Impact of microbiota on the life-history traits of a polyphagous fly

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In memory of my dad

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Thesis summary

The microbiota is a key modulator of fitness and disease resistance in insects. Yet, the extent to which the insect microbiota affects host performance and well-being of current and future generations remains to be elucidated. My thesis aims to better understand direct and transgenerational effects of the microbiota on life-history traits and foraging of the polyphagous fly *Bactrocera tryoni*. I first reviewed the literature to show the state of the art in the field of microbiota research in insects (Chapter 1). Then, I explored the interaction between the insect microbiota and some ecological factors in the early life stage. Results revealed that microbes acquired from both maternal transmission and the environment influence larval food choice whereby larvae that microbiota has been suppressed have a greater preference for diets rich in either protein or sugar (Chapter 2). The results also showed that microbial growth in the larval diet interacts with larval density and diet composition to influence body weight of pupae and adults (Chapter 3). Using axenic lines, I showed that the lack of microbiota negatively affects female fecundity and modulates nutrient intake and body fat reserve differently in males and females (Chapter 4). Transgenerational effects of the microbiota were also observed in offspring that parents' microbiota had been manipulated whereby developmental traits were negatively affected in offspring of axenic parents (Chapter 5). Furthermore, body mass and fecundity decreased in offspring of axenic parents suggesting that the disruption of the microbial communities has long-lasting effect on offspring's fitness (Chapter 5). Lastly, I discuss the significance of my results to the field of ecology and evolution, contextualizing my findings into the broader eco-evolutionary framework (Chapter 6). My thesis increases our understanding of the complex interplay between the insect host and its microbiota, highlighting the profound impacts of the microbiota on host fitness at the developmental and generational levels.

Keywords: fecundity, food choice decision, larval crowding, macronutrients, microbiota, nutrition, transgenerational effects.

Declaration of originality and Statement of Authorship

I certify that this thesis entitled "**Impact of microbiota on the life-history traits of a polyphagous fly**", written in the form of published journal articles, is the result of my original research work with the following acknowledgements.

Chapter 2. The microbiota modulates larval foraging behaviour, development rate and pupal production in *Bactrocera tryoni*

Authors: Juliano Morimoto, <u>**Binh Nguyen**</u>, Shabnam T. Tabrizi, Ida Lundbäck, Phillip W. Taylor, Fleur Ponton and Toni A. Chapman.

Contributions: JM, TAC, BN and FP designed the experiment. JM, BN, STT, and IL performed all experiments. JM and BN analysed the data and wrote the first draft of the manuscript. All authors contributed to the revision of the manuscript. All authors have read and approved the final manuscript.

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Chapter 3. Interactions between ecological factors in the developmental environment modulate pupal and adult traits in *Bactrocera tryoni*

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Chapter 4. Sex-specific effects of the microbiota on adult carbohydrate intake and body composition in *Bactrocera tryoni*

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Chapter 5. Parental microbiota modulates offspring development, body mass and fecundity in *Bactrocera tryoni*

Authors: **<u>Binh Nguyen</u>**, Anh Than, Hue Dinh, Juliano Morimoto, Fleur Ponton

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To the best of my knowledge and belief, this thesis has not previously been submitted for a higher degree to any other universities other than Macquarie University and contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Chapter 1:

General Introduction

Microorganisms represent the earliest and most ubiquitous form of life on our planet (Louca et al., 2019). The widespread application of genetic and genomic approaches in the last decades has unfolded a microbial world astonishing in its ubiquity and diversity. Numerous vertebrate organisms such as fish (Bates et al., 2007; Rawls et al., 2006), amphibians (Rebollar and Harris, 2019), birds (Rawls et al., 2006), mammals (Cebra, 1999) and invertebrates organisms, both aquatic and terrestrial [reviewed in (Louca et al., 2019) and (Engel and Moran, 2013)], have evolved alongside and established life-long relationships with their microbiota. The microbial community, which is composed by a variety of microbes such as bacteria, fungi, virus, protozoa and archaea, can be found in different parts of the host's body from the skin to the digestive and reproductive track (Ley et al., 2006). The skin microbiota or the skin flora is home to a community of microorganisms that protects the body from transient microorganisms (Ross et al., 2019). The microbes associated with reproductive organs are either sexually transmitted or opportunistic and can have significant effects on the reproductive function and fitness of males and females (Rowe et al., 2020). The digestive system, especially the mucosa of the gut, generally harbors the largest amount of microbes (Engel and Moran, 2013; Ley et al., 2006; Louca et al., 2019). The gut microbiota of insects, for example, is structured by a large diversity of microorganisms that can be beneficial, neutral or pathogenic to the host depending on the conditions (Bing et al., 2018; Buchon et al., 2013; Lewis and Lizé, 2015). Some gut microbiota may have the ability to alternate between mutualism/commensalism and parasitism in response to changes in host diets (de Vries et al., 2004). The benign and balanced microbiota of insects has been shown to govern a wide range of functions, contributing to the host's nutrition, physiology, behaviour, reproduction, and pathogen resistance [see for instance (Douglas, 2018; Engel and Moran, 2013; Strand, 2018) reviews]. Notably, the insect microbiota not only affects host development at the parental generation but possibly has long-lasting effects on health and fitness of future generations (Elgart and Soen, 2018). Below, I will discuss some typical

functions of the insect microbiota then review its transgenerational implication on offspring performance.

1.1. The microbiota of insects: an overview

1.1.1. Microbiota and nutrition

As in most animals, the microbiota of insects, especially the gut microbiota, can contribute to food digestion and nutrient provisioning (Ben-Yosef et al., 2015, 2014; Bing et al., 2018; Consuegra et al., 2020; Sannino et al., 2018). However, insects present a wide range in the degree of dependence on their microbiota. Termites, for instance, harbor a highly specific gut flora which digest the plant dry matter lignocellulose to short chain fatty acid (SCFA) acetate that is used as the main energy source for the host (Brune and Dietrich, 2015). On the contrary, some caterpillars in the Lepidoptera order (e.g., *Danaus chrysippus*, *Ariadne merione* and *Anticarsia gemmatalis*) do not rely on gut bacteria for processing food and their gut bacteria show no influence on the host physiology (Phalnikar et al., 2019; Visôtto et al., 2009). Most insects fall somewhere in between this range, whereby the gut microbiota supports for the digestion of low-nutrient foods (Engel et al., 2012) and/or degrades toxins present in food (Ben-Yosef et al., 2015; Kikuchi et al., 2012; Zheng et al., 2016). Interestingly, the gut of the plant sap-feeding insects such as aphids contains no or very low bacteria titre but, instead, harbours intracellular symbionts in a distinct organ called bacteriocyte, and rely on them for a direct supply of amino acids and cofactors (Hansen and Moran, 2014; Poliakov et al., 2011). In other insects like *Drosophila*, the Mediterranean fruit flies (*Ceratitis capitata*) and the olive fruit flies (*Bactrocera oleae*), the nutrient provisioning role of the microbiota varies from producing dietary protein (Ben-Yosef et al., 2015, 2014; Bing et al., 2018; Leitão-Gonçalves et al., 2017) and vitamin (Blatch et al., 2010; Fridmann-Sirkis et al., 2014; Piper et al., 2014; Sannino et al., 2018; Wong et al., 2014) to altering energy-rich molecules (Ben-Yosef et al., 2008; Newell

and Douglas, 2014; Ridley et al., 2012; Shin et al., 2011) or simply being digested and used as a nutrient source (Bing et al., 2018; Daffre et al., 1994; Drew et al., 1983). Studies on *Drosophila melanogaster*, for example, have shown that in axenic larvae (i.e., gut microbiota is absent), individuals experience longer larval development time and have a higher mortality rate compared to the control treatment when vitamin B was removed from the diet [reviewed by (Douglas, 2018)]. Interestingly, reinoculating the axenic treatment with *Acetobacter pomorum*, a common gut bacterium of *Drosophila*, can rescue larval development of individuals raised on diets that lack vitamin B1 (Sannino et al., 2018). Further investigations have revealed that *A. pomorum* is the only symbiont responsible for vitamin B1 provision to the host via its ability to produce this micronutrient (Sannino et al., 2018). Likewise, other studies have shown the involvement of the microbiota in providing protein for *D. melanogaster* and *D. suzukii*, especially in individuals reared on low-protein diets (Bing et al., 2018; Leitão-Gonçalves et al., 2017).

Manipulations of the microbiota-based diets have also been proposed as means to enhance insect performance, especially in new insect pest control programs such as the sterile insect technique [SIT, (Ami et al., 2010; Augustinos et al., 2015; Deutscher et al., 2019)]. In SIT, target insects are reared in millions and sterilised using gamma or x-rays. The sterile males are then released in the field for mating with the wild females, which results in infertile eggs being laid (Ami et al., 2010). For SIT to be effective, the sterilised mass-reared individuals that are released have to be competitive with their wild male counterparts in attracting wild females for mating. The insect gut microbiota influences host quality, fitness and mating preference (Ben-Yosef et al., 2015, 2010; Deutscher et al., 2019). The development of microbiota-enriched diets has shown potential in enhancing performance of larvae and adults reared for SIT programs. In tephritids, for instance, there have been more than ten studies to date investigating the effect of bacterial enrichment diets on the host [see for instance (Aharon et al., 2013; Ami et al., 2010;

Augustinos et al., 2019; Gavriel et al., 2011; Shuttleworth et al., 2019)]. Although substantial changes are not always recorded, positive influence on host performance has been measured [reviewed in (Deutscher et al., 2019)].

1.1.2. Microbiota and behaviour

The microbiota can also modulate the behaviour of animals, including insects (Cryan and Dinan, 2012; Leitão-Gonçalves et al., 2017; Lewis and Lizé, 2015; Zheng et al., 2017). As a neuroactive compound, SCFA produced by the gut microbiota can influence neural and immune pathways, affecting brain function and behavioural traits of the host (Cryan and Dinan, 2012). The gut microbiota of honey bees *(Apis mellifera)*, for instance, shows similar function to the human gut microbiota as it can digest complex carbohydrate components and produce SCFA acetate (Zheng et al., 2017). Moreover, the analysis of the expression level of insulin-like peptide genes shows that microbial metabolism enhances the production and responsiveness of insulin which regulates the behaviour of honey bee (Zheng et al., 2017). A recent study in *D. melanogaster* has also suggested that the interaction between members of the microbiota during co-culture produces SCFA acetate and its metabolic derivatives that influence *Drosophila* egg-laying behaviour (Fischer et al., 2017). Besides, gut bacteria of *Drosophila* have been shown to play a key role on host foraging and food choice decision (Leitão-Gonçalves et al., 2017; Qiao et al., 2019; Wong et al., 2017), kin recognition (Lizé et al., 2014), and mating selection (Sharon et al., 2010) although with conflicting results (Leftwich et al., 2017). These results suggest that the sophisticated alterations in host behaviour induced by microbial modulators can initiate a process that possibly leads to great changes in host ecology and evolution.

1.1.3. Microbiota and immune defence

In addition to influencing host nutrition and behaviour, the microbiota plays protective roles through stimulating host defences against pathogens (Buchon et al., 2013; Raymann and Moran, 2018). For example, *D. melanogaster* larvae that lack a gut microbiota are more susceptible to infection with the pathogen *Candida albicans* than conventional ones (Glittenberg et al., 2011). Similarly, many studies conducted on honey bees and bumble bees have shown important roles of the gut microbiota in protection against infections [reviewed in (Raymann and Moran, 2018)]. In the honey bee *A. mellifera*, in particular, individuals that experienced a gut microbiota disturbance by tetracycline treatment show a significant elevation in mortality rate when infected by opportunistic bacterial pathogens (Raymann et al., 2017). Although there is no evidence for a specific cross-talk between the insect host and its gut microbiota, the innate immune system in the gut does not show any deleterious induction under basal conditions, but allows a rapid elimination of pathogens (Engel and Moran, 2013). Studies on *D. melanogaster* have shown, for instance, that despite commensal and pathogenic bacteria activating similar pathways of the immune response, the level of activation and the amount of damage of the gut epithelium are significantly lower in the case of bacteria from the gut microbiota (Buchon et al., 2009). The protective effect of the gut microbiota may also come through direct elimination of pathogens whereby the gut microbiota alters the gut physicochemical conditions (through changes in pH and levels of digestive enzymes) to create inhospitable environments for pathogens and/or occupy available niches (Behar et al., 2008; Glittenberg et al., 2011; Zheng et al., 2017). Alternatively, the gut microbiota might limit the persistence of pathogens or parasites by competing for available resources or producing antimicrobials (Azambuja et al., 2005; Caragata et al., 2013; Dillon and Charnley, 2002). Indeed, the gut microbiota of the desert locus *Schistocerca gregaria* can produce antimicrobials

from the digestion of plant materials to contribute to the host defence (Dillon and Charnley, 2002).

1.1.4. Microbiota and reproduction

The microbiota has also been shown to influence reproduction in *D. melanogaster* (Elgart et al., 2016; Leitão-Gonçalves et al., 2017; Morimoto et al., 2017), the tephritid fruit flies *Bactrocera tryoni* (Drew et al., 1983), the olive fruit flies *B. oleae* (Ben-Yosef et al., 2010) and the mosquito *Culex pipiens* (Fouda et al., 2001). In some mosquito species from the *Aedes* and *Culex* genera, the bacteria can colonize host reproductive organs and manipulate host reproduction [reviewed by (Minard et al., 2013)]. In particular, bacteria of the genera *Bacillus* and *Staphylococcus* increase fecundity of the mosquito *C. pipiens* although the underlying mechanisms remain to be determined (Fouda et al., 2001). In addition, a recent study in *D. melanogaster* has revealed that when the microbiota was eliminated, host oogenesis and consequently, egg deposition, were suppressed (Elgart et al., 2016). More importantly, these changes are directly linked to the lack of *Acetobacter* (but not *Lactobacillus*) species and appear to be mediated by *Drosophila* Aldh, a group of enzymes responsible for the catabolism of aldehyde substrates in ovaries (Elgart et al., 2016). Nevertheless, future research is required to elucidate the proximate mechanisms by which *Acetobacter* modulates the expression of Aldh and how this in turn affects host reproduction.

1.2. Transgenerational effects of insect microbiota

The microbiota does not only affect host's metabolism, immunity, behaviour and reproduction at one generation, but may also have long-lasting implications on the fitness of descendants (Elgart and Soen, 2018). Insects acquire their microbiota from maternal transmission (i.e., vertical transmission) and/or from the environment (i.e., horizontal transmission) (Elgart and

Soen, 2018; Engel and Moran, 2013). It has been shown for instance that females can coat their eggs with microbes while depositing them, or defecate microbe-rich fecal pellets in the adjacent environment that are ingested by the larvae upon hatching (Estes et al., 2009; Funkhouser and Bordenstein, 2013). Strikingly, females of the stinkbug *Megacopta punctatissima* not only display sophisticated mechanisms for inoculating their eggs in a special "'symbiont capsule" but also show controls over the symbiotic resource allocation through generations (Hosokawa et al., 2007). The microbiota and/or microbial products that offspring inherited from their parents can greatly impact offspring life history traits, especially in the early life stage (Elgart et al., 2016; Farine et al., 2017; Freitak et al., 2014; Morimoto et al., 2017). In *D. melanogaster*, for instance, the gut bacteria *Acetobacter* significantly impacts the development of eggs in ovaries, whilst the transmitted bacteria from parents can modulate odour emission and preference in juveniles (Elgart et al., 2016; Farine et al., 2017). Furthermore, parental microbiota manipulations not only show direct effects on *Drosophila* mating and reproductive behaviour, but reveals transgenerational effects on offspring body mass (Morimoto et al., 2017). It is not yet clear how the parental microbiota influences host germ line and the development of host descendants. However, the transgenerational implications of the microbiota can be mediated either by the effects of the microbiota on parental germline or the impacts of transmitted microbiota on somatic tissues [reviews in (Elgart and Soen, 2018)]. Over multiple generations, these impacts may accumulate and potentially contribute to host phenotypes, adaptation and evolution.

1.3. Integrating the effects of the microbiota on insect fitness and behaviour: a challenge

The microbiota is an important factor of the host nutritional ecology, allowing hosts to extract nutrients from food substrates as well as providing essential vitamins (Behar et al., 2005; Bing et al., 2018; Leitão-Gonçalves et al., 2017; Sannino et al., 2018). It however remains unclear

how the microbiota modulates nutritional signals that affect foraging behavior of larvae and adults. Besides, although larvae are known to acquire and establish their microbiota through vertical transmission (via contaminated eggs) and horizontal acquisition (from environment), there has been little understanding of the contribution of these two transmission routes on development and behaviour (Engel and Moran, 2013). Furthermore, ecological factors such as larval density, diet composition, and the microbes colonizing the diet possibly interact to modulate larval development and adult traits, but no direct experiment has, to date, measured the impacts of this interaction on the performance of larvae and adults. Lastly, given the far-reaching implications of the microbiota on host health and fitness, the parental microbiota may play an essential role on shaping offspring phenotype. Unfortunately, empirical studies targeting the transgenerational effects of host-microbe interactions on life-history traits of descendants are still limited.

1.4. An integrated approach of the effects of the microbiota on life-history traits and feeding behaviour of a polyphagous fly

Here, I addressed these gaps by investigating direct effects and long-term influences of the microbiota on host life history traits and foraging behaviour using the polyphagous fly *Bactrocera tryoni* as a model.

1.4.1. *Bactrocera tryoni* as a model

Bactrocera tryoni (i.e., the Queensland fruit fly, Diptera: Tephritidae) is a major horticultural pest in Australia, infesting a large diversity of fruits and vegetable crops (Moadeli et al., 2017; Sutherst et al., 2000). *B. tryoni* has a short generation time and its life cycle includes four stages: eggs, larvae, pupae and adults (Figure 1). At a temperature of around 26˚C, eggs hatch after 2 days, larvae develop in approximately 6 days inside a fruit and jump out from the fruit when

they reach third instar to pupate in the soil (Figure 1). Adult flies, which emerge from pupae after 10 days, mate and lay eggs at about day 14th post-emergence (Figure 1).

Figure 1. Life cycle of *B. tryoni* (source: https://fruitflyfreeyv.com.au/)

Previous studies have investigated background information on the nutrition, development and infection of adult *B. tryoni* (Dinh et al., 2019; Fanson et al., 2013a; Morimoto et al., 2020) as well as the feeding performance of larvae (Morimoto et al., 2019b, 2018) and adults (Fanson et al., 2013b, 2009; Fanson and Taylor, 2012; Morimoto et al., 2019a). In parallel, the gut microbial community of wild, domesticated and irradiated *B.tryoni* has also been explored (Deutscher et al., 2017, 2014; Majumder et al., 2020, 2019; Morrow et al., 2015; Woruba et al., 2019). Morrow et al. (2015) have found that Enterobacteriaceae and Acetobacteraceae (both belong to Proteobacteria), and Streptococcaceae and Enterococcaceae (both belong to Firmicutes) are the dominant bacterial families in adult flies from wild populations (Morrow et al., 2015). Meanwhile, Majumder et al. (2020) have shown that the bacterial genera of
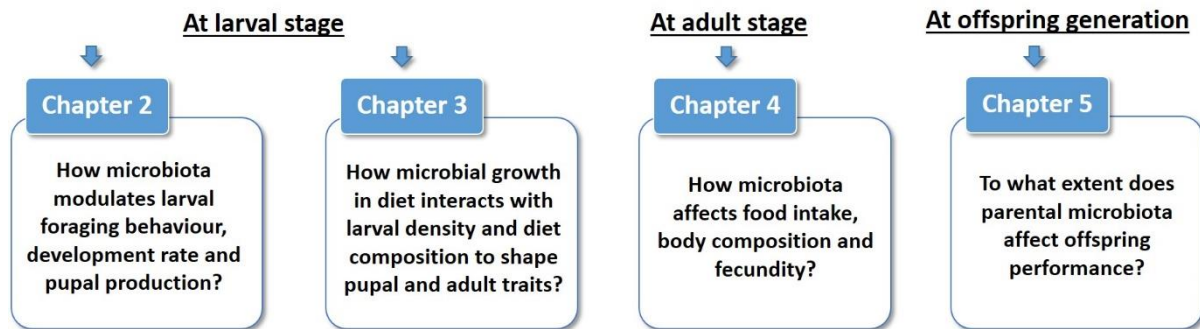
Morganella, Citrobacter, Providencia, and *Burkholderia* were highly abundant in all developmental stages of flies reared in the laboratory on gel diet (Majumder et al., 2020). These studies deliver important information about the composition and structure of the bacterial community of *B. tryoni*, promoting the development of microbiota-based diets that can enhance the performance of mass-reared flies in SIT. Nevertheless, no empirical experiment has so far deciphered the effects of the microbial community on the life history traits and feeding behaviour of *B. tryoni*, as well as, the effects of the parental microbiota on offspring fitness.

1.4.2. Thesis outline

My thesis outline is illustrated in Figure 2. I first examined the effects of the microbiota at larval stage. By manipulating both vertically and horizontally transmitted microbiota, I measured the impacts of microbial exposure on larval foraging, development rate and pupal production (Chapter 2). In parallel, I explored how the microbial growth in the diet interacts with the diet composition and larval density to shape fitness-related traits of individuals (Chapter 3). Results from these studies provide insights into the ecological factors modulating the development of juveniles in holometabolous insects. Then, using axenic lines, I investigated the effects of the microbiota manipulation on fitness-related traits, feeding performance and fecundity of adults (Chapter 4). Transgenerational effects of the microbiota were also measured in offspring of parents that the microbiota had been manipulated (Chapter 5). Insights into the direct and transgenerational effects of the microbiota on host life-history traits in insects advance our knowledge regarding the complex interplay between the host and their microbiota and the long-lasting effect of the host-microbe interaction on future generations.

Impacts of microbiota on the life-history traits of *Bactrocera tryoni*

At larval stage		At adult stage	At offspring generation

Chapter 2

How microbiota modulates larval foraging behaviour, development rate and pupal production?

Chapter 3

How microbial growth in diet interacts with larval density and diet composition to shape pupal and adult traits?

Chapter 4

How microbiota affects food intake, body composition and fecundity?

Chapter 5

To what extent does parental microbiota affect offspring performance?

Figure 2. Thesis outline

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Chapter 2:

The microbiota modulates larval foraging behaviour, development rate and pupal production in *Bactrocera tryoni*

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Commensal microbiota modulates larval foraging behaviour, development rate and pupal production in *Bactrocera tryoni*

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Abstract

Backround: Commensal microbes can promote survival and growth of developing insects, and have important fitness implications in adulthood. Insect larvae can acquire commensal microbes through two main routes: by vertical acquisition from maternal deposition of microbes on the eggshells and by horizontal acquisition from the environment where the larvae develop. To date, however, little is known about how microbes acquired through these different routes interact to shape insect development. In the present study, we investigated how vertically and horizontally acquired microbiota influence larval foraging behaviour, development time to pupation and pupal production in the Queensland fruit fly ('Qfly'), *Bactrocera tryoni*.

Results: Both vertically and horizontally acquired microbiota were required to maximise pupal production in Qfly. Moreover, larvae exposed to both vertically and horizontally acquired microbiota pupated sooner than those exposed to no microbiota, or only to horizontally acquired microbiota. Larval foraging behaviour was also influenced by both vertically and horizontally acquired microbiota. Larvae from treatments exposed to neither vertically nor horizontally acquired microbiota spent more time overall on foraging patches than did larvae of other treatments, and most notably had greater preference for diets with extreme protein or sugar compositions.

Conclusion: The integrity of the microbiota early in life is important for larval foraging behaviour, development time to pupation, and pupal production in Qflies. These findings highlight the complexity of microbial relations in this species, and provide insights to the importance of exposure to microbial communities during laboratory- or mass-rearing of tephritid fruit flies.

Keywords: Nutrition, Larval behaviour, Development, Microbiota

Background

Communities of commensal microorganisms ('microbiota') influence a wide variety of behavioural and physiological traits in their animal hosts [1, 2]. The effects of the microbiota on modulation of blood pressure, diabetes and obesity risks have been shown in vertebrates [3, 4], while the microbiota is known to play numerous fitness-associated roles in a vast diversity of invertebrate hosts [5], from changes in developmental rate, nutrition, reproduction, to kin recognition [6–10] and even mate choice, although with conflicting findings [11, 12]. The microbiota can influence host physiology and behaviour at various developmental stages. Host-microbiota interactions are for instance highly influential at the larval stage in insects [13–17]. In the Fritillary butterfly, *Melitaea cinxia*, the gut microbiota is a key determinant of larval growth rate [18]. In mosquitoes, the lack of gut microbiota results in significantly delayed development and reduced likelihood of larvae developing through to adulthood [19, 20].

The microbiota is often composed by a mix of microbes that have co-evolved with the host and therefore are essential to host survival and fitness (primary obligatory

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symbionts), as well as transient microbes that have not co-evolved with the host but can nonetheless affect hosts' fitness (secondary facultative symbionts) [21, 22]. Both primary and secondary microbes are mainly acquired through (i) vertical transmission via maternal surface contamination of the egg and (ii) horizontal acquisition from the environment [22–25]. In insects, females can contaminate the eggshells of their progeny with their own microbiota, which is then ingested by hatching larvae [5, 26–31]. Through development, the maintenance of microbiota depends on ingestion of microbes from the environment, most often from dietary sources ([32–40]; see also [22, 24] for reviews). Larvae of some insect species can even develop foraging preferences for certain microbiota strains that support their development [10, 41, 42]. Yet, there has been little investigation of how vertically and horizontally acquired microbiota shape development and larval behaviour.

In tephritid fruit flies, the microbiota is an important determinant of health and performance in both larvae and adults [16, 25, 38, 43–47], and manipulations of microbiota communities have been suggested as a mean of enhancing the performance of insects produced for sterile insect technique (SIT) programs [48–50]. For instance, supplementing Mediterranean fruit fly (*Ceratitis capitata*) larvae with the bacterium *Enterobacter sp.* improves pupal and adult productivity and reduces development time without affecting other fitness-related traits such as mating competitiveness [49]. In the present study, we ascertained the importance of vertically and horizontally acquired microbiota in the tephritid fruit fly *Bactrocera tryoni* Froggatt (Diptera: Tephritidae) (aka 'Queensland fruit fly' or 'Qfly'). Previous studies in Qflies have demonstrated the presence of both vertical [30] and horizontal [51] acquisition of microbiota. We manipulated the microbiota of Qfly eggs and larvae to generate treatments comprised of sterile eggs from which larvae were reared in either sterile or non-sterile diet, as well as the control, conventional, treatment of non-sterile eggs reared in non-sterile diet. Our approach therefore manipulated both permanent and transient members of the microbiota simultaneously. The effects of microbial exposure were measured on larval foraging behaviour, development time to pupation and pupal production. The present study investigates not only the importance of the microbiota for larval behaviour, development rate and pupal production in Qfly, but also highlights that manipulation of the microbiota communities acquired horizontally or vertically may provide a valuable means of enhancing mass-rearing of this species for SIT programs.

Methods
Fly stock and egg collection
Eggs were collected from a laboratory-adapted stock of Qfly (> 17 generations-old). The colony has been maintained in non-overlapping generations in a controlled environment room (humidity $65 \pm 5\%$, temperature $25 \pm 0.5\,°C$) with light cycle of 12 h light: 0.5 h dusk:11 h dark: 0.5 h dawn). Adults were maintained with free-choice diets of hydrolysed yeast (MP Biomedicals, Cat. n° 02103304) and commercial cane sugar (CSR® White Sugar), while larvae were maintained using a 'standard' gel-based diet that contains Brewer's yeast (Lallemand LBI2250) [52]; Nipagin used in the gel-based diet was obtained from Southern Biological (Cat no. MC11.2). Eggs were collected in a 300 mL semi-transparent white plastic (LDPE) bottle that had perforations of < 1 mm diameter through which females could insert their ovipositor and deposit eggs. The bottle contained 20 mL of water to maintain high humidity. Females were allowed to oviposit for 2 h, after which eggs were transferred to larval diet.

Experimental procedures
Microbiota manipulation of eggs and larvae
An established protocol was used to manipulate microbial exposure of the eggs and larvae [53]. Briefly, eggs were washed twice in 0.5% Chlorite liquid bleach (Peerless JAL®) for 5 min, followed by one wash in 70% ethanol for 2 min, and three washes in Milli-Q water for 2 min each wash. Using a sterilized brush in a sterile environment, the eggs were then transferred onto either non-sterile standard gel-based diets ('–/+' treatment) or standard gel-based diets supplemented with 50 μg/mL (final concentration) of streptomycin and tetracycline (stock solution: 10 mg/ml for both) (Cat no. S6501 and T3258 from Sigma Aldrich®, respectively) ('–/–' treatment). Finally, eggs with intact microbiota were washed three times in sterile Milli-Q water for 2 min each wash and placed on standard gel-based diets without antibiotics and in non-sterile environment ('+/+' treatment). Antibiotics were dissolved in sterile Milli-Q water in sterile 50 mL tubes to create the stock solutions. The stock solution of tetracycline was warmed to 50 °C to increase solubility. Antibiotics were added to the gel-based diet just before the diet set. To quantify and compare the microbial load of larvae in each treatment, we washed groups of three late 2nd instar larvae 3 times in 80% ethanol for 2 min each wash, followed by 3 washes in PBS buffer for 2 min each wash before homogenising the larvae using Sigma Aldrich® autoclavable plastic pestles (Cat no. Z359947). We plated 30 μL of the homogenate ($N = 5$ replicates per treatment) in de *Man-Rogosa-Sharpe* (Oxoid® MRS, Cat no. CM0361) agar, LB agar (Oxoid® Cat no. 22700025), and *Potato-Dextrose Agar* (PDA) (Oxoid® Cat no. CM0139B) plates ($N = 45$ plates), and incubated for 48 h at 26 °C, after which we counted the number of colonies ('CFU') in the plates. This approach allowed us to quantify culturable bacterial and fungal components of the microbial

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community. For this study, we consider 'vertically acquired microbiota' as the microbiota that is present in the eggs and 'horizontally acquired microbiota' as the microbiota potentially present in the diet and in the surrounding environment. The total CFU *per* replicate *per* larvae was estimated as the sum of colonies in all three plates multiplied by the total volume of homogenate. A non-parametric Kruskal-Wallis test was used to test for differences in CFU counts between treatments. As expected, there was a significant effect of treatment on CFU load of the larvae, in which larvae from treatment +/+ had the highest CFU counts, followed by treatment −/+ with intermediate CFU counts, and treatment −/− with no CFU (Additional file 1).

Developmental time until pupation and pupal production

For each treatment, ca. 50 eggs (SE: ± 0.274) were placed at the centre of 50 mL Falcon tubes that contained 15 mL of standard gel-based diet (40 replicate tubes per treatment). The egg count was achieved by adding 4 μ L of egg-water solution (expected yield of 50 eggs) into the Falcon tubes and then counting the total number of eggs in each Falcon tube under sterile conditions. This approach was needed to avoid contamination of the eggs and diet by airborne microbes (particularly in the −/− treatment); to standardise the methods, we used this protocol for all treatments. When preparing the tubes, diet was poured while warm, and tubes were tilted until diet set in order to generate more surface area of the diet for the larvae. Excess moisture was allowed to evaporate under sterile conditions after which the tubes were sealed. All treatments were maintained in a controlled environment room (humidity 65 ± 5%, temperature 25 ± 0.5 °C) with 12 h light: 0.5 h dusk:11 h dark: 0.5 h dawn cycle.

For collection of pupae, four 50 mL Falcon tubes in which larvae were developing were inserted through 30 mm diameter holes in the lid of a 1.125 L Decor Tellfresh plastic container (12 cm × 9.5 cm × 10.5 cm) so that the top protruded into the plastic container (*N* = 10 replicates per treatment). The plastic containers were sterilized with 70% ethanol, and contained ca. 50 g of autoclaved vermiculite, and laid on their side so that larvae could easily exit from the Falcon tubes to pupate in the vermiculite. No larvae remained in the Falcon tubes at the end of the experiment. This design allowed larvae to pupate in a sterile environment. Pupae were collected by sieving the vermiculite 8, 9 and 10 days after the onset of the experiment, and then holding all collected pupae in 90 mm Petri dishes.

'Pupal production' was calculated as the total number of pupae divided by the number of eggs placed on the diet multiplied by 100 (%). 'Daily pupation percentage'

was measured as the number of pupae collected 8, 9 and 10 days after eggs were placed on the diet divided by the sum of the number of pupae for all days, multiplied by 100 (%). No pupation was observed after 10 days. This allowed us to (1) compare how many pupae were collected each day while standardising for overall pupal production of each treatment group ('daily pupation percentage') and (2) identify the day with the highest pupal production ('peak pupation day'). ANOVA was used to compare treatment groups for pupal production and development time, followed by Student-Newman-Keuls (SNK) posthoc tests. For pupal production, the model contained replicate and treatment as factors in a single model. For developmental time, the model contained replicate, as well as treatment and the linear and quadratic effects of time (and their interactions) as factors in a single model. All statistical analyses were performed using R version 3.4.0 [54]. Figures for developmental time to pupation and pupal production were plotted using the R package 'ggplot2' [55].

Foraging behaviour

The ratio of yeast-to-sugar (Y:S ratios) from the standard gel-based larval diet [52] was manipulated to create 6 diets (280 mg/mL) with yeast-to-sugar (Y:S) ratios of 1:0, 5:1, 1.5:1, 1:1.6, 1:3.4, and 0:1 (for formulations, see Additional file 2). For the experimental diet mixture, we used hydrolysed yeast obtained from MP Biomedicals (Cat no. 02103304) containing ca. 60% protein according to the product data sheet (Datasheet 02103304). Diets made with hydrolysed yeast are translucent which facilitates the counting of the larvae in the foraging patches during the experiment. Sucrose was obtained from MP Biomedicals (Cat no. 02902978). 20 mL of each diet was poured into 90 mm diameter Petri dishes and allowed to set. In addition to the diets, a 1% agar solution that contained the same components as the diets except for yeast and sugar was prepared; 20 mL of the agar solution was poured to cover a 90 mm diameter Petri dish that was used as the 'foraging arena' (*N = 20*). The pH of all diets, including the agar base of the foraging arena, was adjusted to 3.8–4 using citric acid. After setting and 15 min prior to the onset of the experiment, six equally spaced holes were made around the agar base of the foraging arena by perforating it with a 25 mm diameter plastic tube. The plastic tube and all surfaces were sterilised with Ethanol 80% before use. The same tube was used to cut discs from the experimental diets, which were deposited in the holes in order of increasing Y:S ratio.

Larvae were reared in 50 mL Falcon tubes as described previously (i.e., treatments −/−, −/+. +/+). At 4–5 days after egg collection, 25 late 2nd instar larvae from each treatment were collected with a soft brush and placed at the centre of foraging arenas (7 replicates per treatment),

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which were then covered to minimize loss of moisture and placed in a dark room to minimise visual stimuli. The number of larvae on each of the discs of diet and on the agar base between discs was assessed 1 h, 2 h, 4 h, 6 h, and 24 h after larvae were placed in the arena. To analyse larval foraging preference, a multinomial logistic regression model was fitted using the 'multinom' function of the 'nnet' package in R [56] with time, treatment, and their interactions as factors. A multinomial logistic regression measures the relative log-odds of a choice between a reference level (agar base) and a comparative level (each diet).

If relative log-odds > 0, the foraging preference for the diet is higher than to the agar base. If relative log-odds < 0, the foraging preference is higher for the agar base than to the diet. Note that the reference and comparative levels are taken within treatments, that is, the foraging preference for each diet is compared with agar base *within* the treatment. The interaction term measures the statistical significance between two *within* treatment differences in foraging preference for agar base vs. diet. For example, the interaction term measures the difference in relative log-odds of agar base vs. diet 1 within treatment A, and agar base vs. diet 1 within treatment B. The same comparison is applied to all diets. This approach was necessary to account for the non-independence of the data points within each foraging arena over time, and the multiple simultaneous choices of diets presented to the larvae. Statistical inferences of the relative log-odds were made based on the *t*-distribution ($\alpha = 0.05$). Relative log-odds were plotted in Excel version 14.7.3.

Results

The microbiota affects development time and pupal production

Manipulation of microbiota significantly affected pupal production (*Treatment:* $F_{2,11} = 11.710$, *p* = 0.002,
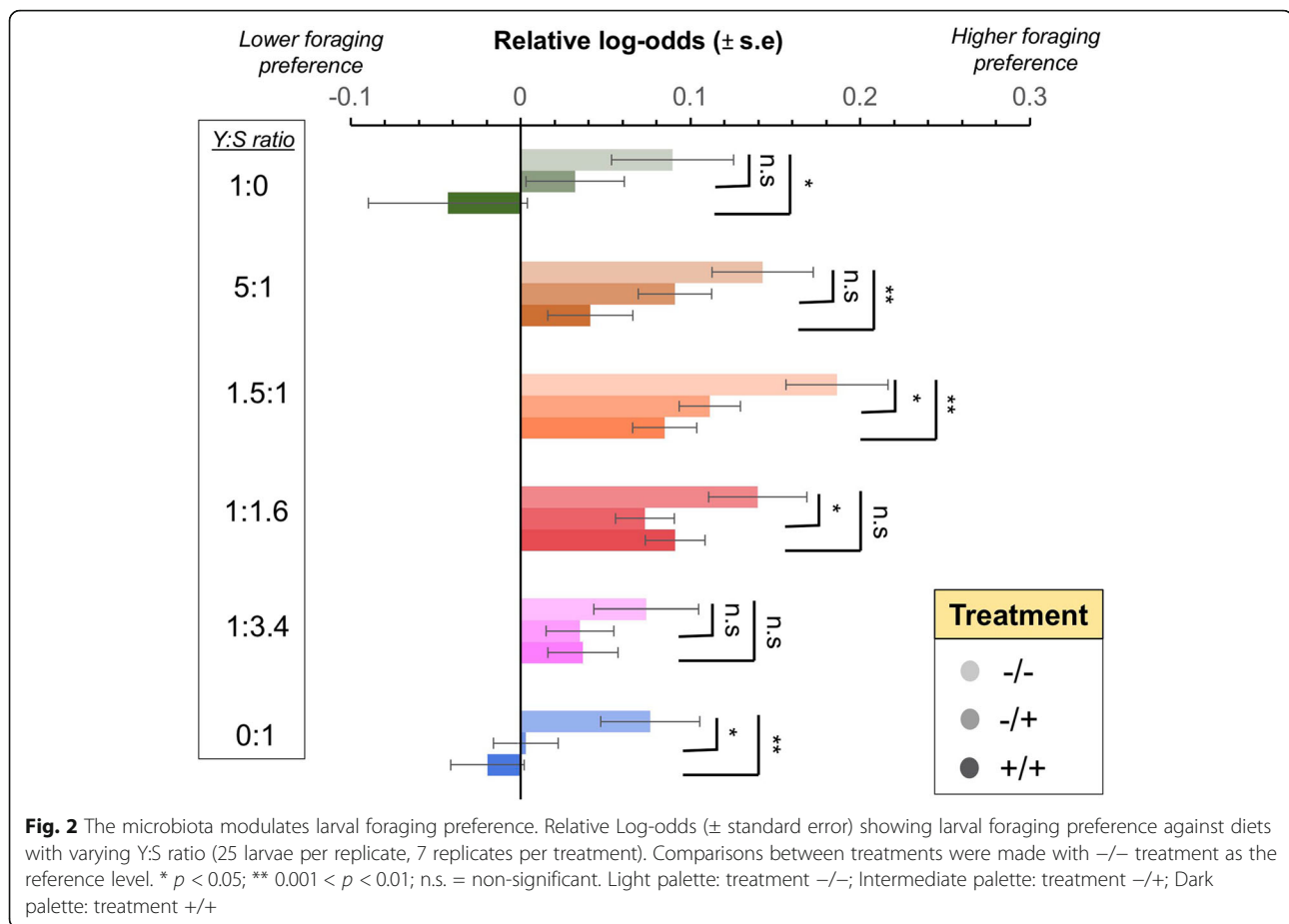
Additional file 2: Table S2), whereby more pupae were produced from treatment +/+ than from treatments −/− and −/+ (Fig. 1 a, Additional file 2: Table S2). There was no significant difference between treatments −/− and −/+ on pupal production (Additional file 2: Table S2). There were also significant interactions between the linear and quadratic effects of time (days after egg collection) and treatment on daily pupation percentage (*Day * Treatment:* $F_{2,35} = 8.315$, *p* = 0.001, *Day^2 * Treatment:* $F_{2,35} = 15.446$, *p* < 0.001, Additional file 2: Table S3), whereby treatments −/− and +/+ had a peak in daily pupation percentage on day 8, after which daily pupation percentage declined in day 9 and 10, whereas treatment −/+ had similar daily pupation percentage on days 8 and 9 before declining sharply on day 10 (Fig. 1 b, Additional file 2: Table S3).

The microbiota affects larval foraging behaviour

Larval foraging preference was assessed by offering larvae a choice amongst 6 diets that varied in yeast-to-sugar ratios (Y:S ratios), including diets that were yeast (protein) biased, balanced, or sugar biased. Larvae of treatment −/− had greater preference to forage in extreme Y:S ratios relative to the agar base than did larvae of other treatment groups (see Additional file 2: Table S4). In particular, larvae from treatment −/− had higher foraging preference for diets of Y:S ratio 1:0 (protein biased) and Y:S ratio 0:1 (no protein) (Fig. 2) than did larvae from treatment +/+. Larvae from treatment −/− also displayed significantly higher foraging preference for balanced diets (i.e., Y:S 5:1 and 1.5:1) in comparison to larvae from treatment +/+ (Fig. 2). On the other hand, absence of vertically acquired microbes for larvae on non-sterile diet (i.e., treatment −/+) influenced preference for foraging on balanced and sugar biased diets
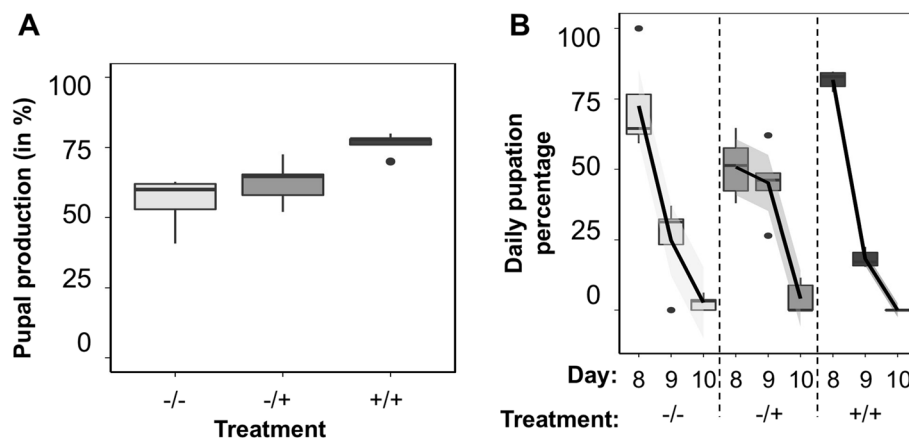
Fig. 1 The effects of the microbiota on pupation in Qfly larvae. **a** Pupal production (in %, from 50 ± 0.274 eggs per treatment). **b** Daily pupation percentage from days 8–10 after the onset of the experiment

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Fig. 2 The microbiota modulates larval foraging preference. Relative Log-odds (± standard error) showing larval foraging preference against diets with varying Y:S ratio (25 larvae per replicate, 7 replicates per treatment). Comparisons between treatments were made with −/− treatment as the reference level. * $p < 0.05$; ** $0.001 < p < 0.01$; n.s. = non-significant. Light palette: treatment −/−; Intermediate palette: treatment −/+; Dark palette: treatment +/+

(Fig. 2 and Additional file 2: Table S4). For instance, treatment −/+ larvae and treatment −/− larvae were significantly different in foraging preference for diets of Y:S 1.5:1, 1:1.6 and 0:1 (Fig. 2, Additional file 2: Table S4). Overall, the foraging preference patterns of larvae from treatments −/+ and +/+ were more similar than to that of larvae from the treatment −/− (Fig. 2).

Discussion

Host-microbiota interactions are highly influential in larval development and adult fitness of many insect species [13–16, 25, 57, 58]. Here, we showed that in Qfly the microbiota affects developmental time to pupation and pupal production, as well as larval foraging behaviour, particularly preference for foraging on diets with extreme nutrient composition. In tephritids, the microbiota modifies the nutritional environment of the larvae [58] and serves as food for adults [25]. Moreover, manipulations of the gut microbiota have been proposed as means to enhance the performance of sterile adult flies released in SIT programs [48–50] since larval nutrition and health is an important determinant of the yield and quality of mass-reared adults. The present study provides insights to the role of vertically and horizontally

acquired bacteria in development and pre-pupal survivorship of Qfly but also provides a starting point for future work aiming at enhancing the quantity and quality of mass-reared Qfly for SIT.

Effects of the microbiota on pupal production

Our data showed that vertically and horizontally acquired microbiota communities were important for development time and pupal production in Qflies. For instance, daily pupation percentage showed a similar pattern of linear decrease over time in treatments −/− and +/+, which was not observed for treatment −/+, suggesting that horizontally acquired microbiota could potentially influence the time until pupation independently of vertically acquired microbiota. It is unclear why larvae from axenic (germ-free) eggs that were exposed to horizontally acquired microbiota (i.e., treatment −/+) showed a delay in pupal production. It is possible that axenic larvae are more susceptible to infection by pathogenic microbes from the environment (see for instance [59–61]) that could have a negative effect on larval development. Despite this, pupal production was significantly lower in treatments −/− and −/+ compared with treatment +/+, revealing that horizontally acquired microbes are

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insufficient to fully rescue pupal production and highlighting the importance of vertically transmitted microbiota for development. The mechanisms through which the microbiota affect pupal production in Qfly is unknown. It is possible that specific strains of the microbiota regulate factors underpinning life-stage transitions of flies. For example, in *Bactrocera dorsalis* gut bacteria of the genus *Enterococcus* have been found to have positive effects, and *Lactobacillus* to have negative effects, on larval development and pupation [62], but the molecular mechanisms of these effects are not known. In Qflies, two yeast strains, *Pichia kluyveri* and *Hanseniaspora uvarum*, have been recently proved to play an important role in pupal production [43], although it is not certain when and from where these fungi are acquired by larvae. These previous findings suggest a complex interplay between the fungal and bacterial components of the microbiota on development [41–43], and open an important avenue for developing approaches that exploit fungi or bacteria, or both, to enhance development in mass-rearing programs. Our results are in agreement with previous literature showing that the microbiota can promote development to pupation in Qflies [43]. It is unlikely that our results were influenced by the sterilization treatment used to remove the microbiota from the eggshells since our findings are broadly consistent with previous literature using axenic (germ-free) models in *Drosophila*, whereby the gut microbiota at early stages of development affects larval development and behaviour, as well as pupal production and adult traits (e.g. [10, 35, 41, 42]), although recently some experimental procedures have been questioned (e.g., [63]).

Effects of the microbiota on larval foraging behaviour
Bacteria that were vertically and/or horizontally acquired affected Qfly larval foraging behaviour. For instance, the number of larvae on foraging patches, rather than the agar base, was relatively high for treatment −/−, intermediate for treatment −/+, and relatively low for treatment +/+ in comparison with other treatments. These patterns were particularly evident for extreme protein- and sugar-biased diets for which the larvae from treatment −/− exhibited much higher preference than did larvae from treatments −/+ and +/+ (Fig. 2). Together, these findings show that vertically and horizontally acquired microbiota can act in combination to regulate larval foraging behaviour patterns. The exact mechanism through which the microbiota modulates Qfly larval foraging behaviour is unknown, although it is possible that microbes modulate nutrient-specific larval foraging behaviour due to their differential carbohydrate and protein metabolism. For instance, a recent study has shown that the gut microbiota can modulate appetite for amino acids in *D. melanogaster* adults [9], although whether the gut microbiota also modulates amino acid appetite in larvae remains unknown. It is also possible that the absence of microbiota may affect metabolic processes and nutrient assimilation in Qfly larvae, as has been found previously in *D. melanogaster* [41, 42]. The total absence of microbiota (−/− treatment) resulted in Qfly larvae with greater tendency to forage in all diets, including those with extreme nutritional values (e.g., Y:S 0: 1). This result might indicate a reduced ability of larvae to discriminate or to balance nutrient intake, and might also suggest a broader nutritional requirement of these larvae compared with larvae that are exposed to vertically and horizontally acquired microbial communities. In addition to influencing larval foraging behaviour, microbiota in the larval diet is also known to alter the diet's nutritional composition. For instance, the microbiota in the diet increases the amino acid content of the substrate where larvae develop, which in turn may affect how larvae balance their dietary preferences [58]. It remains unknown whether these potential effects of the microbiota on larval foraging preferences are carried through to adulthood. Previous studies have shown that laboratory-adapted adult female Qflies are equally attracted to diets with and without microbiota supplementation, suggesting that the modulation of adult dietary preferences could be independent of the microbiota colonising the diet in adult Qflies [64]. However, to our knowledge, there have been no studies that manipulate the microbiota of adult Qflies (instead of the microbiota of the diet) to investigate changes in adult foraging preferences. Thus, future studies using approaches similar to those of the present study but applied to adults are needed in order to shed light into whether the microbiota-associated changes in foraging preferences at the larval stage are also observed in adults.

Conclusion
The present study reveals combined effects of vertically and horizontally acquired microbes on development time, pupal production and larval foraging behaviour in Qflies. These findings contribute to the understanding of fitness-related effects of host-microbial interactions, and provide a starting point for future investigations of how microbiota affects early life stages of this species, as well as guiding development of protocols for enhanced large scale rearing for Qfly SIT programs.

Supplementary information

Additional file 1. Manipulation of the microbiota in Qfly larvae. Total CFU counts of Qfly larvae. Kruskal-Wallis χ^2 = 13.011, df = 2, p = 0.0015 (see Main Text). Light grey: −/− treatment; Intermediate grey: −/+

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treatment; Dark grey: +/+ treatment. Letters indicate statistically significant differences in pairwise Kruskal-Wallis comparisons.

Additional file 2: Table S1 Diet information. The recipes for the diets used in this study. **Table S2** Output of the model investigating the effects of the microbiota on pupal production. Bold – $p < 0.05$. **Table S3** Output of the model investigating the effects of the microbiota on developmental time to pupation. Bold – $p < 0.05$. **Table S4** Complete analysis of the multinomial logistic regression investigating the role of microbiota on larvae foraging preference.

Abbreviations
CFU: Colony-forming units; Qfly: Queensland fruit fly; SIT: Sterile Insect Technique; Y:S: Yeast:sugar ratio

Author's contributions
JM, TAC, BN and FP designed the experiment. JM, STT, BN and IL performed all experiments. JM and BN analysed the data. All authors contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

Availability of data and materials
The raw data used in this study are available in the figures and tables and upon direct request to the lead author.

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

Additional file 1.

Manipulation of the microbiota in Qfly larvae. Total CFU counts of Qfly larvae. Kruskal-Wallis $\chi 2$ =13.011, df=2, p =0.0015 (see Main Text). Light grey: $-/-$ treatment; Intermediate grey: $-/+$ treatment; Dark grey: $+/+$ treatment. Letters indicate statistically significant differences in pairwise Kruskal-Wallis comparisons.
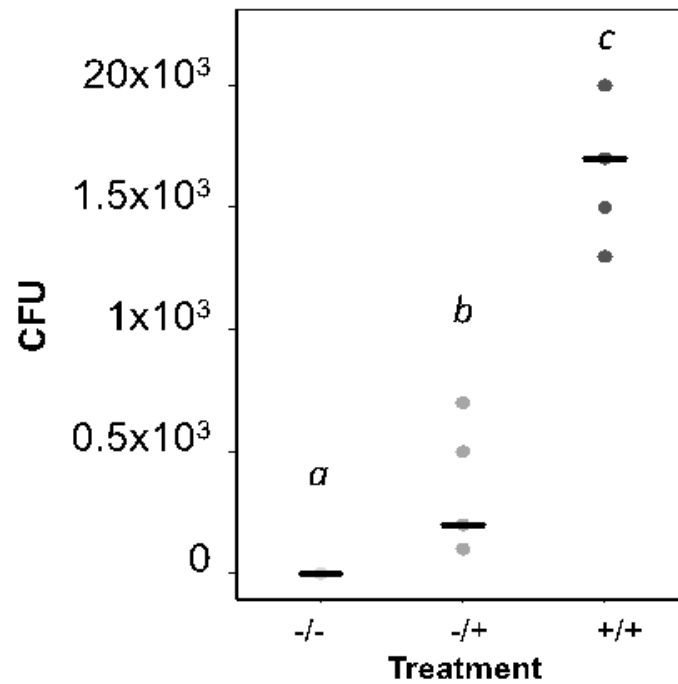
Additional file 2.

Table S1. Diet information (The recipes for the diets used in this study)

Ingredient	Yeast-to-sugar (Y:S) ratios					
	1:0	5:1	1.5:1	1:1.6	1:1.34	0:1
Hydrolyzed Yeast (g)	70	58.33	42	26.92	15.91	0
Sugar (g)	0	11.67	28	43.08	54.09	70
Agar (g)	2.5	2.5	2.5	2.5	2.5	2.5
Citric Acid (g)	4.25	4.25	4.25	4.25	4.25	4.25
Nipagen (g)	0.375	0.375	0.375	0.375	0.375	0.375
Sodium benzoate (g)	0.375	0.375	0.375	0.375	0.375	0.375
Wheat Germ Oil (ml)	0.375	0.375	0.375	0.375	0.375	0.375

MiliQ Water (ml)	250	250	250	250	250	250
Estimated protein-to-Carbohydrate (P:C) ratios	3:1	2.7:1	1:1.15	1:2.7	1.5.7	0:1

Table S2. Output of the model investigating the effects of the microbiota on pupal production. Bold – p <0.05.

Factor	Df	Sum Sq	Mean Sq	F-value	p-value
Replicate	1	0.011829	0.011829	2.4902	0.143
Treatment	2	0.111253	0.055626	11.7103	**0.002**
Residuals	11	0.052252	0.00475		

Post-Hoc		
Treatment	**Mean**	
(+/+)	0.764	a
(-/+)	0.625279	b
(-/-)	0.5570052	b

Table S3. Output of the model investigating the effects of the microbiota on developmental time to pupation. Bold – p <0.05.

Factor	df	Sum Sq	Mean Sq	F-value	p-value
Replicate	1	0	0	0	1.000
Day	1	3.28	3.28	343.303	**<0.001**
Treatment	2	0	0	0	1.000
Day^2	1	0.035	0.035	3.647	0.064
Day* Treatment	2	0.159	0.079	8.315	**0.001**
Day^2*Treatment	2	0.295	0.148	15.446	**<0.001**
Residuals	35	0.334	0.01		

Post-Hoc			
Treatment	**Day**	**Mean**	
(+/+)	8	0.8173405	a
(-/-)	8	0.7258841	a
(-/+)	8	0.5077434	b
(+/+)	9	0.1826595	c
(-/-)	9	0.2477569	c

(-/+)	9	0.4515326	b	
(+/+)	10	0	d	
(-/-)	10	0.026359	d	
(-/+)	10	0.040724	d	

Table S4. Complete analysis of the multinomial logistic regression investigating the role of microbiota on larvae foraging preference.

Relative log odds

Relative to agar

Factors	Y:S ratio	Coefficients	Std.error	p-value
(Intercept)				
	(1:0)	-1.4883	0.2616	
	(5:1)	-1.0636	0.2129	
	(1.5:1)	-1.7807	0.2553	
	(1:1.6)	-0.5291	0.1861	
	(1:3.4)	-0.2258	0.1839	
	(0:1)	0.4033	0.1634	
Time				
	(1:0)	0.0895	0.0359	0.0127
	(5:1)	0.1426	0.0298	0.0000
	(1.5:1)	0.1864	0.0301	0.0000
	(1:1.6)	0.1397	0.0289	0.0000
	(1:3.4)	0.0740	0.0309	0.0165
	(0:1)	0.0763	0.0291	0.0088
Treatment:Conventional				
	(1:0)	0.3765	0.3783	0.3196
	(5:1)	-0.0594	0.3166	0.8511
	(1.5:1)	1.2823	0.3141	0.0000
	(1:1.6)	0.7086	0.2431	0.0036
	(1:3.4)	0.0050	0.2560	0.9845
	(0:1)	0.2752	0.2252	0.2217

Treatment:Gnotobiotic

	(1:0)	-0.0918	0.3717	0.8049
	(5:1)	-0.6044	0.3295	0.0666
	(1.5:1)	0.7986	0.3195	0.0124
	(1:1.6)	0.4740	0.2421	0.0502
	(1:3.4)	-0.0850	0.2507	0.7346
	(0:1)	0.1599	0.2180	0.4632

Time*Treatment:Conventional

	(1:0)	-0.1324	0.0590	0.0249
	(5:1)	-0.1015	0.0389	0.0091
	(1.5:1)	-0.1015	0.0355	0.0042
	(1:1.6)	-0.0486	0.0339	0.1508
	(1:3.4)	-0.0373	0.0371	0.3152
	(0:1)	-0.0959	0.0363	0.0083

Time*Treatment:Gnotobiotic

	(1:0)	-0.0574	0.0461	0.2138
	(5:1)	-0.0516	0.0368	0.1607
	(1.5:1)	-0.0749	0.0351	0.0326
	(1:1.6)	-0.0664	0.0337	0.0488
	(1:3.4)	-0.0390	0.0367	0.2880
	(0:1)	-0.0733	0.0348	0.0354

Chapter 3:

Interactions between ecological factors in the developmental environment modulate pupal and adult traits in *Bactrocera tryoni*

(Published in Ecology and Evolution)

ORIGINAL RESEARCH

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Interactions between ecological factors in the developmental environment modulate pupal and adult traits in a polyphagous fly

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Abstract

1. In holometabolous insects, adult fitness depends on the quantity and quality of resource acquired at the larval stage. Diverse ecological factors can influence larval resource acquisition, but little is known about how these factors in the larval environment interact to modulate larval development and adult traits.

2. Here, we addressed this gap by considering how key ecological factors of larval density, diet nutritional composition, and microbial growth interact to modulate pupal and adult traits in a polyphagous tephritid fruit fly, _Bactrocera tryoni_ (aka "Queensland fruit fly").

3. Larvae were allowed to develop at two larval densities (low and high), on diets that were protein-rich, standard, or sugar-rich and prepared with or without preservatives to inhibit or encourage microbial growth, respectively.

4. Percentage of adult emergence and adult sex ratio were not affected by the interaction between diet composition, larval density, and preservative treatments, although low preservative content increased adult emergence in sugar-rich diets but decreased adult emergence in protein-rich and standard diets.

5. Pupal weight, male and female adult dry weight, and female (but not male) body energetic reserves were affected by a strong three-way interaction between diet composition, larval density, and preservative treatment, whereby in general, low preservative content increased pupal weight and female lipid storage in sugar-rich diets particularly at low-larval density and differentially modulated the decrease in adult body weight caused by larval density across diets.

6. Our findings provide insights into the ecological factors modulating larval development of a polyphagous fly species and shed light into the ecological complexity of the larval developmental environment in frugivorous insects.

KEYWORDS
animal–microbe competition, crowding, density, larval competition, microbiota

1 | INTRODUCTION

Resources acquired at the early stages of development determine the fitness of adults and their offspring (Rowe & Houle, 1996). In holometabolous insects, key ecological factors such as larval density, diet composition, and the microbial community colonizing the diet can modulate larval nutrition and, in turn, influence adult reproductive success, offspring quality, and the survival of groups and populations (Drew & Lloyd, 1989; Fitt & O'Brien, 1985; Morimoto et al., 2019; Sentinella, Crean, & Bonduriansky, 2013; Storer, Wainhouse, & Speight, 1997). While the implications of these ecological factors have been investigated individually or in pairs across many species, challenges remain in better understanding the combined effects of diverse ecological factors in shaping the larval environment (Wertheim, Marchais, Vet, & Dicke, 2002).

Larvae of many insects tend to aggregate in high density (Durisko & Dukas, 2013; Ives, 1991; Taylor, 1961; Taylor, Woiwod, & Perry, 1978) often with positive effects on individual fitness across taxa, including Diptera, Coleoptera, and Lepidoptera (the "Allee effect" [Allee, Park, Emerson, Park, & Schmidt, 1949; Courchamp, Clutton-Brock, & Grenfell, 1999]; see e.g., Appleby & Credland, 2007; Lawrence, 1990; Morimoto, Nguyen, Tarahi Tabrizi, Ponton, & Taylor, 2018; Weaver & Mcfarlane, 1990). Despite this, the positive effects of aggregation depend on diet composition. Nutrient-poor larval diets created by high larval densities have been shown to delay pupation, increase pupal mortality, and result in small adult body size (Gage, 1995; Morimoto, Pizzari, & Wigby, 2016; Stockley & Seal, 2001). This is likely because high larval density decreases the availability of protein (and consequently amino acids) to the developing larvae (Klepsatel, Procházka, & Gáliková, 2018). If larvae are foraging in nutrient-poor diets, the costs of nutrient limitation and competition in larval aggregations can be high and rapidly offset the benefits of aggregation, and thus, larval aggregation patterns tend to be diet-dependent (Morimoto et al., 2018). However, diet quality is defined not only by its nutritional composition but also by its microbial community. This is because microbes in the diet can modulate larval growth by modifying the diet composition, being a direct source of nutrients to the larvae and, in some cases, replenishing the host gut flora (Drew, 1988; Drew, Courtice, & Teakle, 1983; Matavelli, Carvalho, Martins, & Mirth, 2015; Wong et al., 2017). For instance, microbes in the diet increase amino acid availability for tephritid fruit fly larvae (Drew, 1988) while *D. melanogaster* are attracted to diets with microbes that match their own gut microbiota community (Drew, 1988; Wong et al., 2017). In *Bactrocera tryoni*, adults feed on microbes to supplement their nutrition (Drew et al., 1983). Microbes in the diet also influence foraging behavior by releasing odors that attract larvae and gravid females searching for oviposition sites (Durisko & Dukas, 2013; Venu, Durisko, Xu, & Dukas, 2014; Wertheim et al., 2002; Wong et al., 2017), which in turn might influence the density of larvae foraging in a patch at a given time. Thus, the network of interactions between larval density, diet composition, and microbial growth in the diet is complex and certainly shapes larval development. No direct empirical test addressing this complexity has yet been performed, and key questions remain, such as "Can microbial growth in the larval environment mitigate (or accentuate) density- and diet composition-dependent effects on larval development?"; "How does the three-way interaction between larval density, diet composition, and microbial growth affect fitness-related traits of individuals?".

To address these questions, we manipulated larval density ("low" and "high"), larval diet composition through manipulating the ratio of dietary yeast and sugar (Y:S ratio), and preservative content ("low" and "high") in the larval environment of a polyphagous fruit fly pest, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae; aka "Queensland fruit fly"). Low preservative encouraged microbial growth in the diet (Figure S1) and was designed to simulate microbial growth experienced in ripening and decaying substrates commonly experienced by fly larvae (e.g., Matavelli et al., 2015). We tested our predictions arising from previous literature on the single and interactive effects of these three factors on pupal weight, adult emergence, adult body mass, and adult energetic reserves (lipid storage) (see Table 1 for predictions). The wide variety of hosts that are exploited by *B. tryoni* (i.e., 117 hosts known so far; Clarke, Powell, Weldon, & Taylor, 2011) and its status as an effective invader that can readily expand its host range (see [Clarke et al., 2005; Clarke et al., 2011; Vargas, Pinero, & Leblanc, 2015]) makes this species an important target for better understanding how the complex ecological interactions in the larval developmental environment contributes to developmental and adult traits. This study adopts an integrative approach to explore the combined effects of key ecological factors shaping the larval environment. By understanding how larval density, diet composition, and microbial growth interact, our findings provide insights into the ecological factors modulating the ontogeny of many frugivorous insect species.

2 | MATERIALS AND METHODS

2.1 | Fly stock

Eggs were collected from females in our laboratory-adapted stock of *B. tryoni* that was established in 2015 (>20 generations old). The stock has been maintained in nonoverlapping generations, in which adults were provided a free-choice diet of hydrolyzed yeast (MP Biomedicals Cat. n° 02103304) and commercial refined cane sugar (CSR® White Sugar), while larvae were maintained for the last 10 generations using a gel diet formulation (Moadeli, Taylor, & Ponton, 2017) that is based on a liquid diet formulation of Chang, Vargas, Caceres, Jang, and Cho (2006). All stocks and experiments were maintained in a controlled environment room at 65 ± 5% relative humidity and 25 ± 0.5°C, with light cycles of 12 hr light: 0.5 hr dusk:11 hr dark: 0.5 hr dawn in the Department of Biological Sciences at Macquarie University.

2.2 | Experimental diets

We used three diets that varied in yeast-to-sugar ratio (i.e., Y:S ratio). The "standard diet" followed the gel-based diet recipe of

TABLE 1 Hypothesis and predictions tested in this study

Hypothesis	Predictions	References
High larval density is costly	1. Low pupal weight in high larval densities; 2. Low adult weight in high larval densities	Bauerfeind and Fischer (2005), Gage (1995), Morimoto et al. (2016), Stockley and Seal (2001)
High larval density induces nutrient-poor phenotypes that are rescued by microbial growth	3. Relatively minor effects of high larval density on pupal and adult traits when larvae feed on protein-rich diets and/or diets where microbial growth was encouraged in low preservative content diets, because microbes could serve as surplus of protein to the larvae	Klepsatel et al. (2018)
Protein is an essential nutrient for adequate larval development	4. High adult emergence in protein-rich diets	Kaspi, Mossinson, Drezner, Kamensky, and Yuval (2002), Nestel and Nemny-Lavy (2008), Rodrigues et al. (2015), Silva-Soares et al. (2017)
Sugar-rich diets during development increase lipid storage	5. High pupal weight in sugar-rich diets; 6. High percentage of lipid storage for adults raised in sugar-rich diets	Musselman et al. (2011), Na et al. (2013), Nestel, Nemny-Lavy, and Chang (2004)
Microbial growth modifies nutrient composition and serves as nutrient source for the larvae	7. Larvae fed on protein-rich and sugar-rich diets in which microbial growth was encouraged due to low preservative content to have lower body mass than larvae fed on diets in which microbial growth was inhibited due to high preservative content. In particular, larvae fed on protein-rich diets with low preservative content could be leaner and have the lowest pupal and adult weights (Figure 1)	Drew et al. (1983), Fitt and O'Brien (1985)

Moadeli et al. (2017) (see Table S1). We then used the same recipe but modified the amount of yeast and sugar to create a "protein-rich diet" (Y:S ratio 4:1) and a "sugar-rich diet" (Y:S ratio 1:2) (see Table S1 for recipe). When included (see "Experimental design" below), the preservatives were Nipagin (Southern Biological® cat no. MC11.2) and Sodium Benzoate (Sigma® cat no. 18106). Note that although Citric Acid (Sigma® cat no. C0759) is a preservative (Davidson, Taylor, & Schmidt, 2013), it is also needed to control the pH of the media and therefore was used in the diets as

recommended in the original recipe (see Moadeli et al., 2017). Hence, we had two preservative treatments: low and high. There were notable differences in microbial growth on diets with low and high preservatives contents (Figure S1), which supports our assumption that preservative treatments manipulated primarily microbial growth in the diets. In total, we had twelve treatments (three diets × two densities × two preservative treatments). For treatments where preservative content was high, we had *15* replicates per larval density per diet; for treatments where preservative

FIGURE 1 Overview of the experimental design and predictions. Microbial growth arising from low preservative content in the diet was expected to supplement the availability of protein to the larvae. High larval density was expected to reduce overall availability of nutrients (both protein and sugar) as a result of increased larval competition. Orange—protein-rich diet (Y:S ratio 4:1); Blue—standard gel-based diet (Y:S ratio 1.6:1); Magenta—sugar-rich diet (Y:S ratio 1:2)
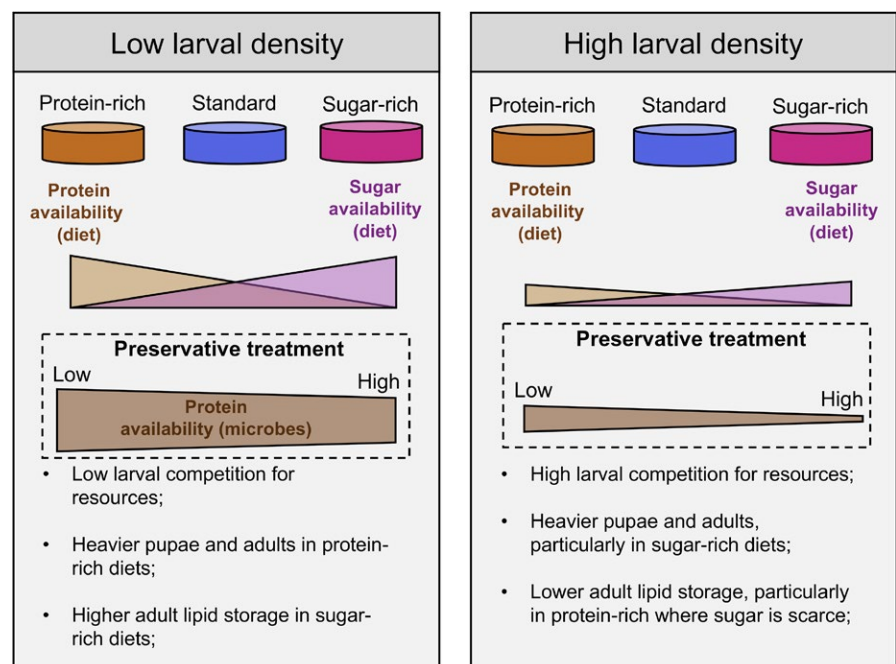
content was low thereby encouraging microbial growth, we had 5 replicates per larval density per diet.

2.3 | Larval rearing

To generate low and high larval densities, 10 and 40 µl of egg–water solution in a final concentration of ca. 16 eggs/ml of diet and 62 eggs/ml of diet, respectively, were placed in 90 mm Petri dishes containing 15 ml of each of the experimental diets, and the Petri dishes were covered. After seven days, when third instar larvae were ready to exit the diet to pupate, the lids were removed and the uncovered Petri dishes were placed into larger plastic containers (16 cm × 14.3 cm × 14.5 cm) that contained ca. 50 g of fine vermiculite for pupation. Pupae were sifted from the vermiculite two to three days after the dishes were placed onto the vermiculite and were transferred to a 90 mm Petri dish for weighing. Next, 40 pupae per replicate per diet composition per preservative treatment per larval density were then transferred in an open 50-ml Petri dish to a 5 L plastic container until adults emerged. Upon emergence, adults were provided water and a free-choice diet of hydrolyzed yeast (MP Biomedicals Cat. nº 02103304) and commercial refined cane sugar (CSR® White Sugar) for three days prior to our assessment of body lipid storage. All flies had unlimited access to water throughout the experiments.

2.4 | Pupal weight and adult percentage of emergence

Pupal weight was assessed by weighing 12–15 randomly selected pupae per replicate per diet per larval density per preservative treatment ($N = 1620$) on a Sartorius® ME5 scale (0.0001 g precision). All pupae were weighed seven days after pupation. Percentage of adult emergence was assessed by counting the number of adults that emerged, dividing by the total number of pupae (i.e., 40) and multiplying by 100 (%).

2.5 | Lipid storage (energetic reserves) quantification

Four to eight three-day-old males and four to eight three-day-old females per replicate per diet composition per larval density per preservative treatments ($N = 300$ males and 300 females) were placed individually in 10-ml glass tubes, freeze-killed (−20°C), and dried at 60°C for three days in a drying oven. Dried bodies were weighed on a Sartorius® ME5 scale (0.0001 g precision). To extract lipids (Ponton et al., 2015), two mL of chloroform (Sigma Aldrich®, Cat no. 288306) was then added to each tube which was then sealed with a rubber plug and held for 24 hr before the chloroform was discarded. The chloroform lipid extraction procedure was repeated three times on consecutive days. Bodies were then dried again at 60°C for three days in a drying oven before we measured body weight after lipid extraction. The percentage of body lipid was calculated as the difference between the dry body weight before and after lipid extraction, standardized by the body weight of each fly before the lipid extraction multiplied by 100 (i.e., percentage of lipid relative to the original dry body weight of each fly).

2.6 | Statistical analyses

All statistical models evaluated the statistical significance of main and interactive effects. We did not exclude nonsignificant interactions because interactions were part of our a priori predictions and therefore needed to be included in the final model. We nonetheless provide the final models of model selection approach in the Supplementary Information (see Tables S2–S6). Note that statistical inferences using model selection or full models converged to the same qualitative results, which corroborates the robustness of our full-model approach. Assumptions of the models were assessed using inbuilt diagnostic plots in the statistical software. The statistical significance of larval density, diet composition, and preservative treatment on pupal weight and adult weight were examined using a generalized linear model (GLM) with Gaussian error distribution as this was the model that best fitted the data (Table 2). The statistical significance of larval density, diet composition, and preservative treatment on the percentage of adult emergence and percentage of body lipid, which are proportion data, were performed using a GLM with binomial error distribution and quasi-extension to control for overdispersion of the data (Table 2). To control for pseudoreplication on the analysis of pupal weight, body weight, and percentage of body lipid, we used the average value per replicate (i.e., within replicate average) as the response variable and included replicate as a covariate in all models (see Table 2). P-values were obtained from F-statistics for all GLM models. All analyses were performed in R (R

TABLE 2 Details of the statistical models used in this study

Dependent variable	Independent variables in the generalized linear model (GLMs)	Error distribution
Average pupal weight	~larval density * diet composition * preservative treatment	Gaussian
Percentage of adult emergence and sex ratio		quasibinomial
Average body weight[a]		Gaussian
Average percentage of lipid stored[a]		quasibinomial

[a]Sexes analyzed separately.

Development Core Team, 2017), and all plots were done using the "ggplot2" package (Wickham, 2009).

3 | RESULTS

3.1 | Larval density, diet composition, and preservative content interact to modulate pupal weight

There was a significant three-way interaction between larval density, diet composition, and preservative treatment on pupal weight ($F_{2,78}$ = 4.297, p = 0.017, Table S3); a decrease in pupal weight between low and high density varied with diet composition, and low preservative content affected pupal weight differently depending on both diet composition and larval density. Specifically, pupal weight was higher in protein-rich and standard diet and lower in sugar-rich diet when preservative content was high but the opposite was observed when preservative content was low (Figure 2).

On average, pupal weight was highest when larvae were reared at low density on the sugar-rich diet when preservative content was low [Mean (SD): 13.26 (0.989)], and the lowest when larvae were reared at high density on the sugar-rich diet when preservative content was low [Mean (SD): 8.82 (1.190)]. It is important to note that the difference in pupal weight between low and high larval density was more accentuated in the sugar-rich diet when preservative content was high (Figure 2). This effect was largely due to a stronger

FIGURE 2　Interaction between larval density, diet, and preservative content on pupal weight. Given in mg. Lines were plotted using the ggplot2 package to guide interpretation of the results. Orange—protein-rich diet (Y:S ratio 4:1); Blue—standard gel-based diet (Y:S ratio 1.6:1); Magenta—sugar-rich diet (Y:S ratio 1:2). "High preservatives"—diets with low preservative content where microbial growth was inhibited; "Low preservatives"—diets with low preservative content where microbial growth was encouraged. Points were "jittered" horizontally to avoid overlapping. Solid lines were drawn with the "loess" method from the "ggplot2" package to highlight trends in the data

decrease in pupal weight from low to high larval density in standard and protein-rich diets when preservative content was low compared with the same decrease in sugar-rich diet when preservative content was low (Figure 2). There were also statistically significant two-way interactions between larval density and diet composition, larval density and preservative treatment, and diet composition and preservative treatment, as well as the main effects of larval density, diet composition, and preservative treatment (Table S3).
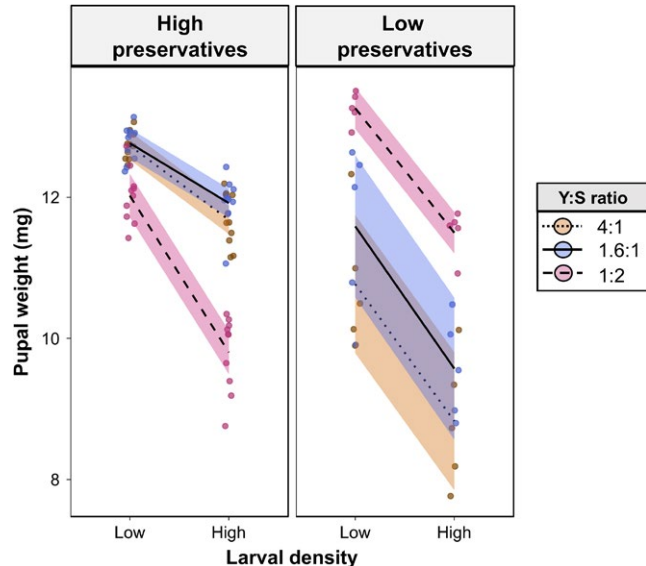
3.2 | Diet composition and preservative content, but not larval density influence adult emergence

The three-way interaction between larval density, diet composition, and preservative treatment was not statistically significant ($F_{1,108}$ = 0.485, p = 0.616, Table S4). However, there was a significant interaction between diet composition and preservative treatment on the percentage of adult emergence ($F_{2,110}$ = 4.729, p = 0.010, Table S4). This effect was driven by a decrease in the percentage of adult emergence in protein-rich and standard diets but a sharp increase in sugar-rich diet when preservative content was low compared with when preservative content was high (Figure S2). There were no significant main effects of larval density or preservative treatment on the percentage of adult emergence, although there was a significant effect of diet composition ($F_{1,116}$ = 5.127, p = 0.007, Table S4) whereby sugar-rich diets had on average lower percentage of adult emergence (Figure S2). There were no significant interactions between larval density and diet composition or larval density and preservative treatment (Table S4). There were also no effects of larval density, diet composition, preservative treatment, or any interactions among these factors, on the sex ratio of emerged adults (Table S4).

3.3 | The interaction between larval density, diet composition and preservative content modulates adult body weight

There were statistically significant three-way interactions between larval density, diet composition, and preservative content on female ($F_{2,48}$ = 3.883, p = 0.027, Table S5) and male ($F_{2,48}$ = 3.819, p = 0.028, Table S5) adult dry body weight. There was also a significant two-way interaction between larval density and preservative treatment ($F_{1,52}$ = 14.190, p < 0.001, Table S5), as well as the main effect of larval density ($F_{1,58}$ = 23.537, p < 0.001, Table S5), preservative treatment ($F_{1,55}$ = 5.845, p = 0.019, Table S5) for females, and a weak but significant two-way interaction between larval density and preservative treatment ($F_{1,52}$ = 4.190, p = 0.046, Table S5), as well as the main effects of larval density ($F_{1,58}$ = 11.201, p = 0.001, Table S5) and diet composition ($F_{1,57}$ = 4.944, p = 0.011, Table S5) for males.

For females, dry body weight decreased from low to high density on all diet compositions when preservative content was low. However, when preservative content was high, a decrease of dry body weight was only observed in sugar-rich diet, whereas dry body weight remained constant or even increased slightly from low

to high larval density in protein-rich and standard diets (Figure 3), respectively.
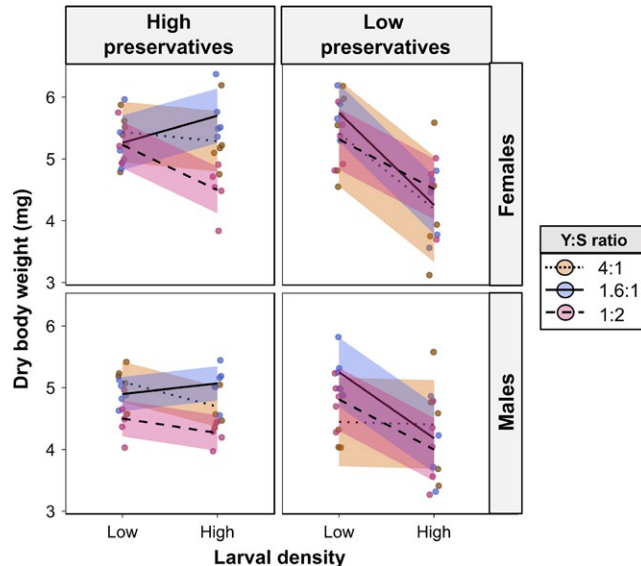
For males, dry body weight also decreased from low to high density when preservative content was low, but only for standard and sugar-rich diets (Figure 3). Dry body weight remained constant between low and high density when on protein-rich diet with low preservative content (Figure 3). However, when preservative content was high, male dry body weight increased slightly from low to high larval density in standard diet, but decreased slightly in protein- and sugar-rich diets (Figure 3).

3.4 | Female, but not male body energetic reserves are modulated by the interaction between larval density, diet composition, and preservative content

In females, there was a statistically significant three-way interaction between larval density, diet composition, and preservative treatment on lipid storage as a percentage of dry mass ($F_{2,48}$ = 5.540, p = 0.006, Table S6). Overall, in females, lipid storage was higher in sugar-rich diet, intermediate in standard diet and lower in protein-rich diet. Effects of larval density on lipid storage in females varied with preservative content; when preservative content was high, there was a slight decrease in lipid storage from low to high larval density in protein-rich diets, but no difference in standard and sugar-rich diets (Figure 4). However, when preservative content was

low, there was a sharp increase in lipid storage from low to high larval density in protein-rich diet, which was absent in standard diet and negative in sugar-rich diet (Figure 4). The two-way interactions between larval density and preservative treatment, as well as the main effects of diet composition, were also statistically significant in the model for female lipid storage (Table S6). The inclusion of nine outliers in the data resulted in the three-way interaction to become borderline nonsignificant (p = 0.063, Table S7), the two-way interaction between diet composition and preservative treatment to become borderline nonsignificant (p = 0.0503, Table S7), and the main effects of microbial growth and the interaction between larval density and diet composition to become weakly statistically significant (p = 0.044 and p = 0.019), respectively; Table S7).
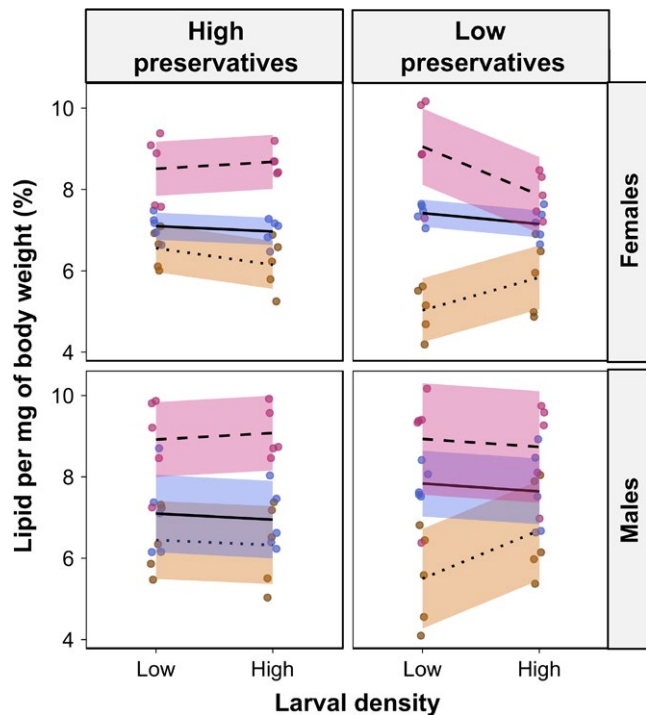
In males, only diet composition had a statistically significant effect on the percentage of lipid stored ($F_{2,56}$ = 33.558, p < 0.001, Table S6), whereby males in sugar-rich diet had higher percentage of lipid stored, intermediate percentage in standard diet, and lower percentage in protein-rich diet (Figure 4). Neither the three-way interaction between larval density, diet composition, and preservative treatment, nor the two-way interactions between larval density and diet composition, diet composition and preservative treatment, and preservative treatment and larval density influenced male percentage of lipid stored (Table S6). The inclusion of three outliers had no qualitative effects on the analysis (Table S7).

4 | DISCUSSION

In this study, we demonstrated how larval density, diet composition, and preservative content in the diet (which influence microbial growth) interact to shape larval development in the tephritid fruit fly _B. tryoni_. We found a significant three-way interaction between larval density, diet composition, and preservative treatment for pupal (Figure 2) and adult weights (Figure 3), as well as for female (but not male) lipid storage (Figure 4). Because preservative treatment had notable effects on dietary microbial growth in our experiments (see Figure S1), we henceforth refer to our findings related to preservative treatment in terms of microbial growth. From our results, general trends can be deduced. First, our results showed that diet composition was a major factor influencing pupal weight as well as lipid storage, although diet-dependent effects were strongly modulated by microbial growth. For example, protein-rich and standard larval diets generated heavier pupae relative to sugar-rich diet when microbial growth was inhibited, but this relationship was reversed when microbial growth was encouraged. These findings corroborate our predictions 3 and 4 (see Table 1) and support our hypotheses that high larval density can induce nutrient-poor environments and that protein is essential for adequate larval development. Males and females from sugar-rich diets stored more lipid in accordance to our predictions 5 and 6, which could suggest that _B. tryoni_ larvae reared on sugar-rich diets express an obese-like phenotype with higher lipid storage as seen in _Drosophila melanogaster_ (Musselman et al., 2011; Na et al., 2013; Rovenko et al., 2015). In contrast, the Mediterranean

FIGURE 3 Interactions between larval density, diet, and microbial growth on adult dry weight. Given in mg. Lines were plotted using the ggplot2 package to guide interpretation of the results. Orange—protein-rich diet (Y:S ratio 4:1); Blue—standard gel-based diet (Y:S ratio 1.6:1); Magenta—sugar-rich diet (Y:S ratio 1:2). "High preservatives"—diets with low preservative content where microbial growth was inhibited; "Low preservatives"—diets with low preservative content where microbial growth was encouraged. Points were "jittered" horizontally to avoid overlapping. Solid lines were drawn with the "loess" method from the "ggplot2" package to highlight trends in the data

FIGURE 4 Interactions between larval density, diet, and microbial growth on adult energetic reserves. Given as % of dry body weight. Lines were plotted using the ggplot2 package to guide interpretation of the results. Orange—protein-rich diet (Y:S ratio 4:1); Blue—standard gel-based diet (Y:S ratio 1.6:1); Magenta—sugar-rich diet (Y:S ratio 1:2). "High preservatives"— diets with low preservative content where microbial growth was inhibited; "Low preservatives"—diets with low preservative content where microbial growth was encouraged. Points were "jittered" horizontally to avoid overlapping. Solid lines were drawn with the "loess" method from the "ggplot2" package to highlight trends in the data

fruit fly (Tephritidae: *Ceratitis captata*) decreased lipid storage when larvae developed in sugar-rich diets (Nestel & Nemny-Lavy, 2008). In sugar-rich diets, female lipid storage decreased from low to high larval density, but in protein-rich diets increased from low to high larval density. If increased lipid storage is detrimental to female *B. tryoni* as it is for *D. melanogaster* (Musselman et al., 2011; Na et al., 2013; Rovenko et al., 2015), then microbial growth and larval density could act in synergy to reduce the negative effects of sugar-rich diet (see below). Further studies are nonetheless needed to provide a better understanding of the relationship between fitness and lipid storage in tephritid fruit flies. Our results also showed that, in general, high larval density had a negative effect on pupal and adult weights, which are in agreement with our predictions 1 and 2 as well as previous studies of diverse insect taxa (Bauerfeind & Fischer, 2005; Blanckenhorn, 1998; Lyimo, Takken, & Koella, 1992; Morimoto et al., 2016; Morimoto, Ponton, Tychsen, Cassar, & Wigby, 2017; Wertheim et al., 2002). This confirms our hypothesis that high larval density is costly for individuals (Table 1). However, this effect was not observed in standard diet when microbial growth was inhibited or in protein-rich diets when microbial growth was encouraged

(see Figure 3). Importantly, our results suggest that the potential surplus of protein availability arising from microbial growth in the diet could be insufficient to overcome the negative effects of high larval density, given that pupal and adult weights were generally lower in high larval density treatments independently of microbial growth. Thus, although microbes may serve as food (Fitt & O'Brien, 1985) and potentially as a source of limiting nutrients in a crowded larval developmental environment (Klepsatel et al., 2018), there seem to exist a more complex relationship between larval feeding and microbial growth that warrants further investigation (see Discussion below on animal–microbe competition).

Our findings can provide insights into the ecological factors that modulate larval development. For instance, our findings corroborated our predictions (Table 1) and showed an overall tendency for larvae developing in sugar-rich diet in high larval density without microbial growth to be lighter (pupae and adults) but fatter (females) than those foraging in protein-rich or standard diets in high density with inhibited microbial growth. Previous studies in other species corroborate these effects of sugar-rich diets in larval development (see for instance Matavelli et al., 2015; Silva-Soares, Nogueira-Alves, Beldade, & Mirth, 2017; Zucoloto, 1987,1991). From our results, we can predict that when larvae encounter a sugar-rich diet (or an unfavorable diet more generally), they would be more likely to disperse in search of diets with higher nutritional value, hence resulting in smaller larval aggregates in unfavorable diets. Given that the nutritional composition of fruits varies across strata within fruits (spatial variation) as well as during the ripening process (temporal variation) (Janzen, 1977; Matavelli et al., 2015), larvae could migrate to and aggregate in different strata within a fruit and potentially (although less likely) move from one fruit to another in more nutritious ripening conditions. Spatial aggregation is known to occur across insect species (Taylor, 1961), including *B. tryoni* (Morimoto et al., 2018); larval movement between fruits remains subject of further investigation. Diet-dependent larval aggregation can influence larval development rate because *B. tryoni* larvae can—like many other species (see Taylor, 1961; Taylor et al., 1978)—benefit from larval aggregation (Morimoto et al., 2018) (see also Discussion on the "Allee effect" below). A recent study has shown that *B. tryoni* larvae tend to aggregate in nutrient-rich diets that support increased larval growth, whereas dispersal is favoured in nutrient-poor diets where high larval aggregation incurs a significant cost to larval growth (Morimoto et al., 2018). Similarly, based on our data, we hypothesized that larvae should forage preferentially on microbe-free protein-rich diets instead of protein-rich diets with microbes, because microbial growth in protein-rich diets has negative effects of larval development and adult traits. This can help understand female oviposition preferences for ripe fruits, in which microbial growth and protein content of the substrate are relatively lower compared with unripe (low protein, low microbial growth) and rotting fruits (high protein, high microbial growth) (Clarke et al., 2011; Rattanapun, Amornsak, & Clarke, 2009).

In nature, *B. tryoni* larvae—as larvae of most Tephritidae—develop in dynamic environments characterized by larval aggregation,

microbial growth accompanying fruit ripening and decaying, as well as patches of food sources with varying nutritional compositions within fruits in one generation and between fruits across generations (Clarke et al., 2011; Deutscher, Reynolds, & Chapman, 2016; Drew, 1988; Drew & Lloyd, 1989; Fitt & O'Brien, 1985). Our findings provide the first direct attempt to understand the complex network of interactions among these factors that determine the quality of the larval development in this species. Our results demonstrate a general negative effect of high larval density on larval development and adult traits. *Bactrocera tryoni* larvae tend to aggregate (Morimoto et al., 2018) and females have evolved mechanisms to mitigate the negative fitness effects of high larval density on their offspring. Adult females decrease egg laying upon encountering substrates already inhabited by larvae (Fitt, 1984). It is still unknown how females modulate oviposition in the presence of larvae but in substrates with different nutritional values. For instance, it will be interesting to know whether high larval density in nutrient-rich and nutrient-poor diets have the similar effects on female oviposition, or whether females are able to fine-tune their oviposition based on both nutritional quality and larval social environment. Another crucial factor underpinning larval development is microbial growth, which our results have shown to be particularly important when larvae are exposed to protein-poor diets. It may be possible that, when protein-rich substrates are scarce, adult females modulate their oviposition behavior so as to oviposit in nutrient-poor but microbial-rich substrates, thereby facilitating larval development; this hypothesis remains to be tested.

The complexity of the larval developmental environment has been investigated within the theoretical framework of the Allee effect. The Allee effect suggests a positive effect of larval aggregation on fitness traits up to a threshold, after which the costs of larval competition offset benefits (Stephens, Sutherland, & Freckleton, 1999). In this context, Wertheim et al. (2002) manipulated the density of larvae (to simulate different larval aggregations) and the number of adults exposed to a fruit substrate prior to inoculation of larvae to test whether the interaction between ecological factors modulated the strength of the Allee effects on *D. melanogaster* larvae. The authors assessed whether fungal growth in the substrate was affected by exposure of fruit to the larvae and adults, and the implications of fungal growth for larval development. Microbial growth had negative effects on larval development, reducing survival of *D. melanogaster* larvae and size of the emerging adults (see both [Trienens, Keller, & Rohlfs, 2010; Wertheim et al., 2002] for similar results). These results are similar to our findings for the protein-rich and standard diets for which microbial growth resulted in lighter pupae, although we did not find the same pattern for adult dry weight or lipid storage. Nonetheless, negative effects of microbial growth on larval development have been suggested as evidence for animal–microbe competition for the food substrate. Such competition can lead to the evolution of toxic compounds that decrease larval survival and growth (Rohlfs & Churchill, 2011; Trienens et al., 2010; Trienens & Rohlfs, 2012; Wertheim et al., 2002). Therefore, in our study, it is possible that harmful microbes could have grown in protein-rich

and standard diets, and their presence resulted in negative effects for the larvae until pupation in these environments. Sugar-rich diets, on the other hand, might have favoured the growth of different—potentially less harmful—microbes that could also serve as an additional source of amino acids for the larvae and promote larval development (as in Drew et al., 1983; Fitt & O'Brien, 1985). This could explain our finding that pupae were heavier in sugar-rich diet when microbial growth was encouraged. Our finding of positive effects of microbial growth in sugar-rich diets are in agreement with some studies suggesting a positive effect of microbial growth on larval development due to the changes in diet composition caused by microbes (Matavelli et al., 2015) as well as studies showing that microbes can be a direct source of amino acids (Drew et al., 1983; Fitt & O'Brien, 1985). It is also possible that some diets allow beneficial microbes from the larvae to grow and serve as food ("self reinoculation") while other diets do not allow this process. If this is true, we would expect some diets to have microbial profiles that are more similar to the larvae microbial community than other diets (Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011) It will be important for future studies to investigate the microbial profile of larval diets with different nutrient compositions because it will provide detailed information of the types of microbes, the potential strength of animal-microbe competition for the food substrate, and their impact on larval development.

The present study suggests that microbial growth can influence effects of protein-rich and sugar-rich diets in ways that minimize the diet-dependent expression of fitness-related traits; for instance, for larvae reared on protein-rich diets microbial growth led to decreased pupal weight and increased female lipid storage whereas for larvae reared on sugar-rich diets microbial growth led to increased pupal weight and decreased lipid storage. Previous studies have not incorporated the combined effects of diet composition, larval density, and microbial growth, and the present study illustrates the additional insights that can be gained by incorporating such complexity in experimental design. More studies are needed for a better understanding of how diverse ecological factors affect larval foraging behavior and developmental rate of holometabolous insects.

5 | CONCLUSION

We found a strong interaction between larval density, diet composition, and microbial growth (through the manipulation of preservative content in the diet) on pupal and adult traits of *B. tryoni*, highlighting the importance of multiple ecological factors in shaping the developmental environment of insect larvae. Given that the developmental environment modulates the expression of life history traits in other invertebrates (Ireland & Turner, 2006; Tavares, Pestana, Rocha, Schiavone, & Guillermo-Ferreira, 2018) and vertebrates (including humans) (Gilbert & Epel, 2009; Gluckman & Hanson, 2006), studies that address how ecology modulates the development of life history traits can help us gain insights into how developmental ecology

influence evolutionary processes and adaptions across the animal kingdom (Gilbert, 2001).

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CONFLICT OF INTERESTS

None to declare.

AUTHORS' CONTRIBUTIONS

JM designed the experiment. JM, BN, FP, and ATT collected data. All authors analyzed the data, provided inputs into the writing of the manuscript, and approved the final version.

DATA ACCESSIBILITY

The raw data used in the study were deposited in Dryad: https://doi.org/10.5061/dryad.qr54869.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Information: 'Interactions between ecological factors in the developmental environment modulate pupal and adult traits in a polyphagous fly'

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Running title: Diet, density, and microbe effects on fitness

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Data accessibility

The raw data used in the study was deposited in Dryad: doi:10.5061/dryad.qr54869

Authors' contributions

JM designed the experiment. JM, BN, FP, ATT collected the data. All authors analysed the data, provided inputs into the writing of the manuscript, and approved the submitted version.

Supplementary Figures
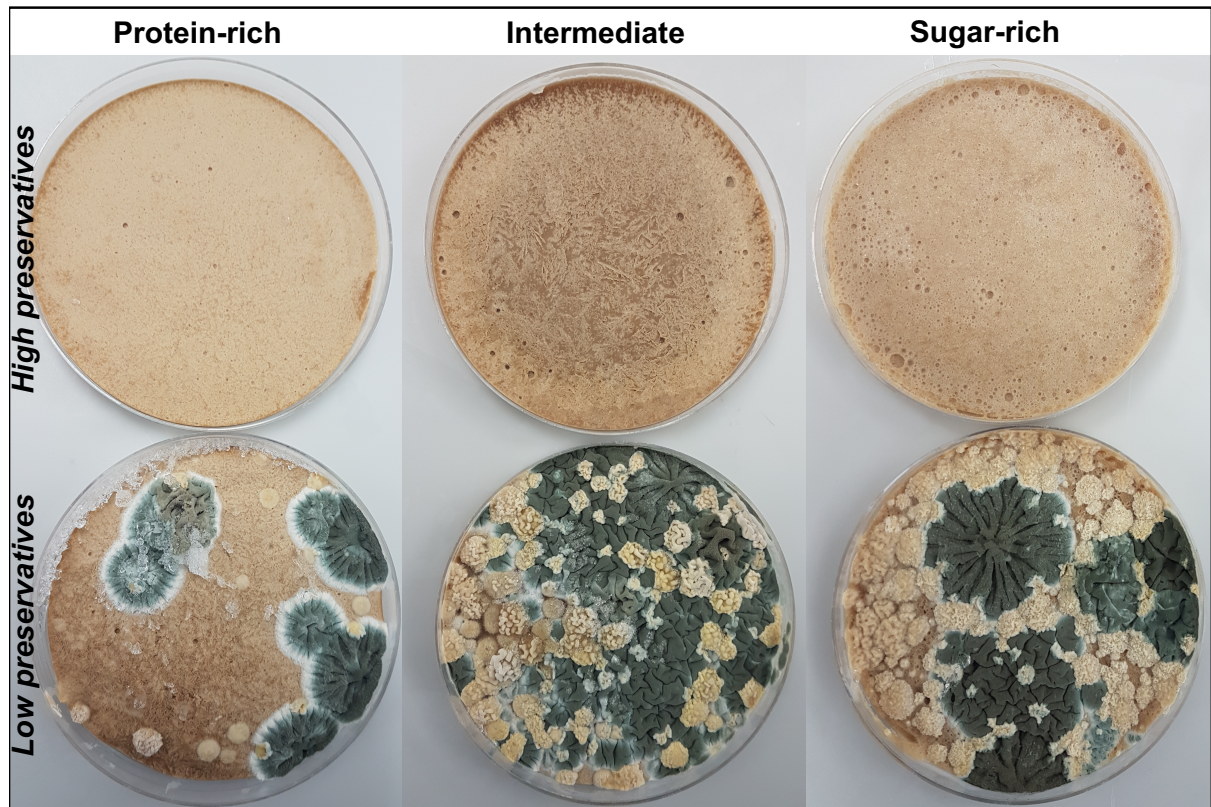
Fig S1 – The microbial growth stimulated by the preservative treatment used in our study. Control diets (i.e., no larvae) with high preservatives content and low preservatives content (high microbe growth) after 6 days incubation at 25 ± 0.5^{o}C and $65 \pm 5\%$ relative humidity. Microbe growth as observed 36-48h after the plates wer
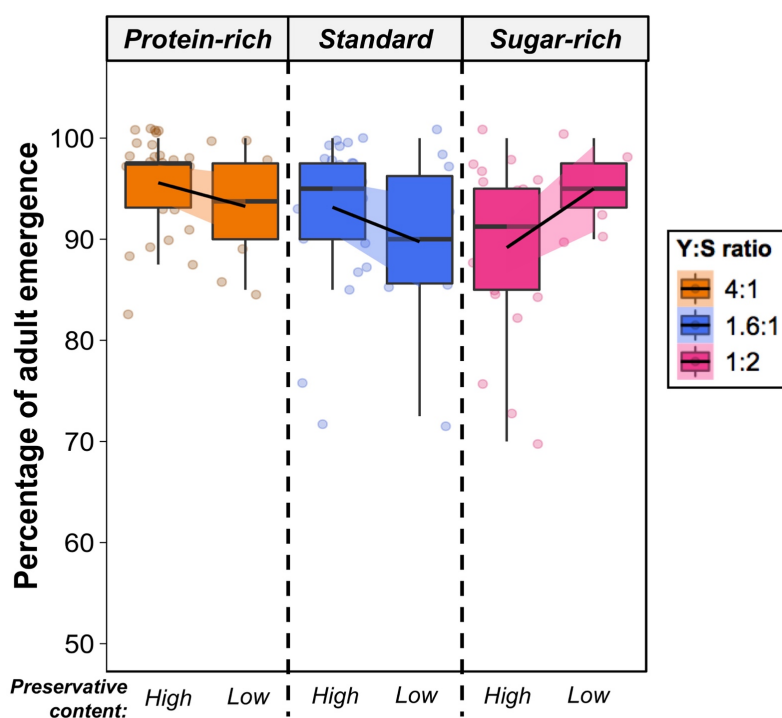
Fig S2 – Diet and preservative content interact to determine the percentage of adult emergence. Given in %. Orange – protein-rich diet (Y:S ratio 4:1); Blue – standard gel-based diet (Y:S ratio 1.6:1); Magenta – sugar-rich diet (Y:S ratio 1:2). Note that because larval density had no statistically significant effect, we omitted this factor from the graph, and lines connect the trend between treatments with high and low preservative contents. Points were "jittered" to avoid overlapping. Solid lines were drawn with the 'loess' method from the 'ggplot2' package to highlight trends in the data.

Supplementary Tables and Statistical Analysis

Table S1 – Diet recipes.

Ingredient	Protein-rich diet	Standard diet	Sugar-rich diet
Brewer's Yeast (g)	260.6	204	108.6
Sugar (g)	65.2	121.8	217.2
Agar(g)	10	10	10
Citric Acid (g)	23	23	23
Nipagin (g)	2	2	2
Sodium benzoate (g)	2	2	2
Wheat Germ Oil (ml)	2	2	2
MiliQ Water (ml)	1000	1000	1000

Model selection

Model selection has been used in complex statistical models with high-level interaction terms (e.g., three-interactions), such as those used in our study. Because we adopted a 'full model approach', it is possible that our high-level interaction terms decrease the power of our analysis, thereby decreasing our ability to detect statistically significant effects. To overcome this, we re-ran our analysis while performing backwards model selection using the 'step' function in R. Model selection was based on AIC values. Table S1 shows the step-by-step model simplification, the final models, and their AIC scores.

Table S2 – Step-by-step model selection for models used in this study.

Model	AIC
Pupal weight	
Final Model: Larval density * Diet composition * Preservative treatment	157.60
Adult emergence$^{\partial}$	
Full Model: Larval density * Diet composition * Preservative treatment	-307.48
Larval density + Diet composition + Preservative treatment + Larval density * Diet composition + Larval density * Preservative treatment + Diet composition * Preservative treatment	-310.48
Larval density + Diet composition + Preservative treatment + Larval density * Preservative treatment + Diet composition + Preservative treatment	-313.51
Larval density + Diet composition + Preservative treatment + Diet treatment * Preservative treatment	-315.37
Final Model: Diet composition + Preservative treatment + Diet treatment * Preservative treatment	-317.30
Body weight (Female)	
Final Model: Larval density * Diet composition * Preservative treatment	106.12
Body weight (Male)	
Final Model: Larval density * Diet composition * Preservative treatment	89.81
Lipid storage (Female)$^{\partial}$	
Final Model: Larval density * Diet composition * Preservative treatment	-425.78
Lipid storage (Male)$^{\partial}$	
Full Model: Larval density * Diet composition * Preservative treatment	-366.80
Larval density + Diet composition + Preservative treatment + Larval density * Diet composition + Larval density * Preservative treatment + Diet composition * Preservative treatment	-368.5
Larval density + Diet composition + Preservative treatment + Larval density * Preservative treatment + Diet composition * Preservative treatment	-370.96

Larval density + Diet composition + Preservative treatment + Diet composition * Preservative treatment	-372.57
Diet composition + Preservative treatment + Diet composition * Preservative treatment	-374.35
Diet composition + Preservative treatment	-375.11
Final Model: Diet composition	-376.99

Final models were selected based on AIC values. $^{\partial}$- Previous 'quasibinomial' models converted to linear models to allow for model selection based on AIC values. No qualitative changes on statistical inferences due to model conversion were detected (Table S3-S6).

There were no qualitatively differences between the statistical significance in final models after model selection and our full-model approach presented in the main text (Tables S3-S6). This suggests that, despite retaining two- and three-way interactions, our full-model approach had enough power to detect statistical significance whenever they existed in the data.

Table S3 – Full model outputs and model selection comparison for the analysis of pupal weight. (Table provided in separate Excel file)

Table S4 – Full model outputs and model selection comparison for the analysis of percentage of adult emergence and sex ratio. Note that to perform model selection, the original *quasibinomial* full model had to be converted into linear model, upon which AIC values could be used for model selection. No qualitative differences in statistical significance of factors from this conversion were observed. (Table provided in separate Excel file)

Table S5 – Full model outputs and model selection comparison for the analysis of female and male adult weights. (Table provided in separate Excel file)

Table S6 – Full model outputs and model selection comparison for the analysis of percentage of body lipid stored in adult flies. Note that to perform model selection, the original *quasibinomial* full model had to be converted into linear model, upon which AIC

values could be used for model selection. No qualitative differences in statistical significance of factors from this conversion were observed. (Table provided in separate Excel file)

Table S7 – Full model outputs and model selection comparison for the analysis of percentage of body lipid stored in adult flies with outliers.

Table S3. Full model outputs and model selection comparison for the analysis of pupal weight.

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			89	160.005		
Larval density	1	54.129	88	105.875	185.671	**<0.001**
Diet composition	2	2.474	86	103.401	4.244	**0.0178**
Preservative treatment	1	16.202	85	87.198	55.577	**<0.001**
Larval density * Diet composition	2	3.051	83	84.147	5.233	**0.0074**
Larval density * Preservative treatment	1	1.417	82	82.73	4.861	**0.0304**
Diet composition * Preservative treatment	2	57.485	80	25.245	98.590	**<0.001**
Larval density * Diet composition * Preservative treatment	2	2.505	78	22.74	4.297	**0.0170**

Table S4. Full model outputs and model selection comparison for the analysis of percentage of adult emergence and sex ratio.

Adult emergence

Full Model (quasibinomial)

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			119	7.5762		
Larval density	1	0.00375	118	7.5725	0.065	0.7995
Diet composition	2	0.59214	116	6.9803	5.127	**0.0075**
Preservative treatment	1	0.00003	115	6.9803	0.000	0.9832
Larval density * Diet composition	2	0.04312	113	6.9372	0.373	0.6893
Larval density * Preservative treatment	1	0.00744	112	6.9298	0.129	0.7204
Diet composition * Preservative treatment	2	0.54621	110	6.3836	4.729	**0.0107**

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
Larval density * Diet composition * Preservative treatment	2	0.05605	108	6.3275	0.485	0.6169

Full Model (linear)

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			119	0.52162		
Larval density	1	0.000255	118	0.52136	0.063	0.8019
Diet composition	2	0.038948	116	0.48242	4.825	**0.0098**
Preservative treatment	1	0.000002	115	0.48241	0.000	0.9835
Larval density * Diet composition	2	0.003573	113	0.47884	0.443	0.6435
Larval density * Preservative treatment	1	0.000502	112	0.47834	0.124	0.7251
Diet composition * Preservative treatment	2	0.038358	110	0.43998	4.752	**0.0105**
Larval density * Diet composition * Preservative treatment	2	0.004066	108	0.43592	0.504	0.6057

Final Model (linear)

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			119	0.52162		
Larval density	-	-	-	-	-	-
Diet composition	2	0.038948	117	0.48267	4.997	**0.0083**
Preservative treatment	1	0.000002	116	0.48267	0.000	0.9832
Larval density * Diet composition	-	-	-	-	-	-
Larval density * Preservative treatment	-	-	-	-	-	-
Diet composition * Preservative treatment	2	0.038358	114	0.44431	4.921	**0.0089**
Larval density * Diet composition * Preservative treatment	-	-	-	-	-	-

Sex ratio

Full model (linear)

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			119	16.427		
Larval density	1	0.10223	118	16.325	0.731	0.395
Diet composition	2	0.31527	116	16.01	1.126	0.328
Preservative treatment	1	0.32401	115	15.685	2.315	0.131
Larval density * Diet composition	2	0.24791	113	15.438	0.886	0.415
Larval density * Preservative treatment	1	0.21942	112	15.218	1.568	0.213
Diet composition * Preservative treatment	2	0.02715	110	15.191	0.097	0.908
Larval density * Diet composition * Preservative treatment	2	0.07718	108	15.114	0.276	0.760

Final model (linear)

Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
NULL			119	16.427		
Larval density	-	-	-	-	-	-
Diet composition	-	-	-	-	-	-
Preservative treatment	1	0.32401	118	16.103	2.374	0.126
Larval density * Diet composition	-	-	-	-	-	-
Larval density * Preservative treatment	-	-	-	-	-	-
Diet composition * Preservative treatment	-	-	-	-	-	-
Larval density * Diet composition * Preservative treatment	-	-	-	-	-	-

Table S5. Full model outputs and model selection comparison for the analysis of female and male adult weights.

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
Females	NULL			59	30.137		

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
	Larval density	1	6.5482	58	23.589	23.5379	**<0.001**
	Diet composition	2	1.2445	56	22.344	2.2367	0.1178
	Preservative treatment	1	1.6261	55	20.718	5.8452	**0.0195**
	Larval density * Diet composition	2	0.148	53	20.57	0.2661	0.7675
	Larval density * Preservative treatment	1	3.9478	52	16.622	14.1906	**<0.001**
	Diet composition * Preservative treatment	2	1.108	50	15.514	1.9913	0.1476
	Larval density * Diet composition * Preservative treatment	2	2.1606	48	13.354	3.8833	**0.0273**
Males	NULL			59	18.857		
	Larval density	1	2.37497	58	16.483	11.2016	**<0.001**
	Diet composition	2	2.09665	56	14.386	4.9445	**0.0112**
	Preservative treatment	1	0.8525	55	13.533	4.0209	**0.0506**
	Larval density * Diet composition	2	0.2444	53	13.289	0.5764	**0.5658**
	Larval density * Preservative treatment	1	0.8892	52	12.4	4.194	**0.0461**
	Diet composition * Preservative treatment	2	0.60307	50	11.797	1.4222	**0.2512**
	Larval density * Diet composition * Preservative treatment	2	1.61972	48	10.177	3.8198	**0.0289**

Table S6. Full model outputs and model selection comparison for the analysis of percentage of body lipid stored in adult flies.

Full Model (quasibinomial)

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
Females	NULL			59	0.149765		

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
	Larval density	1	0.000648	58	0.149116	1.1008	0.2994
	Diet composition	2	0.104094	56	0.045022	88.3697	**<0.001**
	Preservative treatment	1	0.001611	55	0.043411	2.7345	0.1047
	Larval density * Diet composition	2	0.001675	53	0.041737	1.4216	0.2513
	Larval density * Preservative treatment	1	0.000056	52	0.041681	0.0945	0.7598
	Diet composition * Preservative treatment	2	0.006569	50	0.035112	5.5765	**0.0066**
	Larval density * Diet composition * Preservative treatment	2	0.006527	48	0.028586	5.5407	**0.0068**
Males	NULL			59	0.19002		
	Larval density	1	0.000282	58	0.189738	0.1825	**0.6711**
	Diet composition	2	0.103582	56	0.086157	33.5584	**<0.001**
	Preservative treatment	1	0.000162	55	0.085995	0.1047	**0.7477**
	Larval density * Diet composition	2	0.002356	53	0.083639	0.7634	**0.4717**
	Larval density * Preservative treatment	1	0.000497	52	0.083142	0.3219	**0.5731**
	Diet composition * Preservative treatment	2	0.004533	50	0.078609	1.4686	**0.2404**
	Larval density * Diet composition * Preservative treatment	2	0.003472	48	0.075137	1.1249	**0.3331**

Full Model (linear)

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
Females	NULL			59	0.0098648		
	Larval density	1	0.0000433	58	0.0098215	1.1013	0.2992
	Diet composition	2	0.0069273	56	0.0028942	88.1435	**<0.001**
	Preservative treatment	1	0.0001073	55	0.0027868	2.731	0.1049

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
	Larval density * Diet composition	2	0.0001265	53	0.0026604	1.6095	0.2106
	Larval density * Preservative treatment	1	0.0000033	52	0.002657	0.084	0.7731
	Diet composition * Preservative treatment	2	0.0003547	50	0.0023024	4.5129	**0.0160**
	Larval density * Diet composition * Preservative treatment	2	0.0004162	48	0.0018862	5.2955	**0.0084**
Males	NULL			59	0.0129595		
	Larval density	1	0.0000196	58	0.01294	0.1863	0.6679
	Diet composition	2	0.0072184	56	0.0057215	34.3667	**<0.001**
	Preservative treatment	1	0.0000112	55	0.0057103	0.1067	0.7453
	Larval density * Diet composition	2	0.0001369	53	0.0055735	0.6517	0.5257
	Larval density * Preservative treatment	1	0.0000347	52	0.0055387	0.3307	0.5679
	Diet composition * Preservative treatment	2	0.0003013	50	0.0052374	1.4344	0.2483
	Larval density * Diet composition * Preservative treatment	2	0.0001964	48	0.005041	0.9353	0.3995

Final Model (linear)

Sex	Factors	Df	Deviance	Resid df	Resid Dev	F-value	p-value
Females	NULL			59	0.0098648		
	Larval density	1	0.0000433	58	0.0098215	1.1013	0.2992
	Diet composition	2	0.0069273	56	0.0028942	88.1435	**<0.001**
	Preservative treatment	1	0.0001073	55	0.0027868	2.731	0.1049
	Larval density * Diet composition	2	0.0001265	53	0.0026604	1.6095	0.2106

Sex	Factors	Df	Deviance	Resid Df	Resid Dev	F-value	p-value
	Larval density * Preservative treatment	1	0.0000033	52	0.002657	0.084	0.7731
	Diet composition * Preservative treatment	2	0.0003547	50	0.0023024	4.5129	**0.0160**
	Larval density * Diet composition * Preservative treatment	2	0.0004162	48	0.0018862	5.2955	**0.0084**
Males	NULL			59	0.0129595		
	Larval density	-	-	-	-	-	-
	Diet composition	2	0.0072184	57	0.0057411	35.834	**<0.001**
	Preservative treatment	-	-	-	-	-	-
	Larval density * Diet composition	-	-	-	-	-	-
	Larval density * Preservative treatment	-	-	-	-	-	-
	Diet composition * Preservative treatment	-	-	-	-	-	-
	Larval density * Diet composition * Preservative treatment	-	-	-	-	-	-

Table S7. Full model outputs and model selection comparison for the analysis of percentage of body lipid stored in adult flies with outliers

Sex	Factors	Df	Deviance	Resid Df	Resid Dev	F-value	p-value
Females	NULL			59	0.0151235		
	Larval density	1	0.0001565	58	0.014967	2.174	0.1469
	Diet composition	2	0.0097152	56	0.0052518	67.4812	**<0.001**
	Preservative treatment	1	0.0003065	55	0.0049453	4.2583	**0.0445**
	Larval density * Diet composition	2	0.0006171	53	0.0043281	4.2866	**0.0194**

	Larval density * Preservative treatment	1	0	52	0.0043281	0.0003	0.9867
	Diet composition * Preservative treatment	2	0.0004585	50	0.0038696	3.1846	0.0503
	Larval density * Diet composition * Preservative treatment	2	0.0004144	48	0.0034552	2.8783	0.0660
Males	NULL			59	0.0140357		
	Larval density	1	0.0000041	58	0.0140316	0.0374	0.8476
	Diet composition	2	0.0079257	56	0.0061059	35.9553	<0.001
	Preservative treatment	1	0	55	0.0061059	0.0002	0.9893
	Larval density * Diet composition	2	0.0001971	53	0.0059088	0.8941	0.4157
	Larval density * Preservative treatment	1	0.0000828	52	0.005826	0.7512	0.3904
	Diet composition * Preservative treatment	2	0.0003813	50	0.0054447	1.7298	0.1882
	Larval density * Diet composition * Preservative treatment	2	0.0001544	48	0.0052903	0.7005	0.5014

Chapter 4:

Sex-specific effects of the microbiota on adult carbohydrate intake and body composition in *Bactrocera tryoni*

(Manuscript in preparation)

1 **Sex-specific effects of the microbiota on adult carbohydrate intake and body**

2 **composition in a polyphagous fly**

3

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11

Author's contributions

13 BN and FP designed the experiment. BN, HD, and JM performed experiments and analysed

14 data. BN wrote the manuscript. All authors contributed to manuscript revisions.

15

Data accessibility

17 Data will be made available in Dryad upon the acceptance of the manuscript.

18

19 **Keywords:** host-microbe interaction, carbohydrate, reproduction, feeding behaviour, body

20 reserve, fly.

21

22 **Abstract**

23 The microbiota influences hosts' health and fitness. The extent to which the microbiota affects

24 host' foraging decisions and life history traits in both sexes similarly remains however to be

25 fully understood. Our study explored the effects of manipulating the microbiota on feeding

26 performance and phenotypic traits at larval and adult stages of the polyphagous fruit fly

27 *Bactrocera tryoni*. We generated three treatments: control (non-treated microbiota), axenic

28 (removed microbiota), and reinoculated (axenic individuals which had their microbiota re-

29 introduced). We found that axenic larvae and immature (i.e., newly emerged) adults were

30 lighter compared to control and reinoculated individuals. Additionally, we found a sex-specific

31 effect of the microbiota manipulation on carbohydrate intake and body composition of mature

32 adults (10 days old). Axenic males ingested less carbohydrate, had lower body weight and total

33 body fat relative to control and reinoculated ones. Carbohydrate intake was however overall

34 higher in axenic females than in control and reinoculated ones but body weight and lipid reserve

35 were similar across treatments. Axenic females produced fewer eggs than control and

36 reinoculated females. Our findings corroborate the cumulative body of evidence on the far-

37 reaching effects of microbiota in insects and show for the first time a sex-specific effect of

38 microbiota on feeding behaviour in tephritidae. Our results also underline the dynamic

39 relationship between the microbiota and the host as reinoculating microbes restores some traits

40 that were affected in axenic individuals.

41

Introduction

The insect microbiome is composed of a large diversity of microorganisms that can be
beneficial, neutral or pathogenic to the host depending on the environment (Bing et al., 2018;
Buchon et al., 2013; Lewis and Lizé, 2015). A growing body of evidence has shown multiple
effects of the commensal (i.e., healthy and balance) microbiota on insect development,
physiology and reproduction (Douglas, 2018; Engel and Moran, 2013; Lindsay et al., 2020;
Strand, 2018). In the honey bee *Apis mellifera*, for instance, manipulation of the microbiota
has profound impacts on health and disease via effects on host weight gain, metabolism,
hormonal signaling and immune gene expression [reviewed in (Raymann and Moran, 2018)].
In *Drosophila melanogaster*, the presence of the microbiota has been shown to promote
larval growth rate in poor nutritional conditions whereby germ-free larvae exhibit reduced
growth and slower development compared to larvae with an intact microbiota when raised on
imbalanced diets [i.e., with a low yeast concentration, (Shin et al., 2011; Storelli et al.,
2011)]. In parallel, different *D. melanogaster* adult traits are modulated by the microbiota,
such as food choice decision (Leitão-Gonçalves et al., 2017), kin detection (Lizé et al., 2014),
mating duration, reproductive output (Morimoto et al., 2017) and even mating selection,
although with conflicting findings (Leftwich et al., 2017; Sharon et al., 2010).

The microbiota, and more particularly the gut microbiota, has been shown to impact host
development and fitness through its involvement in nutrient acquisition and allocation (Ben-
Yosef et al., 2015, 2014; Bing et al., 2018; Consuegra et al., 2020; Leitão-Gonçalves et al.,
2017; Sannino et al., 2018). For instance, Enterobacteriaceae, the commensal bacteria of the
olive fly *Bactrocera oleae*, enable larvae to digest and develop in unripe olive fruits
containing toxic chemicals and provide adults with amino acids to facilitate protein synthesis
and egg production (Ben-Yosef et al., 2015, 2014). Similarly, studies in *D. melanogaster* and

66

67   *D. suzukii* reveals that the microbiota can buffer for the absence of essential amino acids in

68   artificial diet (Leitão-Gonçalves et al., 2017) or provides key proteins required for the

69   development of flies reared on fresh fruits (Bing et al., 2018). The microbiota can also

70   provide different vitamins such as thiamine [B1, (Sannino et al., 2018)], riboflavin [B2,

71   (Fridmann-Sirkis et al., 2014; Wong et al., 2014)] and folate [B9, (Blatch et al., 2010; Piper

72   et al., 2014)] to its host. Further investigations have shown that supplement of *Acetobacter*

73   *pomorum*, a common gut microbiota of *D.melanogaster*, could rescue the development of

74   larvae grown in no-thiamine diets through its ability to produce this micronutrient and supply

75   it to the host (Sannino et al., 2018).

76

77   While the role of the microbiota on host food exploitation and nutrient allocation is well

78   established, few studies have suggested that the microbiota can influence insects foraging and

79   feeding behaviour (Akami et al., 2019; Leitão-Gonçalves et al., 2017; Qiao et al., 2019;

80   Wong et al., 2017). The gut bacteria *Acetobacter* and *Lactobacilli*, for instance, are key

81   modulators of food choice decisions in *D. melanogaster* (Leitão-Gonçalves et al., 2017; Qiao

82   et al., 2019; Wong et al., 2017). Manipulation of these bacteria modifies host behaviour by

83   increasing *Drosophila* preference towards a diet that is beneficial for the microbes whereby

84   flies that were experimentally colonized with a specific bacterium showed an attraction

85   towards food seeded with the same strain (Qiao et al., 2019; Wong et al., 2017). While there

86   is now clear evidence that the microbiota influences host feeding behaviour and foraging, one

87   question that has not been fully addressed is how the microbiota affects host nutrient intake in

88   both sexes and what are the consequences on the expression of fitness-related traits. Sex is

89   one of the important factors influencing the microbiota, but the association has not yet been

90   sufficiently studied (Beale et al., 2019; Kim et al., 2020). Studies in human and mice have

91   shown that alterations in host metabolism mediated by changes in the gut microbiota can be

92　gender-specific (Kim et al., 2020). In insect, particularly in *Drosophila*, the microbiota-

93　dependent response of the host to diet are not uniform between males and females (Wong et

94　al., 2014). The body mass and some nutritional indices of the males were more robust to the

95　variation in diet compositions compared to that of the females (Wong et al., 2014).

96

97　Here, we investigated the effects of microbiota manipulation on male and female nutrient

98　choice and life-history traits of the polyphagous fruit fly pest *Bactrocera tryoni* (Diptera:

99　Tephritidae). To do this, we developed a protocol to generate and maintain axenic (i.e., germ-

100　free) individuals from eggs to adults using antibiotic-free diet (axenic treatment). In parallel,

101　we generated conventionally reared individuals that hosted a non-modified microbiota

102　(control treatment) and individuals with removed then re-introduced microbiota

103　(reinoculation treatment) to test whether the microbiota-reintroduction can rescue fly

104　performance. By comparing the performance of flies from these treatments, we measured the

105　effects of microbiota manipulation on i) developmental time, body weight and lipid reserve of

106　juveniles and immature (i.e., newly emerged) adults, ii) nutrient balancing and body

107　composition of mature (i.e., 10 day-old) adults, and iii) female fecundity. Because the

108　microbiota is a key modulator of the host development and metabolism (Jing et al., 2020;

109　Pernice et al., 2014; Strand, 2018) and can be a direct source of nutrition to the larvae (Drew

110　et al., 1983), we predicted a negative impact of the microbiota absence (axenic treatment) on

111　the development and body composition of larvae and adults, and female fecundity (prediction

112　1). If prediction 1 was confirmed, and due to the previous literatures which have shown that

113　the microbiota can modulate host feeding behaviour in both larvae (Morimoto et al., 2019;

114　Wong et al., 2017) and adults (Akami et al., 2019; Qiao et al., 2019; Wong et al., 2017), we

115　predicted individuals from the axenic treatment to have a higher food consumption compared

116　to the ones from the control and reinoculation treatments (prediction 2). Our study highlights

117   the interplay between the microbiota and the insect hosts, providing for the first time data on

118   how the microbiota influences male and female food choice, nutritional status and

119   reproductive output of the fruit fly *B. tryoni*.

120

121   **Methods**

122   *Fly stock*

123   *B. tryoni* lab-adapted colony was established in 2015 and has been maintained in non-

124   overlapping generations since then at Macquarie University (NSW, Australia). Adults were

125   provided with a free-choice diet of hydrolysed yeast (MP Biomedicals cat no. 02103304) and

126   sugar (CSR® White Sugar) while larvae were maintained on a gel-based diet (Moadeli et al.,

127   2017). The colony and all experiments hereinafter were maintained in the same control

128   environment room at 25 ± 0.5 ºC, $65 \pm 5\%$ relative humidity and 12: 0.5: 11: 0.5

129   light/dusk/dark/dawn photoperiod.

130

131   *Fly rearing and microbiota manipulation*

132   The axenic treatment was generated by eliminating microbes from the eggs, the diet, and the

133   air. We removed microbes from the egg surface through dechorionation as described in

134   (Koyle et al., 2016) and optimized for *B. tryoni* (see details in supplementary material). The

135   procedure included two washes in 0.5% bleach (White King ®) for 3 min, followed by one

136   wash in 70% ethanol (Sigma, cat no. 64175) for 1 min, and three washes in sterile water. This

137   protocol allowed to eliminate microbes on the egg surface without affecting egg hatching rate

138   (see Figure S1, supplementary material). Eggs (collected for 2h from our lab-adapted colony)

139   were treated following this protocol and batches of 100 eggs were aseptically transferred

140   using a fine paintbrush to 25mL of a sterile gel-based diet. The diet (recipe and suppliers

141   provided in supplementary material, Table S1) was prepared aseptically in a biosafety cabinet

69

142   by mixing brewer's yeast (irradiated at 10 kilogray for 21h by cobalt-60) with nipagin and a

143   freshly autoclaved solution made of water, sugar, wheat germ oil, sodium benzoate, and agar.

144   This mixture was then added in an autoclaved solution of sterile water and citric acid prior

145   being poured in a triple vented petri dish (90mm; Techno Plas, cat no. S6014S10). This diet

146   was prepared one day before the start of the experiment and stored at 4 ˚C.

147

148   The control treatment consisted of the same egg washing procedure using the same batch of

149   eggs but all chemicals were replaced by sterile miliQ water. The reinoculation treatment was

150   generated by reintroducing microbes collected from control eggs to axenic eggs. Briefly, 100

151   control eggs were homogenised in 50 µL sterile Milli-Q water for 2 min by a hand-holding

152   pestle cordless motor (Sigma, cat no. Z359971). This solution was then pipetted onto a pool

153   of 100 axenic eggs that had been deposited on gel diet. The petri dishes that contained these

154   eggs were left open for 5 min for water evaporation. Manipulation of the control and

155   reinoculation treatments were conducted in non-sterile environment. For each treatment, we

156   generated 10 replicates (i.e., 10 petri dishes).

157

158   Larvae of all treatments developed inside petri dishes (90mm; Techno Plas, cat no.

159   S6014S10). One day before pupation, petri dishes that contained third instar larvae of the

160   axenic treatment were surface sterilized using ethanol 70% and aseptically placed in 1.125L

161   cages (Decor Tellfresh, cat no. 136000) containing ca. 20g of autoclaved vermiculite

162   (Ausperl®). The lids of the petri dishes were removed for larvae to pupate in the vermiculite.

163   Cages were housed in a laminar flow workstation (Gelman Science, HLF120, serial no.

164   3176/90) during pupation to avoid air contamination. Pupae were then sieved from

165   vermiculite and transferred to 1.125L sterile cages one day before adult emergence. The

166   control and reinoculation treatments were manipulated in the non-sterile condition using the

167   same steps.

168

169   *Microbial status assessment by culture and nonculture-dependent method*

170   We sampled eggs, third instar larvae, and newly emerged males and females for each

171   treatment (N = 3 replicates per treatment). 100μL of a solution of eggs and sterilised water

172   (contains ca. 1500 eggs) was collected, as well as groups of 20 larvae and individual male

173   and female adults. Larvae and adults were freeze-killed for 2h before subjecting to surface

174   sterilization by the same steps as in the egg washing procedure. We then homogenised eggs,

175   larvae, and adult samples in 0.4, 0.1, and 1mL PBS buffer respectively. After that, we plated

176   25 μL of each homogenate on a petri dish containing 25 mL of either Luria Bertani agar (LB,

177   Life technologies, cat no. 22700-025), or Man-Rogosa-Sharpe agar (MRS Oxoid®, cat no.

178   CM0361), or Potato-Dextrose agar (PDA Oxoid®, cat no. CM0139) using single-use L-shape

179   spreaders (Sigma, cat no. Z723193). The leftover homogenates were stored in -20˚C. We

180   incubated LB dishes at 28˚C for 24-48h, MRS and PDA dishes at 28˚C for 48-72h then

181   counted the number of colony-forming unit (CFU) *per* dish. The CFU count *per* one sample

182   replicate, denoted as *E*, was estimated using the equation

183

184    $E \ = \ (C*V)/P$

185

186   whereby *C* is the sum of CFU that grew on LB, MRS, PDA media divided by three, *P* is the

187   volume plated (i.e., 25 μL), and *V* is the total volume of the homogenate (Koyle et al., 2016).

188

189   To check that our axenic samples were free from unculturable microbes, we quantified the

190   amount of 16S rRNA gene and the internal transcribed spacer (ITS) DNA in the leftover

191   homogenates of axenic treatment (*N total* = 12) by PCR amplification. Corresponding

192   leftover homogenates of control treatment (*N total* = 12) were used as positive control and

193   washing solutions (bleach, ethanol, and sterile water, *N total* = 9) were used as negative

194   controls. The initial PCR amplicons were generated using the AmpliTaq Gold 360 mastermix

195   (Life Technologies, Australia). The PCR conditions and primers were outlined in

196   supplementary material, Table S2. A secondary PCR to index the amplicons was performed

197   with the TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were measured

198   by florometry (Invitrogen Picogreen) and normalised. The eqimolar pool was then measured

199   by qPCR (KAPA).

200

201   *Developmental time*

202   Fly developmental time was observed from petri dishes or cages that host the eggs or pupae

203   (*N total* = 30, 10 replicates per treatment) and was estimated as i) the number of days from

204   egg to larval pupation (egg-larval duration) and ii) the number of days from egg to adult

205   emergence [egg-pupal duration, (Moadeli et al., 2017)].

206

207   *Body weight and lipid reserve*

208   We measured individually the body wet weight of third instar larvae (*N total* = 75, 25

209   individuals per treatment) and immature males and females (*N total* = 120, 20 males and 20

210   females per treatment). Larvae were sampled randomly after "jumping" out of the diet into

211   vermiculite for pupation. Adults were collected 24h after emergence. Samples were freeze-

212   killed in -20˚C for 24h before thawing for 1h then weighing using a precision weighing

213   balance (Sartorius® ME5 scale, d=0.0001g).

214

215   Body fat percentage of the same larval and adult samples (*N total* = 75 and *N total* = 120

216   respectively) were measured following the protocol described in (Ponton et al., 2015).

217   Briefly, samples were oven-dried at 55°C for 48h, then body dry mass was weighed

218   individually by Sartorius® ME5 scale, followed by lipid-extraction in three 24h changes of

219   chloroform (Sigma, cat no. 650498). After the third chloroform wash, samples were left in

220   the fume cupboard (Dynaflow, unit no. FC100316) for 48h for chloroform evaporation before

221   re-drying and re-weighing to determine the fat-free body mass. Body fat mass is the offset of

222   body mass and fat-free body mass. Body fat percentage was calculated as body fat mass

223   divided by body mass, multiplied by 100.

224

225   ***Food consumption***

226   We used a Capillary Feeder (CAFE) assay to investigate the food consumption and feeding

227   choice of adult flies for 10 days. The method has been previously described in (Dinh et al.,

228   2019) and graphically illustrated in supplementary material, Figure S2. In brief, newly

229   emerged males and females from all treatments (*N total* = 138, 23 males and 23 females per

230   treatment) were housed individually in a 70ml clear plastic chamber (Thermofisher, Cat.

231   LBS30005YI) with 6 holes (d < 2mm) on the top, one filled with 50µL of water through a

232   pipette tip, two filled with 30µL glass capillaries (Drummond Microcaps®, Cat no. 1-000-

233   0300), and the rest of the holes were left open for air ventilation. We filled capillaries with

234   either a hydrolysed yeast solution ('protein diet') or a sugar solution ('carbohydrate diet'),

235   both at the concentration of 120g/L. These solutions were prepared aseptically by mixing

236   sterile hydrolysed yeast and sugar with autoclaved mili-Q water in a biosafety cabinet.

237   Hydrolysed yeast and sugar were sterilized by irradiation for 21h at 10 kilograys; this

238   irradiation dose has been confirmed to totally eliminate microbes from food products without

239   affecting their nutritional quality (Hewitt and Leelawardana, 2014; Ley et al., 1969). Mili Q

240   water was supplemented with a commercial blue food dye (final dilution 1:10,000, Queen®

241   brand) prior autoclaving to facilitate the measurement of food consumption in each capillary.

242   The dye has no nutritional value. Mixed solutions were aliquoted and stored at 4 ˚C. We used

243   fresh aliquots to feed flies daily. Flies had *ad libitum* access to water during the experiment.

244

245   The axenic treatment was maintained in the laminar flow workstation and fed aseptically

246   using fresh autoclaved capillaries daily. Maintenance of the control and reinoculation

247   treatments were conducted in parallel in non-sterile environment. To correct for the

248   evaporation rate of solutions in capillaries, we included five empty feeding cages (i.e.,

249   without fly) in sterile condition and five in non-sterile condition. The volume (μL) of

250   solutions consumed by individual flies was measured daily using a digital calliper (serial no.

251   110833) and corrected by evaporation rate of the corresponding conditions. At the end of this

252   experiment, three males and three females from each treatment (*N total* =18) were randomly

253   picked for microbial status assessment by culture-dependent method (Koyle et al., 2016). The

254   rest (*N total* = 120, 20 males and 20 females per treatment) were freeze-killed at -20 ˚C and

255   their body wet weight and total body fat were measured as described above. Cumulative

256   intakes of carbohydrate and protein for 10 days (in μg, *N total* = 120, 20 males and 20

257   females per treatment) were calculated.

258

259   ***Fecundity***

260   Ten newly emerged (0- to 24h-old) females and males were housed in equal sex ratio within a

261   1.125L cage with *ad libitum* food (autoclaved water, irradiated hydrolysed yeast and sugar).

262   We set up 7 replicate cages *per* treatment (*N total* = 21) whereby axenic treatment was

263   maintained aseptically in the laminar flow workstation and non-axenic treatments were

264   maintained in non-sterile condition. As an egg collection device, we used the bottom of a

265   petri dishes (d = 35mm, Corning®, cat no. CLS430165) covered by a thin layer of parafilm

266   (M laboratory®) that had numerous perforations of d <1 mm for females to insert their

267   ovipositor and lay eggs. The device contained 1 mL of sterile water flavoured by natural

268   apple essence (Foodie flavours™, 1mL L^{-1}) to attract females laying eggs. The parafilm for

269   the axenic treatment was subjected to the ultraviolet germicidal irradiation for 15 min for

270   each side. We placed one device per cage. The number of eggs deposited *per* cage was

271   counted daily during 8 days, starting from day 14th post-emergence. The number of dead

272   females was also recorded and taken into account in our statistical models by dividing the

273   number of eggs collected by the number of females in each cage. The final outcome was the

274   average number of eggs produced daily by one female during the collection period (i.e., eggs

275   *per* female *per* day).

276

277   ***Statistical analysis***

278   All analyses were performed in R [version 3.5.2, (R Core Team, 2018)] and figures were

279   plotted using the R package 'ggplot2'(Wickham, 2009) and Excel. Plots are of the raw data.

280   To test for the effects of treatment, sex, and the interaction between treatment and sex on the

281   outcome variable, a Generalized Linear Model (GLM) with Binomial error distribution and

282   *quasi* extension was applied for proportion data (i.e., body fat %) because this was the best

283   fitted model for this data. Similarly, A GLM with Gaussian error distribution was applied on

284   body weight, food intake and fecundity. Data for protein intake were square root transformed

285   prior to analyse to reduce heteroscedasticity. Student-Newman-Keuls (SNK) *post hoc* tests

286   with $p \leq 0.05$ were applied to identify treatments that differ from each other. A non-

287   parametric Kruskal-Wallis test was applied to test for effects of the treatment on data that did

288   not fulfil the assumptions of parametric models, and that include CFU counts, PCR

289   quantifications of 16S and ITS, and developmental time. Dunn *post hoc* tests with $p \leq 0.05$

290   (adjusted with the Benjamini-Hochberg method) were then applied to identify treatments that

291   differ from each other.

292

293   **Results**

294   *Microbial status*

295   Manipulation of the microbiota influenced CFU count (Kruskal Wallis, $\chi^2 = 33.52$, df = 2, p

296   < 0.001). CFU counts of the control and reinoculation treatments were significantly higher

297   than that of the axenic treatment in egg and adult stages but not at larval stage

298   (Supplementary material, Figure S3 and Table S3). CFU count and PCR quantification of

299   16S and ITS supported the sterile status of axenic samples while control samples hosted

300   bacteria and fungi; ITS quantification, however, was very low in control groups and was not

301   significantly different to axenic groups (*16S rRNA*, $\chi^2 = 18.612$, df = 1, p < 0.01; *ITS*, $\chi^2 =$

302   2.087, df = 1, p = 0.149, supplementary material, Figures S3 and S4). Both 16S and ITS were

303   undetected in all the replicates of the washing solutions (Supplementary material, Table S4).

304

305   *The microbiota affects larval and immature adult body weight*

306   The control and reinoculated larvae were about 1 mg heavier than the axenic larvae (*control*:

307   14 ± 0.178 mg; *reinoculation*: 13.76 ± 0.255, *axenic*: 13 ± 0.177 mg; GLM, $F_{2,72} = 5.706$, p

308   =0.005, Figure 1A). Likewise, body weight of immature adults of the control (7.56 ± 0.3 mg)

309   and reinoculation treatments (7.79 ± 0.32 mg) were about 1.2 and 1.4 mg greater compared

310   to that of the axenic counterparts (6.36 ± 0.33 mg; GLM, $F_{2,117} = 9.302$, p < 0.001, Figure

311   1B). Body weight was substantially greater in females than in males (8.49 ± 0.23 mg *versus*

312   5.98 ± 0.21 mg; GLM, $F_{1,116} = 75.109$, p < 0.001, Figure 1B); however, there was no

313   significant interaction between sex and treatment (GLM, $F_{2,114} = 0.018$, p = 0.982). Treatment

314   did not affect the lipid reserve of either the larvae (GLM, $F_{2,72} = 1.67$, p = 0.195, on average

315   30%) or immature adults (GLM, $F_{2,117} = 2.333$, p = 0.102, on average 16%). There was also

316   no effect of sex (GLM, $F_{1,116} = 0.803$, p = 0.372) and of the treatment-sex interaction (GLM,

317    $F_{2,114} = 0.082$, p = 0.921) on lipid reserves of immature adults. Microbiota manipulation did

318   not affect fly developmental time (*Egg-larval duration*: Kruskal Wallis, $\chi2 = 2.522$, df = 2, p

319   = 0.283; *Egg-pupal duration*: Kruskal Wallis, $\chi2 = 0.360$, df = 2, p = 0.835). Egg-larval

320   duration was on average 7.4 $\pm$ 0.02 days while egg-pupal duration took approximately 17.4

321    $\pm$ 0.02 days.

322

323   ***Sex-specific effects of the microbiota on carbohydrate intake and body fat reserve of***

324   ***mature flies***

325   Treatment affected carbohydrate consumption differently between females and males

326   (*Treatment*sex*: GLM, $F_{2,114} = 5.776$, p < 0.01, Supplementary material: Table S5). Control

327   and reinoculated females ingested on average 370 and 750 µg less carbohydrate than axenic

328   females and the absolute intake was significant between the axenic and the reinoculated

329   individuals (*control*: 6457 $\pm$ 216 µg, *reinoculation*: 6073 $\pm$ 173 µg, *axenic*: 6826 $\pm$ 242 µg;

330   GLM, $F_{2,57} = 3.167$, p = 0.049, Figure 2A). The carbohydrate consumption of control males

331   (5885 $\pm$ 155 µg), however, was higher than that of reinoculated (5364 $\pm$ 214 µg) and axenic

332   males (5028 $\pm$ 173 µg; GLM, $F_{2,57} = 5.586$, p < 0.01, Figure 2A). Protein intake was higher

333   in females than males (2535 $\pm$ 68 µg *versus* 1410 $\pm$ 39 µg; GLM, $F_{1,116} = 213.4$, p < 0.01,

334   Figure 2A) but there was no significant influence of the treatment (GLM, $F_{2,117} = 0.143$, p =

335   0.867) or the treatment-sex interaction (GLM, $F_{2,114} = 0.562$, p = 0.571) on protein intake

336   (Figure 2A). Body composition analysis after the feeding experiment showed that body fat

337   percentage was substantially affected by treatment (GLM, $F_{2,117} = 4.466$, p < 0.05), sex

338   (GLM, $F_{1,116} = 18.331$, p < 0.001), and the treatment-sex interaction (GLM, $F_{2,114} = 5.080$, p

339   < 0.01). Mature males of the control and reinoculation treatments contained respectively 3.16

340 and 2.33 % more total body fat than males of the axenic counterparts (GLM, $F_{2,57} = 9.534$, p

341 < 0.001, *control*: 14.53 %, *reinoculation*: 13.7 %, *axenic*: 11.37 %, Figure 2B). In females,

342 however, the trend was different whereby body fat percentage was not significantly different

343 between treatment (GLM, $F_{2,57} = 0.806$, p = 0.452, Figure 2B). Body weight of mature adults

344 was affected by treatment (GLM, $F_{2,117} = 6.235$, p < 0.01) and sex (GLM, $F_{1,116} = 341.7$, p <

345 0.001) but not the treatment-sex interaction (GLM, $F_{2,114} = 0.317$, p = 0.729). Males of the

346 control and reinoculation treatments were about 1 mg heavier than males of the axenic

347 siblings (GLM, $F_{2,57} = 5.217$, p < 0.01, *control*:15 $\pm$ 0.31 mg, *reinoculation*: 15 $\pm$ 0.21 mg,

348 *axenic*: 13.98 $\pm$ 0.29 mg, Figure 2C). In females, body weight was greater in non-axenic

349 treatments compared with axenic treatment although no statistical significance was detected

350 (GLM, $F_{2,57} = 2.029$, p = 0.141, Figure 2C).

351

352 *Axenic treatment reduces egg production*

353 Axenic flies produced on average fewer eggs *per* day than the control and reinoculated flies

354 respectively over the investigated period (GLM, $F_{2,165} = 5.915$, p < 0.01; *axenic*: 40 $\pm$ 2 eggs,

355 *control*: 48 $\pm$ 2 eggs, *reinoculation*: 51 $\pm$ 3 eggs per day, supplementary material, Figure

356 S5A). Fecundity was also influenced by time with the number of egg *per* female reaching a

357 peak of 66 $\pm$ 5 eggs on day 15th post-emergence (GLM, $F_{7,158} = 5.698$, p < 0.001,

358 supplementary material, Figure S5B); however, there was no significant effects of the

359 treatment-time interaction (GLM, $F_{14,144} = 1.379$, p = 0.171).

360

361 **Discussion**

362 In the present study, we measured the effects of microbiota manipulation on *B.tryoni* fitness-

363 related traits and feeding behaviour. We found sex-specific effects of the microbiota on total

364 carbohydrate intake and body fat reserve of mature flies, which confirmed our prediction 2

365   and a part of prediction 1. A healthy microbiota has therefore profound impacts on the

366   performance of *B. tryoni*, with positive effects on body weight and fecundity. Our results also

367   show that beneficial effects of the microbiota can be restored in germ-free individuals

368   through reinoculating the microbiota. These findings advance our understanding of the

369   complex interplay between the insect and its microbiota, highlight the important role of the

370   commensals on animal fitness.

371

372   Our results showed a positive effect of the microbiota on body weight gain of larvae and

373   immature adults, however, lipid reserve was not significantly different across larvae and

374   immature adults of axenic and non-axenic treatments. Previous studies have shown that the

375   microbiota increases body mass of several insect species including fruit flies [*Ceratitis*

376   *capitata* (Hamden et al., 2013), *B. dorsalis* (Khaeso et al., 2018), *B. cucurbitae* (Yao et al.,

377   2017)], honey bee *A. mellifera* (Zheng et al., 2017) and the beetle *R. ferrugineus* (Habineza et

378   al., 2019), through either supplying nutrients, facilitating the digestion of food or modulating

379   gut epithelial renewal (Ben-Yosef et al., 2015, 2014; Engel and Moran, 2013). Recently, a

380   study on the honey bee *A. mellifera* has also revealed a link between commensals, host

381   weight gain and the insulin signaling pathway whereby the commensals may supply amino

382   acids to the host to enhance the production and responsiveness of insulin and consequently

383   increase host body mass (Zheng et al., 2017). Notably, reinoculating axenic eggs by exposing

384   them to the microbiota of control eggs restored body weight as previously suggested in

385   studies on the beetle *R. ferrugineus,* (Habineza et al., 2019) and mice (Bäckhed et al., 2004;

386   Turnbaugh et al., 2006).

387

388   We also showed that the microbiota had different effects on carbohydrate intake and body fat

389   reserve of males and females. Axenic males consumed less carbohydrate and had lower body

390   weight and lipid reserve than control males. In females, however, the trend was different with

391   a greater carbohydrate consumption observed in axenic flies but body composition indices

392   were similar across all treatments. Earlier studies have shown that the microbiota influences

393   insect feeding choice (Akami et al., 2019; Leitão-Gonçalves et al., 2017; Lundgren and

394   Lehman, 2010; Wong et al., 2017) and metabolism (Ben-Yosef et al., 2008; Wong et al.,

395   2014). For instance, in *D. melanogaster*, an elevation of lipid content was observed in axenic

396   flies of both sexes even when host food intake was reduced (Wong et al., 2014). A similar

397   effect has been reported in the beetle *Harpalus pensylvanicus* whereby germ-free adults

398   consumed less food than counterparts that harboured *Enterococcus faecalis*, but no further

399   investigation on the host nutritional state was conducted (Lundgren and Lehman, 2010). On

400   the other hand, in *Bactrocera dorsalis,* when males and females were treated with antibiotics,

401   both sexes responded faster to food drops in foraging arenas, spent more time feeding and

402   ingested more food than control individuals (Akami et al., 2019). A growing body of

403   evidence from mammals and insects suggests an important role of the microbiota on

404   harvesting and storing energy from the diet (Brune and Dietrich, 2015; Chen et al., 2016;

405   Krajmalnik-Brown et al., 2012; Sommer et al., 2016). Our finding agrees with this

406   perspective. In females, non-axenic individuals consumed less carbohydrate but still reached

407   similar body weight and body fat content to their axenic counterparts. Meanwhile in males,

408   the reinoculated flies had higher lipid reserve though they consumed an equal amount of

409   carbohydrate compared to the axenic flies. Similar results have been reported in rodents for

410   which germ-free males had to consume a higher caloric intake to reach the same weight as

411   control individuals; however, body fat percentage of control mice was significantly higher

412   than that of germ-free ones (Bäckhed *et al.*, 2004).

413

414   The different effects of the microbiota on food consumption and nutritional state of males and

415   females *B. tryoni* raise the possibility that the fly-microbe interactions are sex-dependent in

416   some aspects. The microbial composition can differ between sexes in many species, including

417   insects [for instance (Chen et al., 2016; Fransen et al., 2017; Tang et al., 2012)]. This might

418   suggest different types of interactions between male and female hosts and their microbiota

419   and, therefore, different effects of microbiota manipulation on host performance. However, in

420   some tephritids such as *C. capitata*, *B. oleae*, and *Anastrephas*, studies have shown that sex

421   does not influence the composition of the gut microbiota (Augustinos et al., 2019; Koskinioti

422   et al., 2019). We have yet to uncover the mechanism driving the sex-specific effects of the

423   microbiota on carbohydrate intake and body composition in *B. tryoni*. It is possible that the

424   signaling pathways regulating the host metabolism in males and females react differently to

425   the presence/absence of microbial products since several studies in mice have shown that

426   changes in host metabolism mediated by diet-dependent alterations in the gut microbiome can

427   be gender-specific (Bian et al., 2017; Suez et al., 2014). Conversely, the composition of the

428   microbial community and its metabolic traits could be modulated by the physiological

429   differences between males and females, especially during sexual maturity such as puberty in

430   human and mice (Kim et al., 2020) or reproduction in insects (Minard et al., 2018).

431

432   We found that microbiota removal led to lower fecundity, which is in agreement with our

433   prediction 1. This result corroborates a previous finding in the same species where flies

434   rearing on sterile diet produced fewer eggs than the ones reared on non-sterile diet (Drew et

435   al., 1983). In *Drosophila,* while antibiotic-based treatment did not reveal impacts of the

436   microbiota on fecundity (Ridley et al., 2013, 2012), eliminating the microbiota by egg

437   dechorionation and then rear eggs on sterile food induced a strong reduction in egg

438   production (Elgart et al., 2016). How the microbiota affects fecundity is not yet clear,

439   however, insect reproduction is thought to be partly regulated by the nutrient-sensitive TOR

440   (target of rapamycin) pathway (Arsic and Guerin, 2008; LaFever et al., 2010; Ribeiro and

441   Dickson, 2010) and the microbiota can modulate this pathway (Storelli et al., 2011). Thus, it

442   is possible that the microbiota modulates host fecundity by releasing metabolites that directly

443   influence the host nutrient sensing (Leitão-Gonçalves et al., 2017). Specific bacteria in the

444   microbiota community may also play critical roles on egg formation. In *D. melanogaster*, for

445   instance, absence of the gut bacteria *Acetobacter* (but not *Lactobacillus*) suppresses

446   oogenesis through reducing the activity of *Aldh*, an enzyme responsible for the catabolism of

447   aldehyde substrates in ovaries (Elgart et al., 2016). Likewise, in mosquitoes, *Bacillus* and

448   *Staphylococcus* bacteria enhance host fecundity compare to other midgut bacteria, although

449   the mechanisms underlying this effect are yet to be discovered (Fouda et al., 2001).

450

451   Our study provides an antibiotic-free protocol to produce axenic individuals that can be used

452   to investigate the physiological and behavioural effects of host-microbiota interactions across

453   insects. We cannot completely rule out that our protocol for egg dechorionation and aseptic

454   methods to manipulate the microbiota transmitted to eggshells could have had some effects

455   on insect physiology, especially on larval development and pupation (Ridley et al., 2013).

456   However, our results showed that egg hatching success and egg to larval developmental

457   duration were identical across treatments, supporting the idea that egg dechorionation had

458   minor effects on the viability and growth of individuals at the larval stage. We also

459   minimised any further detrimental effects of our protocol by maintaining the axenic treatment

460   in a clean air workstation while feeding flies with sterile, antibiotic-free food. This was not

461   only prevented flies from acquiring microbiota from the diets but also preventing non-

462   specific effects of antibiotic feeding on fly's traits expression (Blum et al., 2013; Nguyen et

463   al., 2019; Ridley et al., 2013). We confirmed the success of this approach by culture-

464 dependent and PCR quantification of the microbiota. Thus, it is unlikely that our findings are

465 a result of our methodology. Our study therefore provides an easy protocol to produce axenic

466 individuals for studies of physiological and behavioural effects of host-microbiota

467 interactions.

468

469 **Conclusion**

470 Our study revealed effects of the microbiota on feeding behaviour, body composition and

471 fecundity of the fruit fly *B. tryoni.* The microbiota promoted body weight gain in juveniles

472 and immature adults, supported fecundity and modulated food consumption as well as

473 energetic reserve in mature adults. Particularly, we found that impacts of the microbiota on

474 host nutrient intake are not similar between males and females and the presence of the

475 microbiota in females may support the host to efficiently harvest and store energy from the

476 diet. These results enhance our understanding of the important role of the microbiota on life-

477 history traits and behaviour of insects, highlighting the fitness implication of the host-

478 microbe interactions.

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Figures

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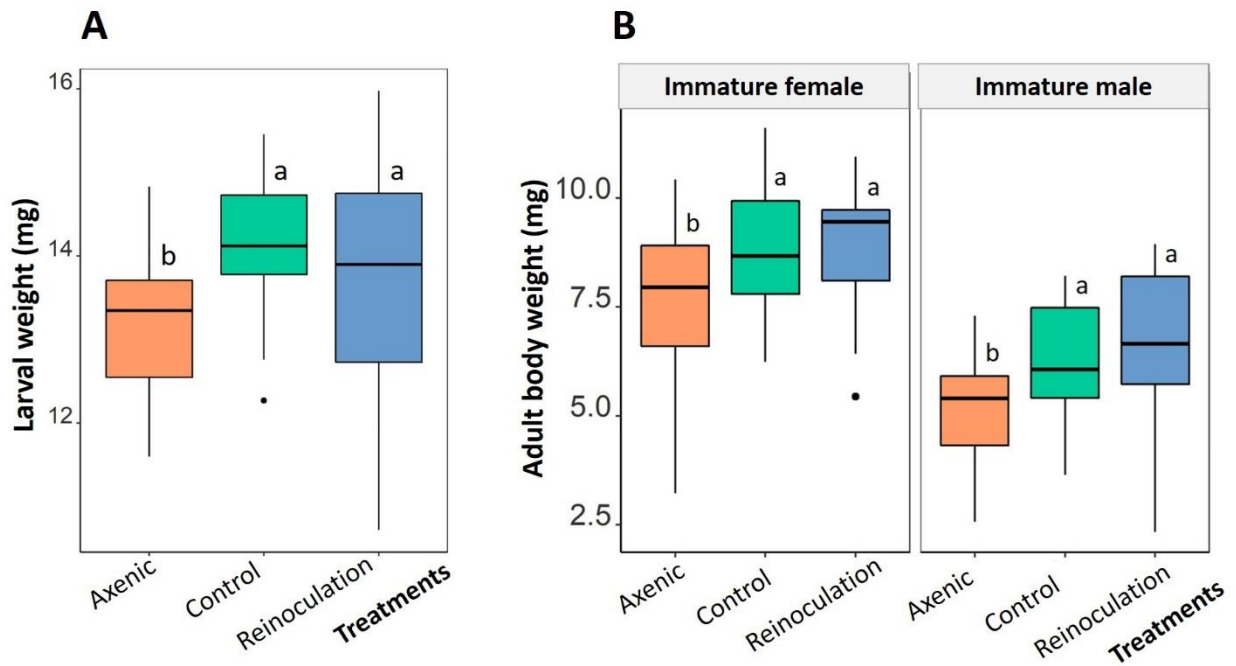
Figure 1. Effects of the microbiota manipulation on body weight of (A) larvae and (B) immature (newly emerged) adults. Different letters indicate a significant difference between the treatments (SNK *post hoc* test, $p \le 0.05$)

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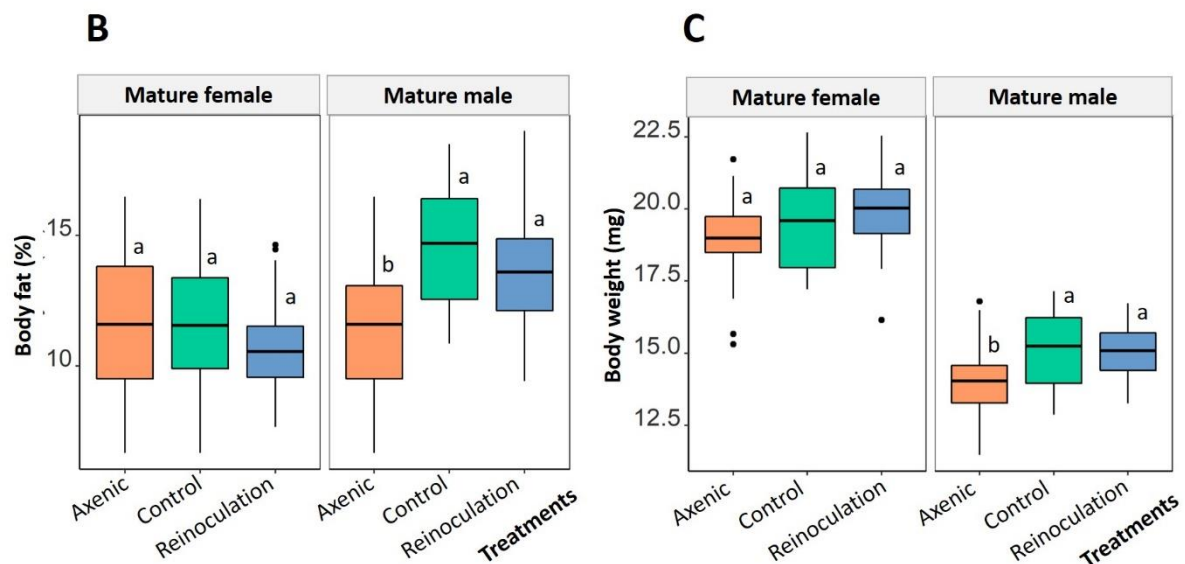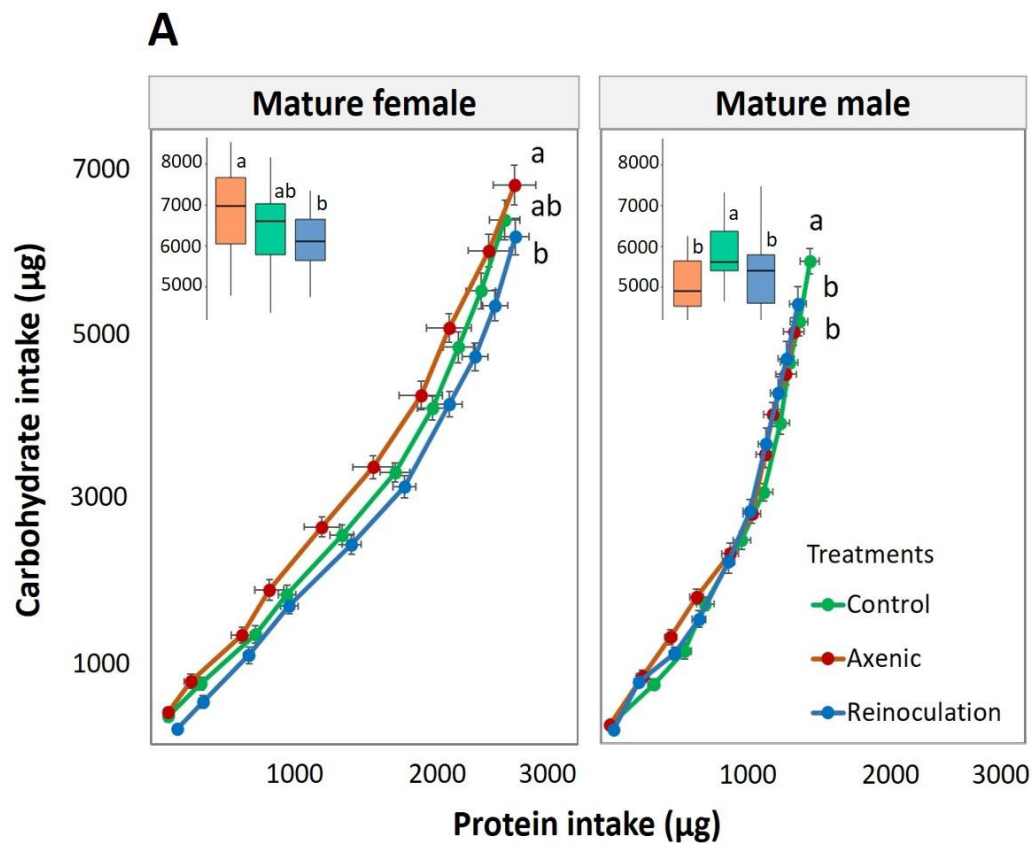
Figure 2. Sex-specific effects of the microbiota manipulation on (A) carbohydrate intake, (B) body weight and (C) total body fat of mature adults. Different letters indicate a significant difference between the treatments (SNK *post hoc* test, p ≤ 0.05)

729 **Supplementary material**

730 Design and results of the egg treatment preliminary test

731 Figure S1. Hatching rate of eggs bleached for different durations (A) and CFU count of

732 control *versus* bleached eggs (B)

733 Figure S2. Schematic representation of the feeding experiment

734 Figure S3. CFU count of fly samples at egg, larval, immature adult (A) and mature adult (B)

735 stages.

736 Figure S4. PCR quantification of 16S and ITS of the axenic and control samples at egg, larval

737 and immature adult stages.

738 Figure S5. Effects of treatment (A) and time (B) on fecundity

739 Table S1– Diet recipe.

740 Table S2– PCR conditions and primer sequences.

741 Table S3 – Output of Kruskal-Wallis and Dunn *post hoc* test of CFU count in egg, larvae,

742 adults (**Bold** – $p < 0.05$)

743 Table S4 – PCR quantification (mean-SE-n) of negative controls

744 Table S5 - Output of ANOVA test of carbohydrate intake (**Bold** – $p < 0.05$)

1   **Supplementary material "Sex-specific effects of commensal microbiota on adult**

2   **carbohydrate intake and body composition in a polyphagous fly"**

3

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10

11   **Data accessibility**

12   Data will be made available in Dryad upon the acceptance of the manuscript.

13

14   **Keywords:** host-microbe interaction, carbohydrate, reproduction, feeding behaviour, body

15   reserve, fly.

16

18   We conducted a preliminary test to determine the optimal bleach washing time that efficiently

19   deleted microbes on the egg surface with minimum effects on *B. tryoni* egg hatching rate. Eggs

20   were collected from our lab-adapted colony for 2h using a 300 mL sterile white plastic bottle.

21   The bottle contained 20 mL of sterile water to maintain humidity and had numerous

22   perforations of <1 mm diameter through which females deposit their eggs. We washed eggs

23   twice in 0.5% bleach (White King ®) for either 5, 3, or 2 minutes, followed by one wash in

24   70% ethanol (Sigma, cat no. 64175) for 1 min, and three washes in sterile mili Q water. The

25   ethanol and water washes were conducted in a biosafety cabinet. 100 eggs (N = 6 replicates

26   *per* treatment) were then transferred aseptically onto a piece of sterile black filter paper

27   (S=15cm^2, Macherey-Nagel, cat no. 104705) embedded on 25 ml sterile agar 1 %, pH 3.5

28   adjusted by citric acid [Sigma, cat no. 77929, (Ling Chang et al. 2006; Moadeli, Taylor, and

29   Ponton 2017)]. 100 control eggs (N = 6 replicates) were pooled from the same egg batch and

30   washed by the same procedure using sterile water. Manipulations for the control treatment were

31   conducted non-aseptically.

32

33   We then measured the number of hatched eggs at 44, 52 and 72h post-deposition. In parallel,

34   the microbial status of fresh washed eggs (N = 6 replicates *per* treatment) was assessed by

35   culture-dependent method as described in (Koyle et al. 2016) using LB, MRS and PDA solid

36   media. A GLM with binomial error distribution and *quasi* extension, followed by SNK *post*

37   *hoc* tests with $p \leq 0.05$, was used to measure the effects of the treatment and time on the

38   percentage of egg hatching. Effects of the treatment on CFU counts of washed eggs were

39   analysed using a Kruskal-Wallis test, followed by Dunn *post hoc* test ($p \leq 0.05$).

40

41　Results revealed that the percentage of egg hatching was almost identical at 44h post-deposition

42　between treatments, but was significantly different at 52 and 72h with eggs bleached for 5 min

43　hatching in a lower number while eggs of the other treatments hatched in similar percentage

44　(GLM: treatment*time: $F_{6,60} = 2.497$, p = 0.032, Treatment: $F_{3,68} = 1.684$, p = 0.179, Time: $F_{2,66}$

45　= 308.061, p < 0.001; Figure S1A). CFU count of control eggs was much greater than that of

46　bleached eggs (Kruskal Wallis, $\chi^2 = 19.272$, df = 3, p < 0.001, *Dunn test*, p < 0.001, Figure

47　S1B). CFU count of 5 and 3 min bleached eggs was zero and that of 2 min bleached eggs was

48　very low (Figure S1B).

49

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60　　　90(2):507–20.

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Supplementary figures
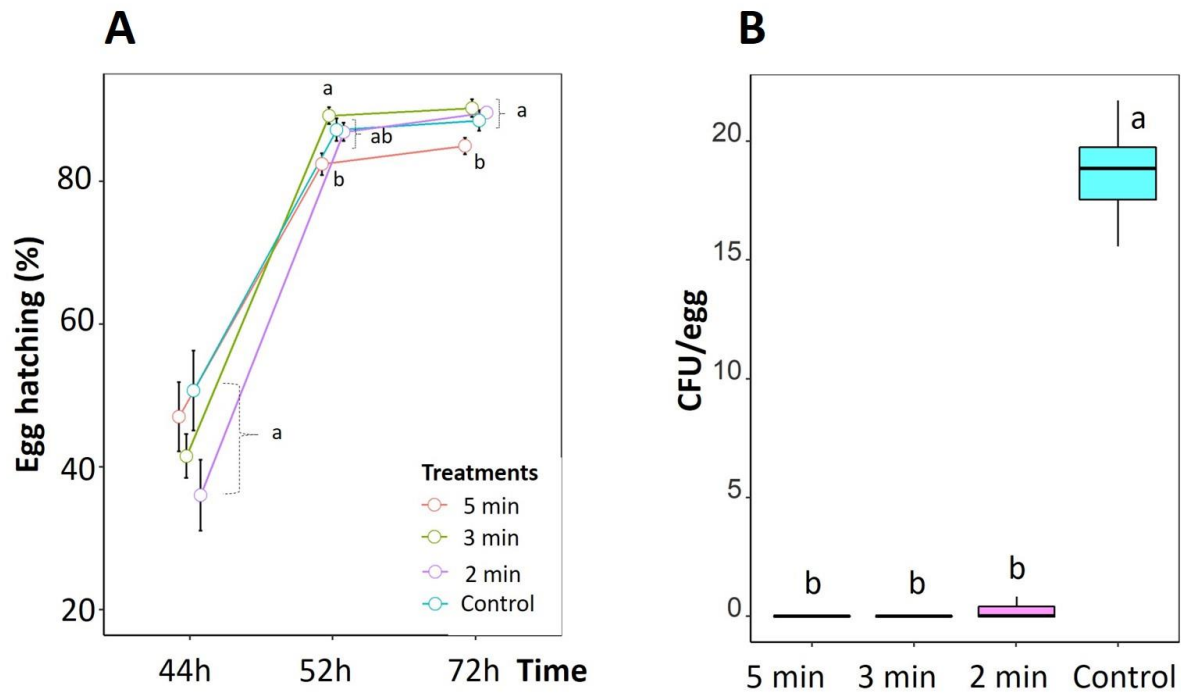
Figure S1. Hatching rate of eggs bleached for different duration (A) and CFU count of control *versus* bleached eggs (B). Different letters indicate a significant difference between the treatments [(A) SNK and (B) Dunn *post hoc* test, $p \leq 0.05$)]
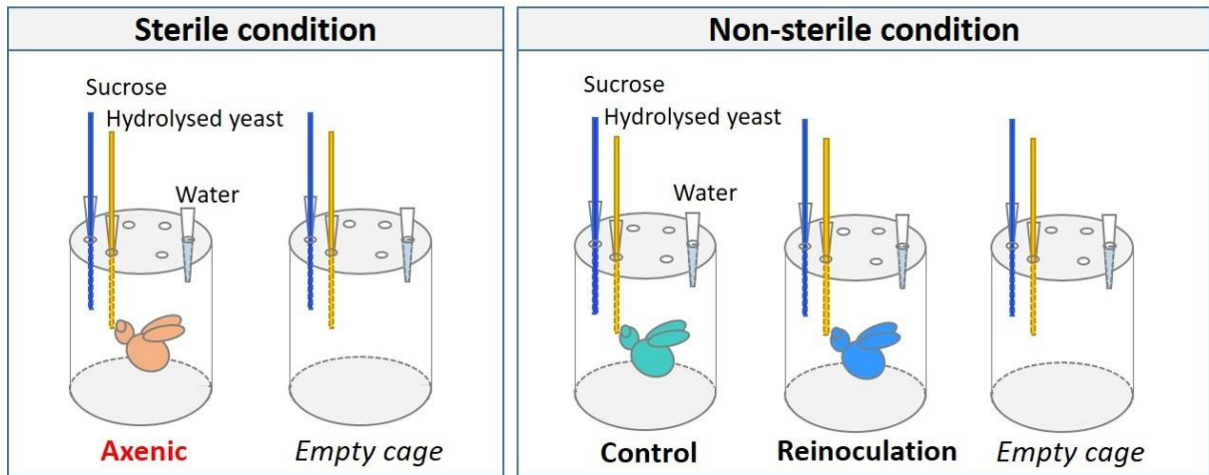
Figure S2. Schematic representation of the feeding experiment

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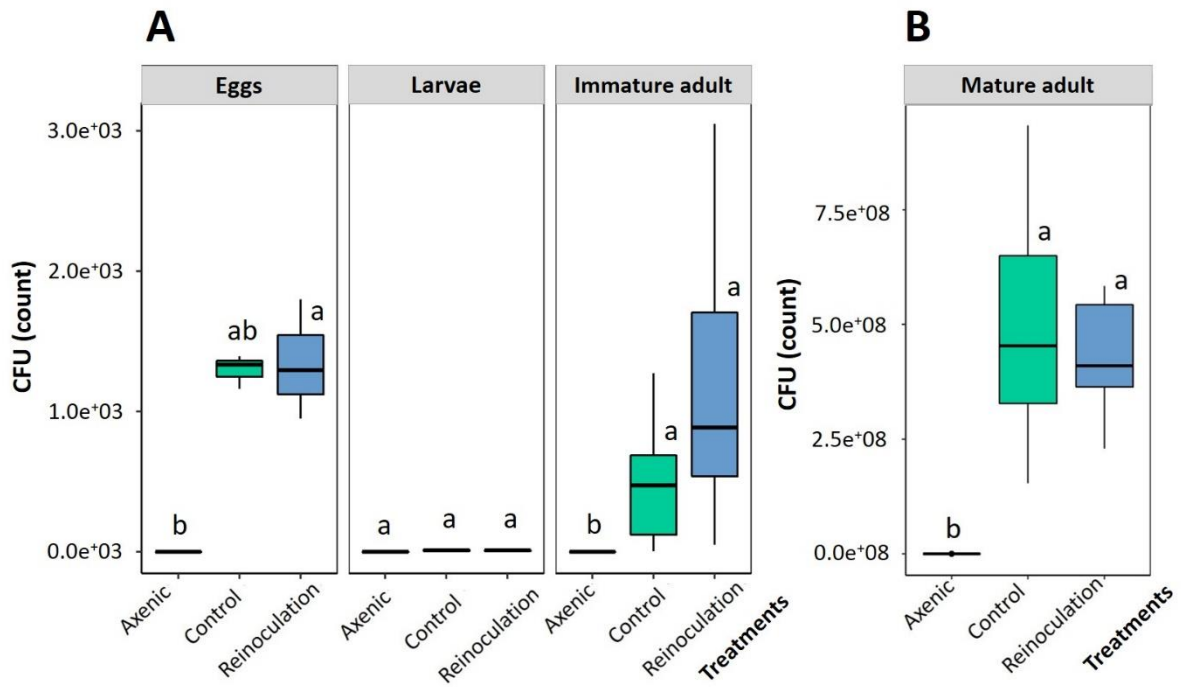
Figure S3. CFU count of fly samples at egg, larval, immature adult (A) and mature adult (B) stages. Different letters indicate a significant difference between the treatments (Dunn *post hoc* test, $p \leq 0.05$)

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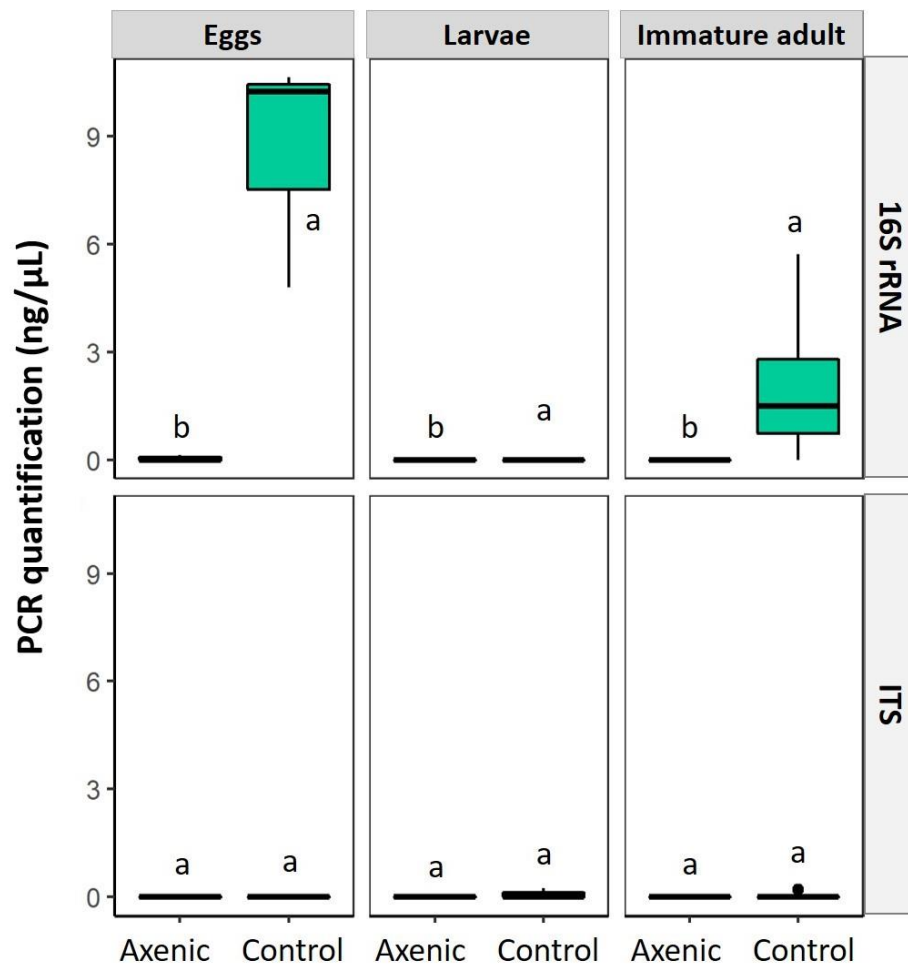
Figure S4. PCR quantification of 16S and ITS of the axenic and control samples at egg, larval and immature adult stages. Different letters indicate a significant difference between the treatments (Dunn *post hoc* test, p ≤ 0.05).

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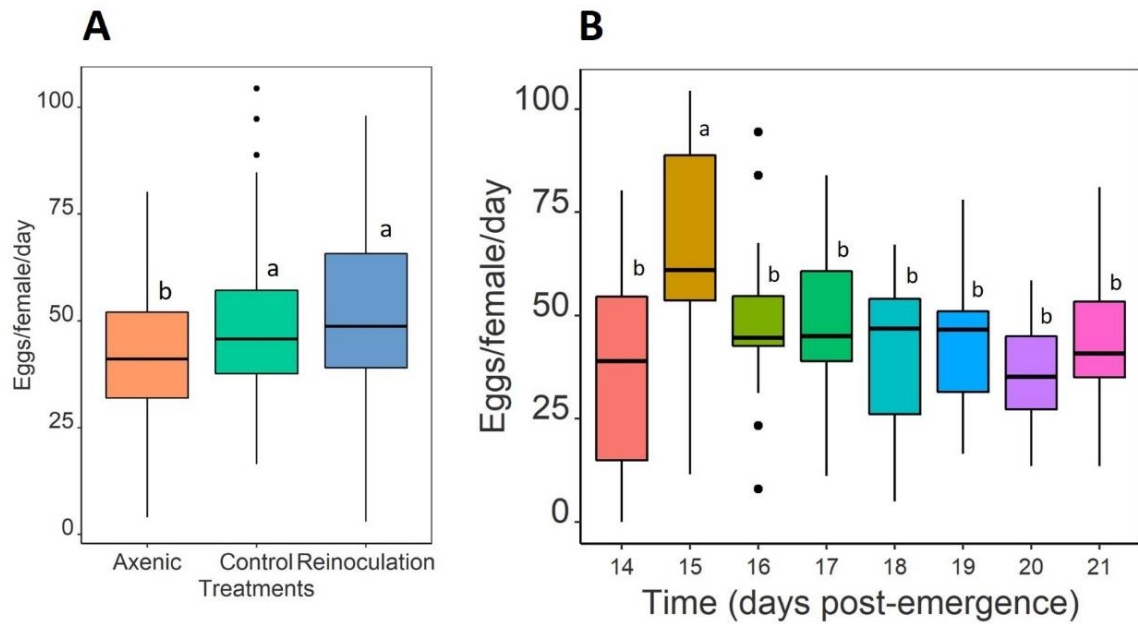
Figure S5. Effects of treatment (A) and time (B) on fecundity. Different letters indicate a

significant difference between the treatments (SNK *post hoc* test, $p \leq 0.05$).

84

Supplementary tables

Table S1 – Diet recipe (for 1000mL)

Ingredient	Amount	Supplier
Brewer's yeast (g)	204	SF Health foods, Australia
Sugar (g)	121.8	Homebrand, Australia
Agar (g)	10	MP Biomedicals
Citric Acid (g)	23	Sigma, Australia
Nipagin (g)	2	Southern Bio- logical, Australia
Sodium Benzoate (g)	2	Sigma, Australia
Wheat Germ Oil (mL)	2	Melrose laboratories PTY LTD, Australia
MiliQ water (mL)	1000	Lab source

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88

Table S2 - PCR conditions and primer sequences

PCR conditions

Target	Cycle	Initial	Disassociate	Anneal	Extension	Finish
16S: V1- V3	29	95 ˚C for 7 min	94˚C for 45s	50˚C for 60S	72˚C for 60S	72˚C for 7 min
ITS1F – ITS2	35	95 ˚C for 7 min	94˚C for 30s	55˚C for 45S	72˚C for 60S	72˚C for 7 min

Primer sequences

Target	16S: V1- V3	ITS1F – ITS2
Forward Primer	AGAGTTTGATCMTGGCTCAG	CTTGGTCATTTAGAGGAAGTAA
Reverse Primer	GWATTACCGCGGCKGCTG	GCTGCGTTCTTCATCGATGC

89

90 **Table S3 – Kruskal-Wallis and Dunn *post hoc* test of CFU count in egg, larval and adult**

91 **stage.**

Stage	χ^2	Df	p-value	P adjusted (Dunn test)
Egg	6.161	2	**0.046**	Axenic-Control: 0.143 Axenic-Reinoculation: **0.046** Control-Reinoculation: 0.45
Larval	6.006	2	0.0496	na
Immature adult	10.194	2	**0.006**	Axenic-Control: **0.041** Axenic-Reinoculation: **0.004** Control-Reinoculation: 0.213
Mature adult	11.794	2	**0.003**	Axenic-Control: **0.004** Axenic-Reinoculation: **0.009** Control-Reinoculation: 1.000

92

93 **Table S4 – PCR quantification (mean $\pm$ SE) of negative controls**

Chemicals	16S rRNA	ITS
0.5% bleach	0 (0)	0 (0)
70% ethanol	0 (0)	0 (0)
MiliQ water	0 (0)	0 (0)

94

95

96

97   **Table S5 - ANOVA analysis of carbohydrate intake (μg)**

Factors	Df	Deviance	Resid. Df	Resid. Dev	F-value	p-value
NULL			119	133913990		
Treatment	2	4104578	117	129809412	2.623	0.077
Sex	1	31592015	116	98217397	40.384	**<0.001**
Treatment*Sex	2	9037393	114	89180004	5.776	**0.004**

98

Chapter 5:

Parental microbiota modulates offspring development, body mass and fecundity *in Bactrocera tryoni*

(Published in MDPI Microorganisms)

Article

Parental Microbiota Modulates Offspring Development, Body Mass and Fecundity in a Polyphagous Fruit Fly

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Abstract: The commensal microbiota is a key modulator of animal fitness, but little is known about the extent to which the parental microbiota influences fitness-related traits of future generations. We addressed this gap by manipulating the parental microbiota of a polyphagous fruit fly (*Bactrocera tryoni*) and measuring offspring developmental traits, body composition, and fecundity. We generated three parental microbiota treatments where parents had a microbiota that was non-manipulated (control), removed (axenic), or removed-and-reintroduced (reinoculation). We found that the percentage of egg hatching, of pupal production, and body weight of larvae and adult females were lower in offspring of axenic parents compared to that of non-axenic parents. The percentage of partially emerged adults was higher, and fecundity of adult females was lower in offspring of axenic parents relative to offspring of control and reinoculated parents. There was no significant effect of parental microbiota manipulation on offspring developmental time or lipid reserve. Our results reveal transgenerational effects of the parental commensal microbiota on different aspects of offspring life-history traits, thereby providing a better understanding of the long-lasting effects of host–microbe interactions.

Keywords: transgenerational effects; gut microbiota; offspring performance; life-history traits; reproductive success

1. Introduction

The experience of parents can influence the behaviour, performance, and fitness of future generations [1–4]. Parental effect is defined as any effect on offspring phenotype that is determined by the genotype or environmental experience of their parents [5,6]. Parental effects can be paternal and/or maternal and have been reported widely in plants [7], insects [8], and vertebrates [5]. Molecular mechanisms responsible for parental effects likely involve epigenetic modifications such as DNA methylation, chromatin modification, and noncoding RNA [9].

The commensal microbiota plays an important role in many physiological functions of its host [10–12], with evidence of transgenerational epigenetic implications on descendants [13–17]. In vertebrates, including humans, the microbiota has been detected in the placenta, amniotic fluid, and meconium, supporting the "in utero colonization hypothesis" which is crucial for the metabolic function, immune development, and further health parameters of neonates during placental development [18]. In insects and, in particular, in tephritid fruit fly species, adult females possess a

symbiont-rich organ called the ovipositor diverticulum, which smears the egg surface with symbionts before the eggs are deposited [19]. Females of the Mediterranean fruit fly *Ceratitis capitata* not only deposit bacteria over the surface of freshly produced eggs but also provide eggs with lysozyme and antibacterial polypeptides which eliminate pathogens while facilitating the development of beneficial bacteria [20–22].

The microbiota and/or microbial products that offspring inherit from their parents can greatly impact offspring fitness, especially in the early life stages [15–17,23]. Research in mammals shows, for example, that signalling from maternal microbial molecules shapes the development and function of the neonatal immune system and the first colonization of the gut microbiota in early life is a critical window for the health and development of offspring [17,24]. In insects, bacteria or bacterial fragments transferred from mothers to eggs can mediate transgenerational immune priming, a phenomenon in which parents prepare their offspring to fight against pathogens that they encountered in their environment [15,23]. Notably, in *Drosophila melanogaster*, manipulations of the gut microbiota directly affect host mating and reproductive behaviour, with transgenerational consequences on offspring body mass [13]. Furthermore, in vertebrates, alterations of the maternal gut microbiota induced by factors such as diet, environmental toxins, or obesity status can influence the establishment of the microbial community and increase metabolic disorders in offspring as a consequence of developing in a detrimental intrauterine environment (see, for instance, [25,26] and [18]). The effects of parental microbiota are, therefore, important in shaping offspring phenotype and fitness, however, empirical studies targeting the transgenerational effects of host–microbe interactions on life-history traits of descendants are still limited.

Previous studies on the polyphagous fruit fly *Bactrocera tryoni* have shown that two yeast strains from the microbiota, belonging to the genera *Hanseniaspora* and *Pichia*, play an important role in development [27] and the microbiota inherited from parents is essential for maximizing pupal production [28]. In the present study, we manipulated the parental microbiota of *B. tryoni* and measured the effects of this manipulation on offspring developmental traits (e.g., developmental time, percentage of egg hatching, pupal production, and adult emergence), body weight and lipid storage of juveniles and adults, and adult fecundity. We generated parental control flies which had an intact commensal microbiota, parental axenic flies for which the commensal microbiota was eliminated, and reinoculated parental flies for which the commensal microbiota was eliminated then reintroduced. Because the axenic treatment did not selectively remove the microbes from the digestive tract, we will use the term "microbiota" manipulation instead of "gut microbiota" manipulation throughout the manuscript. We predicted negative effects of the parental microbiota removal on offspring developmental traits due to the lack of vertically transmitted microbiota (prediction 1). As commensals are key modulators of host metabolism [10,29,30], lacking commensal microbiota at the parental generation may result in offspring with lower body weight and lower body lipid reserve (prediction 2). If prediction 2 was confirmed and because there is a positive correlation between female body size and the number of eggs produced [31–34], we predicted that daughters of parents that host a commensal microbiota produced more eggs than that of axenic parents (prediction 3). The insight gained here into the transgenerational effects of commensal microbiota manipulation on offspring fitness-related traits gives us a better understanding of how animal–microbe interactions can have long-lasting influences on host life-history traits.

2. Materials and Methods

2.1. Fly Stock

A *B. tryoni* lab-adapted colony was established in 2015 and has been maintained for more than 25 generations in non-overlapping generations, whereby larvae were allowed to develop in an artificial gel-based diet [35] and adults fed on ad libitum hydrolysed yeast (cat. no., 02103304, MP Biomedicals),

fine sugar (CSR® White Sugar), and water. All fly stocks and experiments were maintained at 25 ± 0.5 °C, $65 \pm 5\%$ relative humidity, and 12:0.5:11:0.5 light/dusk/dark/dawn photoperiod.

2.2. Experimental Design and Statistical Analyses

2.2.1. Fly Rearing

The experimental design is illustrated in Figure S1. The parental axenic treatment was generated by egg dechorionation as described in [36]. Briefly, eggs were collected for 2 h from the lab-adapted colony, then, dechorionated for 3 min in 0.5% bleach (Peerless JAL®), followed by one wash in 70% ethanol for 1 min and three washes in sterile Milli-Q water. One-hundred treated eggs (N = 10 replicates) were then transferred using a fine paintbrush onto 25 mL sterile gel-based diet (see recipe and suppliers in Supplementary Document: Table S1) in a 90-mm petri dish (cat. no., S6014S10, Techno Plas). The diet was prepared aseptically by mixing irradiated (10 kilograys for 21 h) brewer's yeast and nipagin with freshly autoclaved solution A (water, sugar, wheat germ oil, sodium benzoate, and agar) and B (water and citric acid) in a biosafety cabinet. Axenic eggs were then allowed to develop into axenic adults in sterile conditions with clean air provided by a PCR working station (Airclean® system AC600).

The parental control treatment (N = 10 replicates) was generated from the same batch of eggs as the axenic treatment and treated similarly, except that eggs were washed using water only. The parental reinoculated treatment (N = 10 replicates) was generated by recolonizing the dechorionated eggs with microbes harvested from untreated eggs following these steps: (i) 100 untreated eggs were crushed firmly in 50 µL sterile Milli-Q water for 2 min by a handheld pestle cordless motor (cat. no., Z359971, Sigma), (ii) the solution was pipetted onto a diet that contained 100 dechorionated eggs, and (iii) petri dishes containing reinoculated eggs were left open for 5 min for water evaporation. Manipulations of the parental control and parental reinoculated treatments until adulthood were conducted in a non-sterile environment using the same procedure as for the axenic treatments.

Adults from all parental treatments were provided ad libitum autoclaved water and food (10 kg for 21 h irradiated hydrolysed yeast and sugar). The microbial status was determined in parental axenic and control eggs, larvae, and adults by a culture-dependent method and PCR quantification (Supplementary Document, information 1 and Table S2). Axenic samples were confirmed to be free of germs (Supplementary Document, Tables S3 and S4).

Eggs from 15-day-old axenic, control, and reinoculated flies were then collected to generate the axenic, control, and reinoculation offspring (referred to as axenic, control, and reinoculation treatment, respectively, in Figure 1). Eggs were collected for 24 h from a parental group of 5 males and 5 females (10 groups were generated for each treatment, N total = 30). One-hundred eggs from each group were deposited on a piece of black filter paper (S = 15 cm^2, cat. no., 104705, Macherey-Nagel) settled on the surface of 25 mL non-sterile gel-based diet [35]. The black filter paper was used to assess egg hatching status at 4-day post egg seeding and was then discarded. All manipulations for the axenic, control, and reinoculation treatments in the offspring generation were conducted in a non-sterile environment using non-sterile food. All data below were collected from the offspring generation.
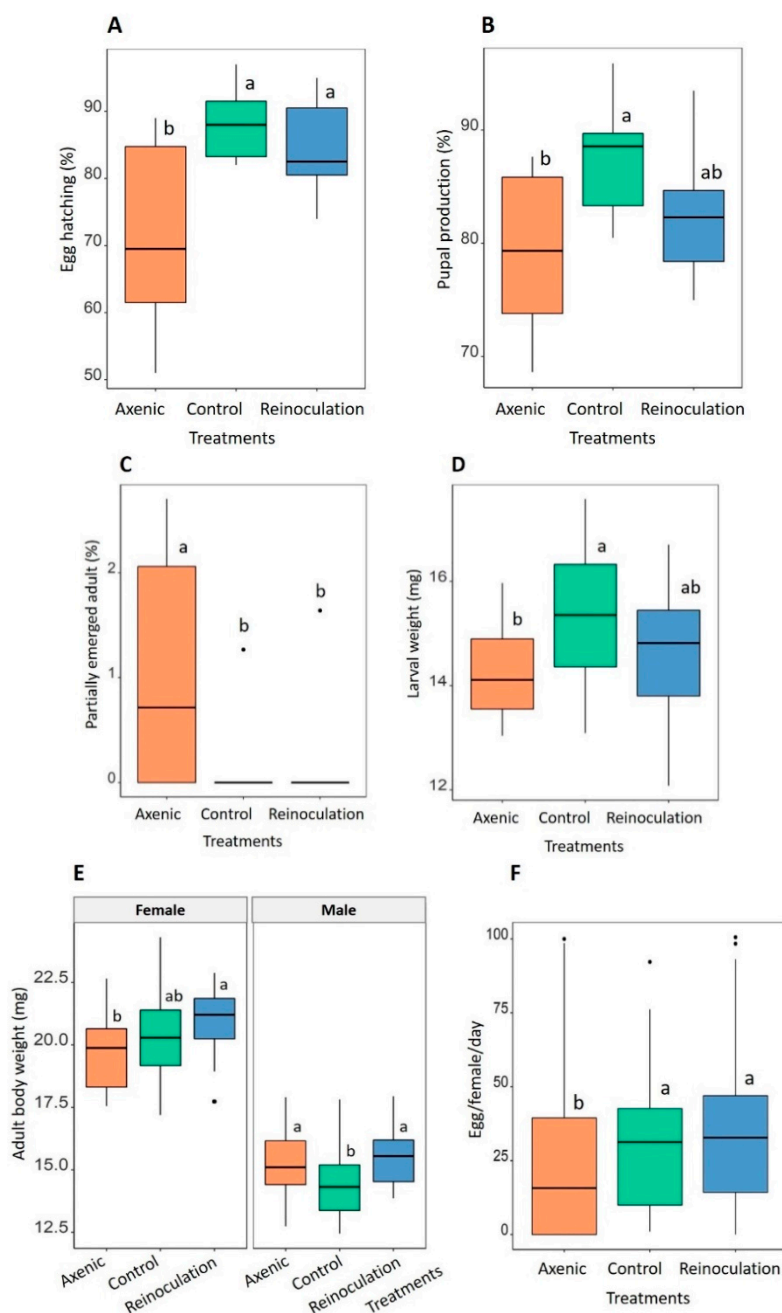
Figure 1. Effect of parental microbiota manipulation on (**A**) percentage of egg hatching, (**B**) pupal production, (**C**) partially emerged adults, (**D**) body weight of larva, (**E**) body weight of adult, and (**F**) female fecundity. Different letters indicate a significant difference between the treatments ((**A,B,D–F**) SNK post hoc test and (**C**) Dunn post hoc test, $p < 0.05$.

2.2.2. Developmental Performance

The percentage of eggs that hatched after 4 days (i.e., fertility, N total = 30 replicates, 10 replicates per treatment) was calculated as $[(N \text{ hatched eggs} / (N \text{ hatched eggs} + N \text{ unhatched eggs})] * 100$. We fitted a generalized linear model (GLM) with a binomial error distribution and quasi extension to test for the effect of the treatment (i.e., parental microbiota manipulation) on the percentage of egg hatching. P values were obtained from F-statistics. Student–Newman–Keuls (SNK) post hoc tests with a significance of 0.05 were applied to identify sample means that were different.

To estimate the percentage of pupal production, petri dishes containing the larvae were transferred to a 1.125 L plastic container (cat. no., 136000, Décor Tellfresh®) that had a 20 cm diameter plastic mesh window on one side and had been filled with 20 mg of vermiculite one day before the larvae started jumping out of the larval diet. Pupae were sieved from vermiculite when all larvae had pupated (i.e., 4 days after the first jump) and the total number of pupae was recorded. The percentage of pupal production (N total = 30) was calculated as (N pupae /N hatched eggs) ∗ 100. A GLM with a binomial error distribution and quasi extension, followed by SNK post hoc tests, was fitted to the data to test for the effect of the treatment on the percentage of pupal production and to compare means between treatments.

Partially emerged flies were individuals that emerged with a portion of their body stuck in the puparium. Percentage of partially emerged flies (N total = 30 replicates) was calculated as (N partially emerged flies /N pupae) ∗ 100. Each replicate had 40 pupae (i.e., N pupae = 40). A Kruskal–Wallis test followed by a Dunn post hoc test was used to detect the significant effect of treatment and compare the mean ranks between treatments.

Developmental time (N total = 30, 10 replicates per treatment) was measured in days as (i) egg–larval duration: from depositing the parental eggs on diet until the first pupation was observed, and (ii) egg–pupal duration: from depositing the parental eggs on diet until the first emergence was recorded. Kruskal–Wallis tests followed by Dunn post hoc tests were used to test for the effect of the treatment on the different variables and compare the mean ranks whenever treatment was significant.

2.2.3. Body Composition

The body wet weight of larvae and adults was measured individually using a precision weighing balance (Sartorius® ME5 scale, d = 0.0001 g). Larvae (N total = 60, 20 individuals per treatment) were sampled post pupation (i.e., 3rd instar larva) to test for the effects of treatment on larval weight. Male and female adults (N total = 120, 20 males and 20 females per treatment) were collected at day 10 post emergence to test for the effects of treatment and sex on adult weight. A GLM model with a Gaussian distribution was fitted to test for the effect of treatment on larval weight, or the effect of treatment, sex, and their interaction on adult weight. Analyses were followed by SNK post hoc tests to compare means between treatments and sex.

We used the same individuals as above to measure the percentage of lipid reserve in larvae (N total = 60) and female and male adults (N total = 120) using a chloroform extraction method [37]. Briefly, samples were dried at 55 °C for 48 h and dry weight measured using a Sartorius® ME5 scale. Body lipid reserves were then extracted in three 24 h changes of chloroform (cat. no., 650498, Sigma). Chloroform was then removed and evaporated by leaving the samples in a fume cupboard (Dynaflow, unit no. FC100316) for 24 h. Samples were then redried and reweighed as previously. Percentage of lipid reserve was calculated as $\frac{Body\ dry\ weight - Lipid\ extracted\ body\ dry\ weight}{Body\ dry\ weight} * 100$. A GLM model with binomial error distribution and quasi extension was fitted to test for the effect of treatment on larval lipid reserve and treatment, sex, and their interaction on adult lipid reserve. Analyses were followed by the SNK post hoc tests to compare means between treatments and sex.

2.2.4. Fecundity

We set up 1.125 L cages with a group of five females and five males (one-day-old, N total = 30 cages, 10 cages per treatment). Flies were provided ad libitum food (Hydrolysed yeast and sugar) and water. The bottom of a 35 mm diameter petri dish (cat. no., CLS430165, Corning®) was used as the egg collection device in each cage. The petri dish contained 2 mL of water flavoured by natural apple essence (Foodie flavours™, 1 mL L^{-1}) and was covered by a thin layer of parafilm (M laboratory®) that has numerous perforations on the surface for females to insert their ovipositors and lay eggs. Egg collection started at day 14 post emergence for 10 days. The number of eggs produced per cage per day was counted and the average number of eggs produced per female per day was estimated as a

proxy of fecundity. No female died during the egg collection period. To test for the effect of treatment on offspring fecundity, we fitted a GLM model with a Gaussian distribution, followed by SNK post hoc tests to compare means between treatments.

3. Results

3.1. Effects of Parental Microbiota Manipulation on Offspring Developmental Traits

We found a significant effect of treatment on the percentage of egg hatching (GLM: $F_{2,27}$ = 8.579, p = 0.0013) with the percentage of egg hatching being about 17% and 13% higher in offspring of the control and reinoculation treatments, respectively, compared to that of axenic treatment (control: 88.2%, reinoculation: 84.5%, axenic: 71.7%, Figure 1A). Similarly, the treatment influenced pupal production (GLM: $F_{2,27}$ = 5.124, p = 0.013). Offspring of the control treatment produced approximately 8% more pupae than that of axenic treatment (87.67% vs. 79.37%), meanwhile, the percentage of pupae produced by offspring of reinoculation treatment (82.38%) was around 3% more than that of the axenic treatment but was not significantly different from both the control and axenic treatments (Figure 1B).

Manipulation of parental microbiota affected the percentage of partially emerged adults (Kruskal–Wallis: χ^2 = 6.521, df = 2, p = 0.038). Pupae from axenic offspring emerged significantly more as partially emerged adults compared to that of the control (1.01% versus 0.12%, Dunn test, p-adjusted = 0.05, Figure 1C) and reinoculation treatments (1.01% versus 0.16%, Dunn test, p-adjusted = 0.046, Figure 1C). The percentage of partially emerged adults was not different between the offspring of the control and reinoculation treatments (Dunn test, p-adjusted = 0.918, Figure 1C). The developmental time of the offspring was not affected by treatment, with egg–larval duration lasting on average 6.9 ± 0.02 days (Kruskal–Wallis: χ_2 = 2.62, df = 2, p = 0.877) and egg–pupal duration 18.3 ± 0.04 days (Kruskal–Wallis: χ_2 = 0.806, df = 2, p = 0.669).

3.2. Parental Microbiota Affects Offspring Body Weight but Not Lipid Reserves

Larval body weight of offspring was influenced by treatment (GLM: $F_{2,57}$ = 4.685, p = 0.013), with larvae from the control treatment being about 1 mg heavier than that of axenic treatment; body weight of larvae from the reinoculation treatment was at intermediate (control: 15.35 ± 0.3 mg, axenic: 14.25 ± 0.19 mg, reinoculation: 14.65 ± 0.27 mg, Figure 1D). The adult body weight of the offspring was significantly impacted by treatment (GLM: $F_{2,117}$ = 4.141, p = 0.018) and sex (GLM: $F_{1,116}$ = 419.3, p < 0.001), however, the interaction between treatment and sex was marginally significant (GLM: $F_{2,114}$ = 2.843, p = 0.062). Females of the reinoculation treatment (20.88 ± 0.3 mg) were approximately 0.5 and 1.2 mg heavier than females of the control and axenic treatments (20.33 ± 0.38 and 19.67 ± 0.31 mg, respectively, Figure 1E). In males, the trend was slightly different whereby males of the control treatment were significantly lighter than males of the reinoculation and axenic treatments (control: 14.45 ± 0.32 mg, reinoculation: 15.53 ± 0.24 mg, axenic: 15.26 ± 0.28 mg, Figure 1E).

The percentage of lipid reserve was, however, not affected by parental microbiota manipulation in both larvae (GLM: $F_{2,57}$ = 0.32, p = 0.728, on average 29.1 ± 0.25%) and adults (GLM: $F_{2,117}$ = 0.353, p = 0.703). Lipid reserve was higher in males (12.4%) than in females (9.84%, GLM, $F_{2,116}$ = 44.46, p < 0.001) with no significant interaction between sex and treatment (GLM: $F_{2,116}$ = 0.847, p = 0.431).

3.3. Parental Microbiota Increases Offspring Fecundity

Treatment influenced the number of eggs produced by offspring (GLM: F_{2297} = 3.703, p = 0.026) with females of the reinoculation and control treatments producing about 7 eggs per day more than females of the axenic treatment (reinoculation: 32 ± 2 eggs, control: 30 ± 2 eggs, axenic: 24 ± 3 eggs, Figure 1F). The number of harvested eggs varied with time (GLM: F_{9288} = 5.62, p < 0.001) but there was no significant interaction between treatment and time (GLM: $F_{18,270}$ = 0.639, p = 0.867). Overall, the number of eggs per day fluctuated from day 14 to day 20 before reaching a peak of

48 ± 4 eggs at day 21 post emergence; after that, egg number decreased gradually to 32 ± 2 eggs per day (Figure S2).

4. Discussion

In this study, the manipulation of parental microbiota had multiple effects on offspring performance. The results confirmed prediction 1 whereby microbiota-deficient parents generated offspring with a lower percentage of egg hatching, lower pupal production, and a higher number of partially emerged adults. We also found evidence of a lighter body weight in larvae and female offspring of axenic treatment, which partly confirmed our prediction 2, however, lipid reserve was not significantly different across offspring of axenic and non-axenic treatments. Reinoculating axenic parents with a microbiota harvested from control parents restored, however, some traits in offspring that were negatively affected by microbiota depletion in parents. Finally, following prediction 3, the fecundity of daughters from axenic parents was lower than that of control and reinoculated parents. It is important to emphasize here that while the treatment used to manipulate the microbiota may have caused nonspecific effects on some traits of the host, previous studies have confirmed that the main effect on insects' performance is due to the absence of microbiota [28,38–40]. In our study, the egg dechorionation method did not interfere with the egg hatching success or the developmental time of parents (Table S5). In addition, the developmental traits measured for the offspring of our reinoculation treatment were not different than that of the control treatment. Thus, the lower developmental and fitness-related traits of the axenic offspring are likely linked to the deficiency in parental microbiota rather than any side effects caused by the egg treatment method.

Our study showed that the offspring of axenic parents had a substantially lower percentage of hatching than that of non-axenic parents, resulting in a lower number of pupae produced. This finding is in agreement with previous research in the pine weevil, showing that the hatching success of eggs laid by mothers with a native microbiota was significantly higher than that of axenic mothers [41]. While the mechanism is unclear, it is possible that the presence or absence of different microbes on the egg surface and in the egg environment (i.e., in the diet in our case) regulates egg hatching as shown in previous studies in insects and nematodes [42–44]. For instance, the close physical contact between common bacteria in the host's intestine and the eggs of parasitic nematodes has been confirmed to regulate egg hatching, with incubations with different bacteria leading to different egg hatching percentages. In addition, reducing the number of bacterial contacts with eggs can significantly decrease hatching percentage [43,44]. In our experiment, we manipulated the microbiota of parents and eggs deposited by axenic and non-axenic parents that were exposed to the same non-sterile diet. However, because eggs did not harbor the same microbial community at the beginning of the experiment (i.e., eggs delivered by axenic parents lack vertically transmitted microbes), the microbiota growing in the diet after egg seeding might had been different [16], thus, likely affecting egg hatching status. This experiment did not allow us to explore the mechanisms involved in the lower hatching percentage of eggs delivered by axenic parents and more investigations are needed. We also found a higher percentage of partially emerged adults in offspring of axenic parents, which, to our knowledge, has never been reported previously. This might be explained by the difference in metabolic status between axenic and non-axenic parents and the resource investment of females on eggs before seeding. Axenic individuals might lack essential nutrients that can be provided by beneficial microbes [12,45], hence, possibly having a lower investment on eggs, which resulted in a long-term implication on offspring quality [46–48]. This is particularly the case for oviparous insects where maternal investment is fixed at the time of egg laying, directly shaping early development with fitness consequences to the offspring [48,49].

Our axenic treatment, where the parental microbiota was removed, resulted in lighter offspring larvae and adult females. This supports previous findings in *D. melanogaster* whereby mating pairs that host different gut bacterial species generate daughters (but not sons) with different body weights [13]. Interestingly, sex-specific effects on body traits have also been observed in *Drosophila* at the parental

generation when the microbiota is removed [40,50]. The mechanisms responsible for these effects are still debated, but some hypotheses argue that females' high energy demand for egg production and the physiological differences between males and females (and thus, their microbiota composition) might explain this [51–53]. It was also surprising in our data that, while parental microbiota manipulation substantially influenced offspring body weight, no significant effect on body fat reserve was found. This result is different to what has been reported before in fruit flies and mosquitoes, at least for the first generation, indicating that the metabolic effect of axenic treatment on lipid metabolism might vary and may be lost at the offspring generation [40,54].

Manipulation of the microbiota at the parent generation also influenced offspring egg productivity with offspring of control and reinoculated parents producing more eggs than that of axenic parents. In insects and other arthropods, it is well established that parental endosymbionts affect offspring reproduction (reviewed by [55]). However, to our knowledge, this is the first time effects of the microbiota on offspring fecundity are reported in insects, though it has been shown to substantially affect the parental reproductive output of many species, including fruit flies [14,56,57], mosquito [54,58], and bean bug [59]. Given that insect fecundity is generally positively correlated with female body size [31–33], the higher number of eggs produced by the offspring of reinoculated parents compared with that of axenic parents might be linked to the greater body weight of females from reinoculated parents. However, because offspring of control parents (that body weight was intermediate) also produced more eggs than offspring of axenic parents and a similar number of eggs than offspring of reinoculated parents, fecundity may also be impacted by other factors such as the association with some specific bacterial species [14,60]. The gut bacterium symbiont *Acetobacter* (but not *Lactobacillus*) in *Drosophila*, for example, has been shown to regulate the activity of enzymes that convert aldehydes to carboxylic acids in the ovaries, which largely affects host oogenesis [14].

In conclusion, our study provides insight into the transgenerational effects of the commensal microbiota in a polyphagous fruit fly, showing that the elimination of the microbial community in parents could lead to fitness consequences in the next generation. Interestingly, when eggs from axenic parents were reinoculated with microbes, hatchings recovered some traits of offspring from unmanipulated parents, underlining the importance of the microbes that are directly transmitted by the mother to its eggs. Overall, the effects of parental microbiota manipulation on offspring reproduction described here extend our understanding of the long-lasting fitness implications of host–microbe interactions to both present and future generations, highlighting the potential evolutionary links between the host and its microbiota, which can drive evolutionary adaptations.

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1 *Supplementary document for article:*

Parental microbiota modulates offspring development, body mass and fecundity in a polyphagous fruit fly

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10 **Supplementary information 1**

11 *Culture-dependent method and PCR quantification to assess microbial status of parental axenic and*
12 *control eggs, larvae, and adults*

13   Samples were collected after egg washing, at third instar larvae, and at one-day-old adult. Eggs were
14 pooled in groups of ca. 1500 eggs (100µL, N = 3) while larvae were collected in groups of 20 (N = 3) and
15 adults were assessed individually (N = 3 males, 3 females). Larvae and adults were freeze-killed for 2h
16 before subjecting to the surface sterilization by one wash for 3 min in 0.5% bleach (Peerless JAL®),
17 followed by one wash in 70% ethanol for 1 min and three washes in sterile miliQ water. Egg, larval,
18 and adult samples were then homogenated in 0.4, 0.1, and 1mL sterile PBS buffer, respectively and
19 25µL of each homogenate was plated on a petri dish contains 25 mL of either Luria Bertani agar (cat.
20 no., 22700-025, Life technologies), or Man-Rogosa-Sharpe agar (cat. no., CM0361, Oxoid®), or Potato-
21 Dextrose agar (cat. no., CM0139, Oxoid®) using single use L-shape spreaders (cat. no., Z723193, Sigma).
22 LB dishes were incubated at 28°C for 24-48h, MRS and PDA dishes at 28°C for 48-72h then the number
23 of colony-forming unit (CFU) *per* replicate was scored. The number CFU *per* egg or larva or adult *per*
24 replicate was then estimated using the equation $E = (C * V)/(P * F)$ [1], whereby C = average of CFU
25 in LB, MRS, PDA dishes; P = volume plated (i.e., 25 µL); V = total volume of the homogenate; F = the
26 number of egg/larvae/adult *per* replicate.

27   The remained homogenates were subjected to PCR to quantified the amount of 16S rRNA gene
28 (represents for the presence of bacteria) and the internal transcribed spacer (ITS, represents for the
29 presence of fungi) with washing chemicals (bleach, ethanol, and sterile water, N *total* = 9) used as
30 negative control. The initial PCR amplicons were generated using AmpliTaq Gold 360 mastermix (Life
31 Technologies, Australia). PCR conditions and primers were outlined in Table S2 below. A secondary
32 PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The
33 resulting amplicons were measured by florometry (Invitrogen Picogreen) and normalised. The
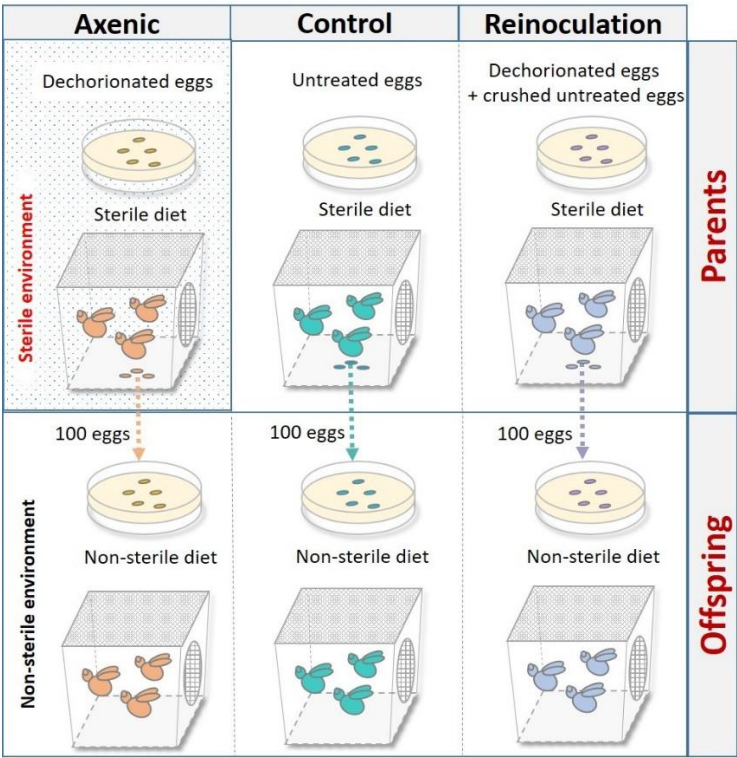34 eqimolar pool was then measured by qPCR (KAPA).

35 **Supplementary figure**

36

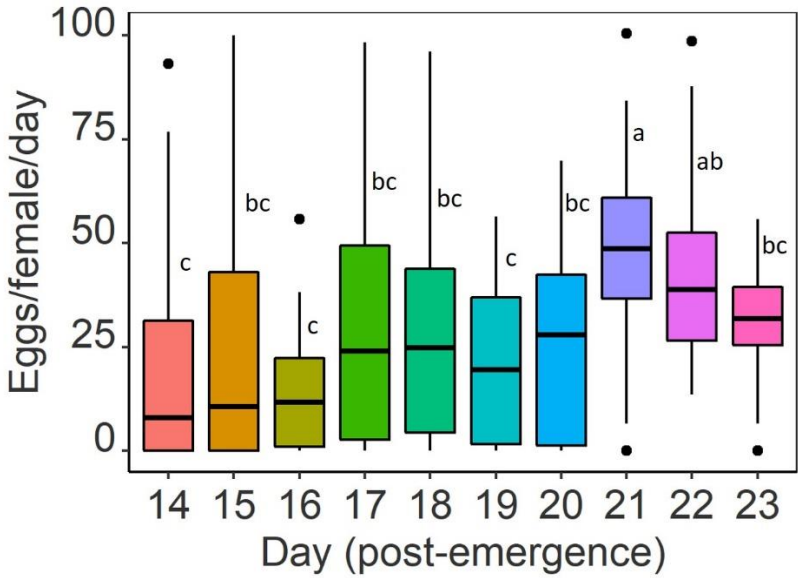Figure S1 – Schematic representation of the experimental design

38

Figure S2. Effects of time on egg production in offspring. Different letters indicate a significant
difference between the treatments (SNK *post hoc* test, p = 0.05)

Supplementary tables

Table S1. Diet recipe (for 1000 mL) and suppliers [2]

Ingredient	Amount	Supplier
Brewer's yeast (g)	204	SF Health foods, Australia

Sugar (g)	121.8	Homebrand, Australia
Agar (g)	10	MP Biomedicals
Citric Acid (g)	23	Sigma, Australia
Nipagin (g)	2	Southern Bio- logical, Australia
Sodium Benzoate (g)	2	Sigma, Australia
Wheat Germ Oil (mL)	2	Melrose laboratories PTY LTD, Australia
MiliQ water (mL)	1000	Lab source

43

Table S2. PCR conditions and primer sequences

PCR conditions

Target	Cycle	Initial	Disassociate	Anneal	Extension	Finish
16S: V1- V3	29	95˚C for 7 min	94˚C for 45s	50˚C for 60S	72˚C for 60S	72˚C for 7 min
ITS1F – ITS2	35	95˚C for 7 min	94˚C for 30s	55˚C for 45S	72˚C for 60S	72˚C for 7 min

Primer sequences

Target	16S: V1- V3	ITS1F – ITS2
Forward	AGAGTTTGATCMTGGCTCAG	CTTGGTCATTTAGAGGAAGTAA
Reverse	GWATTACCGCGGCKGCTG	GCTGCGTTCTTCATCGATGC

45

Table S3. Mean (± SE) of CFU count and PCR quantification of parental samples and negative controls

Treatment	Stage	Sex	n	CFU (per egg/larva/adult)	16S rRNA (per sample)	ITS (per sample)
Parental control	Egg	na	3	1 (0.05)	8.56 (1.89)	0.00 (0.00)
Parental axenic	Egg	na	3	0 (0)	0.00 (0.00)	0.00 (0.00)
Parental control	Larval	na	3	1 (0.21)	0.12 (0.06)	0.136 (0.07)

Parental axenic	Larval	na	3	0 (0)	0.00 (0.00)	0.00 (0.00)
Parental control	Adult	Male	3	150 (150)	2.92 (1.65)	0.09 (0.06)
Parental control	Adult	Female	3	842 (228)	1.22 (0.43)	0.00(0.00)
Parental axenic	Adult	Male	3	0 (0)	0.00(0.00)	0.00(0.00)
Parental axenic	Adult	Female	3	0 (0)	0.00(0.00)	0.00(0.00)
0.5% bleach	na	na	3	na	0.00(0.00)	0.00(0.00)
70% ethanol	na	na	3	na	0.00(0.00)	0.00(0.00)
MiliQ water	na	na	3	na	0.00(0.00)	0.00(0.00)

48

49 **Table S4. Mean (± SE) of egg hatching success and developmental time of parental flies**

Treatment	n	Egg hatching (%)	Developmental time (day)
Parental axenic	10	90.4 (0.37)	17.44 (0.04)
Parental control	10	89.7 (0.97)	17.41 (0.05)
Parental reinoculation	10	88.5 (0.9)	17.44 (0.04)

50 **References**

51 1.	Koyle ML, Veloz M, Judd AM, Wong AC-N, Newell PD, Douglas AE, Chaston JM. 2016 Rearing the Fruit
52	Fly Drosophila melanogaster Under Axenic and Gnotobiotic Conditions. *J. Vis. Exp.* , 1–8.
53	(doi:10.3791/54219)

54 2.	Moadeli T, Taylor PW, Ponton F. 2017 High productivity gel diets for rearing of Queensland fruit fly,
55	Bactrocera tryoni. *J. Pest Sci. (2004).* **90**, 507–520. (doi:10.1007/s10340-016-0813-0)

56

Chapter 6:

General Discussion

This thesis examined the impacts of the microbiota on insect performance at larval and adult stages and measured the extent to which parental microbiota influenced offspring life-history traits. Results obtained here have contributed to our understanding of the complex interplay between insects and microbes. Below, I discuss the main findings of my work and propose future perspectives.

6.1. Effects of the microbiota on larval performance

In animals, in general, and in holometabolous insects, in particular, resources acquired at the early life stage are crucial for the fitness of adults and their offspring (Rolff et al., 2019; Rowe and Houle, 1996). There has been evidence that the microbiota regulates larval development and growth in *Drosophila* (Bing et al., 2018; Shin et al., 2011; Storelli et al., 2011), butterfly (Ruokolainen et al., 2016) and mosquito (Chouaia et al., 2012; Coon et al., 2014). However, there is little information on the influence of the microbiota on larval foraging behaviour, apart from findings suggesting that microbial exposure at early life affects host microbial preference (Wong et al., 2017). During my thesis, I investigated the role of vertically and horizontally transmitted microbiota on larval food choice decision, development rate and pupal productivity using *Bactrocera tryoni* as a model (Chapter 2). I found that both vertically and horizontally transmitted microbes influence larval foraging whereby larvae without any microbiota acquisition chose diets richer in either protein or sugar (Morimoto et al., 2019b). These microbes are also important for larval developmental time to pupation and pupal production of *B. tryoni*. Yet, lacking one or both of these microbial communities resulted in a decrease in pupal production and a delay in pupation (Morimoto et al., 2019b). Microbial modulation of host behavior at larval stage has previously been reported in *Drosophila* (Farine et al., 2017; Qiao et al., 2019; Wong et al., 2017) and in vertebrates such as zebra fish (Davis et al., 2016), and the mechanisms are only now being unravelled. Indeed, studies on germ-free and

gnotobiotic mice have suggested that the proximate mechanisms may comprise alterations of the immune function and production of specific microbial neuroactive metabolites, suggesting a role for the microbiota in the regulation of mood, cognition and anxiety [reviewed by (Cryan and Dinan, 2012)].

In parallel, I tested whether the microbial growth in the diet interacted with the diet composition and larval density to shape larval development and adult traits of *B. tryoni* (Chapter 3). I found a significant interaction between the diet composition and microbial growth on the percentage of adult emergence and, notably, a strong three-way interaction between all factors on body weight of pupae and adults, and on lipid reserve of females but not males (Nguyen et al., 2019). The results showed that although the diet composition was a major factor affecting body weight and lipid storage, diet-dependent effects were significantly modulated by microbial activities in the diet. Microbial growth on sugar-rich diet, for instance, had positive effects on pupal weight suggesting that microbes might serve as an additional amino acid source for larvae and promote larval development (Drew, 1988; Drew et al., 1983; Matavelli et al., 2015). Conversely, for larvae reared on protein-rich diets, the growth of pathogenic bacteria and fungi could have led to a decrease in pupal weight (Nguyen et al., 2019). This study provides additional understanding about the complex interactions between microbial proliferation in the diet, diet composition and larval density in the developmental environment, in addition to some previous work that has explored how diet content and larval crowding modulate larval aggregation (Morimoto et al., 2018) and adult performance (Morimoto et al., 2019a) in *B.tryoni*. Together, the results of Chapter 2 and 3 highlight the role of the microbiota acquired vertically and horizontally and the interaction between the microbiota, larval density and diet components on larval development and adult performance. My results show the importance of the microbiota and other environmental factors in shaping early life traits and fitness in holometabolous insects.

6.2. The microbiota promotes adult weight gain and affects food consumption differently in males and females

Removal of the microbiota has strong effects on the expression of life-history traits and feeding behaviour of adult *B. tryoni*. The data I collected showed positive effects of the microbiota on body weight of young adults and reproductive capability of mature adults (Chapter 4), which are consistent with previous findings in other fruit flies (Elgart et al., 2016; Hamden et al., 2013; Yao et al., 2017), honey bees (Zheng et al., 2017), mosquitoes (Fouda et al., 2001) and beetles (Habineza et al., 2019). Weight gain for hosts with an intact microbiota compared to hosts with an altered microbiota has also been reported in mammalian models such as mice and humans; however, these effects likely depend on the microbiota–diet interaction (Angelakis et al., 2012; Dror et al., 2017). Given that nutrition plays a critical role in the host-gut microbiota interactions (Pasquaretta et al., 2018; Ponton et al., 2011), effects of the microbiota on host body mass are likely related to the contribution of the microbiota to nutrient provisioning. Indeed, a recent study on the honey bee *A. millifera* has shown a link between the microbiota, the host insulin signaling and body weight gain in which the microbiota may enhance the production and responsiveness of insulin and consequently increases host body mass (Zheng et al., 2017).

My results also demonstrated that host-microbe interactions in *B. tryoni* are sex-specific as the microbiota modulates the carbohydrate consumption and body composition of mature adults differently in males and females (see details in chapter 4). How sex contributes to the complex host-microbe interactions is still an open question. Sex, however, is one of the important factors affecting the microbial communities of animals (Chen et al., 2016; Fransen et al., 2017; Kim et al., 2020). Therefore, sex-specific impacts of commensals on host performance can be expected and, in fact, have been described in *Drosophila* (Morimoto et al., 2017; Wong et al.,

2014), fish, mice and human [see (Bolnick et al., 2014) and (Markle et al., 2013)]. In mice, for example, studies have shown that microbial exposures during early life alter sex hormones of individuals, exerting potent effects on autoimmune diseases (a condition in which the immune system mistakenly attacks the body) and these effects are more prevalent in females than males (Markle et al., 2013). My experiment did not allow to explore the mechanisms involved in the sex-dependent influence of the microbiota on the feeding behaviour of *B. tryoni* thus more investigations are needed to go deeper into the proximate mechanisms involved here.

6.3. Transgenerational effects of the microbiota on the life-history traits of offspring

While it is well established that the microbiota can promote insect fitness by contributing to many physiological functions of the host (Douglas, 2015; Engel and Moran, 2013), there is generally a limited understanding of the transgenerational impacts of the microbiota on future generations. My work revealed parental effects with microbiota-deficient (i.e., axenic) parents generating offspring of lower developmental indices, body mass and fecundity relatively to offspring of parents that harbour a microbiota (see details in chapter 5). The mechanisms responsible for these effects are unclear but it is possible that there are differences between the metabolic status of axenic and non-axenic parents, and axenic parents may have a lower investment in eggs (Engel and Moran, 2013; Gould et al., 2018). In oviparous insects, where maternal investment is fixed at the time of egg-laying, egg investment has been shown to have long-term implications on offspring quality (Koch and Meunier, 2014; Wainhouse et al., 2001). In zebra finches (*Taeniopygia guttata*), mother birds with poorer state before mating tend to have offspring with lower fecundity compared to offspring of parents with a better nutritional state (Gorman and Nager, 2004). Likewise, in rodents, female mice inoculated with the beneficial bacterium *Lactobacillus reuteri* gave birth to offspring with a better survival rate (Erdman, 2014). Overall, the transgenerational effects observed on the life-history traits of *B.*

tryoni offspring extend our understanding of the long-lasting implications of host-microbe interactions, which might potentially drive the evolutionary adaptation of the population.

6.4. Perspectives

The study of the microbiota, especially the gut microbiota, and its impacts on host health and fitness is a rapidly moving field of research which attracts broad concerns globally (Doré et al., 2013). Insect models are excellent systems for studying the host-microbe interplay because insect manipulation and maintenance are much easier compared to vertebrates (Dethlefsen et al., 2007). Furthermore, insect life cycles are usually relatively short and experimental processes are inexpensive allowing to explore the far-reaching effects of the host-microbe interactions in multiple generations. While results obtained from insects are not always directly transferable to mammalians, they can bring some new outcomes that possibly inspire more research in mammalian systems. Results from my thesis contribute to a better understanding of direct and transgenerational effects of the microbiota, highlighting its profound implications on host wellbeing at both developmental and generational levels. However, as the molecular mechanisms responsible for these effects remain largely unknown, future studies targeting the relationships between microbe-derived metabolites, host signaling pathways and host physiology are much needed and would provide important knowledge to this exciting topic.

Besides, insects can be serious agricultural pests or disease vectors that negatively affect human health, economic interests or environmental quality (Engel and Moran, 2013). Given that the microbiota is an important determinant of insect fitness, better understanding the ubiquitous relationship between insects and microbes can help to promote the development of new pest management approaches. The sterile insect technique (SIT), for instance, is an environment-friendly method of pest control where millions of fruit flies are reared and sterilised before

adults being releasing in the field. Mass-rearing can negatively impact insect quality and performance, reducing the competitiveness of released sterile males to mate with wild females, hence threatening the effectiveness of SIT (Woruba et al., 2019). In this context, the development of microbiota-based methodologies to improve the performance of mass-reared insects has been proposed. In tephritid fruit flies, in particular, supplementing the diet with a single bacterial strain or a probiotic cocktail has shown positive outcomes on host performance, though substantial changes are not always observed [see for example (Aharon et al., 2013; Ami et al., 2010; Augustinos et al., 2019, 2015; Gavriel et al., 2011; Hamden et al., 2013; Rempoulakis et al., 2018; Shuttleworth et al., 2019); reviewed in (Deutscher et al., 2019)]. The findings of my thesis not only highlight the importance of the microbiota for larval behaviour, development rate and pupal production in *B. tryoni*, but also provide information on the importance of the microbiota acquired vertically and horizontally, which might be implemented to increase the efficiency of SIT programs.

6.5. References

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Appendices

Appendix I. Collaborations

Morimoto, J., **Nguyen, B.**, Tabrizi, S.T., Ponton, F., Taylor, P., 2018. Social and nutritional factors shape larval aggregation, foraging, and body mass in a polyphagous fly. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-32930-0

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Dinh, H., **Nguyen, B.**, Kumar, S., Lundback I., Morrimoto J., and Ponton F., Trans-generational effect of diet on offspring pathogen resistance in Queensland fruit flies (manuscript preparation)

Morimoto, J., Than T. A., **Nguyen, B.**, Ponton, F., Developmental habitat quality effects on individual fitness and population growth: an experimental–computational biology approach (manuscript preparation)

Appendix II. Grants and awards

Travel grant from Project Raising Q-fly Sterile Insect Technique to World Standard (HG14033) AUD$3650 for attending the Congress of the European Society for Evolutionary Biology (ESEB, Aug 2019), Turku, Finland.

Travel grant from Higher Degree Research budget AUD$500 for attending the Australian Society of Microbiology Meeting (ASM, June 2019), Adelaide, Australia.

Best presentation of laboratory-based research (Runner up), Higher Degree Research Conference, Biological Sciences Department, Macquarie University, Australia, June 2019.

Appendix III. Academic and outreach presentation

"Trans-generational effects of commensal microbiota on pupal and adult traits of a polyphagous fly"

Poster presentation, the Australian Society of Microbiology Meeting (ASM), Adelaide, Australia, June 2019.

"Trans-generational effects of commensal microbiota on pupal and adult traits of a polyphagous fly"

Poster presentation, the Congress of the European Society for Evolutionary Biology (ESEB), Turku, Finland, August 2019.

"Effects of commensal microbiota on the development and behavior of the Queensland fruit fly"

Oral presentation, Higher Degree Research annual conference, Biological Sciences Department, Macquarie University, Australia, June 2017-2019.

"Ain't nobody eating my food! Food choice in fruit fly larvae"

Oral presentation, Presbyterian ladies' College science summit, Sydney, Australia, 23 February 2019.