926X evaluation using amplicon sequencing

The blocker performance was further evaluated using two 16S rRNA gene libraries constructed from the same 47 Triatominae DNA templates (extraction protocol is described in 2.2 section of the main text). While the "*regular*"16S rRNA gene library was amplified solely with double barcoded 515F/926R primer pair of the EMP protocol [2], the "*blocked*" library also employed the novel 18S rRNA gene blocking primer (metadata are provided in Additional File 1). Each library contained two negative controls (PCR water template and blank extraction control) and a single positive control from a previously sequenced *R. prolixus* adult isolated from our laboratory colony [6]. The PCR products were amplified as described above, cleaned with AMPure XP (Beckman Coulter) magnetic beads, pooled and additionally purified using Pippin Prep (Sage science) (see 2.5 section of the main text). Amplicons subjected to this trial were sequenced with 300 cycle Nano V2 chemistry in a multiplexed low output run of Illumina MiSeq. Altogether, the run contained ribosomal amplicon pools retrieved from 192 samples intended for other studies.

Data processing and analyses

The raw data comprised 897, 897 high quality paired reads. Since Illumina technology cannot currently read through the full length of 18S rRNA amplicons retrieved with 515F/926R primers (app. 740bp), we opted for stitching R1 and R2 reads using *fastq_join* script of USEARCH v9.2.64 [7].

Reads were demultiplexed, joined and quality filtered, and the dataset was clustered as described in 2.6 section of the main text. Taxonomy was assigned to the representative sequences using the BLAST algorithm [8] against the SILVA 132 SSU database [9].

Results and Conclusion

Out of 47 template pairs (amplified with and without the novel 926X blocking primer), 2 pairs produced extremely low amounts of data (29 and 62 total reads) and were not further analysed. The negative controls of the "*regular*" library contained 44 and 8 reads, while those of the "*blocked*" library comprised 6 and 104 reads (all the reads were assigned to 8 OTUs representing *Chrysobacterium*, two *Geobacillus* OTUs, two Thermaceae OTUs, *Deinococcus*, *Bacillus* OTU38 and *Sphingomanas*). The single positive control in the "*blocked*" library with 3251 total reads comprised an expected profile of previously sequenced *R. prolixus* [6], i.e. 80.9% *Enteroccoccus*, 6.9% *Bacillus* OTU15, 2.1% *Arsenophonus* and 10.1% of non-bacterial reads. Recalculated as 90%, 7.6%, and 2.4% of the bacterial reads, the profile mirrors those of the positive controls used in our main experiment (section 3.1 of the main text).

The read number retrieved per sample from the "*regular*" library was 4505 (±126). The "*blocked*" library produced notably lower numbers or reads per sample (873 ±195), Figure 3A. However, the proportion of 16S rRNA reads was extremely low in the "*regular*" library (on average 2% ±2) compared to 29% ±23% in the "*blocked*" library; Figure 3). On average, implementing the 926X blocking primer increased the bacterial read yield by 27%. In other words, we present 10% or higher improvement of 16S rRNA gene amplification in over 73% of our samples (Figure 3).

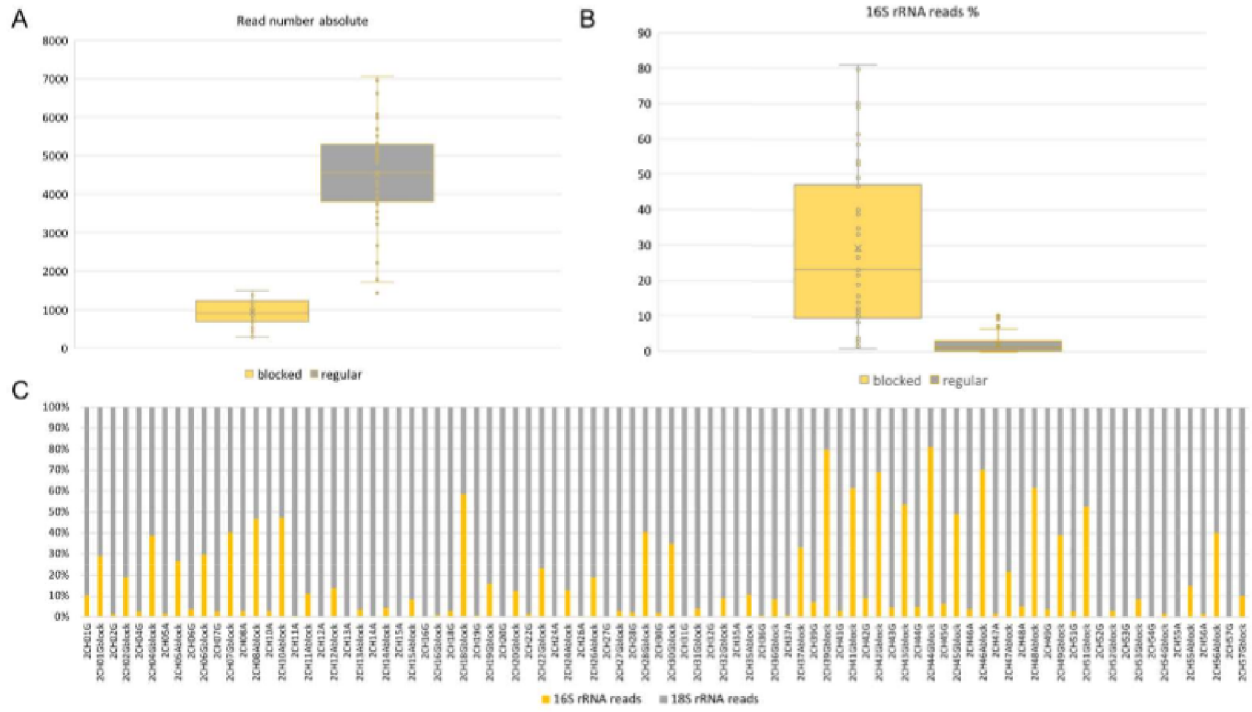

Figure 3. Improvement of 16S rRNA gene amplification using 926X blocking primer. Absolute read number retrieved after data processing for the samples from the "*regular*" library and "*blocked*" library (**A**). Proportion of 16S rRNA reads in the "*regular*" library and "*blocked*" library (**B**). Proportional values of 16S rRNA and 18S rRNA reads retrieved for each of the samples in the "*regular*" library and "*blocked*" library (organized in paired order from left to right; **C**).

We assume that the difference between the number of reads retrieved here from the "*regular*" and "*blocked*" library stems from non-equimolar proportion between the two pooled libraries rather than the 926X blocking primer directly reducing read numbers. We support our assumption with the results presented in the main body of this study (see 3.1 section of the main text). There, we have implemented the 926X blocking primer in a highly multiplexed library of 480 samples and did not experience any particular reduction of data retrieved with a regular output mode of Illumina MiSeq (V3 chemistry 600 cycles).

While our *in silico* prediction suggests potentially more general use of the 926X blocking primer, further validation with various templates, especially those containing a high proportion of eukaryotic DNA, should be performed. So far, we have tested its performance with positive results (enhancing 16S rRNA gene amplification) in our current projects with Anoplura, Diptera (Hippoboscidae), and Sternorrhyncha (Aleyrodidae); data not shown.

Data availability

All metadata for the samples used in this evaluation trial are provided in Additional File 1. Demultiplexed data have been deposited in ENA under following accession number: PRJNA657483

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Chapter 6

Discussion and Conclusions

The previous chapters of this thesis investigated several aspects of insect-associated microbiomes, the factors influencing their composition, and their role in community ecology. Through these studies, my thesis significantly contributes to insect-microbiome research by examining microbiome differentiation against a wide variety of factors, demonstrating the intricacies of microbiomes within different insect hosts, and beginning to incorporate the microbiome into traditional community ecology. From the chapters presented here, several patterns regarding microbiome communities have emerged beyond the specific conclusions of each chapter:

- Firstly, that insect life stage significantly influences microbiome profiles (Chapters 3-5), in both holometabolous (e.g. *Drosophila*) and hemimetabolous insects (e.g. *Triatoma*) (Hammer & Moran 2019). This is highly notable because holometabolous and hemimetabolous insects develop in distinctive ways, but life stage is very important for microbiome composition in both.
- Secondly, that it is vital for researchers of free-living organisms to consider their symbiotic microbes and determine what role those symbionts have in influencing host ecology. Chapter 2 discussed the metacommunity approach to stimulate research ideas on this topic. This is especially important within the context of complex, interacting host communities, like those in Chapters 3 & 4.
- Thirdly, the importance of controlling for diet in field studies of microbiomes. Diet is a well-known influencer of microbiome community composition (Turnbaugh *et al.* 2009; Muegge *et al.* 2011; Colman *et al.* 2012; David *et al.* 2014; Yun *et al.* 2014). Thus, in my chapters, we explicitly made an effort to control diet (banana bait for *Drosophila* in Chapters 3 & 4 and collecting triatomines from the same rodent species in Chapter 5). A common theme of microbiome communities is a high degree of among-individual variability because microbiomes can be influenced by so many different factors, so controlling for any one of these factors - particularly diet and location - can allow us to see other deterministic patterns in wild insect microbiomes.
- Fourthly, the importance of building up the taxonomic and functional catalogues of bacterial diversity. The focal insect species within this thesis were all new for microbiome study. Bacterial species, both free-living and host-associated, are estimated to make up the vast majority of undescribed

species on Earth (Torsvik *et al.* 2002), and thus each new host profiled represents a major advance in cataloguing the alpha taxonomy of the biosphere.

The most prominent conclusion from the chapters presented here is that host life stage is an incredibly important factor for determining insect microbiome composition, in both holometabolous and hemimetabolous insects. Hammer & Moran (2019) recently reviewed the central features of holometabolous insect development with regard to their gut microbiomes, which emphasises how much the gut microbiome can change across different developmental stages. Logically this makes sense because the insect is undergoing complete metamorphosis, thereby 'resetting' its gut physiology and microbiome. The results in Chapter 4 were particularly notable in this regard, where we found a surprisingly large difference between pupae and larvae of the same species that had been kept in identical micro-environments and fed the same diet for multiple generations. Therefore, even in a scenario where the *Drosophila* microbiomes had undergone long-term restriction, there was still a strong difference between life stage. With hemimetabolous insects, the incomplete metamorphosis between life stages creates a less distinct picture and makes it more likely for certain bacterial taxa to persist between nymphal stages. Further work on hemimetabolous insects is required to determine how stochastic these microbiome changes are, or whether it is primarily a product of the specific region that a microbe resides in (i.e. foregut, midgut, or hindgut). In Chapter 5 we found a complex and unexpected ontogenetic pattern in the microbiomes of multiple triatomine species. Triatomines are a good example of insects with particularly complex ontogenetic cycles, and their microbiomes reflect this. Most studies of triatomine microbiomes have just focused on adults - thereby ignoring 6 out of 7 total ontogenetic stages, and not providing a complete biological picture. Thus the main takeaway is to never underestimate the importance of ontogeny, regardless of organism!

A second important contribution, most prominently from Chapter 2, is the importance of including symbiotic microbes in insect community studies. The results from Chapters 3 and 4 nicely illustrate how flexible insect-associated microbiomes

can be, even when working with the same study system in the same place, thus adding incentive to focus on microbiomes within the context of insect community ecology. The perspective in Chapter 2 was to provide a way into large-scale ecology for microbial biologists. Other studies have presented links between metacommunity ecology and host-microbiome research (Mihaljevic 2012; Burns *et al.* 2016; Halliday *et al.* 2017; Miller *et al.* 2018; Miller & Bohannan 2019), so the specific goal in ours was to provide a more practical approach by providing a guided publication on how to model insect host-symbiont interactions. As more and more studies emerge documenting the importance of host-associated microbiomes, techniques like these will become more necessary. Moran (2002) and Douglas (2010) both described hosts as 'evolutionarily addicted' to their microbes, because microbes have been ubiquitous in the environment for the entire history of all eukaryotic organisms. Thus to study organismal ecology and evolution *is* to study bacterial symbiosis, knowingly or not. We cannot sufficiently understand an organism without considering the symbiotic microbes and their genes. For instance, human gut bacteria collectively contain 300x as many metabolic genes as the human genome (Qin *et al.* 2010). That incredible array of functional diversity cannot be ignored if we are to fully understand how human guts function.

Another major contribution of this thesis is documenting the alpha diversity of insect microbiomes. Many species studied within these chapters had not had their whole microbiome sequenced prior to the work presented here. This may seem trivial, but we can't study something if we don't know what it is. We encountered these sorts of issues with *Dietzia* in Chapter 5. Our study is the first to document *Dietzia* from triatomines in large proportions, which limits our ability to unravel its function in triatomine microbiomes but also positions this taxon as a candidate for culture and further analysis. Comparatively, in Chapters 3 & 4, our *Drosophila* microbiomes contained dominant taxa that one would generally expect to find from other studies on closely related species, which could be explained by the disparity in literature on these two insect taxa - much remains unknown about triatomine microbiomes, whereas *Drosophila* have been comparatively well studied. Indeed, one of the limitations of this thesis, and insect-microbiome work in general, is our knowledge of the species within the community. We require a significantly greater comprehension

of microbial alpha diversity and functional diversity to properly understand host-associated microbiomes (Blow *et al.* 2020; Jacoby *et al.* 2021). Yet because bacteria do not fit neatly into any existing species concept, our taxonomic understanding has lagged behind, relative to other organisms. For example, different strains of *Escherichia coli* exhibit up to 30% genome variation and we still refer to them as the same species (that's 10x more genome variation than exists between humans and chimpanzees; Lane 2015). Similarly, two strains of *Streptomyces* have identical 16S rRNA gene sequences, but completely different metabolomes (Antony-Babu *et al.* 2017). These two examples highlight the difficulties we have categorising bacteria, which simultaneously raises the importance of attempting to create a broad catalogue of bacterial taxa.

Methodologically, this issue ties together well with the known limitations of 16S rRNA gene sequencing. Genetic metabarcoding is largely taxonomic and provides limited insight into function, or active/inactive status in a community. Some studies work around this by comparing DNA/RNA sequencing on the same community, to get a measure of the proportion of taxa involved (Meyer *et al.* 2018). Thankfully, modern molecular techniques are rapidly developing, and technologies are becoming cheaper, enabling increased usage of metagenomics, metatranscriptomics, and metaproteomics. This results in larger datasets which will allow us to analyse patterns at broader scales (more samples in single sequencing runs), investigate microbiome composition in greater depth (more depth of reads from new sequencing processes), further investigate transmission of microbes (and their genes), and examine the mechanisms behind the different functions of microbial taxa (by analysing enrichment of functional pathways) (Hatzenpichler *et al.* 2020). If I was to start the doctorate process all over again, knowing what I know now, I would want to follow the technology and emphasise the necessity of genomic sequencing of the hosts and their microbiomes, to get a better picture of the functional pathways in insect-microbiome symbiosis. This is potentially where the holobiont concept can become useful, because it enables us to think about host organisms and their symbiotic microbes in a more fluid way, thereby combining them into a single entity from a functional perspective (Zilber-Rosenberg & Rosenberg 2008; Gilbert *et al.* 2012; Shropshire & Bordenstein 2016).

Moving forward, I anticipate that host-microbiome research will continue to develop at a rapid pace, particularly when we start investigating non-bacterial elements of organismal microbiomes, i.e. symbiotic viruses, archaea, eukaryotes, more broadly. Many of these microbiome components have been overlooked, but (Seelbinder *et al.* 2020) found that three months after antibiotic use, the gut bacteria community had mostly recovered but the gut fungal community had not, and thus the gut had not fully recovered its pre-antibiotic range of functions. Moreover, the interactions amongst the symbiotic fungal community shifted from mutualism to competition. In a similar vein, Zhu *et al.* (2018) found that symbiotic polydnaviruses were crucial for trophic interactions between parasitoids. Thus we are starting to discover the functional relevance of these other symbiotic partners to insect hosts (also see Gao *et al.* 2020). I anticipate that similar studies on other host organisms will uncover a range of important relationships between hosts and symbiotic eukaryotes, archaea, and viruses, and I expect there to be a lot more of these studies.

Looking further forward, as mentioned in the Introduction (Section 1.5), Vellend (2010) proposed that ecology and evolution are influenced by the same four processes operating on different timescales (dispersal, selection, drift, and diversification). This synthesis is analogous to the arguments presented by Jonathan Chase and others about spatial scale (i.e., the processes are the same, but the scale of analysis determines which ones appear dominant). I believe host-associated microbiomes/holobionts represent a unique opportunity to study these concepts in explicit detail. Firstly, these host-associated communities are much more spatially explicit (e.g. if focusing on the organismal microbiome of *Drosophila melanogaster*, one whole fly equals the whole microbiome, by default). Secondly, by dealing with a eukaryotic host and symbiotic bacteria, one is studying organisms with radically different generation times, which results in different rates of evolution. Thus the boundaries between ecological and evolutionary time are more blurred than usual. Ecology suffers enormously from the "disease of context" (Lawton 1999). In community ecology, on occasions when microbiomes have been considered they have often been treated as a host trait. Host filtering of the microbiome is a heritable trait, in the sense that any single host microbiome cannot be colonised by every single

possible bacterium (Capunitan *et al.* 2020). Obligate endosymbionts can also be treated as a host trait because they are omnipresent within hosts but treating the whole microbiome in this manner fails to acknowledge the intricacies (e.g. interactions amongst microbes) of the other microbiota present. Generalising their importance masks enormous amounts of variation and papers over unique and elaborate aspects of the microbiome that have important ecological and evolutionary ramifications, especially when examining facultative endosymbionts in invertebrates. The combination of stochastic and deterministic selection pressure, variable rates of evolution, and stochastic microbiome community colonisation will undeniably make disentangling these factors incredibly difficult but no less relevant for our understanding of nature.

Overall, whilst we have come a long way in understanding insect microbiomes in the last 25 years, we are still at the tip of the iceberg. The development of this field has been incredibly rapid and shows no signs of slowing down, and the ramifications for community ecology have been enormous. From my dissertation, we have learned that insect life stage is crucial for determining microbiome composition, and that controlled sampling from natural environments provides new information on insect microbiome communities and their potential interplay with the host. From a broad perspective, these interactions have clear ramifications for the survival (or not) of host insects, thus affecting population, community, and species-level dynamics. I hope that future research will continue along this path and give the ecological and evolutionary influence of microbial symbiosis the consideration it warrants.

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APPENDIX: Curriculum Vitae

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CURRENT POSITION

Dec 2016 - Current **Ph.D. in Entomology, University of South Bohemia**
Supervisors: Dr. Jan Hrček & Prof. Owen Lewis

ACADEMIC TRAINING

Oct 2016 - Feb 2017 **Barcoding Technician, Department of Zoology, Oxford University**
Supervisor: Prof. Owen Lewis

Oct 2015 - Sep 2016 **M.Sc. Hons. Ecology, Evolution & Conservation, Imperial College London**
Supervisors: Dr. Sarah Knowles & Dr. Lauren Cator

Sep 2012 - July 2015 **B.Sc. Zoology, Queen Mary, University of London with First Class Honours**
Supervisors: Dr. Robert Knell & Dr. David Hone

RESEARCH OUTPUT

PUBLICATIONS & MANUSCRIPTS (Listed chronologically; self in bold)

1. **Brown, J.J.**, Mennicken, S. ...., Hrček, J. & de Bello, F. (2019). A novel method to predict dark diversity using unconstrained ordination. *Journal of Vegetation Science*. 30 (4), 610-619.
2. **Brown, J.J.**, Mihaljevic, J.R., Des Marteaux, L. & Hrček, J. (2020). The metacommunity concept for horizontal transmission of insect associated heritable symbionts. *Ecology & Evolution*. 10 (3), 1703-1721.
3. **Brown, J. J.,** Rodríguez-Ruano, S. M. ...., Nováková, E. (2020) Ontogeny, species identity, and environment dominate microbiome dynamics of wild kissing bugs (Triatominae). *Microbiome.* 8, 146.
4. Jeffs, C. T., Terry, J. C. D. ..., **Brown, J. J.,** ..., Hrček, J. & Lewis, O. T. (2021) Molecular analyses reveal consistent food web structure with elevation in rainforest *Drosophila* - parasitoid communities. *Ecography*. 44 (3), 403-413.

5. Lue, C-H., Buffington, M. L. ..., **Brown, J. J.,** ..., Hrček, J. (2021) DROP: Molecular voucher database for identification of *Drosophila* parasitoids. *bioRxiv.* 10.1101/2021.02.09.430471.

CONFERENCE PARTICIPATION (listed chronologically)

1. Making London Nature Smart Symposium, London, UK, September 2015.
2. 2nd Joint Congress of Evolution, Montpellier, France, August 2018.
3. British Ecological Society Annual Meeting, Birmingham, UK, December 2018 – Poster: "The metacommunity concept for horizontal transmission of insect associated heritable symbionts".
4. Unifying Tropical Ecology (Joint BES/GTO), Edinburgh, UK, April 2019 - Presentation in symposium "Tropical mountains: ecological trends across elevation", title: "Climatic shifts alter the structure of translocated rainforest *Drosophila*-parasitoid communities".
5. Gordon Research Conference "Animal-Microbe Symbioses as Nested Ecosystems", Vermont, USA, June 2019. - Poster: "Making symbiont-insect metacommunities a reality".
6. Ecological Society of America Annual Meeting "Harnessing the ecological data revolution", Salt Lake City, USA, August 2020. - Virtual Presentation: "Ontogeny, species identity, and environment dominate microbiome dynamics of wild kissing bugs (Triatominae)".

Other Contributions (self in bold)

1. Rodríguez-Ruano, S.M, **Brown J.J.** ..., Nováková, E. Chagas Disease in the United States: Do vector microbiomes matter? (Gordon Research Conference "Tropical Infectious Diseases", Texas, USA, March 2019.

PROFESSIONAL RESEARCH EXPERIENCE

Extensive terrestrial field work experience (~15 months) in Australia, USA, Brunei, Canada, and UK, performing a variety of tasks, including food-web translocation experiment, parasitism rate experiments, species identification, camera trapping, fossil removal, assessing ecosystem productivity, biodiversity estimates, and behavioural surveys.

Molecular lab experience performing DNA extractions, confirmation and Multiplex PCRs, gel electrophoresis, Sanger sequencing, DNA metabarcoding through the Illumina MiSeq platform. Processing metabarcoding sequencing reads and analysing bioinformatic data in QIIME. Performing statistical analysis in R.

Long-term research stays at James Cook University in Australia (Feb.-July 2017; March-April 2018), Northern Arizona University in USA (July-Aug. 2019).

SCHOLARSHIPS & FELLOWSHIPS

IBERA 2020 from the Czech Academy of Sciences, valued at ~2000 USD.

AWARDS & PRIZES

Best Presentation at PhD Student Conference Jan. 2020, Czech Academy of Sciences.
Best PhD Student Publication in 2020, Czech Academy of Sciences.

PUBLIC & ACADEMIC SERVICE

Professional memberships

- British Ecological Society (since 2017)
- Australian Herpetological Society (since 2017)
- European Society for Evolutionary Biology (since 2018)
- Tucson Herpetological Society (since 2018)

Peer reviewer - Journals

Biocontrol, Eco Health, Ecological Entomology

Peer reviewer - Grant Proposals

1 proposal for the Hungarian National Research, Development and Innovation Office in 2020.
4 proposals of GAJU student grants at University of South Bohemia

Invited talks

Murdock Lab, University of Georgia, September 2020

Outreach

Posters and articles for the British Ecological Society, Nov. 2018 on nocturnal biodiversity.
Supervision of National Insect Week events for local schools, at Silwood Park, Imperial College London, July 2016.

Broad research interests: Parasite ecology, microbiomes, vectors of pathogens and disease, species interactions in ecological networks, holobiont concepts & horizontal gene transfer, herpetology.
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