Microbiome structure of a wild *Drosophila* community along tropical elevational gradients and comparison to laboratory lines

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Abstract

While the biogeography of free-living microbial communities is well-studied, community turnover along environmental gradients in host-associated communities is not well understood. In particular, patterns of host-microbiome diversity along elevational gradients remain largely uncharacterized. Because elevational gradients may serve as natural proxies for climate change, understanding these temperature-influenced patterns can inform our understanding of the threats facing hosts and their microbes in a warming world. In this study, we analysed microbiomes from pupae & adults of four *Drosophila* species native to Australian tropical rainforests. We sampled wild individuals at high and low elevation along two mountain gradients, to determine natural diversity patterns, and sampled laboratory-reared individuals from isofemale lines established from the same localities, to see if any natural patterns would be retained in the lab. In both environments, we controlled for diet to help elucidate other deterministic patterns of microbiome composition. Microbiome community composition differed radically between laboratory-reared and field-caught flies but did not significantly differ across elevation. We found some notable taxonomic differences in *Drosophila* microbiomes between different species and elevations. We also found similar microbiome composition from both types of provided food, and we therefore suggest the significant differences in richness are the products of environments with different bacterial species pools. We conclude that elevational differences in temperature are not a major factor in determining *Drosophila* microbiome composition and we caution against determining microbiome composition from lab-only specimens, particularly long-term cultures.

Importance

Bacteria form microbiome communities inside many different hosts. These communities vary widely in diversity, and form differently, depending on a wide variety of factors. Diet is often an important factor for microbiome composition. We controlled diet in our study by providing the same food sources. We looked at how host-microbiome communities differed in four *Drosophila* species over two mountain gradients in tropical Australia, to see if temperature-driven differences in elevation affected host-microbiomes. We also compared these results to individuals kept in the laboratory to understand how different settings changed microbiome communities. We found that field samples had substantially greater microbiome diversity than those from the lab and found minimal differences in microbiome communities over elevation. Our study shows that environmental sources of bacteria matter for *Drosophila* microbiome composition, and caution should be exercised when interpreting microbiome results from lab-only studies.

Introduction

Patterns of diversity over environmental gradients like latitude, elevation, or environmental degradation have long been of interest in community ecology and are of renewed interest for studying the potential consequences of climate change (Fierer et al., 2011; Wang et al., 2011; Pärtel et al., 2016; Thompson et al., 2017). Most studies have focused on animals and plants to investigate these patterns, but bacterial communities are receiving increased attention. Some studies suggest free-living 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). Fierer et al. (2011) showed that soil bacteria did not change significantly in diversity when sampled across an elevational gradient, in contrast to trends documented in most other taxa. Subsequent studies have found inconsistent patterns in bacterial communities sampled

72 from streams and soils across elevational gradients, with differences usually being attributed
73 to changes in pH and C:N ratio (Wang et al., 2011; Shen et al., 2015; Siles and Margesin, 2016;
74 Meyer et al., 2018).
75
76 Many insects maintain intimate communities of symbiotic microbes (their 'microbiome').
77 Insect microbiomes can play important roles in host health, digestion, thermal regulation, and
78 protection against natural enemies (reviewed in McLean et al., 2016; Corbin et al., 2017;
79 Brown et al., 2020a). In turn, many factors can influence insect microbiome composition,
80 some host-associated (e.g. diet, insect species identity, ontogeny, and parent-to-offspring
81 transmission), others abiotic (e.g. local environment and temperature) (Colman et al., 2012;
82 Yun et al., 2014; Xie et al., 2015; Martinson et al., 2017; Nováková et al., 2017; Bing et al.,
83 2018; Park et al., 2019; Brown et al., 2020b). Symbioses between insects and bacteria have
84 been particularly well investigated (Douglas, 2016), notably because their microbiome
85 communities tend to be less complex than those of vertebrates (Woodhams et al., 2020).
86 However, in contrast to environmental microbial communities, the effect of elevational
87 change on insect-associated microbiome communities has yet to be investigated in-depth.
88 The most conspicuous aspect of a change in elevation is a difference in mean temperature,
89 creating different abiotic environments that can be used as a proxy for climate change
90 scenarios (Wadgymar et al., 2018; Nottingham et al., 2019). We would expect to see
91 differences in microbiome composition because development is temperature-dependent in
92 both insects (Economos and Lints, 1984; James et al., 1997; Kinjo et al., 2014; Tochen et al.,
93 2014; Brankatschk et al., 2018) and bacteria (Ratkowsky et al., 1982; Pettersson and Bååth,
94 2003; Tsuji et al., 2017). Thus, at different elevations and in climate change scenarios, insect-
95 associated microbiomes could develop differently.

Many microbiome studies have been performed on lab-reared organisms, and many studies have sampled organisms from the field, but few have done both. Some studies suggest that microbiome richness is not appreciably different between the lab and the field (Hegde et al., 2018), whilst others find that captive animals have significantly reduced microbiome richness, and significantly altered taxonomic composition, compared to wild animals (Morrow et al., 2015; Bost et al., 2018a; Hegde et al., 2018; Dada et al., 2020). Model organisms such as mice, *Drosophila melanogaster,* and mosquitoes have had their microbiomes sequenced as part of laboratory studies. The main 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, zoos, or wildlife parks (Amato et al., 2013; Nelson et al., 2013; Hammer et al., 2014; Kohl et al., 2014; Clayton et al., 2016; Staudacher et al., 2016; Adair et al., 2020; Dada et al., 2020). For many free-ranging animal species, lab vs field comparisons are not feasible due to the difficulty of bringing wild animals into a laboratory, but insects offer a tractable model system to directly compare lab and field microbiotas from the same host species.

Drosophila spp. are established models for studying 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 including 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

120 endosymbionts (*Wolbachia* and *Spiroplasma*) that influence host immunity and protect
121 against natural enemies, including pathogenic fungi, nematodes, and parasitoids (Chrostek et
122 al., 2013; Hamilton and Perlman, 2013; Haselkorn et al., 2013; Xie et al., 2014; Yadav et al.,
123 2018). *Drosophila* bacterial microbiomes are of moderate-to-low diversity, making them
124 relatively simple to characterise. This, combined with the well-studied nature of *Drosophila,*
125 makes them ideal candidates for investigating insect-associated microbiomes over
126 elevational gradients, and in a field vs laboratory setting.

127

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

144 find high among-individual variation and hypothesised that species identity, elevation, and
145 environment (i.e. lab vs field) would be the primary causes of difference in host microbiome
146 community composition (Chandler et al., 2011; Staubach et al., 2013; Adair et al., 2018, 2020).
147
148 **2. Material & Methods**
149 *2.1 Study Sites*
150 The Australian Wet Tropics World Heritage Area (WTWHA) is a 450 km long, narrow section
151 of rainforest along Queensland's northeast coast between Cooktown and Townsville (15-19'S,
152 145-146.30'E). Samples were collected from two altitudinal gradients: Paluma Range Road
153 (within Paluma Range National Park 19°00'S, 146°14'E) and Kirrama Range Road (within
154 Girramay National Park 18°12'S, 145°50'E). The Paluma gradient ranges from 59 m to 916 m
155 above sea level (a.s.l.) and the Kirrama gradient ranges from 92 m to 770 m a.s.l (Jeffs et al.,
156 2021). We chose sites at high, middle, and low elevations (Paluma: 880m, 350m, 70m;
157 Kirrama: 730m, 390m, 70m) to capture a ~5°C temperature range (mean temperatures 21°C
158 at high elevation, 26°C at low elevation; Jeffs et al., 2021).
159
160 *2.2 Sample Collection and Selection*
161 To ensure a sufficiently comparable dataset, we selected stratified subsets of samples from
162 the field and laboratory. We primarily collected pupae because they are a sessile and long-
163 lasting life stage, allowing us to account for discrepancies in development rate between
164 species. We also collected a small number of adult flies to compare microbiome composition
165 between life stages. Based on the results of Jeffs et al., (2021) which identified the wild
166 *Drosophila*-parasitoid food web to species level with COI metabarcoding and Multiplex PCR

methods, we selected 214 field samples of the four most common species (to control for species identity) that occurred at all elevations along both transects: *D. rubida, D. pseudoananassae, D. pallidifrons,* and *D. sulfurigaster*. Eight individuals of *D. rubida* were parasitised, enabling us to examine if there are any changes in microbiome richness or unique microbial taxa associated with a developing parasitoid. We subsequently 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. We additionally 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, to ensure we could compare *Drosophila* microbiome samples to a dietary reference and determine how controlling for diet influenced our *Drosophila* microbiome communities.

Samples of *Drosophila* pupae were collected from banana-baited bottle traps placed at low, middle, and high elevation sites along both altitudinal gradients. Each bottle trap had a piece of cardboard to assist *Drosophila* larvae in pupation. 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). On the day of sampling, these cards were removed and sealed in Ziploc® bags. The pupae we collected had only fed on banana bait. Pupae from each card were sampled by placing the card on a white plastic dinner plate and adding distilled water, using a small paintbrush to remove all pupae. Each individual pupa was placed into an individual well in 96-well PCR plates and preserved in 100% ethanol. Adults were collected using an aspirator, 2 days after provision of fresh

191 banana bait and placed into individual vials in 100% ethanol. Laboratory-reared pupae were
192 collected with forceps from standard fly food. Adults were collected with an aspirator and
193 sexed, then placed in individual vials with 100% ethanol. Laboratory isofemale lines were
194 established from the same populations sampled in the field (i.e., they were collected at the
195 same sites and shipped live to the lab in Czech Republic, one year after collection of the field
196 samples used in this experiment). Isofemale lines were kept in the lab for between 18-30
197 months by the time of sampling. Complete sample breakdown is shown in Table 1.
198
199 *2.3 Library Preparation & Sequencing*
200 Field sample DNA was extracted using GeneAid Blood and Tissue kits for host-parasitoid
201 identification (published in Jeffs et al., 2021). Lab and bait samples were extracted using the
202 same single column method according to manufacturer instructions, with one extraction
203 negative control accompanying every 29 samples. All samples were subsequently moved to
204 96-well plates in a randomised order. DNA templates were stored at -75ºC. These templates
205 were used for amplification of ~400 bp of the V4/V5 hypervariable region of the 16S rRNA
206 gene according to Earth Microbiome Project standards (EMP;
207 http://www.earthmicrobiome.org/protocols-and-standards/16s/). Sample multiplexing was
208 based on the EMP-proposed double barcoding strategy using the recommended
209 modifications (12 bp Golay barcodes included on the forward primer 515F, and additional 5
210 bp barcodes on the reverse primer 926R). We also added a custom 18S rRNA gene blocking
211 primer (named 926X) to counteract the low specificity of EMP primers towards the 16S rRNA
212 gene (details in Brown et al., 2020b). PCR amplification was confirmed with gel
213 electrophoresis. PCR products were purified with AMPure XP (Beckman Coulter) magnetic
214 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™). The library comprised four plates of samples. In each sequencing plate there was one ENC, two NCs, and two PCs - one even mock community and one staggered mock community. The library was 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).

2.4 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, demultiplexed and merged the reads which resulted in a final amplicon length of 357 bp. We then 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 1132 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 identity threshold (e.g., Fig. S8).

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 of positive detection of OTUs in a sample to 2000 reads, because all the negative controls had a total number of reads beneath this number. This 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, to standardize the dataset for diversity analyses. These procedures resulted in a dataset of 117 OTUs and 343 samples. We used Shannon index and Bray-Curtis dissimilarity as quantitative measures of alpha and beta diversity, respectively, 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).

With negative controls removed from the data, we had a mean 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 sequence outcomes (Rodríguez-Ruano et al., 2018; Brown et al., 2020b).

3. Results

3.1 Microbiomes across elevation

Elevation had a small but significant effect (NMDS ordination, mean stress ≈ 0.22, PERMANOVA, R^2 = 0.067, $p \leq 0.001$; Fig. S4) when comparing pupae samples from the different field sites, suggesting that differences in temperature and geographic location (because of elevation) 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 Shannon index values (Fig. 1). We found this significant trend in both pupae and adult *Drosophila* (Fig. S5/S6). 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* (Fig. S1).

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 in this dataset). *D. rubida* microbiomes had a much greater relative abundance of *Providencia* than the other three species. The other dominant bacterial genera were evenly distributed throughout all four *Drosophila* species sampled here, including *Acinetobacter,* which was the most dominant genus 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, nor was there any notable change in the abundance of dominant bacteria. Examination of microbiome composition indicated that there were no bacterial genera unique to parasitised samples.

In contrast to the field, there was much stronger species-specificity in the lab-reared samples (PERMANOVA R^2 = 0.292, p ≤ 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 durations to ensure we characterized the insect community fully. The field sample results suggest that this had minimal influence on microbiome composition, in comparison to the dominant patterns we identified (3% variation explained by length of exposure vs 7% or more by other factors). In the lab, the number of generations a fly line had been in the lab was not significant when tested with alpha diversity metrics but was a significant factor in NMDS analysis. Yet it only explained 1.5% variation and there was no discernible difference in bacterial genera composition. Surprisingly, there was no significant difference in microbiome composition of laboratory food samples from Czech Republic and the banana bait that we used in the field in Australia. Both are completely dominated by *Acetobacter* and *Lactobacillus,* with some lab food samples containing *Gluconobacter* (Fig. S7). In lab-reared flies, these 3 genera dominated the microbiomes of pupae and adults (Fig. S1). In the field, however, *Acetobacter* and *Lactobacillus* were not the most dominant genera. There was still some congruence because these taxa were still present

330 at high relative abundance, but their relative abundance was proportionally lower because
331 field-caught fly microbiomes were much richer.

332

333 **Discussion**

334 We examined *Drosophila* microbiome community-level patterns across multiple elevations,
335 species, environments, and life stages. We specifically focused on elevation as a potential
336 factor influencing microbiome composition, due to the lack of prior investigation and the
337 natural variation in temperature that elevation gradients provide. Our results reveal
338 significant differences in community dissimilarity between high and low elevation across both
339 gradients but these differences are small, based on the amount of variation explained by
340 PERMANOVA tests. This finding is likely a result of the species sampled here being ubiquitous
341 across elevation and not forming sufficiently distinct populations at high and low sites, and
342 because the ~5°C temperature shift between our sites is not strong enough to drastically alter
343 microbiome composition. This result was unexpected, because there is well-documented
344 evidence of both insects and bacteria developing differently according to differences in
345 temperature of this magnitude (Pettersson and Bååth, 2003; Kinjo et al., 2014; Tochen et al.,
346 2014; Tsuji et al., 2017; Brankatschk et al., 2018; Cooper et al., 2021). Despite previous studies
347 demonstrating a lack of change in bacterial diversity across elevation (Fierer et al., 2011;
348 Wang et al., 2011), we expected to find a difference in *Drosophila*-associated microbiomes
349 because an insect's internal environment is very different from soil or streams. Naturally, diet
350 might differ along an elevational gradient based on the fruits that develop in different
351 environments. By standardising diet, we showed that changes can be expected in different
352 *Drosophila* species due to non-dietary forces. Furthermore, the high disparity in microbiome
353 composition between banana bait samples and field-reared pupae suggests that diet is not

354 always the most important variable structuring *Drosophila* microbiomes, either. By
355 standardising diet, we expected some diet-induced homogenisation to occur in *Drosophila*
356 microbiomes. Instead, we found an overall lack of congruence. From a broad perspective,
357 changing global temperatures may not result in large changes to insect-associated
358 microbiomes, at least in insects like *Drosophila*.

359

360 The most pronounced differences in microbiome composition were between individuals
361 raised in the laboratory and those raised in the field. Multiple factors coalesce to explain this
362 distinction. Firstly, lab and field individuals were exposed to agar-yeast fly food medium and
363 banana, respectively, thus their dietary sources were different. However, the food sources
364 themselves have very similar microbiome profiles, suggesting the dietary source had little
365 influence. The bacterial community from lab food matches well with the microbiomes found
366 within pupae and adult flies. This would be expected, because it shows a well-established
367 pathway of insect microbiome colonisation - they ingest food and acquire the bacteria
368 associated with that food source. Yet in the field, fly microbiomes do not correspond well
369 with the bacterial communities found on banana bait samples, the microbiomes are much
370 richer in the field. We can therefore infer that the differences are mostly due to significant
371 differences in microbiome colonisation from environmental bacterial species pools (Cornell
372 and Harrison, 2014; Kohl, 2020). The flies sampled from the lab come from a highly regulated
373 environment, with a specific and consistent food source provided into heat-sterilised glass
374 vials, so the only 'available' bacteria for colonising their microbiomes comes from the food
375 and vertically inherited bacteria (e.g., *Wolbachia* in *D. pseudoananassae*). In contrast, the
376 bacterial species pool in Australian tropical rainforest comprises much greater diversity and
377 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. We found consistent diversity in wild *Drosophila* microbiomes - suggesting that bacteria are not being selectively removed from communities and that low-biomass microbiomes are predominantly colonised from the wider environment. Bottle traps were visited by other organisms, which could have functioned as a source of bacteria indirectly transmitted to the *Drosophila* sampled in this study.

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 congruence 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 is unlikely to explain the discrepancy. With adult flies we cannot 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 is clear that different food sources could explain 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, particularly those from cultures maintained across multiple generations, as these are highly unlikely to be representative of natural microbiomes (Fig. S1) (Bost et al., 2018b; Dada et al., 2020). For example, we found that species-specificity was greater in lab-reared flies, but this result is likely a product of the heavily reduced microbiome richness in lab samples and not reflective of natural species-specific differences.

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 (7% variation explained in the field; 29% in the lab) but it did not explain as much variation as the results from Adair et al., (2020) - who found species identity explained 42% and 70% variation in two different sets of *Drosophila*. This 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 (e.g. *Anopheles albimanus;* Dada et al., 2020). Since the four species we sampled are all frugivorous, sympatric *Drosophila* species, it is unlikely that

426 *Drosophila* diversification played much of a role in generating the microbiome differences we
427 found here (especially because this study was not multi-generational).
428
429 The discrepancy in microbiome diversity found between lab and field flies suggests that
430 *Drosophila* are not heavily reliant on a diverse bacterial microbiome, because a core group of
431 specific bacterial taxa has not been consistently maintained between species or between
432 environments. Hammer et al., (2019) raised compelling points about bacterial microbiome
433 functionality and demonstrated multiple invertebrate species that appear to have no resident
434 gut microbiome. In other insect species, host transmission of extracellular symbionts (like
435 those in the gut) have been hypothesised to result in long-term associations between insect
436 and microbe (Sanders et al., 2014; Kwong et al., 2017; Sinotte et al., 2020). The long-term
437 laboratory survival of these four *Drosophila* species (minimum 24 generations) with radically
438 different microbiome composition compared to the field suggests that they do not fit the
439 hypothesis of long-term association between host and microbe, nor that their symbiotic
440 relationship with bacteria is highly specialised (Leftwich et al., 2017). However, our findings
441 from the lab partially corroborate those of Henriques et al., (2020) who found a dominant
442 functional role for *Lactobacillus* and *Acetobacter* in lab-reared *Drosophila.* Specifically, the
443 presence of *Lactobacillus* can stimulate *Acetobacter* to produce and excrete Isoleucine in
444 *Drosophila* deprived of essential amino acids, suggesting that the high relative abundance of
445 *Acetobacter* and *Lactobacillus* in our *Drosophila* species reflects a functioning microbiome
446 (Henriques et al., 2020).
447
448 A crucial factor in this study was ensuring each field sample received the same food source,
449 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 life cycle from egg to pupa within a baited trap and thus 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 useful 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, such as diet.

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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

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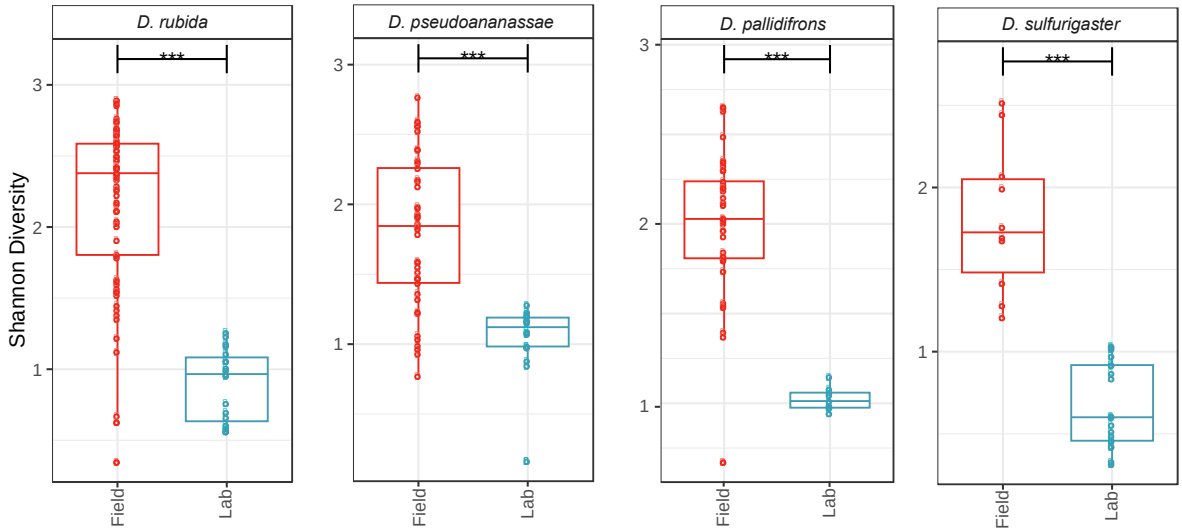


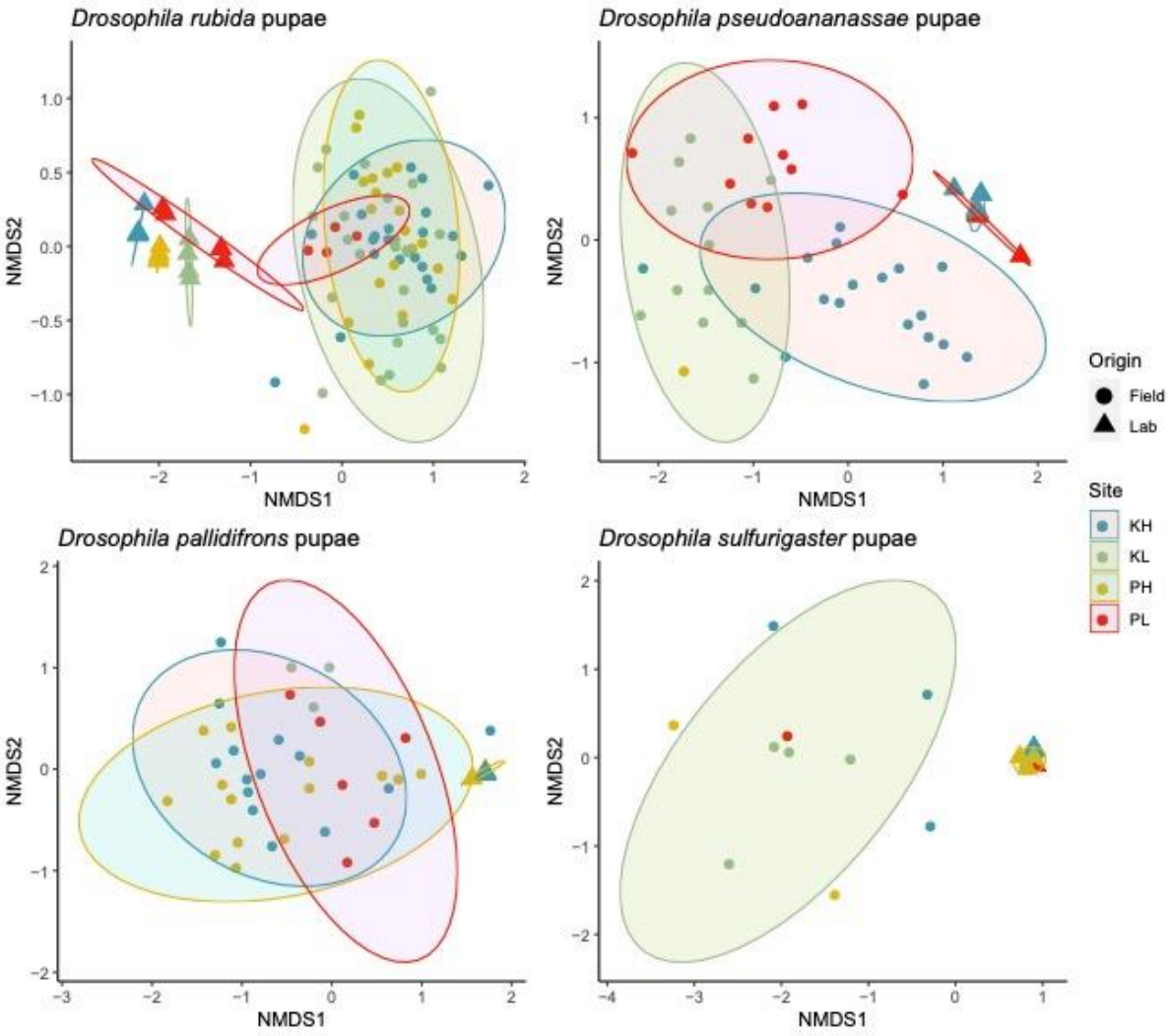
728

729 **Figure 1: Comparison of Shannon index values for pupal samples from each species of**

730 ***Drosophila* in the lab and the field.** A) = *D. rubida*, B) = *D. pseudoananassae,* C) = *D.*

731 *pallidifrons,* D) = *D. sulfurigaster.* Field samples are shown in red; lab samples are shown in

732 blue. Three asterisk (***) denotes highly significant result ($p \leq 0.001$).



733

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

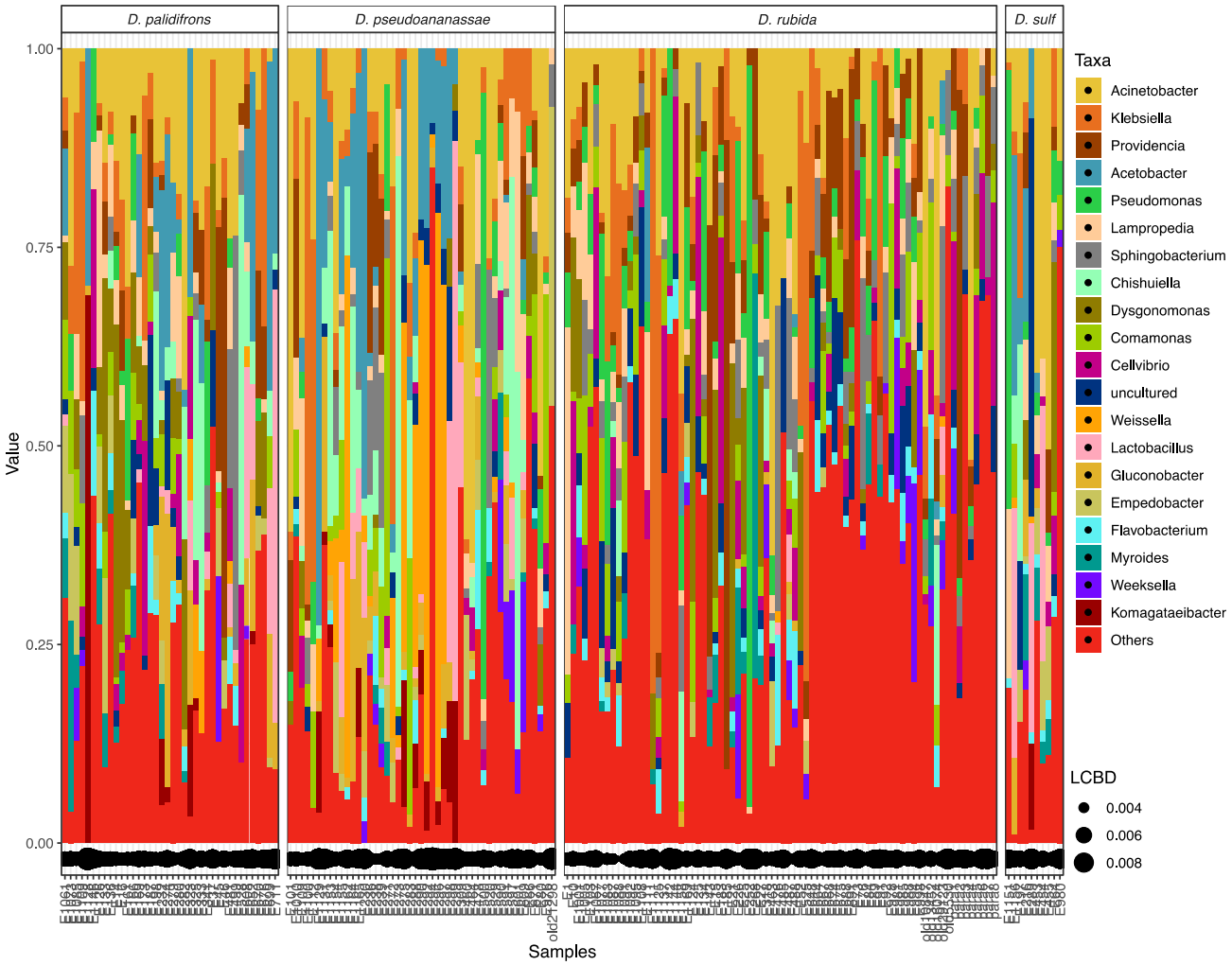


Figure 3: The top 20 bacterial genera for field-reared pupal samples of all four *Drosophila* species. Each individual column represents an individual sample. Relative abundance is on the y axis. LCBD = Local Contribution to Beta Diversity.