Developmental Stage-Specific Microbiota Profile of a Polyphagous Fly

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Running Title: Microbiome and developmental stages of Qflies

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Abstract

Gut bacteria play a key role in insect fitness, but the changes in gut microbiome profile across developmental stages of holometabolous insects remains little explored. Understanding changes in the microbiome across life stages is an important step toward understanding the associated shifts in functional relationships and trade-offs. Here, we characterised the microbiome of larvae, pupae, and adults of the highly polyphagous fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) using next-generation sequencing. We sampled individuals from colonies that had been recently introduced to the laboratory environment from naturally infested fruits at generations one ('G1') and five ('G5'). Alpha diversity increased across developmental stages at both G1 and G5, with maximum diversity in adults. Community composition changed across developmental stages and between generations. In G1, larval and pupal microbiomes were dominated by the genus *Asaia* whereas adult microbiomes were dominated by *Enterobacter*. In G5, larval and pupal microbiomes contained a high relative abundance of *Asaia*, but pupae also had a high relative abundance of *Staphylococcus* and *Burkholderia*, and there were no dominant patterns in adults. Our findings provide insights into the developmental stage-dependent microbiome associations of a polyphagous fly, and how host-symbiont interactions change at each life stage through the transition from nature to laboratory environments.

Keywords: microbiome; metamorphosis; symbiosis; endopterygota

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Introduction

Holometabolous insects undergo remarkable morphological changes during development. While physiological factors that influence these changes have been studied in detail [1,2], we still have limited understanding of how symbiotic relationships with microbes change across insect life stages [3-6]. Recent studies have suggested that, just as the host morphology is drastically altered between life stages, the composition of the insect microbiome can also undergo radical shifts [4,5,7]. These shifts in microbiome composition have been associated with a range of host functions, impacting development, nutrition, and host immunity [6,8,9]. For instance, in *Drosophila melanogaster*, development is severely impaired when the microbiome is highly modified or eliminated, indicating an integral functional role of bacteria in the healthy functioning of these flies. Specific symbiotic relationships between *D. melanogaster* and bacteria, particularly *Acetobacter pomorum* and *Lactobacillus plantarum*, can rescue normal development, and are key for development of larvae in nutritionally poor diets [6,9] as well as fitness in adulthood [10]. Thus, the microbiome composition at the early life stages can play an important role on growth, development, and fitness.

Recent studies have shown that life stage transitions are associated with compositional and functional changes in the microbiome. For instance, the composition and predicted functionality of the Egyptian cotton leafworm *Spodoptera littoralis* microbiome shifts through development in concert with morphological changes, with adult microbiomes being enriched in genes involved in replication and energy metabolism [4]. Similarly, microbiome diversity and composition change across life stages in mosquitoes (*Anopheles gambiae*), whereby the bacteria present in each mosquito life stage are essential for normal development [11,12]. To date, however, the scope of our understanding about microbiome changes during insect development is very limited. While a few studies of cosmopolitan fruit flies such as the polyphagous Medfly (*Ceratitis capitata*) and the diet specialist olive fruit fly (*Bactrocera oleae*) exist, knowledge on other tephritid fruit fly species is lacking [13,14]. Better understanding of developmental changes in microbiome will enable significant advances in

understanding of the ecology of these globally important flies, with potential applications for mass-rearing of fruit flies in biological control programs [14,15].

In the present study, we addressed this knowledge gap by collecting fruits from nature that were infested with eggs and larvae of the polyphagous fruit fly *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) and allowing flies and their microbiome to adapt to the laboratory environment over five generations. We used 16S Next Generation Sequencing (NGS) to investigate the compositional changes of the microbiome across developmental stages (larvae, pupae, adults) at G1 and G5. Previous studies of other insects have reported differences between life stages in microbiome composition and diversity, although the direction of changes in diversity and the bacterial taxa affected were species-specific [7,8,16,17]. Based upon these previous studies, we predicted that the diversity of the *B. tryoni* microbiome would increase as development progresses and that composition would be distinct between developmental stages. We also predicted that specific bacterial operational taxonomic units (OTUs) would drive these compositional differences across generations as the colonies adapted to the laboratory environment. The findings of this study provide a first insight into the changing composition of the gut microbiota across life stages and through generations in the laboratory.

Materials and Methods

Fruit samples and fly colonies

Pomegranates, apples, and quinces were collected from multiple locations across the states of New South Wales and Victoria (Australia). Infested fruits were stored in buckets (60L, 447 x 236 x 663 mm, Award, Australia) containing fine vermiculate (250 g) in a control environment laboratory (25 ± 0.20°C, 65 ± 3% RH and 11: 1: 11: 1 light: dusk: dark: dawn) at Macquarie University, Australia. Approximately 400 adult *B. tryoni* were obtained from these fruits. Two replicate populations, each of ca. 200 flies, were placed in mesh cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm), and

maintained in the controlled environment room for five generations. At each generation, eggs were collected using a perforated plastic bottle (150 mL) that contained ca. 5 mL of water; eggs were washed from the bottle and seeded into the carrot-based diet (see Supplementary Table 2), in which larvae fed *ad libitum* until development was completed. Adults were provided a free choice diet of hydrolysed yeast (MP Biomedicals, Cat. no 02103304) and commercial sucrose (CSR® White Sugar).

Sample collection and preparation

For G1 and G5 we sampled third instar larvae ($N = 6$), pupae ($N = 6$), adult males ($N = 6$) and females ($N = 6$) flies (15-16 days old) from each of the two replicate populations. There was no statistical difference between the microbial profile of adult males and females (R2 = 0.008, p = 0.25) and we therefore pooled the sexes (i.e., 'adults') for the analysis. All samples were surface sterilized following [18]. The guts of adult flies were removed under a stereomicroscope (Leica MZ6, Leica®, Germany). Using sterile pestles, we homogenised larvae, pupae, and guts from adults in a solution of Brain Heart Infusion (BHI) broth (Oxoid Ltd, UK, Lot # 1656503) with 20% Glycerol (Sigma Aldrich®, Lot # SHBG2711V) and stored the homogenised solution at -80°C for analyses. Samples were processed by Australian Genomics Research Facility (AGRF). DNA extraction was performed using DNeasy PowerLyzer PowerSoil Kit-100 (Qiagen) following the manufacturer's protocol. We amplified through PCR the V1-V3 16S rRNA region [19] for amplicon library preparation and microbial profiling. Amplicon sequencing was performed using Illumina MiSeq platform with 2 x 300 base pairs paired-end chemistry.

Data analysis

Raw sequences were demultiplexed by the AGRF. Demultiplexed sequences were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2) software (version 2018.4) using DADA2 to remove low-quality regions and chimeric sequences [20,21]. Reverse reads were truncated to 280bp

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based upon quality scores. DADA2 also performed dereplication by combining identical sequences to construct an amplicon variant table. Taxonomy was assigned using the QIIME2 q2-feature-classifier plugin and a Naïve Bayes classifier trained on the SILVA 99% OTU database [20,22]. Analyses of amplicon sequence data were performed in the RStudio statistical package (version 1.1.453) [23]. We used 'phyloseq' package in R to perform analysis of alpha diversity using multifactor analysis of variance with a post hoc Tukey honest significant difference (HSD) test [24], non-metric multidimensional scaling (NMDS), and to perform permutation multivariate analysis of variance (PERMANOVA) with 999 permutations on Bray Curtis distance matrices [25]. Differentially abundant OTUs were identified using the 'DESeq2' package [26].

Results and Discussion

In total, 177 samples generated 2,835 unique sequence variants (mean of 48 ± 32 s.d.) which were assigned to 347 OTUs (mean of 10 ± 8 s.d). We found a clear trend of increasing alpha diversity (Shannon), richness (Chao1), and evenness (Simpson) as development progressed from larvae to adults (Figure 1). Adult microbial diversity was significantly higher than larval microbial diversity at both G1 and G5, and was significantly higher than pupal microbial diversity at G1 (Table 1). Pupae had higher alpha diversity than larvae, although differences were not significant (Figure 1a-b). While shifts in microbial diversity between life stages have been observed in other insect species, these studies found larval diversity to be higher than adult and pupae [7,16]. However, low microbial diversity has been previously noted in domesticated *B. tryoni* larvae, potentially indicating that the microbial diversity of domesticated *B. tryoni* larvae is lower than in larvae of other insects [27]. The larval microbiome is almost completely dominated by OTUs assigned to the genus *Asaia* (Figure 2b-c). Despite there being 24 OTUs that were shared between the larvae, pupae and adults in G5 and only 9 in G1 (Figure 2c-d), the bacterial taxa of the shared microbiome were relatively consistent, whereby the shared microbiome in G1 and G5 was dominated by OTUs assigned to the families *Enterobacteriaceae*, *Streptococcaceae*, *Enterococcaceae*, *Acetobacteraceae*, and *Staphylococcaceae*.

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OTUs assigned to the families *Orbaceae* and *Burkholderiaceae* were also shared between all life stages in G5. As indicated by clear differences in taxonomy between life stages in Figure 1b, the overall microbial composition was significantly different between the larvae, pupae, and adult flies at both G1 and G5 (Table 1), and is expressed in non-multidimensional scaling plots in Supplementary Figure 1. As studies in other species indicate that specific microbial groups are important for host fitness, we expected the composition of the *B. tryoni* microbiome to differ across life stages due to the distinct host benefits these bacteria provide at each developmental stage. Thus, while our results are in agreement with previous literature showing that the overall composition of the microbiome differs between life stages in insects, the results also suggest that the types of microbes that change are species-specific [7,8,16,28].

There were some differentially abundant OTUs between life stages of *B. tryoni* (Table 2, Supplementary Figure 2). OTUs from the *Enterobacteriaceae* were significantly higher in adult flies than in the pupae and larvae at both G1 and G5 (Table 2). This result corroborates a previous study that reported high levels of *Enterobacteriaceae* in both wild and laboratory *B. tryoni* adults [29]. The consistently high levels of *Enterobacteriaceae* noted across generations in the present study and across populations in [29] suggests that members of this family are preferentially promoted by adult flies, and hence that they are likely important for host biology. *Enterobacteriaceae* have been linked to nitrogen-fixation in other fruit flies [30], and therefore the high levels of these bacteria in adult *B. tryoni* may be promoted due to the nutritional benefits. Pupae were associated with higher levels of *Staphylococcaceae* than were either the adult flies or the larvae (Table 2). Similarly high levels of *Staphylococcus* have also been previously reported in *D. melanogaster* pupae [7]. The pupae in generation 5 were also associated with higher levels of *Burkholderia* than were larvae or adults (Table 2). The levels of an OTU assigned to the genus *Asaia* (family *Acetobacteraceae*) were significantly higher in the pupae and larvae than the adult at both generations (Table 2). In agreement with our study, the microbiome of domesticated *B. tryoni* larvae from multiple populations have

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168 previously been reported to be dominated by *Asaia* [27], indicating that this genus is likely important
169 for larval development. *Acetobacter pomorum,* another member of the *Acetobacteraceae*, is essential
170 for development of *D. melanogaster* larvae [6]. Given that *Asaia* appears to be consistently present in
171 *B. tryoni* larvae, and that other members of the *Acetobacteraceae* are important for larval
172 development in other insects, we speculate that this genus is central to larval development in *B.*
173 *tryoni*.
174
175 **Conclusion**
176 The overall composition and diversity of the *B. tryoni* microbiome is distinct at larval, pupal and
177 adult life stages. Changes in the microbiome across life stages are driven by shifts in specific
178 bacterial groups, suggesting that these particular microbes may be key for success at each life stage.
179 This posits a starting-point for a better understanding of the microbiome changes associated with
180 life-stage transitions in *B. tryoni* and in other holometabolous insects. Given that holometabolous
181 insects are the most abundant and diverse group of insects – and which includes species in rapid
182 decline (e.g., bees) as well as major pests (e.g., Medfly) – our findings have the potential to aid
183 future efforts into understanding and manipulating the microbiome for improving individual
184 performance or as a tool for biological control.
185
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192 bioinformatics.
193

Competing interests

The authors have no conflict of interests to declare.

Data accessibility

Raw sequencing data will be made freely available in Dryad upon the acceptance of the manuscript.

Authors' contributions

RM, TC, FP and JM designed the experiment. RM and JH collected the data. JH, IL and JM analysed the data. PWT, TC, FP, JM supervised the project. All authors provided inputs into the writing of the manuscript, and approved the submitted version.

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297 **Tables**

298 **Table 1. Matrix of pair-wise microbiome comparison across life stages.**

	Generation	R2	p-value
Larvae vs. Pupae	G1	0.11	**0.0195**
	G5	0.78	**0.0014**
Larvae vs. Adult	G1	0.88	**0.0014**
	G5	0.48	**0.0014**
Pupae vs. Adult	G1	0.23	**0.0014**
	G5	0.62	**0.0014**

299 NOTE – Comparison of the overall microbial community composition of life stages within each
300 generation. Pairwise PERMANOVA with 999 permutations was performed on a Bray-Curtis
301 dissimilarity matrix. Comparisons for which $p < 0.05$ are presented in bold.

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Table 2. Matrix of significantly differentially abundant OTUs between life stages for each generation.

		Generation 1	
	Larvae	Pupae	Adult
Larvae	-	-	*Asaia*
	-	-	*Asaia*
Pupae	*Pseudomonas*	-	-
	Staphylococcus	-	*Staphylococcus*
Adult	[1]*Enterobacter*	[1]*Enterobacter*	-
	[1]*Klebsiella*	[1]*Klebsiella*	-
	[1]*Providencia*	[1]*Providencia*	-
	Staphylococcus	-	-
	[1]*Unassigned*	[1]*Unassigned*	-
		Generation 5	
	Larvae	Pupae	Adult
Larvae	-	-	*Asaia*
	-	-	*Asaia*
Pupae	*Burkholderia*	-	*Burkholderia*
	Staphylococcus	-	*Staphylococcus*
Adult	*Aeromonas*	*Aeromonas*	-
	Burkholderia	-	-
	[1]*Citrobacter*	[1]*Citrobacter*	-
	[1]*Enterobacter*	[1]*Enterobacter*	-
	Gilliamella	*Gilliamella*	-
	Lactococcus	*Lactococcus*	-
	[1]*Unassigned*	[1]*Unassigned*	-

NOTE – Matrix detailing OTUs that were significantly ($p<0.05$) different in abundance between life stages for G1 and G5 in a pairwise manner. Titles of rows indicate the life stage where the OTU detailed is significantly higher in relative abundance (i.e. OTUs in the larvae rows have significantly higher relative abundance than pupae or adult samples). [1] OTUs assigned to the family *Enterobacteriaceae*.

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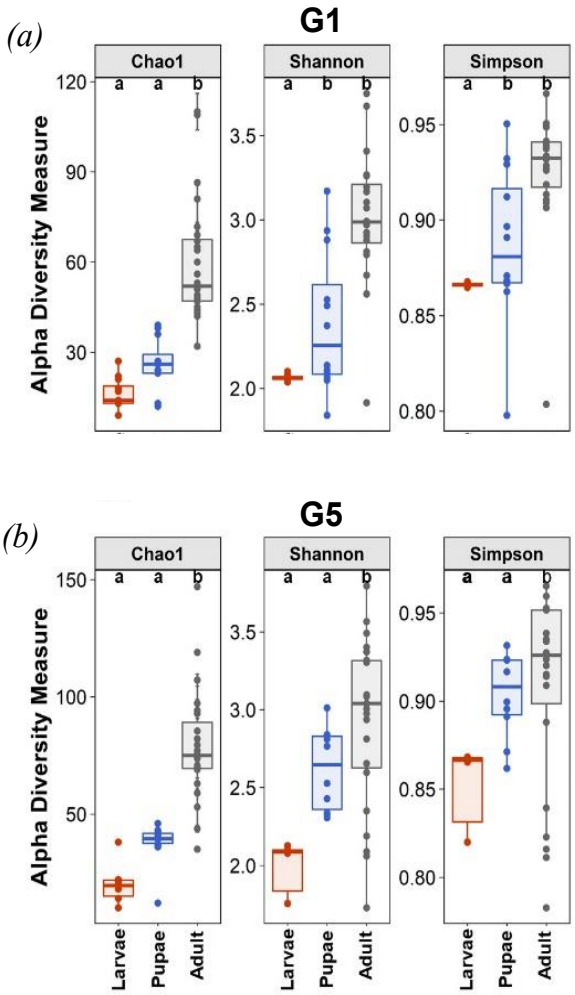
310 **Figures**



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312 **Figure 1. Microbiome diversity across life-stages.** (*a-b*) Alpha diversity (Shannon), richness

313 (Chao1), and evenness (Simpson) of larvae, pupae, and adult microbial communities for G1 (*a*) and

314 G5 *(b)*. Different letters denote significant ($p < 0.05$) differences between treatments by ANOVA

315 with Tukey's HSD test.

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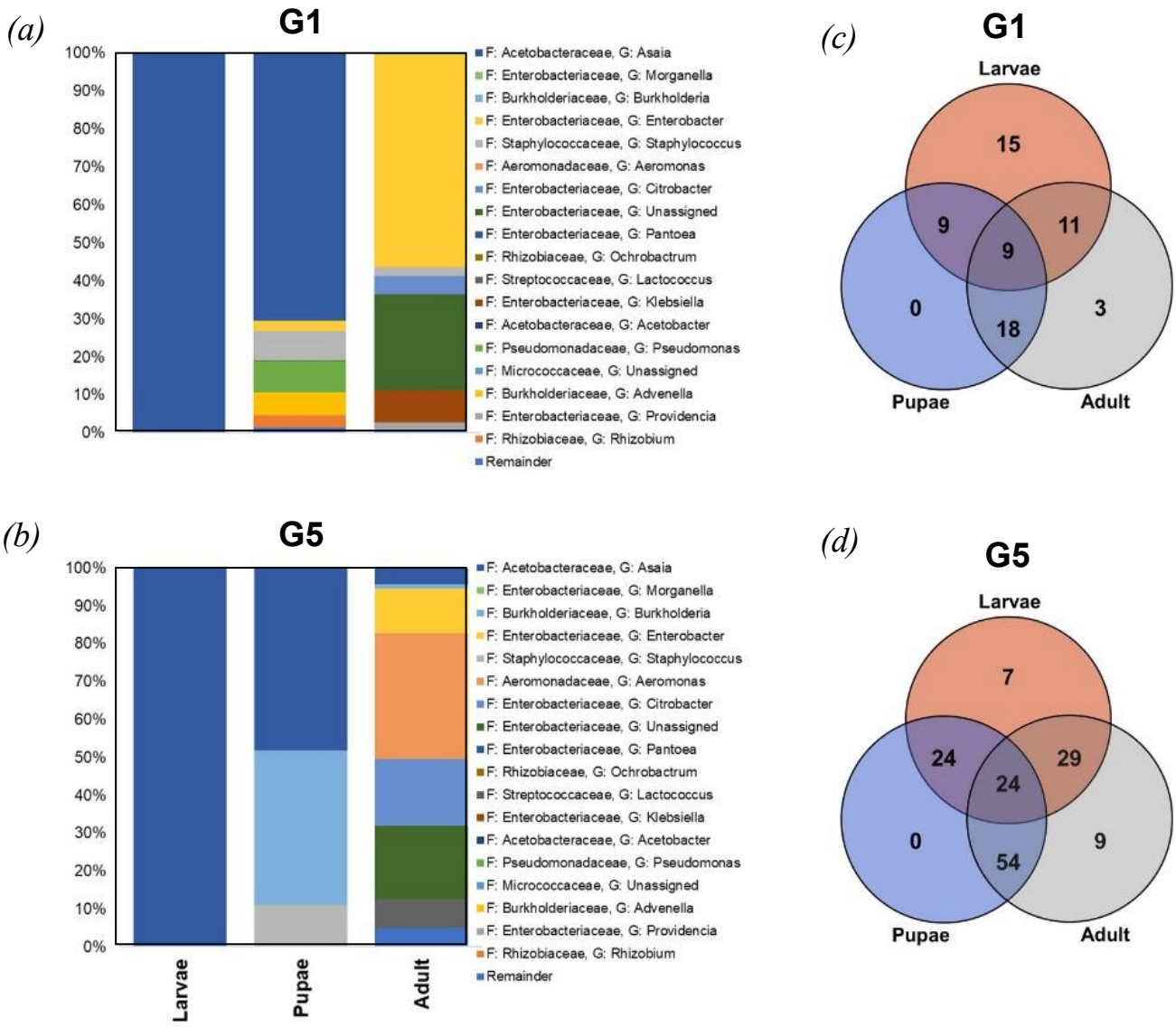

317

318 **Figure 2. Microbiome composition across life-stages.** *(a-b)* Microbial composition (mean relative
319 abundance of OTUs) for larvae, pupae, and adult samples for G1 *(a)* and G5 (*b)* grouped by genus
320 with assigned family detailed. Genera comprising less than 1% of the population are grouped under
321 remainder. *(c-d)* Venn diagrams detailing unique and shared OTUs for larvae, pupae, and adult for
322 G1 (*c)* and G5 *(d).*

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