An integrated approach of the interactions between nutrition and resistance to infection in fruit flies

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Thesis summary

While it is well established that nutrition affects a large range of life history traits, our understanding of the effects of diet on immunity and resistance to infections remains limited. This thesis focused on better understanding the effects of macronutrients, protein and carbohydrate, on resistance to infection using the fruit fly, *Bactrocera tryoni*. The results revealed that infected female flies shifted their diet choice to a carbohydrate-biased diet after septic infection with a pathogenic bacterium, which promoted their survival after infection and contained the growth of pathogen population. Moreover, the dietary macronutrient balance had long-term effects which can be observed across developmental stages and generations. Dietary manipulations at larval stage greatly affected a number of adult traits including the fly's ability to resist infection. Interestingly, these effects were sex-specific with infected females- but not males- surviving better the infection at adult stage when fed a sugar-biased larval diet compared to those fed a protein-biased larval diet. This sex-specific effect of the diet composition was also observed in offspring that parents had been fed different macronutrient ratios. Surprisingly, when both parents were fed unbalanced diets, their sons, and not their daughters, had a greater survival after infection. In contrast, when only mothers experienced unbalanced diets, their sons resisted less well the infection. Oral immune priming was also investigated but no evidence for positive effects of priming on the survival of *B. tryoni* after infection was observed. This work highlights the complexity of the interactions between nutrition and resistance to infection, not only at the individual level but also at developmental and generational levels. Better understanding the nutritional components that influence resistance to infection is an important challenge with broad implications spanning from health to organismal science.

Keywords: Diet quality, Infection, Macronutrient, Nutritional self-medication, Oral priming, Transgenerational effect

Declaration of originality

I certify that the thesis entitled "**An integrated approach of the interactions between nutrition and resistance to infection in fruit flies**" written in the form of published journal articles has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis 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

1.1. Nutrients, immunity and infection

According to the World Health Organization (WHO), malnutrition can be defined as deficiencies, excesses, or imbalances in the energy and/or nutritional intake of an individual. Malnutrition, directly or indirectly, accounts for 53% death that are associated with infectious diseases in children under five years of age in developing countries (WHO, 2005), with the causal relationships between malnutrition, immunity and infection being well recognized in numerous studies [see review in (Cunningham-Rundles et al., 2005; França et al., 2009; Katona and Katona-Apte, 2008; Kumar Chandra, 1983; Miller, 1987; Scrimshaw and SanGiovanni, 1997)].

Under-nutrition is believed to be a primary cause of immunodeficiency (Calder and Jackson, 2000; Katona and Katona-Apte, 2008). The most common and studied form of undernutrition is Protein-Energy Malnutrition (PEM). Experimental studies using animal models, such as mice and rats, have shown great negative impacts of PEM on both the innate and adaptive immune systems. Protein-Energy Malnutrition is known to not only reduce the biological function of macrophages (Redmond et al., 1991a) and B lymphocytes (Petro et al., 1994; Redmond et al., 1991b) but also to cause a reduction in the number of T cells (Woodward, 2009) and granulocyte white blood cells (Paßlack et al., 2017). Moreover, antibody production (Amaral et al., 2006; Bell, 1975; Grimble and Mcillmurray, 1973a, 1973b; Michael and Bundy, 1992), blood monocyte phagocytic activity (Paßlack et al., 2017) and macrophage apoptosis (Rivadeneira et al., 2001) are also decreased in PEM individuals.

Not only PEM impairs immunity, it also greatly increases susceptibility of individuals to infections (Schaible and Kaufmann, 2007) and, therefore, is an important risk factor for many infectious diseases. There is evidence from both human and experimental animal studies that individuals under PEM are more susceptible to infections by the bacterium responsible for tuberculosis, *Mycobacterium tuberculosis* (Cegielski and McMurray, 2004; Evans, 2005; Hood, 2013; Rodriguez et al., 2005) and other opportunistic pathogens (Duggal et al., 2012) [see also (Hagel, 2003; Hagel et al., 1995; Ing et al., 2002)].

On the other hand, over-nutrition is associated with metabolic disorders that also impair the immune function and increase susceptibility to infectious diseases (Samartín and Chandra, 2001). An extensively studied aspect of over-nutrition is obesity which usually results from an imbalance between energy intake and energy expenditure (Samartín and Chandra, 2001). Obesity has been found to be a risk factor of many types of infections including pneumonia (Falagas et

al., 2009; Huttunen and Syrjänen, 2013), influenza [see for instance, (Louie et al., 2011; Viasus et al., 2011; Yu et al., 2011)], periodontitis (Saito et al., 1998) and nosocomial infections (Choban et al., 1995; Kaye et al., 2011). Mechanism underlying the causal relationship between obesity and infection in vertebrates involves an impaired cross-talk between adipocytes [i.e., cells involve in fat storage that control the whole body metabolism (Ali et al., 2013)] and leukocytes (i.e., immune cells) that ultimately leads to dysregulations of the immune systems (Huttunen and Syrjänen, 2013).

There have been extensive studies correlating malnutrition to the incidence of diseases and numerous investigations of the impacts of malnutrition on immune systems, exploring the interactions between nutrition and infection remains however complex because:

i. In addition to the total quantity of food eaten (i.e., under- or over-nutrition), the ratio between food components is also an important modulator of host resistance and immune responses to infections (Ponton et al., 2011). Although some investigations have already shown the effects of the balance between diet components on immunity and resistance to infections in insect models [see for instance (Graham et al., 2014; Lee et al., 2006; Ponton et al., 2018; Povey et al., 2014, 2009)], this still remains to be fully understood.
ii. During an infection, nutrition affects both the host and the pathogens. From the host's perspective, mounting and maintaining immune responses is energetically costly (McKean et al., 2008; Sheldon and Verhulst, 1996). From the pathogen's perspective, both amount and specific components of host food can affect their growth (Bedhomme et al., 2004; Hall et al., 2009; Pulkkinen and Ebert, 2004). Variations in the nutritional status of the host during an infection can therefore lead to different outcomes of the host-pathogen interactions (Pike et al., 2019).
iii. Studies in insect models have shown that immune traits have their own specific nutritional requirements (Cotter et al., 2011) with no specific diet composition maximizing all traits simultaneously.

To capture this complexity, it is necessary to have a comprehensive and integrative approach of the interactions between nutrition, immunity and resistance with dietary manipulations that include ratios of nutrients.

1.2. Long-term effects of nutrition

Nutrition not only affects individuals that directly consumed food but has also long-term effects that can be observed across developmental stages and even across generations (i.e., transgenerational effects of diet) (Brookheart and Duncan, 2016; Grueber et al., 2018).

1.2.1. Effects of nutrition in early-life stages

Nutritional conditions experienced early in life are critical to shape individuals' life-history traits. In human, foetal undernutrition is linked to a number of non-communicable diseases later in life, including coronary heart disease, diabetes, obesity and hypertension (Barker, 1998; Deboer et al., 2012; Roseboom et al., 2006). Over nutrition (i.e., high protein) in children from 12 months to 13 years may also contribute to an increased risk of adult obesity (Hörnell et al., 2013). In birds, environmental conditions experienced in early-life stages play an important role in shaping growth and adult immunity (Butler and McGraw, 2011; Naguib et al., 2006, 2004; Tella et al., 2001). In holometabolous insects, nutritional resources acquired at larval stage are particularly important for survival during metamorphosis (Arrese and Soulages, 2010; Mirth and Riddiford, 2007) and affect several life-history traits including development time (Colasurdo et al., 2009; May et al., 2015; Roeder and Behmer, 2014; Telang et al., 2007; Zwaan et al., 1991), adult body size (Boggs and Freeman, 2005; Colasurdo et al., 2009; Morimoto et al., 2019a, 2017; Zwaan et al., 1991), lifespan (Bauerfeind and Fischer, 2009; May et al., 2015; Runagall-Mcnaull et al., 2015), and adult reproductive performance (Colasurdo et al., 2009; Dmitriew and Rowe, 2011; Kaspi et al., 2002; Morimoto et al., 2017, 2016).

Nutrition early in life also affects resistance to infection in later developmental stages. It has been shown, for instance, that the diet experienced by frog tadpoles (*Osteopilus septentrionalis*) affects adult resistance via modulating antibody production, parasite establishment and gut microbiota (Knutie et al., 2017a, 2017b). In insects, the constitutive components of innate immunity including phenoloxidase activity, haemocytes number, and melanisation are greatly reduced in adults that experienced nutritional deprivation at larval stage (Rolff et al., 2017; Suwanchaichinda and Paskewitz, 1998). The survival of infected adults is also modulated by the nutritional conditions at early developmental stages (Kelly and Tawes, 2013; Linenberg et al., 2016).

Despite these advances, research on the effects of nutrition early in life on adult immunity and resistance remains quite limited. More investigations are needed to better understand the influence of the quality of juvenile diet on adult capability to fight against infections.

1.2.2. Effects of parental nutrition

The importance of maternal diet has been well recognized as stated by the WHO "Good maternal nutrition- The best start in life" (WHO, 2016). Numerous studies have focused on the long-term effects of maternal diet on the susceptibility to non-communicable diseases (e.g., diabetes, heart disease, Alzheimer's disease and obesity) in offspring (WHO, 2016). Using experimental

models, especially invertebrate models, more details about the contribution of the nutritional conditions experienced by the parent(s) on offspring life-history traits have been revealed. Many offspring traits were found to be modulated by the parental diet, including body size and body weight (Triggs and Knell, 2012; Valtonen et al., 2012; Vijendravarma et al., 2010), developmental time (Matzkin et al., 2013; Valtonen et al., 2012; Vijendravarma et al., 2010), egg-to-adult survivorship (Prasad et al., 2003), reproduction (Matzkin et al., 2013), body composition of protein, glycogen and triglycerides (Buescher et al., 2013; Matzkin et al., 2013). Interestingly, the results from these studies have also emphasized the importance of the paternal diet on offspring phenotype (Aldrich and Maggert, 2015; Buescher et al., 2013; Öst et al., 2014; Valtonen et al., 2012). These observations challenge the previous idea that maternal diet had greater effects than paternal diet due to the tendency of mothers to invest more resources in reproduction and care of offspring (Magiafoglou and Hoffmann, 2003; Mousseau and Fox, 1998; Tallamy, 1984; Zeh and Smith, 1985). Furthermore, a study in Drosophila has shown that a diet manipulation in both parents has complex effects on offspring traits. For instance, offspring developmental time is shorter than in control condition when only one parent is malnourished while it is rather slower than control condition when both parents are malnourished (Valtonen et al., 2012). This suggests the importance of not only considering the relative contribution of the diet of each parents on progeny performance but also the possibility of their interactive effects (Valtonen et al., 2012).

Effects of maternal nutrition on offspring immunity has received a great attention over the last decades. In invertebrates, the maternal nutritional status directly influences offspring immunity. For instance, the phenoloxidase activity and the number of haemocytes in offspring were found to be reduced when their mothers experienced a poor diet (Triggs and Knell, 2012). Interestingly, the same effect was observed in offspring from fathers under poor nutritional conditions (Triggs and Knell, 2012), suggesting contributions of both, maternal and paternal, diets on offspring immunity. Other examples of the effects of parental diet on resistance in invertebrates include offspring of the Indian meal moth (*Plodia interpunctella)* surviving better viral infection (Boots and Roberts, 2012) and *Daphnia magna* offspring being less susceptible to pathogenic bacteria (Ben-Ami et al., 2009; Mitchell and Read, 2005) when their mothers underwent food stress.

While the effects of maternal diet on offspring pathogen resistance have been widely studied, the effects of paternal or both parents' diet remain to be explored. Moreover, studies on the transgenerational effects of parental diet have mainly focused on investigating effects of variation in food quantity posing the need to examine the transgenerational effects of variation in diet quality (i.e., balance between diet components).

1.3. Immune priming

The invertebrate immune system lacks the adaptive immune arm, that is specific to vertebrates (Dempsey et al., 2003; Iwasaki and Medzhitov, 2015). Innate immune responses are non-specific but have the ability to quickly respond to infections caused by a wide range of pathogens (i.e. bacteria, viruses, fungi, and parasites) (Hoffmann and Reichhart, 2002). Recent studies have demonstrated that the innate immune system is however more complicated than traditionally considered with some memory abilities (Kurtz, 2005; Kurtz and Franz, 2003). This phenomenon is widely recognised as "immune priming" in invertebrates [for review, see (Milutinović et al., 2016; Moret and Siva-Jothy, 2003)]. A higher survival after infection of primed individuals compared to non-primed individuals when re-infected by the same pathogen has been commonly used to detect immune priming [e.g., (Futo et al., 2017; Milutinović et al., 2013; Moret and Siva-Jothy, 2003; Roth et al., 2009; Sadd and Schmid-Hempel, 2006)]. Other changes were observed in primed individuals including an increased number of haemocytes (López et al., 2014; Zhang et al., 2014), a higher phenoloxidase (PO) activity (Lin et al., 2013; Shi et al., 2014), an enhanced reactive oxygen species activity (Mikonranta et al., 2014) or a lower pathogen load (Thomas and Rudolf, 2010).

Priming not only has protective effects within life-stages but can also have long-term protection against infections across life-stages (ontogenic priming) or across generations (transgenerational priming). Within-developmental stage immune priming has been observed in, for instance, bumble bees (Sadd and Schmid-Hempel, 2006), red flour beetles (Roth et al., 2009), moths (Tidbury et al., 2011), silkworms (Miyashita et al., 2014), vinegar flies (Pham et al., 2007), and mosquitoes (Contreras-Garduño et al., 2014). Also, exposure of parents to pathogens can protect their offspring from reinfection of the same pathogen as evidenced by studies in mealworm beetles (Dubuffet et al., 2015) (Martinaud et al., 2012), red flour beetles (Dubuffet et al., 2015; Eggert et al., 2014; Freitak et al., 2009; Little et al., 2003; Martinaud et al., 2012; McNamara et al., 2014; Schulz et al., 2019; Tate and Graham, 2015; Tidbury et al., 2011; Trauer and Hilker, 2013), daphnias (Little et al., 2003), field crickets (McNamara et al., 2014), and moths (Tidbury et al., 2011; Trauer and Hilker, 2013). Despite great physiological changes between life-history stages, immune priming at specific stage (i.e., larval stage) can also protect hosts at later stage (i.e., adult stage) as demonstrated in the flour beetle *Tribolium confusum* (Thomas and Rudolf, 2010), the red flour beetle *Tribolium castaneum* (Khan et al., 2016) and the mosquitoes *Aedes aegypti* (Moreno-García et al., 2015).

Immune priming is a complex process that may vary with many different factors: (i) Priming effects might depend on the developmental stage of the animal when primed and when the

outcome of immune priming was measured [review in (Tetreau et al., 2019)]. Different outcomes might be observed due to differences in metabolism, physiology, and immunity between developmental stages (Tetreau et al., 2019); (ii) Effects of immune priming are not always similar in both sexes. For instance, a sex-dependent immune response has been observed in adult *Aedes aegypti* mosquitoes primed with bacteria at larval stage: males showed a higher phenoloxidase activity, whereas, females had a greater antimicrobial activity and Nitric Oxide production relative to non-primed individuals (Moreno-García et al., 2015); (iii) Different priming routes (septic vs. oral infection) induce different physiological and immunological processes (Behrens et al., 2014; Milutinović et al., 2014; Roth and Kurtz, 2008); and (iv) Active infection (live bacteria) and inactive infection (heat-killed pathogen or culture supernatant) might also result in differences in outcomes of immune priming. Moreover, our understanding of effects of nutrition on the effectiveness of immune priming (e.g., ability to kill pathogen) is still limited and requires more investigation. Future studies should consider all of these factors in experimental designs to study effect of diet on immune priming.

1.4. Study models

Invertebrates, mostly insects, were first used as research models in the late 1800s, leading to discoveries in almost every areas of biology and medicine (Wilson-Sanders, 2011). The use of insects as host models of opportunistic pathogens has numerous benefits: (i) Insects have short developmental time and are inexpensive to rear; (ii) The infection process is simpler and quicker compared with mammals; (iii) The lack of adaptive immunity in insects allows the study of the interaction between pathogen and innate immunity separately; and (iv) The parallels in pathogenesis [review in (Scully and Bidochka, 2006)] as well as the high conservation of innate immunity and metabolism pathways between insects and mammals (Herranz and Cohen, 2017; Magor et al., 1999; Teleman, 2010) provide great advantages to study nutrition-host-pathogen interactions in insects.

1.4.1. *Bactrocera tryoni*

In this thesis, I used the fruit fly *Bactrocera tryoni* (Froggatt; Diptera: Tephritidae) as a system model to explore the interactions between nutrition and resistance to infection. *Bactrocera tryoni* is a holometabolous insect (i.e., complete metamorphosis) with four developmental stages: eggs, larvae, pupae and adults (Fig.1). Third instar larvae jump from their food source to pupate into the soil. Adult flies start mating and ovipositing 14 days after emergence.

Bactrocera tryoni is a major horticultural pest insect infesting a large diversity of fruits and vegetable crops (Drew et al., 1978; Bateman, 1991; Hancock et al., 2000). It is widely distributed

in Eastern Australia and has invaded some South Pacific islands (Drew et al., 1978). *Bactrocera tryoni* is a good experimental invertebrate model to explore important fundamental questions about the effects of nutrition on innate immunity and resistance since it is easy to rear in the lab, has a short generation time and there is background information on the feeding behaviour of adults (Fanson et al., 2013a, 2013b, 2012, 2009; Fanson and Taylor, 2012) and larvae (Morimoto et al., 2019b, 2019c, 2018; Nguyen et al., 2019). Furthermore, increasing our knowledge on the biology, ecology and physiology of *B. tryoni* is essential to develop sustainable methods to control their populations in the field.

Figure 1. Life cycle of the fruit fly, *Bactrocera tryoni* (source: https://fruitflyfreeyv.com.au/)

1.4.2. *Serratia marcescens*

Serratia marcescens is a Gram-negative bacterium which is classified as an opportunistic pathogen in the family of the *Enterobacteriaceae* (Hejazi and Falkiner, 1997). *Serratia marcescens* is commonly used to experimentally infect insects such as flies (*Drosophila melanogaster*) [e.g., (Cronin et al., 2009; Duneau et al., 2017; Nehme et al., 2007; Valtonen et al., 2012)], field crickets (*Gryllus texensis*) [e.g., (Adamo et al., 2010, 2008; Kelly and Tawes, 2013)], moths (*Parasemia plantaginis*) (Mikonranta et al., 2014) and mosquitoes (*Anopheles gambiae*) (Stathopoulos et al., 2014) to study host-pathogen interactions. This bacterium can be found almost everywhere in the environment and can survive and grow under extreme conditions such as in disinfectants, antiseptics and double- distilled water (Hejazi and Falkiner, 1997).

Serratia marcescens is a concern for public health since it is increasingly responsible for nosocomial infections (i.e., hospital-acquired infections) (Khan et al., 2015) that result in increasing incidence of multidrug-resistant strains (WHO, 2016). Better understanding about the interaction between *S. marcescens* and its hosts can help to manage infections caused by this bacterial species.

1.5. Study objectives

Macronutrients, particularly protein and carbohydrate, are major energy yielding food components (Simpson and Raubenheimer, 2012). Nutritional research using the Geometric Framework for Nutrition has revealed that the ratio between protein and carbohydrate in the diet (PC ratio) is a critical modulator of key life-history traits including development, lifespan and reproduction in vertebrates and invertebrates (Simpson and Raubenheimer, 2012). In invertebrates, reproduction and longevity are influenced by the dietary macronutrient composition and maximised at different PC ratios [see for instance, (Fanson et al., 2009; Fanson and Taylor, 2012; Grandison et al., 2009; Lee, 2015; Lee et al., 2008)], with different protein and carbohydrate requirements for males and females as seen in *B. tryoni* (Fanson et al., 2013), Drosophila (Jensen et al., 2015; Lee et al., 2013), caterpillar *Spodoptera litura* (Lee, 2010) and crickets *Teleogryllus commodus* (Maklakov et al., 2008). The PC ratio also influences fly development in a sex-specific manner with low PC ratios influencing egg-to-adult viability in a positive way in males but in a negative way in females (Andersen et al., 2010). Moreover, there have been some investigations of the relationship between the PC ratio and infection in insect models, with the PC ratio influencing the ability of the host to resist infections (Graham et al., 2014; Lee et al., 2006; Ponton et al., 2018; Povey et al., 2014, 2009). Despite these advances, the interactions between nutrition, immunity and pathogen still remain to be fully explored. Moreover, our understanding about the long-term effects of the PC ratio on infection outcome and immune priming is very limited. In my thesis, I will address these gaps by measuring both direct effects (at same developmental stage) and long-term effects (across developmental stages or across generations) of the dietary PC ratio on pathogen resistance (chapter 2, 3, and 4) and oral priming (chapter 5) (see Fig. 2 for thesis outline).

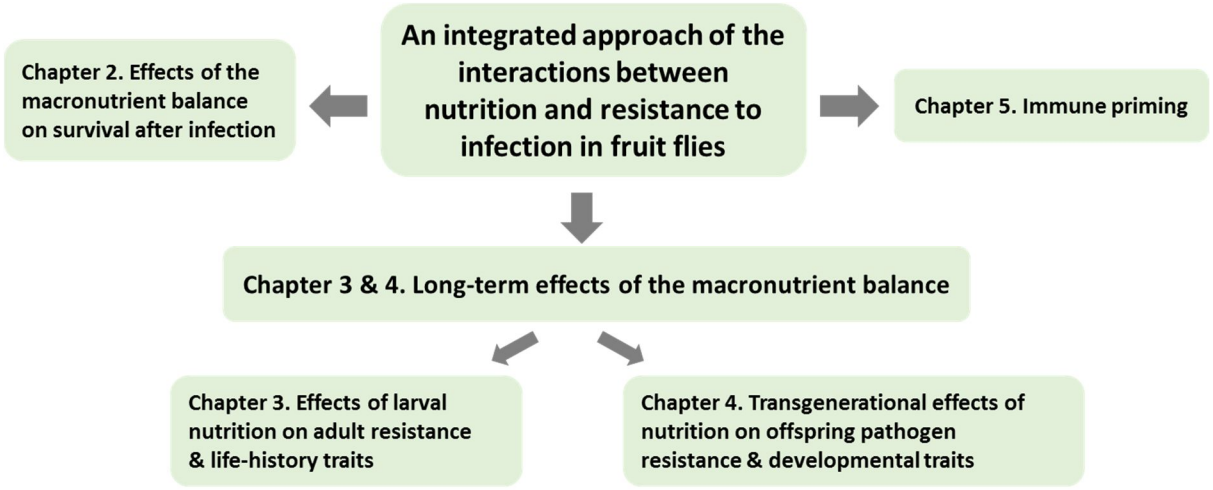

Figure 2. Thesis outline

In chapter 2, I investigated the relationships between the dietary macronutrient ratio and septic infection in female fruit flies. Flies were injected with *S. marcescens* and offered a choice between a sugar and a yeast solution (sugar and yeast are the only source of carbohydrate and protein used in the study, respectively) to examine whether flies shifted their nutritional choice when infected. I then explored the consequences of a change in diet choice on the survival of flies after infection, within-host pathogen proliferation and lipid body reserves by restricting flies to single diets varying in the macronutrient ratio. This chapter intended to better understand the direct interactions between nutrition, pathogen resistance and host physiology during an infection.

In chapter 3, the dietary macronutrient ratio was manipulated during larval stage. I measured the effects of the larval diet manipulation on (i) bacterial load, survival and food intake of adult male and female flies following septic infection with *S. marcescens*, (ii) developmental traits including egg hatching rate, pupation rate, emergence rate and developmental time, and (iii) physiological traits of the adults including body weight, body lipid and protein reserves. Results from this study contributed to better understand the carry-over effects of environmental conditions experienced in early life on the subsequent life-history traits of an individual.

In chapter 4, I investigated the effects of parental diet on offspring traits. The ratio of macronutrient in the diet of mothers, fathers or both parents was manipulated. I tested the transgenerational effects of the diet manipulations on (i) the disease resistance of male and female offspring by measuring their survival following an infection with *S. marcescens,* and (ii) developmental traits including egg hatching success, pupation rate, emergence rate, larval weight, pupal weight and larval body lipid reserves. The results from this work provided useful

insights into the long-term effects of parental diet on the pathogen resistance of female and male offspring.

In chapter 5, I tested for ontogenic immune priming. Larvae were orally exposed to either heat-killed or live bacteria, and injected with the same bacterium at adult stage. Within - developmental stage immune priming was also examined. For this purpose, adult flies were fed either heat-killed or live bacteria and then injected with the same bacterium two days following priming. In both experiments, the survival rate of challenged flies was measured. This work provided the first investigation of the effects of oral priming at adult stage and across developmental stages in fruit flies. While there was no direct measurement of the effects of food composition on oral priming, this work constitutes a first step in exploring immune priming in tephritid flies.

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Chapter 2

Interactive effects of adult nutrition and pathogen infection on diet choice and disease resistance in adult fruit fly

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Macronutrients and infection in fruit flies

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ABSTRACT

Nutrition and infection are closely linked. While it is now well established that hosts can modulate their nutrition after being infected, the extent to which this change in foraging provides the host with a greater fitness remains to be fully understood. Our study explored the relationships between dietary choice, macronutrients intake [i.e., protein (P) and carbohydrate (C)], infection, survival rate and growth of pathogenic bacterial population in the true fruit fly *Bactrocera tryoni*. Results showed that flies injected with the bacterium *Serratia marcescens* decreased their macronutrient intake and shifted their diet choice to carbohydrate-biased diet compared to naïve individuals. Interestingly, flies injected with either PBS (i.e., sham-infected) or heat-killed bacteria also reduced food intake and modulated diet choice but only for a day after injection. When infected flies were restricted to the diet they selected (i.e., PC 1:8), they survived better the infection than those restricted to a protein-biased diet (i.e., PC 1:5). In addition, we did not observe any growth of pathogen load in infected flies fed PC 1:8 for the first 3 days post-infection. Finally, a decrease in lipid body reserves was found in flies injected with live bacteria and, interestingly, this loss of body lipid also occurred in flies injected with heat-killed bacteria, but in a diet-dependent manner. Our results indicated that *B. tryoni* flies modulated their macronutrient intake and decreased the negative effects of the infection on their survival ("nutritional self-medication") the first days following the infection.

1. Introduction

Animals use pharmaceutical compounds to self-medicate after infection, and such anti-pathogen self-medication has been observed widely among diverse groups (Jacobus et al., 2013; Shurkin, 2014). Insects, for instance, ingest toxic products such as pyrrolizidine alkaloids, honeys with a higher antibiotic activity or ethanol when infected (Singer et al., 2009; Gherman et al., 2014; Milan et al., 2012). Besides, animals can also modulate the composition of their diet through ingesting different quantities and ratios of nutrients after infection (Kyriazakis et al., 1994, 1996; Cosgrove and Niezen, 2000), and there has been growing interest in measuring the effect of infections on feeding choice in the last decade. As an example, caterpillars of the African cotton leafworm (*Spodoptera littoralis*) and African armyworm (*Spodoptera exempta*) shift their diet choice and ingest a greater protein-to-carbohydrate (PC) ratio than non-infected individuals when either virally or bacterially infected. This change in feeding provides caterpillars with a higher survival after infection (Povey et al., 2009, 2014; Lee et al., 2006).

Infected hosts not only shift diet preference but also reduce food intake [i.e., "anorexia" (Adamo et al., 2010; Ayres and Schneider, 2009)]. It has been suggested that anorexic responses after infection can enhance host's immune function through reducing metabolite digestion (Adamo et al., 2010). While an anorexic response during infection is defined as an overall decrease in total food consumption, it can also be characterized as a decrease in the intake of specific nutrients. For instance, *S. exempta* larvae that have been virally infected, reduced their carbohydrate intake while keeping a constant level of protein consumption (Povey et al., 2014).

The interactions between nutrition, immunity and resistance to infection are however complex (Ponton et al., 2011, 2013). Cross modulations between nutrition and immunity are dependent on different factors such as host developmental stage and genotype (Kutzer et al., 2018; Grimm and Steinle, 2011). Another complexity arises from the specific immune responses induced by different types of pathogens. Hosts can respond to specific infection not only by activating different immune pathways [review in (Lemaitre and Hoffmann, 2007)], but also by adjusting the immune strategy through resistance which is the host ability to clear pathogen, and/or tolerance defined as the host ability to limit negative impacts caused by an infection (Råberg et al., 2007; Best et al., 2008). Nutrients can either promote or reduce host tolerance and resistance to a specific infection. Low protein reduced tolerance in mice infected with helminths (Clough et al., 2016), and in *Drosophila* infected with *Escherichia coli* (Kutzer and Armitage, 2016), whereas, an

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increased tolerance was observed in bacterially infected beetles fed a low fat/high protein diet compared to those fed a high fat/low protein diet (Miller and Cotter, 2018). In Drosophila, when flies were fed a diluted diet, a greater resistance to *Lactococcus lactis* was observed (Burger et al., 2007), while a lower resistance to *Listeria monocytogenes* was measured (Ayres and Schneider, 2009).

Integrating approaches on how nutrition affects resistance and tolerance to infection and how the host modulates its nutrition in response to challenges such as injuries, immune activation, and infections with live pathogens is essential for getting a comprehensive understanding of host-pathogen interactions. Geometric approaches of nutrition (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 2012) are now allowing quantitative measurement of the effects of nutrients and dietary caloric content on host and pathogen populations. Applying this approach to different model species will give us a broader understanding of the animal nutritional immunology as well as insight into the mechanisms involved.

In this study, we investigated the relationships between nutrition and infection for the first time in *Bactrocera tryoni* (Froggatt; Diptera: Tephritidae, "Q-fly") adult female flies infected with the pathogenic opportunistic Gram-negative bacterium, *Serratia marcescens*. The main aims were (i) to test whether flies shifted their macronutrient (i.e., protein and carbohydrate) intake when infected with either live pathogens or immune challenged through injection with heat-killed pathogens and PBS; and (ii) to measure the consequences of dietary change on host survival after infection, within-host pathogen proliferation and lipid body reserves. We intended here to better understand the interactions between nutrition, immune activation, injury recovery, growth of pathogen population and host physiology in adult female fruit flies.

2. Materials and methods

2.1. Fly strains

Fly stock was maintained on a gel-based diet at larval stage (Moadeli et al., 2017) and a 1:3 ratio of hydrolysed yeast to sugar (Y:S) at the adult stage [Sugar (CSR® White Sugar), Hydrolysed yeast (MP Biomedicals Cat. no 02103304)]. All experiments were carried out in controlled environment rooms at Macquarie University (NSW, Australia) under 25 °C and 65% humidity with a 12-h light/dark cycle. Eggs were collected using an ovipositional device that contained 30 ml of water to maintain humidity and had numerous puncture holes through which females could oviposit. Eggs were collected for 2 h and 250 µl eggs (around 3500 eggs) were then transferred into rearing trays (17.5 cm long, 12 cm wide, 4 cm deep) using a 1000 ml pipette. Each rearing tray contained 150 ml of larval gel diet at room temperature. Five days after seeding eggs, the rearing trays were placed into 12 L plastic containers filled with 500 ml of fine vermiculite to allow larvae to pupate. Pupae were collected and kept on a mesh cage (Megaview Bugdorm, 47.5 × 47.5 × 47.5 cm). Within 24 h after emergence, female flies were sorted, transferred to12 L plastic cages and provided *ad libitum* food. We used 5-day-old female flies in all experiments.

2.2. Bacterial preparation and infections

Serratia marcescens (ATCC 13880, Thermo Scientific) was spread on Nutrient Agar (Oxoid, CM0003) plates and incubated at 26 °C in a microbiological incubator (Biobase). Single colonies were then cultured into 5 ml of sterile Nutrient Broth (Oxoid, CM0001) incubated overnight (approximately 16 h) at 26 °C with shaking at 200 rpm. The bacterial culture was centrifuged at 10,000 g at 4 °C for 2 min. The supernatant was discarded, and the pellet washed twice using 1X Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Cat. No P4417) to remove any trace of culture media. The culture was centrifuged at 10,000 g at 4 °C for 2 min between each washing step. The bacterial cells were resuspended in sterile PBS and optical density (OD_{600}) was measured using a spectrometer (Eppendorf). Cells were then diluted in PBS to achieve a target concentration of $OD_{600} = 0.025$. The number of colonies was checked by spreading 10 µl of each bacterial solution on Nutrient Agar plates (10 replicates). The plates were then incubated at 26 °C for 24 h and the number of colonies counted. The actual concentration of bacterial cells at $OD_{600} = 0.025$ was on average 4.29×10^7 cells/ml. The heat-killed bacteria solution was prepared by incubating concentrated bacterial cultures (1 ml of $OD_{600} = 3$) in a water bath (Thermoline) at 90 °C for 20 min. The solution was then centrifuged for 2 min at 10,000 g, and washed two times in PBS. We then diluted the solutions in PBS to an $OD_{600} = 0.025$ and stored them at −80 °C. We checked that all cells had been killed by spreading 10 µl of the heat-killed bacterial solution on a nutrient agar plate (5 replicates), that was incubated at 26 °C for 24 h.

Groups of 10 female flies were cold anesthetized in a −20 °C refrigerator for 2 min, then transferred to a petri dish placed on a dry bath (Product code: MK20) at −10 °C. Injections were performed by using a 10 µl syringe (NanoFil) to inject 0.2 µl of bacterial solution (yielding a dose of approximately 8580 cells) into the fly's coxa of the third right leg. The bacterial suspension was kept on ice throughout the injection and mixed up thoroughly after every two injections in order to keep the solution homogenous. We used flies injected with heat-killed bacteria to measure effects of immune activation on feeding behaviour and flies injected with PBS to measure effects of injury. Naive flies were left unmanipulated.

2.3. Choice experiment

To measure flies nutritional choice, we used a Capillary Feeder (CAFE) assay as previously described (Ja et al., 2007) and adapted to Q-flies (Fanson et al., 2009). Flies were housed individually in a clear plastic chamber (diameter = 4 cm, height = 6 cm) and were offered two 30 µl capillaries (Drummond Microcaps), one filled with a sugar (CSR® White Sugar) liquid diet and the other with a yeast (MP Biomedicals Cat. No. 02103304) liquid diet, both at a final concentration of 120 g/l. Water was also provided to the fly via 200 µl-pipette tips.

Sugar and yeast liquid diets were prepared by mixing sugar or hydrolysed yeast into distilled water at 80 °C on a hot plate. The diets were then dispensed into 30 µl-capillaries using a 200 µl pipette. Each capillary was filled with 16 µl of liquid diet and the meniscus was marked with permanent marker. A blue food colouring (Brand: Queen®) was added to the sugar diet to facilitate visualization. Food consumption was measured daily for 9 days, and capillaries were replaced every single day. Nutrient intake was calculated by measuring the length of the remaining food with a digital calliper (accuracy ± 0.02 mm). Ten plastic chambers without any fly inside were set up to measure the evaporation level of both diets. Food consumption was corrected for evaporation by subtracting the evaporation value from the consumption value and then the result was converted to volume of diet eaten (µl). The hydrolysed yeast used in this study was assumed to be the only source of protein available for the flies, containing approximately 62.1% protein and 1% carbohydrate. Final food intake (µg) was calculated based on these values.

2.4. No-choice experiment

The no-choice experiment set up was similar to the choice experiment's one, except that flies were fed with two capillaries of a same single diet. The two chosen diets were a balanced diet with a protein-to-carbohydrate ratio of 1:5 (i.e., PC 1:5) and a carbohydrate-biased diet with PC 1:8. Food consumption was measured daily for nine days.

2.5. Survivorship on single diets

Groups of 20 flies injected with PBS, or heat-killed bacteria or live

bacteria, were kept in 1.25-L cages (10 cm x 10 cm) and fed single diets (i.e., PC 1:8 or PC 1:5). Food was provided to the flies by soaking a cotton ball with 2 ml liquid diet that was placed in a 30 mm Petri dish. Injections with PBS and heat-killed bacteria were included as treatments. We excluded naïve flies from this experiment because following the no-choice experiment we found that naïve flies did not die on either diet throughout the duration of the feeding experiment. Dead flies were counted and removed daily from the cages and food was replaced daily. During the experiment, each treatment was performed in 3 replicates (total n = 60) simultaneously.

2.6. Bacterial load

We measured the bacterial load of flies fed on single diets. To ensure that *S. marcescens* was not present in the digestive tract of Q-flies in our colony, 10 flies were crashed individually in 100 µl PBS, 50 µl of the solution was plated onto Nutrient Agar supplemented with Tetracycline (30 µg/ml, Sigma), and incubated at 26 °C for 24 h. We did not observe any *S. marcescens* colonies. The bacterial colony forming unit was estimated only in flies injected with live bacteria. The bacterial load was measured 1, 3, 5, 7, and 9 days post-infection for each diet treatment. Individual flies were homogenized using a micro pestle in 1.5 ml Eppendorf tubes containing 200 µl of PBS, serially diluted to 1:10, 1:100, 1:1000 in PBS. A volume of 20 µl from each dilution was plated onto Nutrient Agar supplemented with Tetracycline, incubated at 26 °C and the bacterial load measured after 24 h.

2.7. Total lipid measurement

The method for total lipid measurement was described in (Ponton et al., 2015). After injection, flies from different diet treatments (PC 1:5 versus 1:8) were collected 1, 3, 5 and 7 days post-infection for body lipid measurement. Flies were snapped freeze at −20 °C, bodies transferred into 6 ml glass tubes (Sigma-Aldrich) and dried in a drying oven (Binder) at 50 °C for 48 h. Dry body weight was measured using a microbalance (Sartorius, accuracy ± 0.001 mg). The glass tubes were then filled with 1 ml of Chloroform (Sigma-Aldrich Cat. No 650498) and tightly closed to avoid any evaporation. Chloroform was discarded and replaced every day, for 3 days. Fly bodies were dried again for 48 hrs at room temperature in a fume hood (Dynasafe), and for 48 hrs at 50 °C in a drying oven (Binder). Lipid-free dried bodies were weighted using a microbalance. The percentage of body lipid was calculated by subtracting the lipid-free dry body weight to the initial dry body weight and dividing the difference by the initial body weight multiplied by 100. Six to 10 replicates (i.e, individual flies) were performed for each diet treatment at each time point (see raw data for more detail about the replicates number).

2.8. Statistical analyses

All analyses were performed in R version 3.2. 2 (R Development Core Team, 2011) and data were plotted using SPSS (version 23). Normality was assessed using Shapiro–Wilk test where applicable. Homoscedasticity was assessed using Levene's test.

To analyse cumulative and daily intakes of protein and carbohydrate, we fitted generalized linear models (GLM) with Gamma error distribution to test for the effects of treatment, time and their interaction. Because many flies did not eat the first day of the experiment, the first data point was the cumulative intakes for the two first days. P-values are given from F-tests. We used Tukey post hoc tests to determine differences in mean total cumulative protein and carbohydrate intakes (i.e., cumulative intakes for nine days) between treatments. The same analytical procedure was applied to the data of the no-choice experiment, diet was added as a third factor in the model.

To analyse flies' survival in the no-choice experiment, we fitted GLM with Gamma error distribution to test for the effects of treatment, diet and their interaction. We fitted GLM with Binomial distribution to test for the effects of time, treatment, diet and their interactions on flies' survival rate for the 6 first days and the last 3 days of the no-choice experiment. P-values are given from χ^2 tests.

To analyse bacterial load, we fitted GLM with Gamma error distribution to test for the effects of time, diet and their interaction, followed by a Tukey post hoc test with significance level of 0.05. P-values are given from F-tests.

Percentage of body lipid was square root transformed and analysed using a GLM with Gaussian distribution to test for the effects of diet, treatment, time and their interactions. P-values are given from F-tests. We used Tukey post hoc tests to determine differences in mean percentage of body lipid between treatments.

3. Results

3.1. Infection by Serratia marcescens induced a shift in diet choice and a decrease in macronutrient intake

Cumulative intakes of protein and carbohydrate were significantly influenced by the interaction between treatment and time (GLM; Protein, Treatment: $F_{3,736} = 178.850$, $P < 0.001$; Time: $F_{1,735} = 334.060$, $P < 0.001$; Treatment × Time: $F_{3,\ 732} = 20.589$, $P < 0.001$; Carbohydrate, Treatment: $F_{3,737} = 32.780$, $P < 0.001$; Time: $F_{1,736} = 1053.480$, $P < 0.001$; Treatment × Time: $F_{3,733} = 22.291$, $P < 0.001$). Overall, flies injected with live *S. marcescens* ate cumulatively less protein and carbohydrate than naïve flies, and flies injected with either PBS or heat-killed bacteria (Fig. 1). These observations were time-dependent since food intake was different between all injected flies (PBS, heat-killed bacteria and live bacteria) only from the second day of the experiment (Fig. 1). At the end of the experiment, flies injected with live bacteria ingested on average 54% less protein and 26% less carbohydrate than flies from the other 3

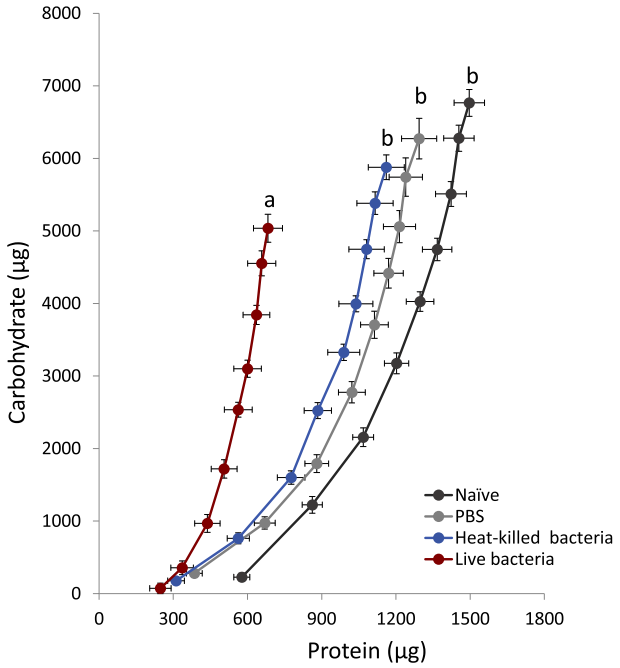

Fig. 1. Cumulative macronutrient intakes in the diet choice experiment. Cumulative carbohydrate and protein intake for naïve flies (n = 19), flies injected with PBS (n = 17), heat-killed bacteria (n = 22) or live bacteria (n = 13) was measured for 9 consecutive days (each data point representing 1-day increment). Plots show means and standard error of the means for protein (horizontal) and carbohydrate (vertical) cumulative intake. Letters indicate significant differences in carbohydrate and protein intakes between infection treatments assessed by Tukey post hoc tests.

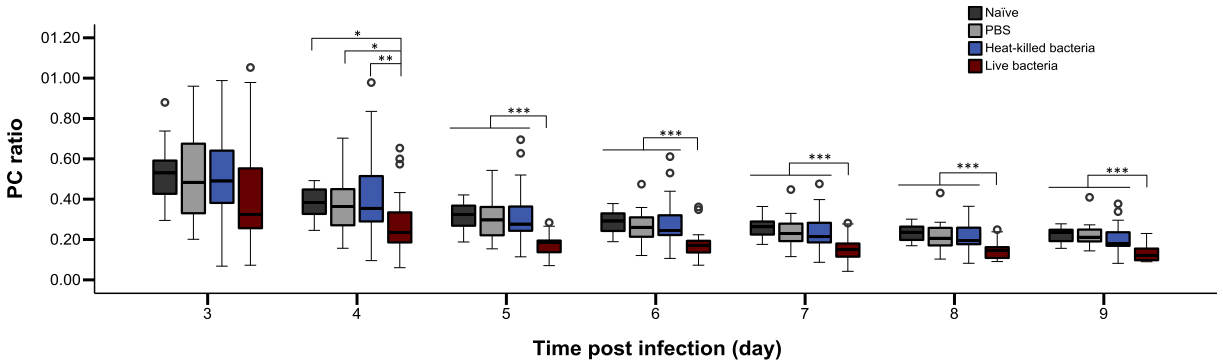

Fig. 2. Dietary protein-to-carbohydrate (PC) ratio in the diet choice experiment. Daily dietary PC ratio ingested by naïve flies (n = 19), flies injected with PBS (n = 17), heat-killed bacteria (n = 22) or live bacteria (n = 13). Box-and-whisker plots show median and interquartile range (IQR); whiskers show either 1.5 × IQR of the lower and upper quartiles or range. Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001).

treatments (Fig. 1).

The PC ratio (i.e., ratio between cumulative intake of protein and cumulative intake of carbohydrate) was calculated from day 3 to leave enough time for flies to balance their diet. We found a significant effect of time and treatment on the PC ratio (GLM; PC ratio, Treatment: $F_{3,559} = 15.407$, $P < 0.001$; Time: $F_{1,558} = 270.722$, $P < 0.001$; Treatment × Time: $F_{3,555} = 1.244$, $P = 0.293$). Overall, the PC ratio ingested by flies injected with live bacteria was lower than the PC ratio ingested by flies from the other treatments, except for the first time point (Fig. 2). Furthermore, for all treatments, PC ratio tended to decrease during the first 5 days of the experiment (Fig. 2). At day 9, infected flies ingested a PC ratio of around 1:8, while flies from the other treatments ingested a PC ratio of around 1:5 (Fig. 2).

3.2. Infected flies had a greater survival rate when fed a carbohydrate-biased diet

Longevity of flies injected with PBS, heat-killed and live bacteria was followed when flies were restricted to two single diets (PC 1:8 or PC 1:5) for 9 days. We found a significant effect of the interaction between treatment and diet on flies' survival (GLM; Treatment: $F_{2,313} = 272.619$, $P < 0.001$; Diet: $F_{1,315} = 2.853$, $P = 0.092$; Treatment × Diet: $F_{2,311} = 4.180$, $P = 0.016$). Flies injected with live bacteria died at a significant greater rate compared to those injected with PBS and heat-killed bacteria (Fig. 3). However, while diet did not influence the survival rate of flies injected with PBS and heat-killed bacteria, flies injected with live bacteria (Fig. 3) survived better the infection on PC 1:8 compared to PC 1:5 (Fig. 3). This effect was nevertheless dependent on time. Survival rate for infected flies fed PC 1:8 was higher only during the first 5 days post-infection (GLM for effect of diet on the survival rate of infected flies that died during the first 5 days of the experiment, df = 1, Residuals df = 109, Residuals deviance = 109.65, P = 0.0031; Fig. 3). No effect of diet was detected after 5 days post-infection (GLM for effect of diet on the survival rate of infected flies that died after the first 5 days of the experiment, df = 1, Residuals df = 84, Residuals deviance = 104.7, P > 0.05, Fig. 3).

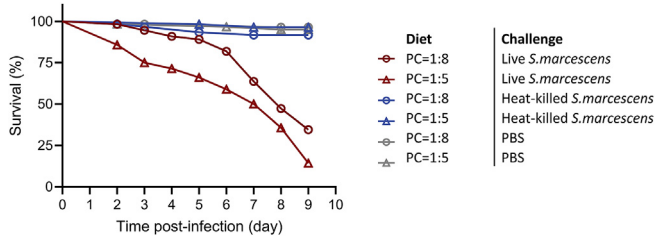

Fig. 3. Effect of diet on flies' survival after injection. Percentage of survival of flies injected with live bacteria (red), heat-killed bacteria (blue) or PBS (grey) when fed two diets varying in the protein-to-carbohydrate (PC) ratio: PC 1:8 (circle) vs PC 1:5 (triangle). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Flies balanced their carbohydrate intake when restricted to single diets

Total protein and carbohydrate cumulative intakes were measured for flies restricted to PC 1:8 or PC 1:5. Treatment and diet significantly influenced protein intake (GLM; Treatment: $F_{3,89} = 19.084$, $P < 0.001$; Diet: $F_{1,88} = 122.15$, $P < 0.001$; Treatment × Diet: $F_{3,85} = 2.703$, $P = 0.051$), whereas, only treatment significantly influenced total cumulative carbohydrate intake (GLM; Treatment: $F_{3,89} = 16.257$, $P < 0.001$; Diet: $F_{1,88} = 0.502$, $P = 0.481$; Treatment × Diet: $F_{3,85} = 0.700$, $P = 0.555$). Overall, flies infected with live bacteria ate a lower amount of protein and carbohydrate compared to naïve flies and flies injected with either PBS or heat-killed bacteria, regardless of diets (Fig. 4A and B). Interestingly, flies from all treatments fed PC 1:5 ingested more protein than those fed PC 1:8 (Fig. 4A). However, we did not observe any significant difference in the ingested amount of carbohydrate between flies fed PC 1:5 and PC 1:8 (Fig. 4B).

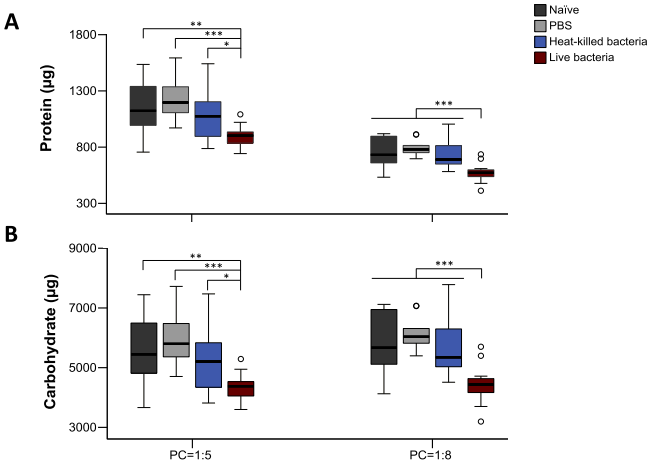

Fig. 4. Macronutrient intake in the no-choice experiment. Quantity of protein (A) and carbohydrate (B) consumed by flies for 9 days when restricted to a single diet, PC 1:8 (naïve flies n = 11, flies injected with PBS n = 11, heat-killed bacteria n = 12 or live bacteria n = 15) or PC 1:5 (naïve flies n = 11, flies injected with PBS n = 11, heat-killed bacteria n = 11 or live bacteria n = 12). Box-and-whisker plots show median and interquartile range (IQR); whiskers show either 1.5 × IQR of the lower and upper quartiles or range. Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001).

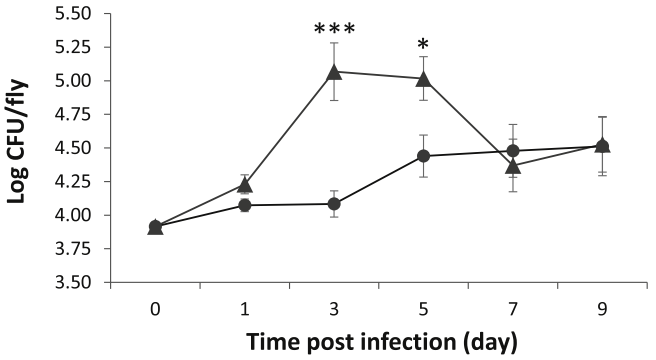

Fig. 5. Bacterial load of infected flies in the no-choice experiment. Bacterial load of flies injected with live bacteria when restricted to a single diet [PC 1:8 (circle) or PC 1:5 (triangle)]. Bacterial load was measured at 0, 1, 3, 5, 7 and 9 days post-infection. Plots show means of bacterial loads and standard error of the means (n = 10 to 11 flies). Significant differences of bacterial load between two diets were assessed by Tukey post hoc tests (*P < 0.05, ***P < 0.001).

3.4. Bacterial load was lower on carbohydrate-biased diet over the first 5 days post-infection

Bacterial load was followed for infected flies restricted to PC 1:8 or PC 1:5 at 0, 1, 3, 5, 7 and 9 days post-infection. The interaction between diet and time influenced bacterial load (GLM; Time: $F_{1,118} = 31.233$, $P < 0.001$; Diet: $F_{1,119} = 10.286$, $P = 0.001$; Time × Diet: $F_{1,117} = 8.942$, $P = 0.003$). Interestingly, bacterial load was significantly lower in flies fed PC 1:8 compared to flies fed PC 1:5 at 3 and 5 days post-infection (Fig. 5). In flies fed PC 1:5 diet, bacterial load increased from day 3 to day 5 post-infection (post hoc test, $P < 0.05$) but then decreased at day 7 and 9 post-infection (post hoc test, $P > 0.05$; Fig. 5). Bacterial load in flies fed PC 1:8 remained unchanged until day 3 post-infection (post hoc test, $P > 0.05$, Fig. 5), and increased from day 5 post-infection (post hoc test, $P < 0.05$, Fig. 5).

3.5. Lipid body reserves were lower in infected flies

Body lipid reserves were measured in flies injected with live bacteria, heat-killed bacteria, PBS as well as in naïve flies at 1, 3, 5 and 7 days post-injection. Flies were restricted to either a PC 1:8 or a PC 1:5 diet. We found a significant effect of the interactions between diet and treatment and diet and time on the percentage of body lipid reserves (Table S2). Percentage of lipid was lower in flies injected with live *S. marcescens* compared to the other treatments for both diets (Fig. 6). However, while the percentage of body lipid was not different between naïve flies and flies injected with either PBS or heat-killed bacteria on PC 1:8, flies injected with heat-killed bacteria had a lower percentage of body lipid than naïve flies on a PC 1:5 diet (Fig. 6). In naïve flies and flies injected with either PBS or live bacteria, we found no significant difference in the percentage of body lipid between diet treatments (Tukey post hoc test $P > 0.05$, Fig. 6). Flies injected with heat-killed bacteria showed, however, a lower percentage of body lipids on PC 1:5 compared to PC 1:8 (Tukey post hoc test $P < 0.05$, Fig. 6). Finally, while the percentage of body lipid (data pooled for all treatments) was similar between the different time points on diet PC 1:8; it tended to decrease at day 7 on PC 1:5 diet (Fig. S1).

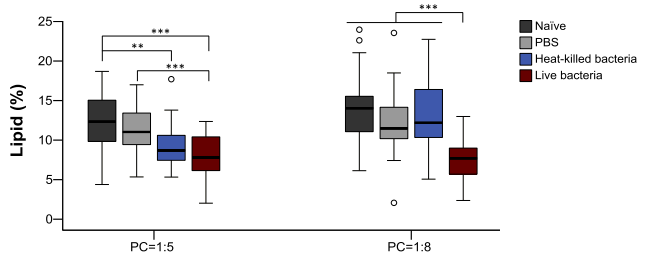

Fig. 6. Effect of diet and infection treatment on percentage of body lipid under diet restriction. Percentage of body lipid for naïve flies, flies injected with PBS, or heat-killed bacteria or live bacteria when restricted to a single diet (PC 1:8 or PC 1:5). Box-and-whisker plots show median and interquartile range (IQR); whiskers show either 1.5 × IQR of the lower and upper quartiles or range. Tukey post hoc tests were used for pairwise comparisons (**P < 0.01, ***P < 0.001).

4. Discussion

Using experimental designs derived from the nutritional geometry approach (Simpson and Raubenheimer, 2012), this experiment investigated the separate and interactive effects of macronutrients on the relationship between nutrition and survival to bacterial infection in *B. tryoni* flies. Flies injected with live bacteria ingested less protein and, to a smaller extent, less carbohydrate than naïve flies and flies injected with either PBS or heat-killed bacteria. Hence, infected flies not only decreased their total food intake but also shifted their feeding towards a carbohydrate-biased diet. Remarkably, flies survived better the infection and contained the growth of pathogen populations the first days following the injection when restricted to a carbohydrate-biased diet. Stimulation of immunity through injection of PBS or heat-killed bacteria also induced changes in flies' feeding but only one day after injection. Lipid reserves of infected flies were lower compared to the other treatments, while, in flies injected with heat-killed bacteria, lipid reserves were reduced compared to naïve flies in a diet dependent manner. This suggests a link between carbohydrate metabolism, storage of fatty acid and immunity in *B. tryoni*.

4.1. Infection modulates feeding behaviour in adult flies

Flies injected with live bacteria selectively decreased their macronutrient intake, what we might call "selective anorexia". They shifted their dietary PC ratio towards a carbohydrate-biased diet. These results are consistent with previous findings showing that infected caterpillars of the arctiinae moth *Grammia incorrupta* increased their intake of low-protein food when immune challenged through exposition to tachinid fly parasitoids or beads injection (Mason et al., 2014). Also, adult female *Drosophila melanogaster* have been shown to shift their nutrient balance to a carbohydrate-biased food after infection with the bacterium *Micrococcus luteus* (Ponton et al., 2018). Different modifications of feeding have however been found in caterpillars of other insect species that, when infected, ingested a protein-biased diet (Povey et al., 2009, 2014; Lee et al., 2006). Considering the complexity of host-pathogens interactions, changes in feeding might be pathogen-, host-, or pathogen/host interaction-dependent. Pathogens used in previous studies included virus, gram-positive bacteria, fungi and macroparasite, while here flies were infected with a gram-negative bacterium. Different pathogens activate different immune pathways (reviewed in (Lemaitre and Hoffmann, 2007)) in the host and might therefore interfere differently with host physiology. The timing of the infection (i.e., different developmental stages) might be another important factor influencing host nutritional response. Because nutritional needs change throughout development whereby juveniles select a diet to optimise growth and adults to optimise lifetime reproduction (Simpson et al., 2012), the relationship between infection, nutrition and physiology is expected to be different.

When restricted on single diets, flies from all treatments adjusted their food consumption and maintained carbohydrate intake, which implies that flies ingested different amounts of protein (see also Lee et al., 2008). Thompson et al. (2005) have shown that naïve *Manduca sexta* larvae ate an excess of food in order to ingest an adequate amount of a deficient nutrient as the dietary nutrient ratio shifts. In contrast,

infected larvae ate lower amounts of food in order to avoid consuming a surplus of specific nutrients (Thompson et al., 2005). Our data are different since, when given a low-protein diet (i.e., PC 1:8), uninfected flies ate a lower amount of protein and consume the same amount of carbohydrate than flies fed a more balanced diet (PC 1:5). In contrast, when infected flies were offered a PC 1:5 diet, they ingested an excess of protein to achieve the same quantity of carbohydrate as flies fed PC 1:8 diet. We suggest that the benefits of maintaining an adequate carbohydrate intake is prioritised over the costs of eating an excess of protein when flies are infected.

4.2. The macronutrient balance affects survival and pathogen load

Our results confirm that, in addition to the quantity of macronutrients eaten, the ratio of macronutrients in the diet is an important modulator of host response to bacterial infections. Flies restricted to a carbohydrate-biased diet (i.e., PC 1:8) survived better the infection than flies fed a more balanced diet (i.e., PC 1:5) the first days following bacterial injection. Given that infected flies ingested similar amount of carbohydrate on both diets, the excess in protein eaten by individuals on PC 1:5 diet might partly account for the increase in mortality rate [but see Wang et al. (2016)]. We also found that the number of pathogens in infected hosts fed PC 1:8 was maintained while it increased in flies fed PC 1:5 during the first 5 days following the infection. The average time required to contain or clear an infection is an important factor that determines the probability to survive an infection (Duneau et al., 2017) and might explain why flies fed PC 1:8 had a higher survival rate.

It has been previously shown that survival after infection and pathogen resistance in infected animals can be age-dependent (Fang et al., 2010; Apidianakis and Rahme, 2009; Klinge et al., 2009; Reinarz et al., 1971). In our study, flies from all treatments tended to decrease their dietary PC ratio with time. Older flies might therefore ingest a diet that would provide them with a better survival after infection. These results raise the question of whether the links between nutrition and survival rate after infection are age-dependent. Furthermore, our results are limited since despite an increased survival for infected individuals on carbohydrate-biased diet, flies did not reach sexual maturity and were, therefore, enable to reproduce. Future studies should focus on the interaction between infection, immune stimulation and nutrition for adult flies at different biological (i.e., different days after emergence) and physiological (i.e., mature versus non-mature flies) ages.

4.3. Involvement of carbohydrate metabolism in infection

In this experiment, we found that the bacterial load of flies fed PC 1:8 did not increase the first 3 days following the infection. Two main hypotheses can explain this result: (1) pathogens required more protein to grow or (2) the host's immune system was more stimulated on carbohydrate-biased diets and contained the infection. Noticeably, hosts might have resisted the infection but were not able to totally clear the pathogens from their bodies. While we do not have data to support any of these hypotheses, it has been shown that Drosophila flies fed a carbohydrate-biased diet display an up-regulation of anti-microbial peptides expression (Ponton et al., 2018). Activation of immune transcription can be independent of immune pathways through molecules involved in energy balance and nutrients sensing (Becker et al., 2010). For instance, the transcription factor FOXO can activate antimicrobials (Becker et al., 2010), and interact with key molecules across metabolic pathways such as Target of rapamycin (TOR) and AMP-Activated Protein Kinase (AMPK) complexes (Hay, 2011) that respond to nutrient balance (Simpson and Raubenheimer, 2009). The interactions between nutrition and immunity can therefore be mediated directly through nutrient signalling pathways.

In addition, infected flies had lower lipid energetic reserves than naïve and PBS-injected individuals (Fig. 6). It is interested to notice that percentage of body lipid in flies injected with heat-killed bacteria was similar to that in naïve flies when fed PC 1:8 but showed a decrease when fed PC 1:5, having lipid reserves similar to flies infected with live bacteria (Fig. 6). Wasting process is therefore dependent on diet quality when immunity is stimulated by injection of dead pathogens. Percentage of body lipid reserves was however not different between diet treatments when flies were injected with live bacteria, suggesting that stimulation of immunity through an active infection might affect the metabolism and energetic reserves in a way that the host cannot compensate through feeding.

The Insulin/IGF (IIS) signalling pathway plays an essential role in the regulation of glucose metabolism (review in (Saltiel and Kahn, 2001) and interacts with immunity (McCormack et al., 2016; Libert et al., 2008; Evans et al., 2008). Interestingly, Dionne et al. (2006) have shown that suppressing wasting via inhibition of FOXO activity promoted survival of infected flies (Dionne et al., 2006). Given that infected flies fed a PC 1:8 diet survived at a higher rate compared to those fed a PC 1:5 diet, we predicted a lower level of wasting in flies fed a PC 1:8 diet the few days following the infection. However, we did not detect any difference in the percentage of body lipid between two diet treatments. The higher survival rate in Dionne's work might be linked to tolerance immune responses because no difference in bacterial load was observed (Dionne et al. (2006). In our study, the higher survival rate in flies fed diet PC 1:8 might, in contrast, be linked to higher resistance.

In conclusion, infected flies modulated their macronutrient intake and selected a diet that decreased the negative effects of the infection on their survival to contain the growth of pathogen population, at least for the first few days following the infection. We call this modulation of feeding following infection “nutritional self-medication”.

Author contribution

HD and FP conceived and designed the experiment. HD, VM and STT conducted experiment. HD, VM, STT and FP analysed the data and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.05.002.

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Supplementary materials

Figure

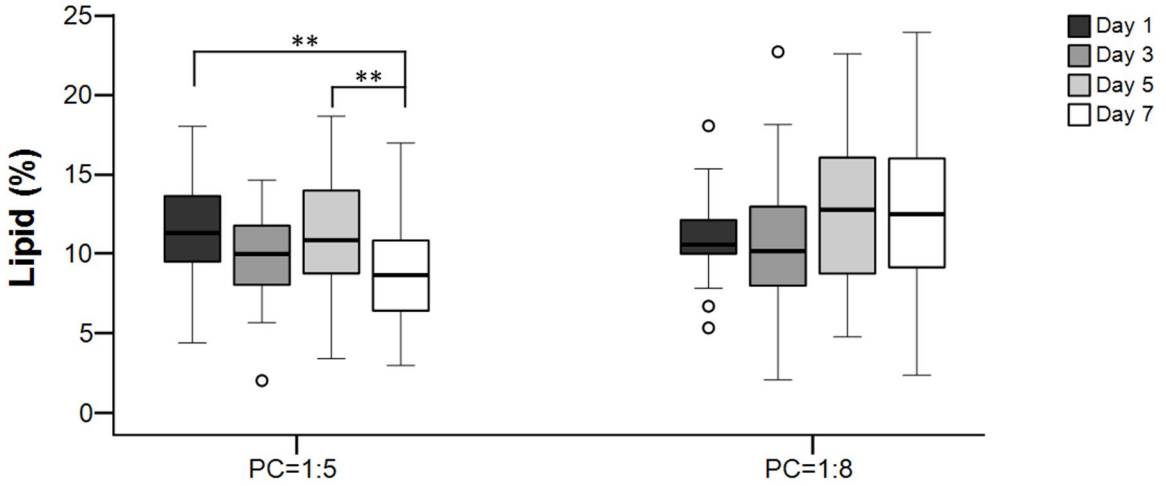

Figure S1. Percentage of body lipid in the no-choice experiment. Percentage of body lipid for flies fed two single diets (i.e., PC 1:5 or PC 1:8). Box-and-whisker plots show median and interquartile range (IQR); whiskers show either 1.5 × IQR of the lower and upper quartiles or range. Tukey post hoc tests were used for pairwise comparisons (**P < 0.01)

Tables

Table S1. Results from Generalized linear model (GLM) to access effects of treatment, diet, time and their interactions on daily total macronutrient intake in the no-choice experiment. Significant effects are shown in bold.

Tested effect	df	Residuals df	F	P
Treatment	3	1010	35.209	**<0.001**
Time	1	1013	195.584	**<0.001**
Diet	1	1014	5.928	**0.015**
Treatment × Time	3	1003	17.904	**<0.001**
Treatment × Diet	3	1006	1.806	0.144
Time × Diet	1	1009	9.718	**0.002**
Treatment × Diet × Time)	3	1000	1.764	0.152

Table S2. Results from GLM to access effects of treatment, diet, time and their interaction on percentage of body lipid reserve. Significant effects are shown in bold.

Tested effect	df	Residuals df	F	P
Treatment	3	310	28.830	**<0.001**
Time	1	313	0.097	0.755
Diet	1	314	16.847	**<0.001**
Treatment × Time	3	303	2.494	0.060
Treatment × Diet	3	306	3.251	**0.022**
Time × Diet	1	309	17.601	**<0.001**
Treatment × Diet × Time	3	300	0.452	0.716

Chapter 3

Effects of larval macronutrient balance on adult pathogen resistance and life history traits in fruit fly

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Sex-specific effects of protein-rich diet promotes higher bacterial load and lower survival after infection in a polyphagous fly

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Author's contributions

H.D. designed and carried out the experiments and performed the statistical analyses. I.L. carried out the experiments, collected and analysed the data. S.K. carried out the experiments and analysed the data. A.T.T. helped in setting up the experiments. J.M. performed the statistical analyses. F.P. conceived and designed the experiments and performed the statistical analyses. All authors contributed to the writing of the manuscript and gave final approval for publication.

Competing interests

The authors have no competing interests to declare.

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Abstract

Nutrition is a central factor influencing immunity and resistance to infection, but the extent to which nutrition during development affects adult responses to infections is poorly documented. Our study investigated the effects of larval diet on the survival, pathogen load, and food intake of adult fruit flies after bacterial septic infection. Effects on development and adult physiological traits were also measured. We found a strong sex-specific effect of larval diet on survival post-infection; with a greater survival rate and lower bacterial load for infected females- but not infected males- fed the sugar-biased larval diet compared with females fed protein-biased larval diet. This might be linked to the different macronutrient intakes that were observed between infected males and females when given a choice between diets. While macronutrient intake was comparable between larval diets for infected males, infected females reared on the sugar-biased larval diet ingested less food than those on the protein-biased larval diet. Larval diet also affected developmental time, adult body weight and body reserves. Our results highlight the importance of the interaction between developmental diet and sex on infection outcomes in adulthood.

Keywords: Bacterial load, Infection, Larval diet, Macronutrient, Nutrition, Resistance

1. Introduction

Environmental conditions during development can influence many aspects of adult phenotype and fitness. In humans, under- or over-nutrition at foetal stage can increase predisposition to metabolic disease at adult stage (see review in [1]). In birds, when conditions are unfavourable during early development, growth and adult immunity are affected ([2–6], but also see [7,8]). In holometabolous insects, the nutritional resources acquired at larval stage are crucial for survival during metamorphosis [9,10]. When faced with food restrictions and unbalanced diets at larval stage, insects show a delayed development [11–16], lower adult body size [12,14,16–20], and a decreased lifespan [21,22] (but see [11] where *Drosophila* adult life span is increased when fed a protein-restricted diet during larval stage). Adult reproductive performance is also affected by larval diet restriction with lower courtship levels, lower number of matings, lower investment in reproductive organs and a decrease in the total offspring production compared to individuals that were fed *ad libitum* at larval stage [14,16,17,20,23–25].

Nutrition during development affects not only life history traits but also resistance to infection. In insects, a food shortage at larval stage strongly reduces immune activity as observed in adult damselflies, *Lestes viridis* [26]. Similarly, nutritional deprivation during development decreases the melanisation of foreign bodies in adults *Anopheles gambiae* [27]). Larval food quality (i.e., yeast-to-sugar ratio) also influences the expression of antimicrobial peptide genes (Diptericin A and Metchnikowin) in adult *Drosophila*, with an increase in expression level when the yeast-to-sugar ratio in the larval diet was increased [28]. The effects of larval diet on immune-challenged adults have recently been described in female mosquitoes *Anopheles coluzzii* [29]. However, it remains unclear which aspect of the larval diet (quality or quantity) induces differences in pathogen load and survival rate of the infected individuals. Interestingly, in hemimetabolous insects, Kelly et al. (2013) found a sex-specific effect of juvenile diet on the survival of adult crickets, *Gryllus texensis,* when infected with the pathogenic bacterium, *Serratia marcescens* using defined diets (i.e., low- or high-protein diets) [30]. However, crickets were fed the same experimental diet at both juvenile and adult stage, and, therefore, it is difficult to decipher between the effects of juvenile and adult diet on adult resistance in this system.

Studies on the effects of diet during development on adult immunity and resistance to infection, especially when adults are immune challenged, are still scarce or have been only partial for three main reasons. First, diet manipulations during development have focused on the quantity of food available and there have been very few studies investigating how diet quality might affect adult resistance. While juveniles might be food-restricted when in high density or very poor

environmental conditions, the nutritional environment is more likely to vary in the composition of nutrients that generates unbalanced food. Second, the nutritional requirements of immune responses can be different between sexes [31,32]. Hence, it remains to be tested if pathogen resistance in both adult males and females is affected similarly by the juvenile diet. Lastly, individuals might compensate for unfavourable juvenile nutritional conditions by shifting their diet at adult stage. Exploring the effects of variation in the quality of juvenile diet on adult nutritional responses and body energetic reserves would give us insights into the extent of the effects of developmental diet on adult physiology.

In the present study, we manipulated the dietary macronutrient ratio (yeast-to-sugar ratio, YS ratio) during the development of the holometabolous fruit fly *Bactrocera tryoni*. We investigated the effects of larval diet manipulation on (i) bacterial load, survival and food intake of adult males and females following septic infection with the pathogenic bacterium *S. marcescens* [33], (ii) developmental traits (i.e., percentage of egg hatching, percentage of pupation, percentage of emergence and developmental time), and (iii) physiological traits (i.e., adult body weight, body lipid and protein reserves). Given that the nutritional requirements of immune responses are sex-specific [31,32], we predicted that larval diet would affect pathogen resistance of males and females differently. Moreover, a positive correlation between expression level of adult antimicrobial peptide genes and protein level in larval food observed in *Drosophila melanogaster* [28] leads us to predict that adult from a larval high protein diet might resist better the infection.

2. Materials and Methods

(a) Fly stock

Fly stock was maintained on a gel-based diet at larval stage [34] and a 1:3 ratio of hydrolysed yeast (MP Biomedicals Cat. no 02103304) to sugar (CSR® White Sugar) (YS) provided separately at adult stage. Flies were reared in a controlled environment room under the conditions of 25^0C and 65% humidity, with a 12-hour light/dark cycle at Macquarie University ARC Centre for Fruit Fly Biosecurity Innovation (North Ryde, NSW, Australia).

(b) Diet preparation

Three larval diets varying in the yeast-to-sugar (YS) ratio were prepared (ingredients are listed in Table S1). The balanced diet is considered to be an optimized diet that has been used routinely to rear *B. tryoni* in the laboratory (YS 1.67:1) [34]. We manipulated the relative amount of yeast and sugar according to [35] to generate unbalanced diets including a “protein- biased diet” (YS 5:1) and a “sugar-biased diet” (YS 1:3.4). These diets have been found to modulate the development and adult traits of *B. tryoni* flies [35]. All ingredients were mixed into warm water and the final

volume (250 mL) achieved by adding distilled water. Citric acid was added to adjust the pH of the diet solution to 3.5 at room temperature. Diet plates were prepared by pouring 25 mL of larval gel diet into 100 mm Petri dishes and stored at room temperature.

(c) Bacterial infection

Serratia marcescens (ATCC 13880, Thermo Scientific) was inoculated into 5 mL of sterile Nutrient Broth (Oxoid, CM0001) and incubated overnight (approximately 16 hrs) at 26^0C with shaking at 200 rpm. The bacterial culture was centrifuged at 10,000g at 4^0C for 2 min. The supernatant was discarded, and the bacterial pellet washed twice using 1X Phosphate Buffered Saline (PBS) (Sigma-Aldrich, Cat. No P4417) to remove any trace of the medium. The bacterial pellet was resuspended to a target concentration of $OD_{600} = 0.025$ in sterile PBS.

One-day-old flies were cold anesthetized in a -20^0C freezer for 2 minutes and then placed on a Petri dish on a dry bath (Product code: MK20) at -10°C. Injections were performed using a 10µL syringe (NanoFil) connected to a microinjector (World Precise Instrument) with a delivery speed of 50 nL/sec. A volume of 0.2 µL of the bacterial solution, yielding a dose of approximately 1680 cells, was injected into the fly's coxa of the third right leg. PBS-injected (i.e., sham-injured) flies were used as injured controls.

(d) Bacterial load

Bacterial load was measured in infected flies with females and males being individually crushed in 100 µL of PBS, and serially diluted to 1:10 and 1:100. A volume of 10 µL from each dilution was plated onto Nutrient Agar supplemented with 30 µg/mL Tetracycline (Sigma) [33] and incubated at 26^0C for 48 h. Given that the bacterial load during the early phase of the infection might be an important factor determining the survival of infected hosts [36], bacterial load was measured at 6, 24, 48 and 96 h post-infection (PI). We performed 10 replicates (i.e., 10 individual flies of each sex) for each larval diet at each time point. *Serratia marcescens* was not present in our fly stock. This was checked by crushing individual flies (12 individual flies of each sex) in 100 µl PBS and 25 µl of the solution was plated onto Nutrient Agar supplemented with 30 µg/mL Tetracycline, and incubated at 26 °C for 48 h. We did not observe any *S. marcescens* colonies.

(e) Survival after infection

Adult flies (males and females) were injected with either PBS or live bacteria. Injected flies were then maintained in groups of 25 in 1.25-liter cages (10 cm × 10 cm × 12.5 cm) and provided with food and water *ad libitum*. Dead flies were counted and removed daily from the cages. We initially limited the experimental time to 4 days post-infection (PI) in which we measured

bacterial load. The low mortality rate after 4 days PI led us to extend the timeframe of the survival experiment until 15 days PI. We ran 3 replicates per larval diet.

(f) Food intake

The method to measure and calculate food intake was previously described in [33]. Briefly, individual flies (males and females) were fed sugar (CSR® White Sugar) and yeast (MP Biomedicals Cat. No. 02103304) solutions provided separately in two 30 µL capillaries. The final concentration of both solutions was 160 g/L. The hydrolysed yeast used in this study was the only source of protein available to the flies, containing approximately 62.1% protein and 1% carbohydrate. Final macronutrient (i.e, protein and carbohydrate) intakes (µg) were calculated based on these values.

(g) Development traits

Eggs were collected from the fly stock colony for 2 h using an ovipositional device that consisted in a plastic bottle with numerous puncture holes and filled with 30 mL of water to maintain humidity. Using a paint brush, 100 eggs were transferred to a black filter paper previously soaked in distilled water before being placed onto the diet surface. The plates were then covered with their lids and kept under controlled environment laboratory conditions during the experiment. Nine replicates per larval diet were performed simultaneously. The number of unhatched eggs was counted 4 days post-seeding under a stereomicroscope. After counting, the black filter paper and unhatched eggs were removed from the diet plates. Lids of the diet plates were removed seven days post-seeding; plates were placed on 50 mL of autoclaved fine vermiculite to allow larvae to jump outside the plates and pupate. The total number of pupae was then recorded and pupae placed into partially netted 12.5 litre plastic cages for emergence. The number of pupae that did not emerge was recorded over four days.

(h) Adult dry body weight

One-day-old adult flies were stored at -20^{0}C. Carcasses were then dried at 55^{0}C for 48 h (Binder drying oven). Dry weight was measured using a microbalance (Sartorius, accuracy ±0.001mg) for 30 individual flies of each sex per larval diet.

(i) Adult body lipid reserves

Body lipid reserves were extracted in three, 24-h changes of chloroform as previously described [33]. At the end of the third chloroform wash, lipid-free bodies were re-dried and re-weighed to calculate lipid content. We performed 15 replicates (i.e, 15 individual flies of each sex) per larval diet. It is noted that our method using chloroform to extract lipid might also extract lipoproteins.

However, the proportion of lipids extracted by this method might be largely greater than that of lipoproteins and thus it may not influence the results.

(j) Adult body protein reserves

After lipid extraction, fly bodies were crushed in 300 µl 0.1M NaOH and centrifuged at 8000 rpm for 30 sec. 100 µl of supernatant was collected in new Eppendorf tubes and diluted 1:10 time. Five µl of the diluted solutions were transferred to 96-well plates and allowed to react with 200 µl of Bradford reagent (Sigma-Aldrich). Plates were incubated for 5 min at room temperature, and absorbance was measured at 595 nm using a spectrometer (Eppendorf). We ran 15 biological replicates (i.e., 15 individual flies of each sex) per larval diet. Each sample was run in 3 technical replicates. The Bradford assay was calibrated using a standard curve generated from six different concentrations of IgG from rabbit serum (0.2, 0.15, 0.1, 0.05, 0.025 and 0 µg/µl) (Sigma-Aldrich, Cat # I5006).

(k) Statistical analyses

We fitted Generalized Linear Models (GLM) with quasibinomial distribution to test for the effects of larval diet on percentage of egg hatching, percentage of pupation, percentage of emergence, body lipid reserves, and body protein reserves. A GLM with Gaussian distribution was used to assess (i) the effects of time, diet and their interactions on bacterial load of infected flies with the bacterial number being log-transformed to achieve normal distribution; (ii) the effects of larval diet, time, injection treatment and their interactions on the cumulative intake of carbohydrate and protein as well as protein-to-carbohydrate (PC) ratio of adult flies; and (iii) the effect of larval diet on the dry body weight. P-values were obtained from F tests.

To test for the effects of larval diet, time PI and their interaction on the survival of adult flies during the first 4 days and the first 15 days PI, we fitted a GLM with binomial distribution, with P-values obtained from χ^2 tests. Because only few flies died in the PBS-injected treatments (6 dead flies out of 439), the analysis was performed only for infected flies (i.e., injected with live bacteria).

Following GLM, Student–Newman–Keuls (SNK) tests were used for multiple comparisons. Significances were considered at $P<0.05$. Analyses of adult traits were performed separately for males and females. Statistical analyses were performed in R [37], and plots were done using BM SPSS Statistics 25.0.

3. Results

(a) The interaction between larval diet and time after infection influences adult bacterial load

The bacterial load of both females and males was significantly influenced by the interaction between larval diet and time PI (Table S2). At 24 h PI, the bacterial load was higher in flies fed the protein- and sugar-biased larval diets compared to flies fed the balanced diet (Fig.1). At 96 h PI, the bacterial load of flies fed the protein-biased diet was greater compared to that of flies fed either the balanced or the sugar-biased larval diet (Fig.1).

(b) The sugar-biased larval diet positively influences the survival of females after infection

During the first 4 days PI, the survival of both infected females and males was only affected by time PI (Table S3). During 15 days PI, however, we observed a significant effect of larval diet on the survival of infected females- but not infected males (Table S3). At 15 days PI, survival of infected females from the sugar-biased larval diet was significantly higher compared to those from the balanced and protein-biased larval diets (Fig.1).

(c) The nutritional choice of adult immune-challenged flies is influenced by the larval diet in a sex-dependent manner

As expected, the interaction between time and injection treatment significantly affected the cumulative carbohydrate intake of females and males (Table S4). We also found a significant effect of the interaction between larval diet and injection treatment on cumulative carbohydrate intake of males- but not females (Table S4). In males, carbohydrate amounts ingested by infected individuals were comparable between larval diets at 4 days PI (Table S5, Fig. 2). However, sham-injured males from the sugar-biased larval diet ingesting less carbohydrate compared to sham-injured individuals from the protein- biased and balanced larval diets (Table S5, Fig. 2). In females, carbohydrate amounts ingested by infected individuals fed the sugar-biased and balanced larval diets were lower in comparison with infected females on protein-biased larval diet (Table S5, Fig. 2). When females were sham-injured, carbohydrate intake was lower in individuals fed sugar-biased larval diet relative to those fed either protein-biased or balanced larval diet (Table S5, Fig. 2).

The interactions between time and injection treatment as well as larval diet and injection treatment significantly affected the cumulative protein intake of females and males (Table S4). At 4 days PI, lower protein intakes were observed in infected females on the balanced and sugar-biased larval diets relative to those reared on the protein-biased larval diet (Table S5, Fig. 2). Sham-injured females on the sugar-biased larval diet ate less protein than those on the balanced and protein-biased larval diets (Table S5, Fig. 2). In males, despite a trend of infected individuals eating less protein when fed protein-biased diet al larval stage, we could not detect any significant difference

in protein intake between larval diets in both sham-injured and infected males at 4 days PI (Table S5, Fig. 2).

The ingested PC ratio of both females and males were only affected by the injection treatment (Table S4). At 4 days PI, the sham-injured flies ingested a higher PC ratio (PC~1:2) compared to infected flies (PC~1:3) (Fig S1).

(d) Larval sugar-biased diet negatively affects developmental time but not percentage of egg hatching, percentage of pupation and percentage of emergence.

Larval diet did not influence percentage of egg hatching (GLM, $F_{(2,24)}$=0.01, P=0.905), percentage of pupation (GLM, $F_{(2,23)}$=1.940, P=0.166) and percentage of emergence (GLM, $F_{(2,23)}$=1.111, P=0.346). Percentage of egg hatching and percentage of pupation were around 90% in all larval diets (Fig. S2). Percentage of emergence observed across larval diets was around 98% (Fig. S2). Larval diet had however a significant effect on the egg-to-adult developmental time (GLM, $F_{(2,23)}$=33.896, P<0.001). Developmental time was longer for the larvae fed the sugar-biased larval diet (~20 days) compared to those fed the balanced and protein-biased larval diets (~18 days) (Fig.3).

(e) Unbalanced larval diets negatively influence adult body weight

The dry body weight of both females and males was significantly influenced by larval diet (GLM; Female: $F_{(2,77)}$ = 5.321, P=0.007; Male: $F_{(2,86)}$ = 6.842, P=0.002). The adults from both the sugar and protein-biased larval diets were lighter than adults from the balanced larval diet (Fig.4A).

(f) Adult body lipid composition is higher on larval sugar-biased diet

Larval diet significantly influenced the body lipid reserves of both females and males (GLM; Female: $F_{(2,35)}$ = 8.074, P=0.001; Male: $F_{(2,37)}$ = 21.313, P<0.001). Body lipid reserves were greater in adult flies from the sugar-biased larval diet compared to flies from the balanced and protein-biased diets (Fig. 4B).

(g) Sex-dependent effects of larval diet on adult body protein composition

Larval diet composition significantly influenced the body protein reserves of males but not females (GLM; Male: $F_{(2,37)}$=7.454, P=0.002); Female: $F_{(2,35)}$=1.564, P=0.223). The body protein reserves of males from the sugar-biased diet were lower compared to those of males from the protein-biased and balanced larval diets (Fig. 4C).

4. Discussion

In the present study, we examined the effects of the macronutrient composition of larval diet on adult resistance to infection as well as on some developmental and physiological traits. When adult flies were challenged with *S. marcescens,* we observed a higher bacterial load in both males and females fed protein-biased larval diet; however, only females showed a lower survival rate after infection. This result suggests a sex-dependent effect of larval diet on pathogen resistance, which is likely to be linked to a difference in food intake observed between both sexes after infection. Indeed, while amounts of protein and carbohydrate consumed by infected males were comparable between larval diets, infected females from the sugar-biased larval diet ingested lower amounts of carbohydrate and protein compared to individuals from the protein-biased diet.

Larval diet also influenced development and body energetic composition. A longer developmental time was observed in larvae fed the sugar-biased diet. In adults, body weight was lower in flies fed either the protein- or sugar- biased larval diets relative to those fed the balanced diet. In addition, flies fed the sugar-biased larval diet had higher body lipid reserves at adulthood. Effect of larval diet on body protein reserves was restricted to males, with protein reserves being lower in males fed the sugar-biased larval diet compared to those fed the other larval diets.

(a) *Larval diet influences adult pathogen resistance and macronutrient intake following infection with the bacterium S. marcescens*

The bacterial load of infected flies was influenced by the nutritional conditions experienced at larval stage. This might be due to two reasons. First, pathogens require energy for growing and thus, the allocation of within-host energy reserves is essential to this process [38–42]. Here, we found that the total body reserves of protein and/or lipid were modulated by larval diets. Second, early-life nutrition affects host immune responses at later developmental stages which might modulate the number of pathogenic cells in the host. In mosquitoes and *Drosophila*, poor larval conditions (i.e., starvation or protein-restriction) have been shown to alter the expression of adult immune-related genes [28, 43]. Finally, larval diets might affect the bacterial growth dynamics of infected hosts. According to Duneau et al., (2017), growth of within-host bacterial population can be divided into three phases [36]. In a first early phase, the bacterial population grows exponentially without being controlled. In a second phase, hosts start controlling bacterial proliferation. In the third phase, if the hosts successfully control growth of pathogen, they are likely to survive the infection. In contrast, if the hosts fail to control their pathogen

population, they will die. It is likely that flies fed the carbohydrate-biased diet started controlling their pathogen population earlier (i.e., 24hr PI) than flies fed the protein-biased and balanced larval diet (i.e., 48hr PI). Furthermore, flies fed the protein-biased larval diet likely failed to control their bacterial load as it increased again at 96hr PI.

Despite differences in the bacterial load between larval diets, the survival of infected flies was similar during the first 4 days post-infection. Infected flies might only start dying when the number of pathogenic bacteria reaches a certain level defined as "bacterial load upon death" [36], and this level may not have been reached only few days after the infection. At 15 days PI, however, infected females that were reared on the sugar-biased larval diet survived at a greater rate compared to those kept on the protein-biased larval diet. Several hypotheses can explain these results. First, a lower bacterial load at the early stage of infection might have slowed down the time required to reach the "bacterial load upon death" in infected females fed the sugar-biased larval diet. Second, it has been previously shown that infected flies reduce total food intake and shift diet choice towards a carbohydrate-biased diet which promote their survival after infection [33]. Here the ingested PC ratio was not different between larval diets; however, infected females from the sugar-biased larval diet ingested lower amounts of carbohydrate and protein relative to those fed protein-biased larval diet, which might explain their greater survival after infection (positive effects of anorexia on host defence (see [44–49]). Third, female flies fed the sugar-biased larval diet might have invested more in immunity at the expense of other life-history traits. This is supported by studies in birds and insects showing the negative correlation between immune function and developmental time [50–52]. For instance, female moths reared on sugar-biased larval diet allocate a lower proportion of their mass to the development of their ovaries (i.e., invest less on reproduction) compared to those on protein-biased diets [14]. Hence, female flies fed the sugar-biased larval diet potentially allocate more resources to their immunity over their developmental time and reproduction. Also, fat content serves as a crude estimate of the size of the fat body, the major immune responsive tissue in insects [53]. Given that body lipid reserves were higher in flies fed the sugar-biased larval diet, we speculate that their immune system was more efficient at fighting the infection. However, it is noted that higher percentage of lipid may simply reflect that cells have more fat droplets. Further explorations of the immune status, reproductive output, and longevity of females kept on the different larval diets would provide insights into the effects of juvenile nutritional environment on resource allocation and potential trade-offs between immune traits and other life-history traits.

Early-life environment, including nutrition, can be used to predict future adult environment, and individuals can develop proper behaviours to respond to environmental challenges in later developmental stages [54]. The higher survival rate of infected females on sugar-biased larval diet might suggest that unbalanced diets at larval stage can act as a cue for higher disease risk in adulthood and flies on this diet might invest more in immune defence (see also [29–30, 55-57]).

Unlike what we observed in female flies, the survival rates of infected adult males were comparable between larval diets despite a difference in bacterial load. While it is difficult at this stage to explain why the effect of larval diet on survival rate is sex-specific, previous studies have shown that the diet composition can influence immunity differently in males and females. For instance, in fruit flies and crickets while both protein and carbohydrate intakes affect phenoloxidase (PO) activity and encapsulation ability in females, only protein intake influences these immune traits in males [31,32]. Also, the magnitude of the effects of larval diet composition on PO activity and nitric oxide production in adults can be different between male and female mosquitoes, *Aedes aegypti*, with a stronger effect in females [58]. In parallel, larval diet can influence differently other adult traits in both sexes. In butterfly, *Melitaea cinxia*, larval food stress negatively affects reproductive output of females- but not males [59]. Also, developing on a high yeast diet only benefits life span of female *Drosophila* [60]. While there are evidences of sex-specific effects of diet on adult traits, the physiological mechanisms at the basis of these differences remain to be investigated.

(b) ***Effect of larval diet on development traits***

Percentage of pupation and percentage of emergence were not different between flies from the different larval diets, suggesting that the unbalanced larval diets chosen here do not affect larval ability to survive metamorphosis. However, we found a significant effect of the larval diet on egg-to-adult development time, and interestingly, this was only observed in larvae fed the sugar-biased diet. This result is in accordance with previous observations in the forest tent caterpillar, *Malacosoma disstria* [14,61]. The negative effect of the sugar-biased larval diet on development time is likely caused by the low protein level. Insect growth and metamorphosis are controlled by the insulin/target of rapamycin (TOR) signalling pathways [62–64], which are triggered by high levels of amino acids [65,66]. Indeed, inhibition of the amino acid transporter gene has been shown to result in a lengthened development time [67]. Measuring the insulin/TOR activity in larvae fed the experimental diets would give insights into their

metabolic state and deepen our understanding of the links between low-protein feeding and delayed developmental time.

5. Conclusion

The present study highlights sex-specific effect of the larval diet composition on the survival of adult fruit flies after infection. Protein-rich larval diet promoted higher bacterial load and lower survival in female flies. The effects of larval diet on the developmental and physiological traits of adults were also demonstrated. Better understanding the carry-over effects of environmental conditions experienced in early life on individuals life history traits and populations dynamics is a central question in Ecology [68]. Answers to this question can further assist protection of endangered species, especially in the context of dramatic environmental changes that potentially lead to decreases in food availability and changes in food composition as well as introductions of infectious disease to wildlife populations [69,70].

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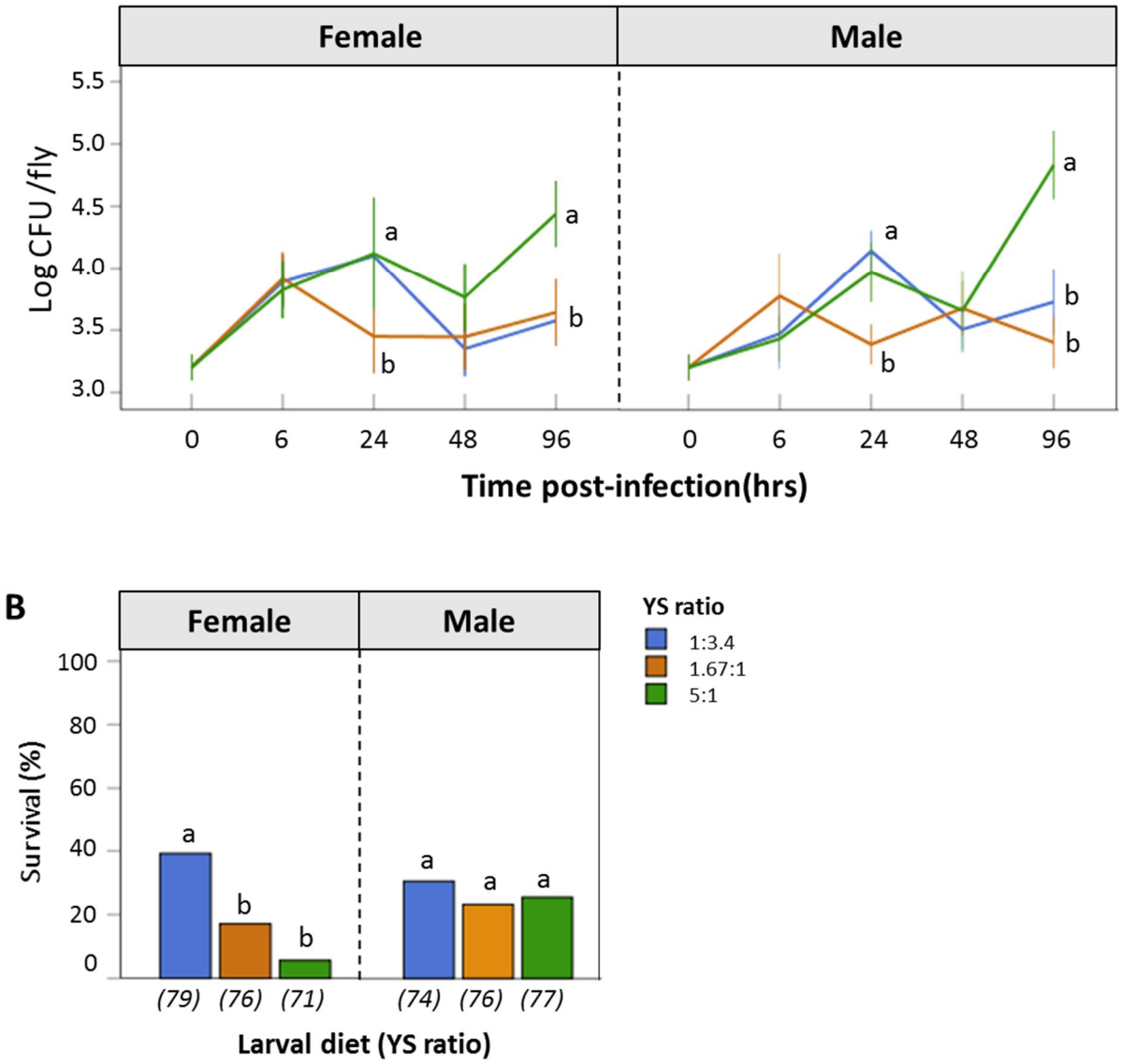
572 **Figures**



573

574 **Figure 1.** Effect of larval diets on flies' ability to resist the infection.

575 (A) Effect of larval diets on bacterial loads measured at 0, 6, 24, 48 and 96 h post-infection.

576 Larval diets: Blue - YS 1:3.4 (sugar-biased); Orange - YS 1.67:1 (balanced); Green - YS 5:1

577 (protein- biased). Data are given as mean ± SE. (B) Effects of larval diets on the survival rate of

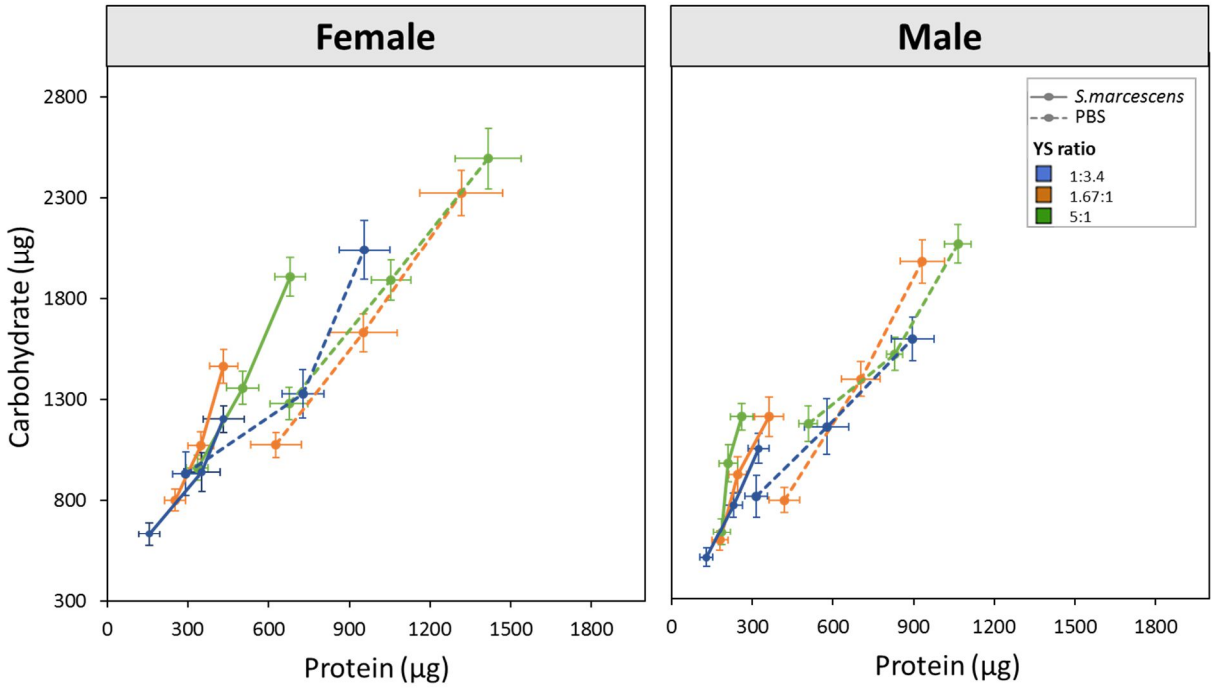
578 infected females and males at 15 days post-infection. Numbers in parentheses below the bars

579 indicate number of flies in each treatment. In both (A) and (B), different letters indicate

580 significant differences between larval diets, assessed by SNK test at $P<0.05$.

581

582



583

584 **Figure 2.** Cumulative macronutrient intakes of male and female flies. Macronutrient intakes
585 were measured for 4 consecutive days for infected and sham-injured male and female flies fed
586 three larval diets varying in the yeast-to-sugar ratio (YS ratio). Each data point represents 1-day
587 increment. Plots show means and standard errors of the means for protein (horizontal) and
588 carbohydrate (vertical) cumulative intake.

589

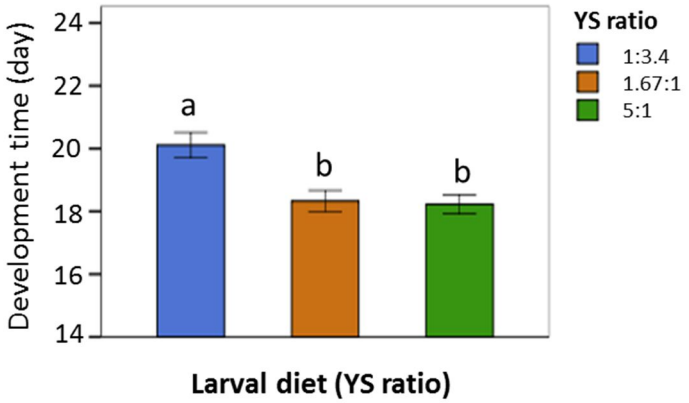


Figure 3. Effect of larval diet on egg-to-adult developmental time. Effect of larval diets varying in the yeast-to-sugar ratio (YS ratio) on egg-to-adult developmental time. Bars show means of developmental time, and error bars show standard errors. Different letters indicate significant differences between larval diets, assessed by SNK test at $P<0.05$.

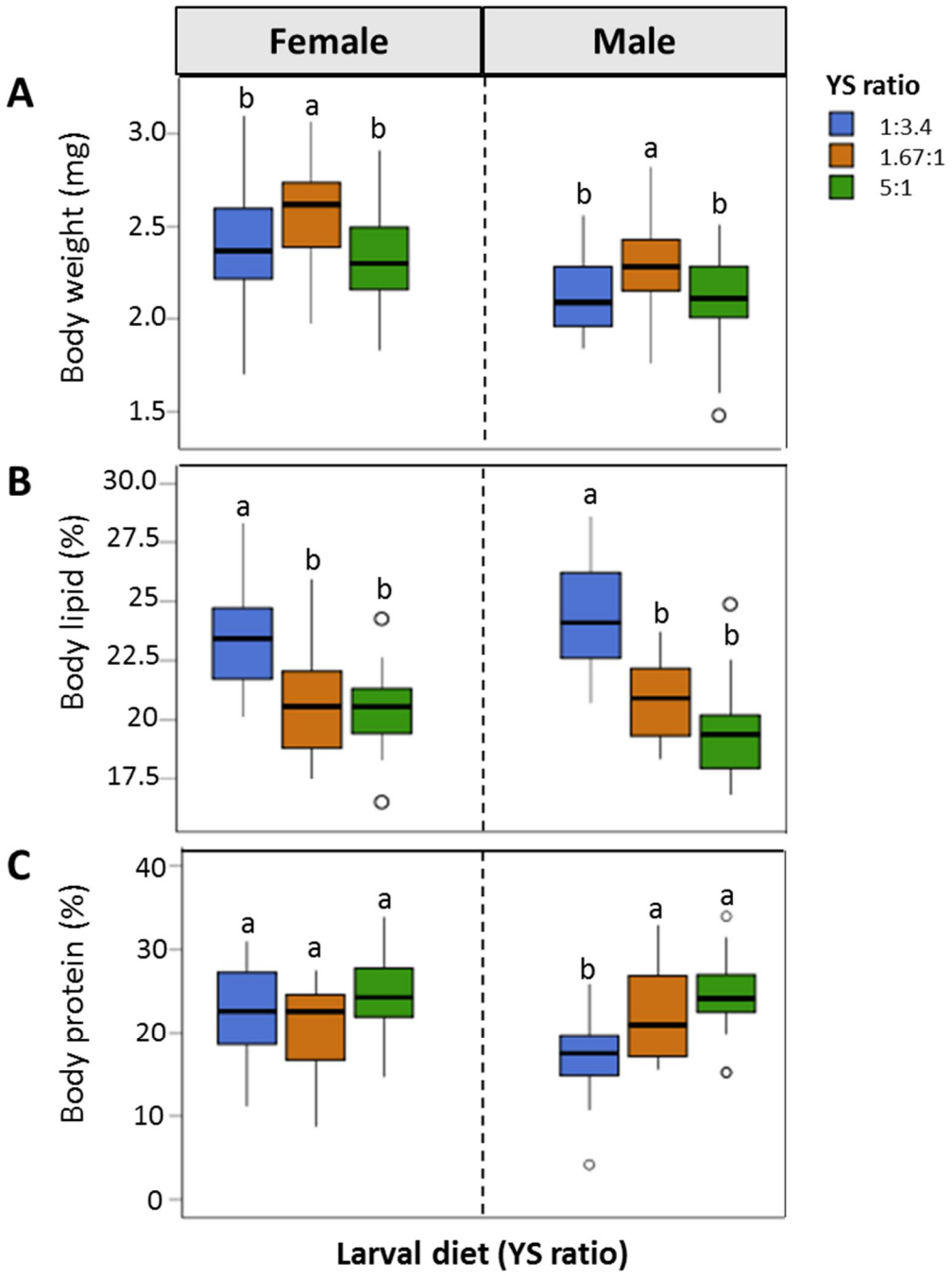


Figure 4. Effect of larval diet on adult body weight, lipid and protein body reserves. Body weight (A), lipid body reserves (B) and protein body reserves (C) were measured in male and female flies fed larval diets varying in the yeast-to-sugar ratio (YS ratio). Different letters indicate significant differences between larval diets, assessed by SNK test at $P<0.05$.

603 **Supplementary materials**

604 **Table S1.** Recipes of the larval diets used in the study

Ingredient	Diet		
	Protein-biased	Standard	Carbohydrate-biased
Brewer yeast (g)	58.33	43.8	15.91
Sugar (g)	11.67	26.2	54.09
Agar (g)	2.5	2.5	2.5
Nipagin (g)	0.375	0.375	0.375
Sodium benzoate (g)	0.375	0.375	0.375
Wheat Germ oil (µl)	375	375	375
Water	Final to 250 ml		
pH	Adjust to pH of 3.5 using Citric acid		

605

606

Table S2. Statistical analyses for bacterial loads of infected flies. Binomial GLM results to test effects of larval diet, time post-infection and their interaction on bacterial loads of infected males and females. Significant differences are in bold.

Tested effect	Female						Male					
	Df	Deviance	Resid. Df	Resid. Dev	F	P	Df	Deviance	Resid. Df	Resid. Dev	F	P
NULL	-	-	149	221.889	-	-	-	-	149	225.173	-	-
Larval diet	2	16.31252	147	205.577	9.442	**<0.001**	2	14.682	147	210.491	11.168	**<0.001**
Time	4	60.1616	143	145.415	17.411	**<0.001**	4	56.246	143	154.244	21.391	**<0.001**
Larval diet * Time	8	28.79641	135	116.619	4.167	**<0.001**	8	65.501	135	88.743	12.455	**<0.001**

Table S3. **Statistical analyses for the survival of infected flies.** Binomial GLM results to measure for the effects of larval diet, time post-infection (PI) and their interaction on survival of infected males and females during the first 4 days and during the first 15 days post-infection. Significant differences are in bold

	Tested effect	Female					Male				
		Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
4 day PI	NULL	-	-	225	144.283	-	-	-	226	130.623	-
	Larval diet	2	0.398	223	143.885	0.814	2	7.590	224	123.033	0.072
	Time PI	1	73.435	222	70.450	**<0.001**	1	65.902	223	57.130	**<0.001**
	Larval diet *Time	2	<0.001	220	70.450	1.000	2	<0.001	221	57.130	1.000
15 days PI	NULL	-	-	225	225.626	-	-	-	226	217.431	-
	Larval diet	2	14.849	223	210.778	**<0.001**	2	0.401	224	217.031	0.531
	Time PI	1	173.321	222	37.457	**<0.001**	1	126.257	223	90.774	**<0.001**
	Larval diet *Time	2	<0.001	220	37.457	1.000	2	<0.001	221	90.774	1.000

Table S4. Statistical analyses for cumulative intakes of carbohydrate and protein, and protein-to-carbohydrate ratio. GLM results to measure for the effects of larval diet, time, injection treatment and their interactions on the cumulative intakes of carbohydrate and protein and protein-to-carbohydrate ratio of females and males during the first 4 days post-infection. Significances are in bold.

	Tested effect	Female						Male					
		Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
Carbohydrate intake	NULL	-	-	240	87764458	-	-	-	-	245	64999206	-	-
	Larval diet	2	6498751	238	81265707	27.757	**0.000**	2	4050077	243	60949129	22.518	**0.000**
	Treatment	1	15877635	237	65388072	135.630	**0.000**	1	15507586	242	45441542	172.442	**0.000**
	Time	1	35997755	236	29390317	307.500	**0.000**	1	21862101	241	23579441	243.103	**0.000**
	Larval diet *Treatment	2	74616	234	29315701	0.319	0.727	2	549010	239	23030432	3.052	**0.049**
	Larval diet *Time	2	322778	232	28992923	1.379	0.254	2	388601	237	22641831	2.161	0.118
	Treatment*Time	1	1989098	231	27003825	16.991	**0.000**	1	1404686	236	21237145	15.620	**0.000**
	Larval diet *Treatment*Time	2	195737	229	26808088	0.836	0.435	2	193653	234	21043492	1.077	0.342
Protein intake	NULL	-	-	240	46007077	-	-	-	-	245	25900543	-	-
	Larval diet	2	1877894	238	44129183	12.129	**0.000**	2	702057	243	25198486	12.261	**0.000**
	Treatment	1	15497939	237	28631244	200.192	**0.000**	1	12413135	242	12785351	433.563	**0.000**
	Time	1	8349475	236	20281769	107.853	**0.000**	1	3999001	241	8786350	139.676	**0.000**
	Larval diet *Treatment	2	688907	234	19592862	4.449	**0.013**	2	478249	239	8308101	8.352	**0.000**
	Larval diet *Time	2	50231	232	19542631	0.324	0.723	2	11333	237	8296768	0.198	0.821
	Treatment*Time	1	1781960	231	17760671	23.018	**0.000**	1	1557390	236	6739378	54.396	**0.000**
	Larval diet *Treatment*Time	2	32572	229	17728099	0.210	0.810	2	39834	234	6699544	0.696	0.500
Protein-to-carbohydrate ratio	NULL	-	-	242	14.359	-	-	-	-	245	11.455	-	-
	Larval diet	2	0.029	240	14.331	0.292	0.747	2	0.025	243	11.430	0.371	0.691
	Treatment	1	2.803	239	11.528	57.431	**0.000**	1	3.342	242	8.088	99.411	**0.000**
	Time	1	0.029	238	11.499	0.596	0.441	1	0.001	241	8.087	0.042	0.838
	Larval diet *Treatment	2	0.136	236	11.363	1.393	0.250	2	0.024	239	8.063	0.359	0.699
	Larval diet *Time	2	0.072	234	11.291	0.734	0.481	2	0.060	237	8.003	0.892	0.411
	Treatment*Time	1	0.006	233	11.285	0.131	0.717	1	0.037	236	7.966	1.098	0.296
	Larval diet *Treatment*Time	2	0.011	231	11.274	0.111	0.895	2	0.100	234	7.866	1.490	0.227

Table S5. Statistical analyses for total intakes of carbohydrate and protein. Results from SNK tests to assess differences in total carbohydrate and protein intake between larval diets at 4 days post-infection. Significant differences are indicated with different letters. Larval diets: YS 1:3.4 (sugar-biased); YS 1.67:1 (balanced); YS 5:1 (protein-biased).

		Carbohydrate							Protein					
		Diet	Mean	SE	N	min	max	SNK test	Mean	SE	N	min	max	SNK test
Female	*S.marcescens*	1.67:1	1464.185	84.393	14	893.6621	2015.015	b	433.0726	51.692	14	104.9852	802.9541	b
		1:3.4	1203.221	65.416	12	747.6106	1525.354	b	432.7489	77.321	12	134.4618	963.5077	b
		5:1	1906.397	97.078	19	1214.523	2695.015	a	679.9354	57.612	19	142.0314	1141.785	a
	Sham-injected	1.67:1	2324.954	110.940	12	1630.154	2838.4	a	1315.98	154.823	12	264.8618	2233.477	a
		1:3.4	2041.161	148.986	12	1409.519	2831.2	b	955.5386	94.100	12	300.0006	1430.585	b
		5:1	2494.667	150.900	12	1726.4	3742.4	a	1417.528	122.610	12	587.3231	1838.4	a
Male	*S.marcescens*	1.67:1	1200.677	98.693	16	462.1541	1913.477	a	362.254	53.290	16	99.56953	843.3231	a
		1:3.4	1044.585	73.181	18	587.324	1735.918	a	323.59	39.987	18	104.1237	703.2	a
		5:1	1201.674	65.157	15	768.8615	1616.616	a	260.0125	42.681	15	16.73906	592.2462	a
	Sham-injected	1.67:1	1967.775	110.385	12	1376.862	2411.323	a	932.2154	82.341	12	372.5538	1296.246	a
		1:3.4	1583.998	108.850	9	1071.815	2106.892	b	896.2941	78.136	9	559.8154	1237.354	a
		5:1	2056.41	98.891	12	1509.785	2626.708	a	1064.574	50.238	12	850.9541	1507.323	a

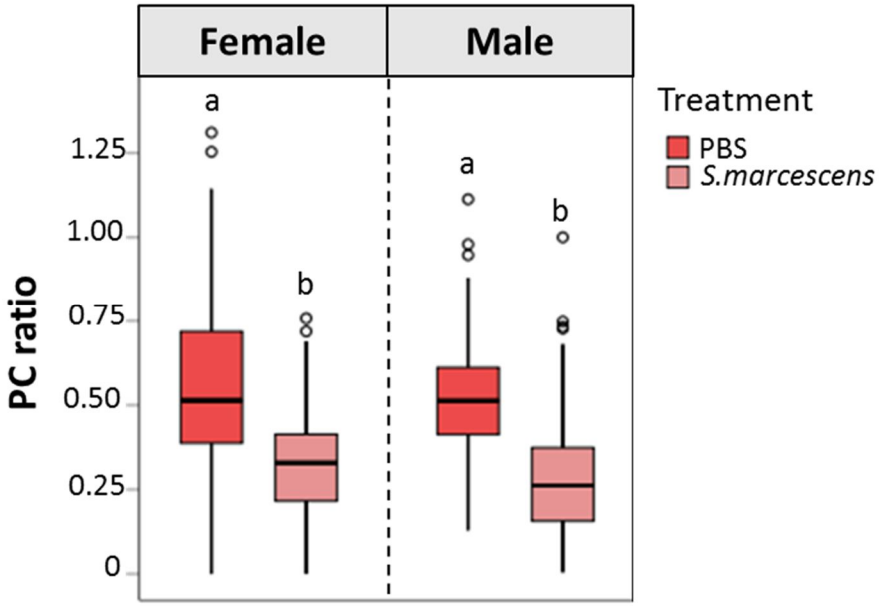


Figure S1. Effect of injection treatment on dietary protein-to-carbohydrate ratio (PC ratio) of female and male flies at 4 days post-infection. Adult females and males were either sham-injured (i.e., PBS) or injected with live bacteria (*Serratia marcescens*). Individuals were offered a choice between a yeast and sugar solution. Food intakes were measured for 4 days post-infection. Different letters indicate significant differences between larval diet treatments, assessed by SNK test at $P<0.05$.

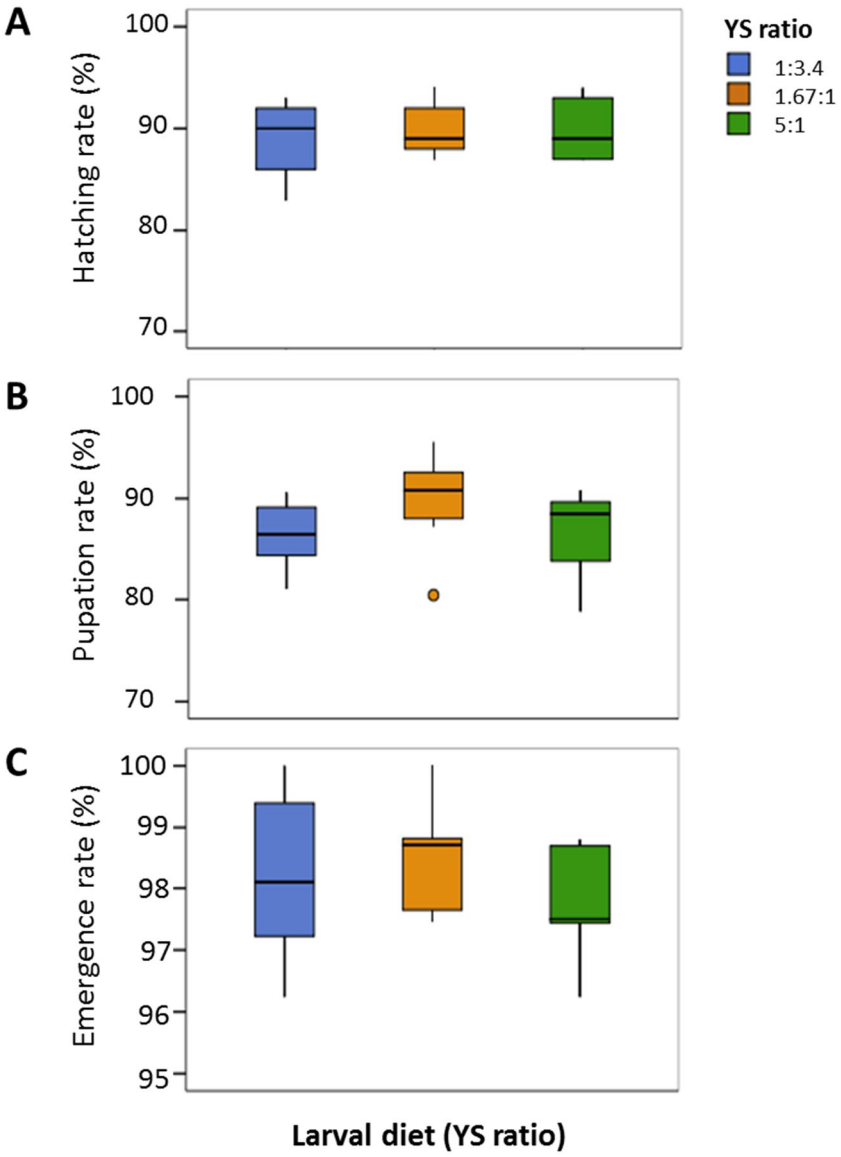


Figure S2. Effect of larval diet on developmental traits. Larval diet had no effect on the percentage of egg hatching (A), percentage of pupation (B) and percentage of emergence (C. Significance was assessed by SNK test and was considered at $P<0.05$.

Chapter 4

Transgenerational effects of macronutrient balance on offspring pathogen resistance and life history traits in fruit fly

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ABSTRACT

Environmental conditions experienced by parents can influence next generations, with parental nutritional conditions playing an important role in shaping offspring phenotypes. However, our understanding of transgenerational effects of parental diet on the pathogen resistance of offspring is poorly documented. In this study, we addressed this knowledge gap by manipulating the diet quality of parents (i.e., mother, father, or both) and measuring transgenerational effects on offspring development and survival after pathogen immune challenge. Our results showed that maternal, but not paternal, diet had a sex-specific effect on offspring resistance, with sons (but not daughters) from mothers fed either carbohydrate- or protein-biased diets being less resistant to infection than sons from mothers fed balanced diets. Interestingly, this effect was reversed when the diet of both parents was manipulated, whereby sons from parents fed either carbohydrate- or protein-biased diets had higher survival upon pathogen infection than sons from parents fed balanced diets. Diet manipulation of mother, father, or both parents had no effect on offspring developmental traits with the exception of egg hatching rate, which decreased when mothers were fed a protein-biased diet. The results emphasize the complexity of the transgenerational effects of parental diet on offspring pathogen resistance and provide insights into the long-lasting implications of parental nutrition to future generations' fitness.

INTRODUCTION

Environmental conditions experienced by parents can have long-term effects on the next generations (Burton and Metcalfe, 2014). It has been observed in both vertebrate and invertebrate that parents in good physiological conditions (e.g., good nutritional condition or favourable temperature) tend to produce offspring with higher fitness relative to the offspring of parents with low physiological conditions (Grafen 1988; Mousseau and Fox, 1998; Qvarnström and Price, 2001; Bonduriansky and Head, 2007). However, the environmental conditions experienced by parents can also serve as cue to predict conditions that offspring will experience (i.e., "transgenerational phenotypic plasticity") (West-Eberhard 2003;Pigliucci 2001; Whitman and Agrawal, 2009). The outcome of transgenerational phenotypic plasticity depends on whether environmental conditions experienced across generations are stochastic or predictable (Burgess and Marshall, 2014). Offspring can gain benefits from acquiring information of their potential environment from their parents when parental environment is a reliable predictor of the offspring environment [e.g., (Gluckman and Hanson, 2004; Galloway and Etterson, 2007; Marshall and Uller, 2007; Bateson, Gluckman and Hanson, 2014; Burgess and Marshall, 2014; Murren *et al.*, 2015). In contrast, a mismatch between the offspring' and parents' environments can potentially reduce offspring fitness [see e.g., (Gluckman, Hanson and Beedle, 2007; Raubenheimer, Simpson and Tait, 2012); but also (Uller, Nakagawa and English, 2013) showing only weak evidence for greater offspring performance when parental and offspring environments are matched compared with when they were mismatched].

Amongst key environmental factors experienced by parents, nutrition is likely an essential factor shaping the phenotype of the offspring (Bonduriansky and Day, 2009). In insects, parental diet influence many offspring traits including body size and body weight (Vijendravarma, Narasimha and Kawecki, 2010; Triggs and Knell, 2012; Valtonen *et al.*, 2012); developmental time (Vijendravarma, Narasimha and Kawecki, 2010; Valtonen *et al.*, 2012; Matzkin *et al.*, 2013); egg-to-adult survivorship (Prasad *et al.*, 2003); egg hatching success (Bonduriansky, Runagall-McNaull and Crean, 2016); reproduction (Matzkin *et al.*, 2013) and body composition (Buescher *et al.*, 2013; Matzkin *et al.*, 2013). Parental diet can also affect the next generation at the genomic level through a decrease in the number of ribosomal RNA and changes in expression levels of metabolic and chromatin-defined genes, which potentially result in cancers and diabetes in the offspring (Buescher *et al.*, 2013; Öst *et al.*, 2014; Aldrich and Maggert, 2015)

Immunity and resistance to infections are important physiological traits that are known to be influenced by manipulations of the parental diet (Table S1). For instance, in Indian meal moths *Plodia interpunctella*, parents fed poor foods had offspring with lower phenoloxidase activity

and haemocyte counts compared with offspring from parents fed normal diets (Triggs and Knell, 2012). Moreover, in *Drosophila melanogaster*, expression of immune genes were lower in sons of fathers fed low-protein diets compared to sons of fathers fed high-protein diets (Zajitschek, Zajitschek and Manier, 2017). When offspring were immune-challenged, parental diet also influenced their immune response levels with consequences on offspring pathogen resistance. For instance, in the Indian meal moth *Plodia interpunctella*, larvae from mothers reared in poor diets (i.e., diets with high cellulose content) are less resistant to viral and fungal infections than larvae from parents reared in rich diets. This effect was due to lower levels of phenoloxidase activity and a higher infection rate (Boots and Roberts, 2012) (Kangassalo *et al.*, 2015). In the mosquito *Aedes aegypti,* daughters from parents kept on a poor diet had higher viral load compared to daughters from parents in rich diet, an effect that persisted even when the offspring was given rich diets (Zirbel, Eastmond and Alto, 2018). However, in *Drosophila melanogaster,* no difference on adult survival after bacterial infection has been detected between offspring from parents fed standard diets and offspring from parents fed yeast-poor diets (Valtonen et al., 2012). Also, in the *Melitaea cinxia* butterfly, maternal diet (*ad libitum* vs. starvation) did not have a significant effect on the total number of haemocytes, granular cells and oenocytoids of offspring when infected with the parasitoid *Cotesia melitaearum* (Saastamoinen, Hirai and van Nouhuys, 2013). Together, these findings highlight the complexity of the effects of parental diet on offspring pathogen resistance.

To date, however, investigations in this field are still partial because manipulations of parental diet have mainly focused on energy-limited regime, which excludes measuring the effect of diet composition on offspring traits (Table S1). In addition, studies have mainly focused on manipulating the diet of mothers or both parents simultaneously, overlooking the effects of fathers' diet. Yet, there is increasing evidence showing that the environment experienced by fathers can prime offspring basal immunity (Zanchi et al., 2011; Eggert, Kurtz and Diddens-de Buhr, 2014) and increase offspring resistance to infections (Roth et al., 2010). Here, we investigated transgenerational effects of nutrition by manipulating the dietary protein-to-carbohydrate balance in the diet (hereafter referred to as 'PC ratio') of mothers, fathers, and both parents simultaneously, using the fruit fly *Bactrocera tryoni* as model system. The transgenerational effects of the diet manipulations were measured on (i) disease resistance of the adult offspring by recording the survival of male and female offspring after an infection with the pathogenic bacterium *Serratia marcescens*; (ii) development traits including egg hatching rate, pupation rate, emergence rate, larval weight, pupal weight and larval body lipid reserves. Knowledge gained from this study contributes useful insights into important questions of ecology regarding effects of previous environmental condition on animals' life history traits.

MATERIALS AND METHODS

1. Fly stock

Flies used in this study originated from a *Bactrocera tryoni* stock colony that was maintained on a gel-based larval diet (Moadeli, Taylor and Ponton, 2017) and raised as adult with *ad libitum* hydrolysed yeast and sugar [Sugar (CSR® White Sugar); Hydrolysed yeast (MP Biomedicals Cat. no 02103304)]. The stock colony was maintained at 25^0C and 65% humidity with a 12-hours light/dark cycle for 25 generations.

2. Diet preparation

We used four single liquid diets varying in the protein-to-carbohydrate ratio (PC 1:8, 1:5, 1:3, and 1:1) (Dinh *et al.*, 2019). A choice diet where solutions of hydrolysed yeast and sugar were offered to the flies separately was included as a control diet. Based on previous published literature, we considered PC 1:3 as a balanced diet for *B. tryoni* [see (Fanson *et al.*, 2009)], PC 1:8 and 1:5 as carbohydrate-biased diets and PC 1:1 as a protein-biased diet. Diets were prepared by mixing hydrolysed yeast (MP Biomedicals Cat. no 02103304) and/or sugar (CSR® White Sugar) in warm distilled water using a hot plate set at 80^0C. All diets were made to a final concentration of 120 g/L. The hydrolysed yeast used in this study contains 62.1% protein and 1% carbohydrate.

3. Diet manipulation

We performed three experiments where the diet of mothers, fathers, or both parents was manipulated at adult stage, and the effects on offspring life-history traits were measured. The experimental design is described in Fig. S1. In experiment 1, we manipulated the diet of mothers by assigning groups of 60 two-day old females to one of five diet treatments (PC 1:8, 1:5, 1:3, 1:1, or choice diet), whereas, fathers were given a choice diet. In contrast, in experiment 2, we manipulated the diet of fathers (same treatments as above) while mothers were fed a choice diet. In experiment 3, we manipulated the diet of both parents (same treatments as above). Food was provided to the flies via a cotton ball soaked in the liquid diets. Flies from all experiments were fed on the experimental diets for 12 days and allowed to mate in groups of 45 females and 45 males. Eggs used to generate offspring were collected for 24h using an ovipositional device that consisted in a plastic bottle with numerous puncture holes and filled with 5 mL of water to maintain humidity and used to generate offspring. Offspring were reared on standard diet at both larval and adult stage.

4. Pre-adult traits

Percentage of egg hatching, percentage of pupation and percentage of emergence

Groups of 100 eggs were randomly selected from egg pool collected from each cage and transferred to 100 mm Petri-dishes containing 15 mL of standard larval gel-based diet (Moadeli, Taylor and Ponton, 2017). Petri-dishes were then transferred to 1.75 L containers filled with 50 mL vermiculate for pupation. Each diet treatment was run at least 6 replicates. To test for the effects of maternal diet on pre-adult survival, we recorded the number of eggs that did not hatch after 4 days of seeding, the number of pupae and the number of newly emerged flies.

Larval weight, pupal weight and larval body lipid

Ninety µl of eggs mixed with water were transferred to diet trays containing 150 mL of gel-based diet. Five days after seeding, the diet trays were placed in 12.5 L containers with 500 mL vermiculite to allow the larvae to jump and pupate. Three diet trays were prepared for each diet treatment. We collected 20 jumping larvae from each diet tray, yielding total of 60 larvae per diet treatment, for measurements of body weight and body lipid reserves. The rest of the larvae was allowed to develop into pupae. Seven days after the first larvae jumped, 60 pupae per diet treatment (20 larvae per replicate) were collected and pupal weight measured. The rest of the pupae was transferred in 12.5 L cages and allowed to develop into adults.

Body weight of individual larva and pupa was measured using a microbalance (Sartorius, accuracy ± 0.001mg). For body lipid reserves measurement, we used the protocol described in (Dinh *et al.*, 2019). Briefly, 15 jumping larvae were snap frozen at -20^{0}C, bodies transferred into individual 6 mL glass tubes (Sigma-Aldrich) and dried in a drying oven (Binder) at 50^{0}C for 48 h. Dry body weight was measured using a microbalance (Sartorius, accuracy ±0.001mg). Body lipid reserves were then extracted in three 24h washes of chloroform (Sigma-Aldrich Cat. No 650498). At the end of the third chloroform wash, lipid-free bodies were re-dried and re-weighed to calculate lipid content.

5. Survival of adult flies after septic infection with *S. marcescens*

Serratia marcescens (ATCC 13880, Thermo Scientific) was cultured on Nutrition broth (Oxoid, CM0001) overnight (approximately 12 h) at 26^{0}C, 200 rpm. The liquid culture was centrifuged at 10,000 x *g* for 2 min at 4^{0}C to remove residues of the culture medium, and the pellet was washed twice using 1X Phosphate Buffered Saline (PBS, Sigma). The bacterial cells were resuspended in sterile PBS and the solution diluted to achieve an optical density (OD_{600}) of 0.025. Injections were performed on two-day old flies. A group of 15-20 flies were cold anesthetized at -20^{0}C for two minutes and kept on dry bath (Product code: MK20) at -10°C during the injection. Bacterial cells were injected in the fly at the coxa of the third right leg using

a MP4 microinjection system (World Precision Instruments). The injection volume was 0.2 μL dispensed at the rate of 50 nL/sec, corresponding to approximately 2000 bacterial cells/ fly. PBS-injected flies were used as controls for injury. After injection, flies were kept in a 1.5L plastic cage and fed a choice diet. Survival was recorded for six days post-infection (PI). We did three replicates for each diet treatment, 18-22 flies/replicate.

6. Statistical analysis

We fitted Generalized Linear Models (GLM) with quasibinomial distribution to test for effects of diet manipulation in mothers, fathers, or both parents on percentage of egg hatching, percentage of pupation and percentage of emergence and larval body lipid reserves. A GLM with Gaussian distribution was used to assess for the effect of diet manipulation in mothers, fathers, or both parents on pupal weight and larval weight. Effects of diet, sex, infection treatment and their interactions on the survival of adult offspring were tested by fitting a GLM with a binominal distribution, with significance assessed by $\chi 2$ test. All analyses were performed in R (R Development Core Team, 2017), and all plots were done using BM SPSS Statistics 25.0.

RESULTS

1. Experiment 1: Maternal diet manipulation

Effects of maternal diet on offspring pathogen resistance: At six days PI, we only found a significant effect of the infection treatment on the survival of adult offspring (Table S2, Fig. S2). However, there was a significant effect of the two-way interaction between maternal diet and sex on the survival of adult offspring at 4 days PI (Table S3). To gain greater insights into this result, we analyzed seperatedly the survival of males and females. We found that the maternal diet significantly affected the survival of male offspring (GLM, Resid. Dev = 557.650, Resid. Df=647, P=0.023) but not female offspring (GLM, Resid. Dev = 552.871, Resid. Df=640, P=0.563). The survival of infected sons that mothers were reared on the choice diet was greater than the survival of sons from mothers fed PC 1:8, 1:5 and 1:1, but was not significantly different than those from mothers fed PC 1:3 (Fig.1).

Pre-adult survival: Maternal diet significantly affected percentage of egg hatching (GLM, $F_{(4,26)}$=9.401, P<0.001). Eggs from mothers fed the protein-biased diet (PC 1:1) had a lower hatching percentage compared to those from the other diet treatments (Fig. 2). There was no significant effect of the maternal diet on percentage of pupation (GLM, $F_{(4,24)}$=1.964, P=0.132) and percentage of emergence (GLM, $F_{(4,24)}$ =2.570, P=0.064).

Pupal and larval body weight: Maternal diet had no significant effect on larval weight (GLM, $F_{(4,296)} = 1.742$, P=0.141) and pupal weight (GLM, $F_{(4,298)} = 1.549$, P=0.188).

Larval body lipid reserves: The body lipid reserves of offspring larvae was not affected by maternal diet (GLM, $F_{(4,90)} = 0.726$, P=0.576).

2. Experiment 2: Paternal diet manipulation

Effects of paternal diet on offspring pathogen resistance: At 6 days PI, the survival of adult offspring was only affected by the infection treatment (Table S4), with PBS-injected flies surviving at a greater rate compared to infected flies (Fig. 3, Fig. S3).

Pre-adult survival: Paternal diet did not affect percentage of egg hatching (GLM, $F_{(4,40)} = 0.852$, P=0.500), percentage of pupation (GLM, $F_{(4,40)} = 0.328$, P=0.856) or percentage of emergence (GLM, $F_{(4,40)} = 0.329$, P=0.857).

Larval and pupal weight: Paternal diet did not influence larval weight (GLM, $F_{(4,297)} = 1.575$, P=0.181) and pupal weight (GLM, $F_{(4,294)} = 2.091$, P=0.082).

Larval body lipid reserves: There was no effect of paternal diet on the body lipid reserves of offspring larvae (GLM, $F_{(4,86)} = 0.999$, P=0.412).

3. Experiment 3: Parental diet manipulation

Effects of parental diet on offspring pathogen resistance: The survival of adult offspring was significantly influenced by the three-way interaction between parental diet, sex, and infection treatment (Table S5, Fig. S4). To better understand the result, we ran four seperate GLMs to test for the effect of parental diet on the survival rate of non-infected males, non-infected females, infected males, and infected females. We found that parental diet did not influence the survival of non-infected males (Binominal GLM, Resid. Dev = 119.796, Resid. Df=284, P=0.099), non-infected females (Binominal GLM, Resid. Dev = 122.898, Resid. Df=291, P= 0.123) and infected females (Binominal GLM, Resid. Dev = 388.933, Resid. Df=310, P= 0.798), but significantly affected the survival of infected male offspring (Binominal GLM, Resid. Dev = 316.853, Resid. Df=300, P= 0.002). The survival rates of infected males of parents fed PC 1:8, 1:5 and 1:1 were greater than the survival rates of infected males of parents fed the choice diet and PC 1:3 diet (Fig.4).

Pre-adult survival: Parental diet had no effect on percentage of egg hatching (GLM, $F_{(4,34)} = 0.749$, P=0.566), percentage of pupation (GLM, $F_{(4,34)} = 1.882$, P=0.136) and percentage of emergence (GLM, $F_{(4,34)} = 2.117$, P=0.100).

Larval and pupal weight: Parental diet did not influence larval weight (GLM, $F_{(4,294)} = 0.964$, P=0.428) and pupal weight (GLM, $F_{(4,294)} = 2.091$, P=0.082).

Larval body lipid reserves: Parental diet had no effect on the body lipid reserves of offspring larvae (GLM, $F_{(4,99)} = 0.706$, P=0.589).

DISCUSSION

In this study, we investigated how parental diet (i.e., mother, father, or both) influence offspring developmental traits and adult survival after septic infection with the pathogenic bacterium *S.marcescens*. We found that paternal diet did not affect the survival of infected offspring; however, maternal diet had a sex-specific effect on offspring survival with sons from mothers fed unbalanced diets being less resistant to infection than the ones from mothers fed balanced diets. When the diet composition was manipulated for both parents, effects were rather surprising. Infected sons from parents fed unbalanced diets survived at a greater rate after infection compared to those from parents fed balanced diets. Parental diet manipulation hardly affected offspring developmental traits, with only a lower hatching percentage observed when mothers were fed a protein-biased diet.

Our results showed a sex-dependent effects of mothers' or both parents' diet on offspring pathogen resistance. Previous studies have shown that parental diet can affect differently the age and size at maturity of male and female offspring (Zizzari, van Straalen and Ellers, 2016). A sex-dependent effect of parental diet on offspring disease resistance is therefore possible. While our knowledge of the mechanisms involved in sex-specific effects are still limited, they might involve sex-specific transfer of genetic material. This is supported by a study in Drosophila showing specific transfer of ribosomal DNA from fathers to daughters but not to sons when fathers were fed a protein-rich diet (Aldrich and Maggert, 2015).

Transgenerational effects can be adaptive with offspring having greater fitness in environments similar to those experienced by their parents [see for instance, (Gluckman and Hanson, 2004; Galloway and Etterson, 2007; Gluckman, Hanson and Beedle, 2007; Marshall and Uller, 2007; Raubenheimer, Simpson and Tait, 2012; Bateson, Gluckman and Hanson, 2014; Burgess and Marshall, 2014; Murren *et al.*, 2015)]. Our results showed, however, that offspring from parents fed unbalanced diets had a greater survival despite the mismatch between the nutritional conditions of parents and offspring (i.e., parents were fed unbalanced diets and offspring were fed a balanced diet). We can speculate in this case that parents in poor nutritional conditions might have primed their offspring to better survive the infection [see also (Boots and Roberts, 2012) (Ben-Ami, Ebert and Regoes, 2009) (Mitchell and Read, 2005)]. The differences between

maternal and paternal environments might be an important factor when explaining transgenerational plasticity. If the environmental conditions experienced by mothers and fathers are different, this may cause a conflict in the parental strategies to maximize their offspring performance. On the other hand, when both parents experienced the same condition, they may transfer the same environmental information to their offspring. In our results, we found opposite transgenerational effects when diets of both parents and only mothers were manipulated. This reflexes the complexity of the effects of parental diet on offspring pathogen resistance that cannot be predicted by measuring the single effects of paternal and maternal diet. However, it is consistent with previous observations on the effects of parental diet on offspring developmental time in *Drosophila melanogaster* (Valtonen *et al.*, 2012).

Our study, together with previous work in *Galleria mellonella* moth and *Drosophila melanogaster*, reported no effect of paternal diet on the survival of offspring during infection (Kangassalo et al., 2015) (Valtonen et al., 2012). Although effects of paternal poor diet on offspring phenoloxidase activity and expression level of immune genes have been observed in *Plodia interpunctella* moths (Triggs and Knell, 2012) and *Drosophila melanogaster* (Zajitschek, Zajitschek and Manier, 2017), it is noted that changes in immunity components does not necessarily lead to a change in survival (Adamo, 2004). Measuring different immune traits in offspring in addition to their survival rate might help to better understand these effects.

Sucessful host defence involves resistance (i.e., clear pathogen) and tolerance (i.e., reduce the damage of the infection on its health) mechanisms (Miller and Cotter, 2018; Kutzer and Armitage, 2016; Ayres and Schneider, 2009). Effects of an infection on host defence can be pathogen-specific. For instance, it has been shown in *Drosophila melanogaster* that while Salmonella typhimurium infection involves resistance mechanism, Listeria monocytogenes infection involves tolerance mechanism. Given that parental diet can influence both tolerance and resistance in offspring, measuring the bacterial load and fitness traits (e.g., fecundity, growth) of infected offspring would provide helpful information of the mechanism underlying the effects of parental diet on offspring's survival after infection.

Lastly, we found that parental diet hardly affected offspring developmental traits. These findings are consistent with previous observations in a neriid flies, *Telostylinus angusticollis* showing that only egg hatching success was affected by maternal diet (Bonduriansky, Runagall-McNaull and Crean, 2016). The absence of effects of paternal/parental diet on egg hatching success might be due to abundant resources available at larval and adult stage for the offspring. Also, eggs were collected from groups of flies and thus hatching success from individual mothers could not be measured. It would be interesting for future studies to explore the transgenerational effect of diet

290 on the variability in hatching success across individual mothers. Furthermore, measuring the
291 effects of parental diet on offspring traits under different offspring dietary conditions can provide
292 useful insights into transgenerational plasticity. Furthermore, transgenerational effects of diet can
293 persist through several generations (Deas, Blondel and Extavour, 2019; Tariel, Plénet and
294 Luquet, In press), with different effects between generations (Deas, Blondel and Extavour,
295 2019). Future studies investigating the effects of diet manipulation on multiple offspring traits
296 for more than one generation can provide further information regarding the long-term impacts of
297 nutrition.

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FIGURES

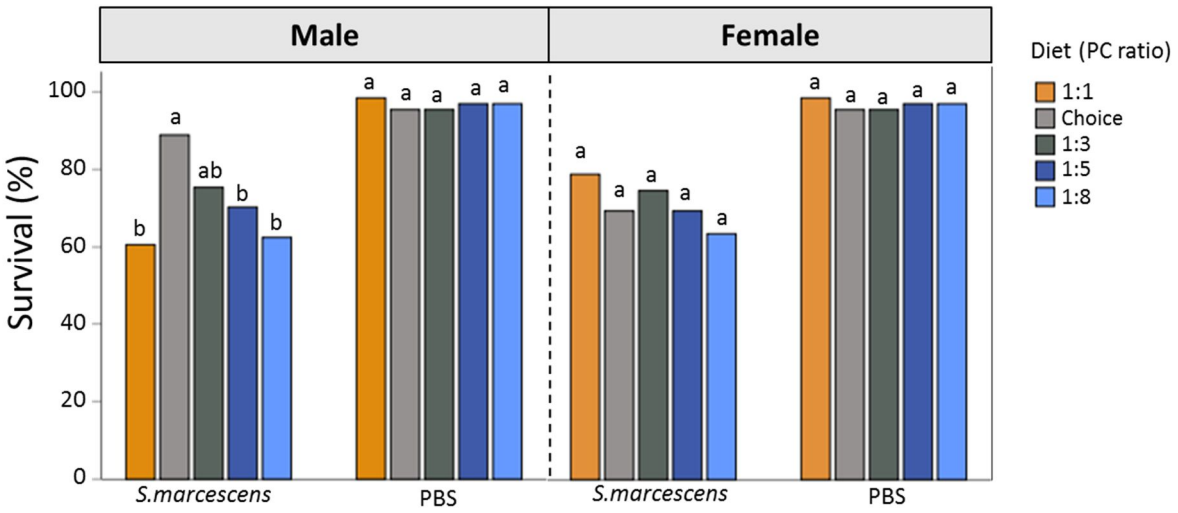


Figure 1. **Effect of maternal diet manipulation on offspring survival 4 days following septic infection.** Mothers were fed one of 5 diets varying in the protein-to-carbohydrate ratio (PC ratio) and offspring injected with either PBS or the bacterium *Serratia marcescens*. Bars indicate overall survival of adult offspring injected with either PBS or *S.marcescens* at day 4 post-infection. Different letters above bars indicate significant difference of survival between maternal diet treatments. Significance was determined by SNK test and was considered at $P<0.05$.

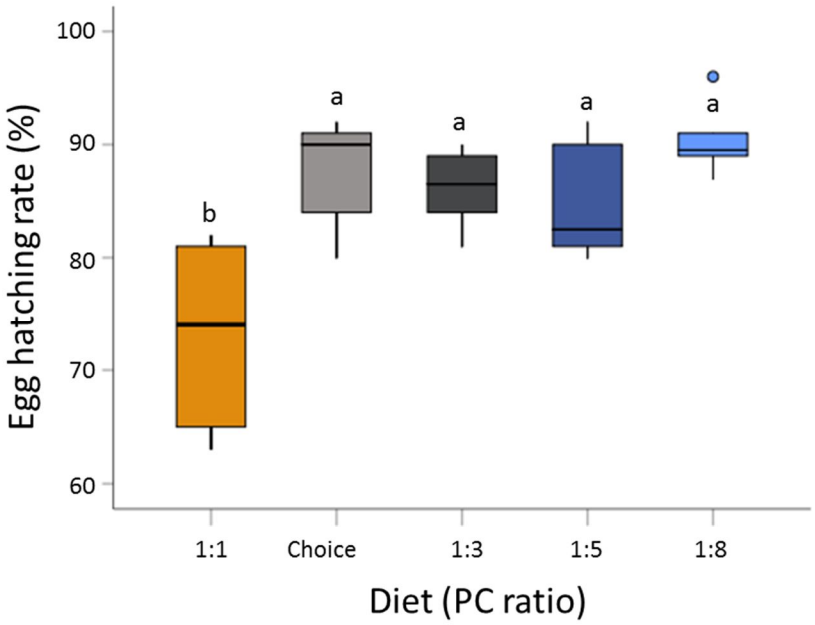
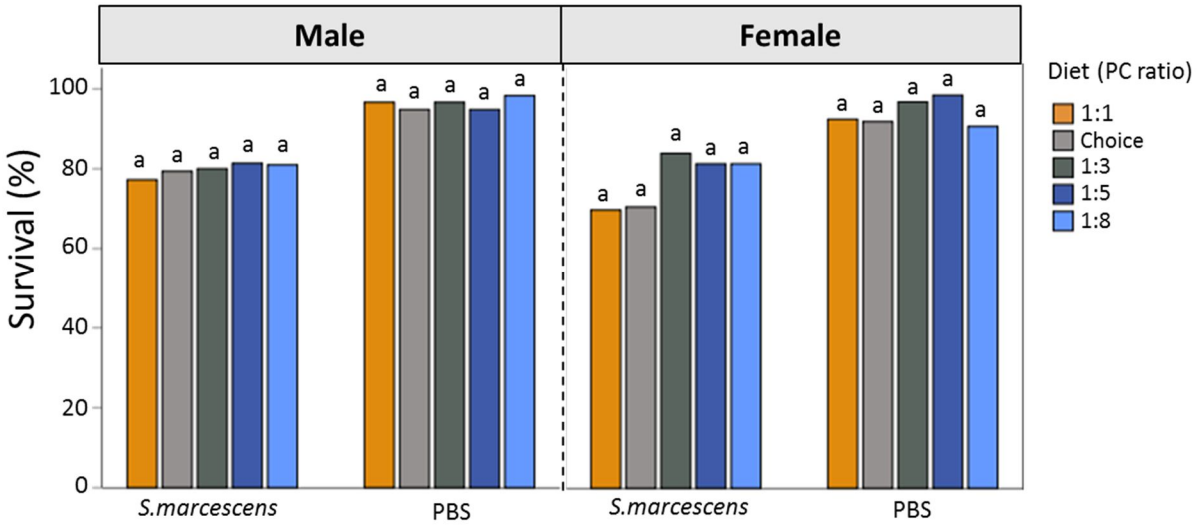


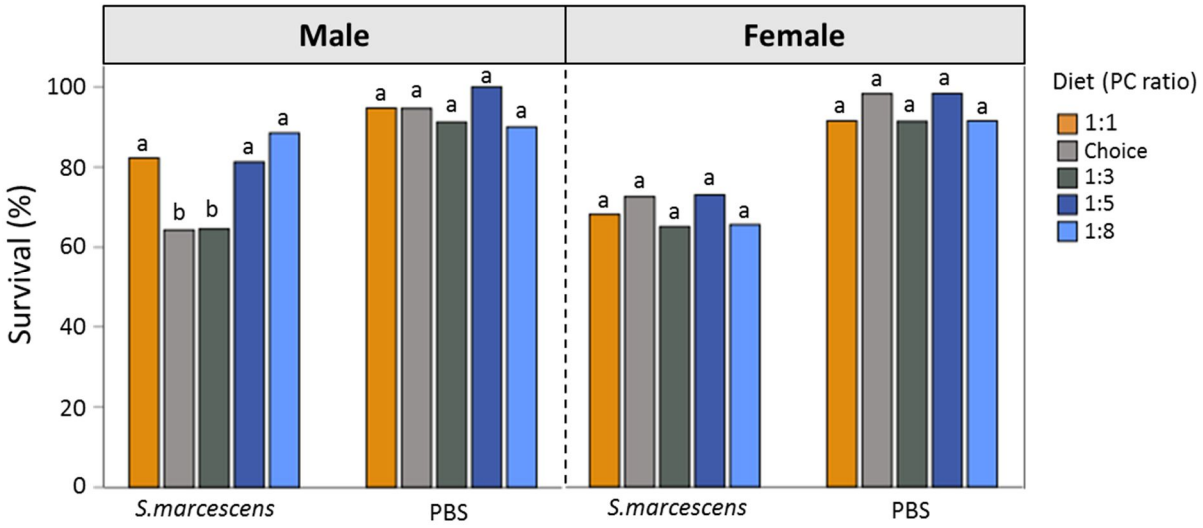
Figure 2. Effect of maternal diet manipulation on hatching rate. Mothers were fed 5 diets varying in the protein-to-carbohydrate ratio (PC ratio) and egg hatching rate was measured. Box-and-whisker plots show median and interquartile range (IQR); whiskers show either 1.5 × IQR of the lower and upper quartiles or range. Letters above bars indicate significant difference of egg hatching rate between diet treatments. Significance was accessed by Student-Newman-Keuls (SNK) test and was considered at $P<0.05$.



459

460 **Figure 3**. **Effect of paternal diet manipulation on offspring survival 6 days following septic**
461 **infection.** Fathers were fed one of 5 diets varying in the protein-to-carbohydrate ratio (PC ratio)
462 and offspring injected with either PBS or the bacterium *Serratia marcescens*. Bars indicate overall
463 survival of adult offspring injected with either PBS or *S.marcescens* at day 4 post-infection.
464 Different letters above bars indicate significant difference of survival between paternal diet
465 treatments. Significance was determined by SNK test and was considered at $P<0.05$.

466



467

468 **Figure 4**. **Effect of parental diet manipulation on offspring survival 6 days following septic**
469 **infection.** Both mothers and fathers were fed the same of one of five diets varying in the protein-
470 to-carbohydrate ratio (PC ratio) and offspring injected with either PBS or the bacterium *Serratia*
471 *marcescens*. Bars indicate overall survival of adult offspring injected with either PBS or
472 *S.marcescens* at day 4 post-infection. Different letters above bars indicate significant difference of
473 survival between parental diet treatments. Significance was determined by SNK test and was
474 considered at $P<0.05$.

SUPPLEMENTARY MATERIALS

Table S1. Summary of current studies on effects transgenerational effects of diet on offspring pathogen resistance

Study model	**Diet manipulation**	**Diet type**	**Immune challenge in offspring**	**Measured parameters**	**Results**	**Reference**
Indian meal moth *Plodia interpunctella*	Mother	Diets containing indigestible bulking agent	Granulosis virus (PiGV)	PO activity & proportion of infection	Lower proportion of infection and higher PO activity in offspring from mother fed poor diet (i.e., high bulking agent)	Boots and Roberts, 2012
Greater wax moth, *Galleria mellonella*	Mother, father	Diets containing indigestible bulking agent	*Beauveria bassiana*	Survival rate	- Maternal diet: offspring from high maternal diet survived fewer days after infection than larvae with low-nutrition maternal diet. - Paternal diet: no significant effect	Kangassalo *et al.*, 2015
Fritillary butterfly *Glanville fritillary*	Mother	Food-deprived	*Cotesia melitaearum*	Parasitoid load, encapsulation rate, total number of haemocytes, granular cells and oenocytoids	- Offspring of food-deprived mother has more living parasitoids at early stage after infection - No effect of maternal diet on other measured immunity traits	Saastamoinen, Hirai and van Nouhuys, 2013
Mosquito *Aedes aegypti*	Both parents simultaneously	Low nutrition (4 g oak leaves + 0.06 g crickets) vs high nutrition (12 g oak leaves + 0.2 g crickets)	Dengue virus	Viral load	Daughters from poor parental diet had higher viral load compared to high diet	Zirbel, Eastmond and Alto, 2018
Drosophila melanogaster	Mother, father and both parents simultaneously	Yeast-poor vs yeast-rich diet	*Serratia marcescens*	Survival rate	No effect of maternal/paternal/parental diet on offspring survival	Valtonen *et al.*, 2012

476 **Experiment 1: Maternal diet manipulation**

477

478 **Table S2.** Results of a Binomial GLM to test for the effects of maternal diet, sex, infection
479 treatment and their interactions on the survival of adult offspring at 6 days post-infection.
480 Significance are in bold.

Tested factor	Df	Deviance	Resid. Df	Resid. Dev	P
NULL	-	-	1297	1371.489	-
Maternal diet	4	4.772	1293	1366.716	0.311
Sex	1	0.312	1292	1366.405	0.577
Infection treatment	1	279.132	1291	1087.273	**0.000**
Maternal diet ×Sex	4	3.464	1287	1083.809	0.483
Maternal diet ×Infection treatment	4	2.676	1283	1081.133	0.613
Sex ×Infection treatment	1	0.588	1282	1080.545	0.443
Maternal diet ×Sex ×Infection treatment	4	3.163	1278	1077.382	0.531

481

482 **Table S3.** Results of a Binomial GLM to test for the effects of maternal diet, sex, infection
483 treatment and their interactions on the survival of adult offspring at 4 days post-infection.
484 Significance are in bold.

Tested factor	Df	Deviance	Resid. Df	Resid. Dev	P
NULL	-	-	1297	1125.736	-
Maternal diet	4	7.411	1293	1118.325	0.116
Sex	1	0.012	1291	935.648	0.915
Infection treatment	1	182.666	1292	935.659	**0.000**
Maternal diet ×Sex	4	8.297	1283	925.280	**0.008**
Maternal diet ×Infection treatment	4	2.071	1287	933.577	0.723
Sex ×Infection treatment	1	0.389	1282	924.891	0.533
Maternal diet ×Sex ×Infection treatment	4	6.215	1278	918.676	0.184

485

Experiment 2: Paternal diet manipulation

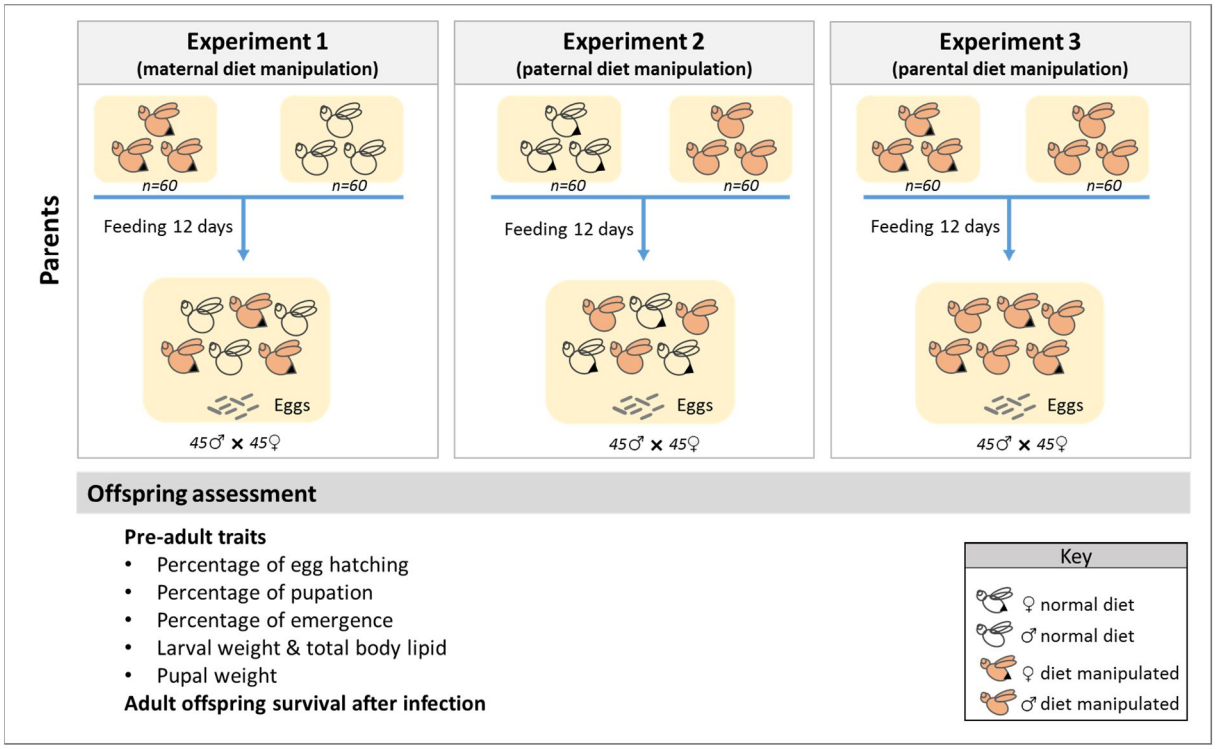
Table S4. Results of a Binomial GLM to test for the effects of paternal diet, sex, infection treatment and their interactions on the survival of adult offspring at 6 days post-infection. Significance are in bold.

Tested factor	Df	Deviance	Resid. Df	Resid. Dev	P
NULL	-	-	1244	977.759	-
Paternal diet	4	6.101	1240	971.659	0.192
Sex	1	1.186	1239	970.473	0.276
Infection treatment	1	80.264	1238	890.208	**0.000**
Paternal diet ×Sex	4	3.231	1234	886.977	0.520
Paternal diet ×Infection treatment	4	1.008	1230	885.969	0.909
Sex ×Infection treatment	1	0.506	1229	885.463	0.477
Paternal diet ×Sex ×Infection treatment	4	3.863	1225	881.601	0.425

Experiment 3: Parental diet manipulation

Table S5. Results of a Binomial GLM to test for the effects of parental diet, sex, infection treatment and their interactions on the survival of adult offspring at 6 days post-infection. Significance are in bold.

Tested factor	Df	Deviance	Resid. Df	Resid. Dev	P
NULL	-	-	1204	1095.986	-
Parental Diet	4	6.920	1200	1089.066	0.140
Sex	1	3.120	1199	1085.946	0.077
Infection treatment	1	109.135	1198	976.811	**0.000**
Parental diet ×Sex	4	11.837	1194	964.974	**0.019**
Parental diet ×Infection treatment	4	2.572	1190	962.402	0.632
Sex ×Infection treatment	1	1.145	1189	961.257	0.285
Parental diet ×Sex ×Infection treatment	4	12.774	1185	948.483	**0.012**



498

499 **Figure S1. Experimental scheme.** Single parent (mothers or fathers) or both parents were fed
500 one of five diets varying in the protein-to-carbohydrate ratio (PC ratio) for 12 days and allowed
501 to mate. Eggs from each mating events were collected to generate offspring. Offspring were
502 reared on standard diet at both larval and adult stage. The effects of diet manipulation of
503 mothers, fathers or both parents were assessed by measuring developmental traits and pathogen
504 resistance in adult offspring.

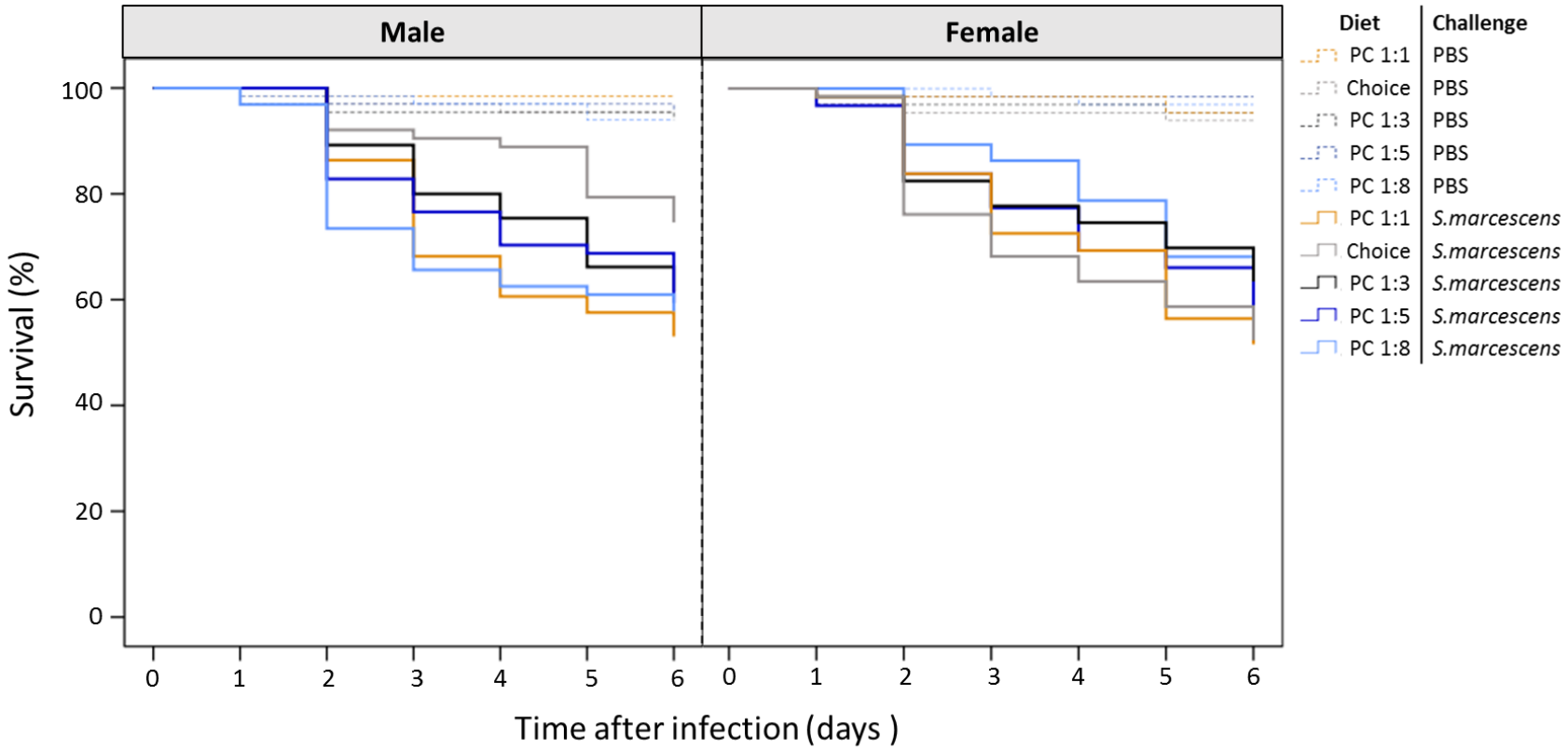


Figure S2. **Effect of maternal diet manipulation on offspring survival after septic infection.** Mothers were fed one of 5 diets varying in the protein-to-carbohydrate ratio (PC ratio) and offspring injected with either PBS or the bacterium *Serratia marcescens*. Survival was followed 6 days after infection.

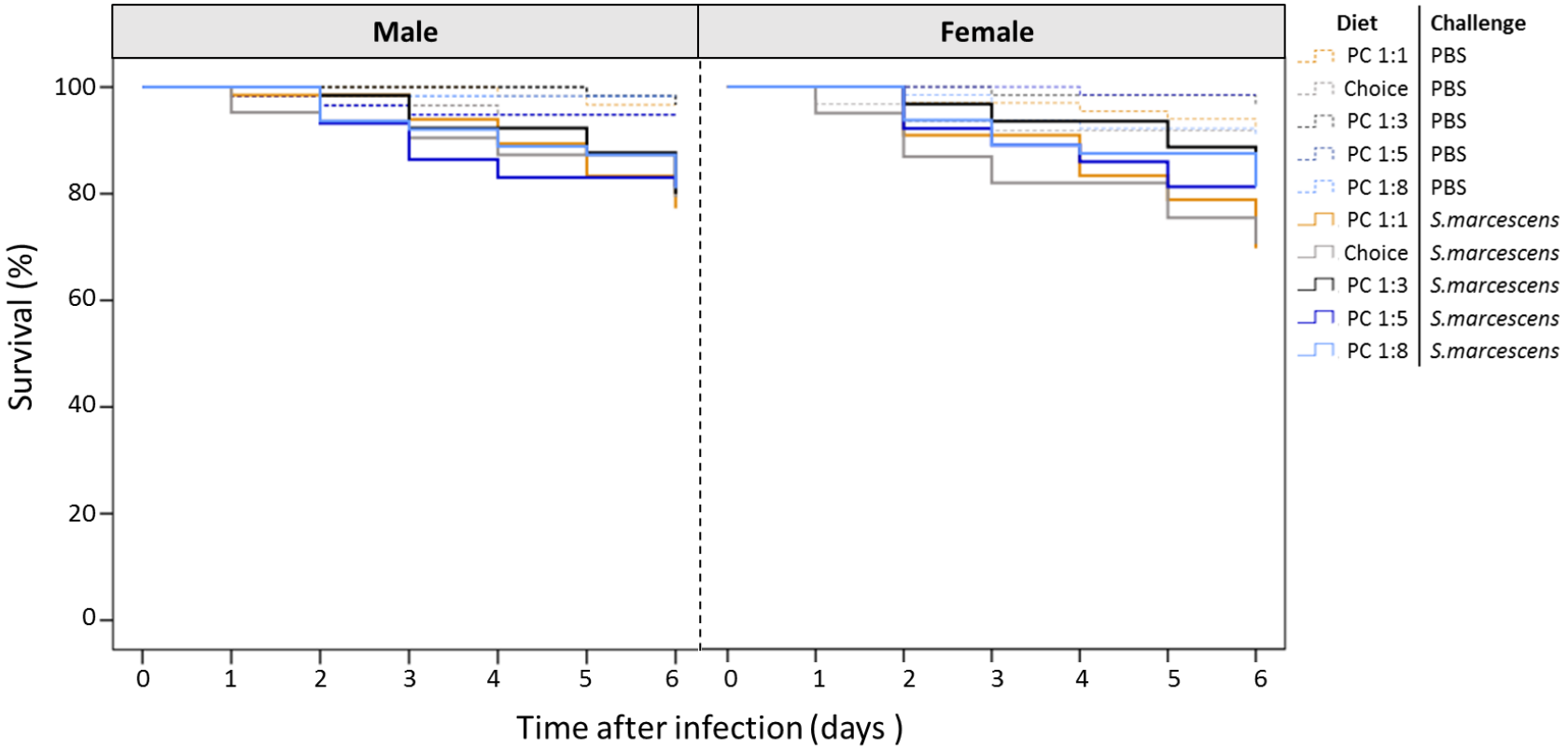


Figure S3. **Effect of paternal diet manipulation on offspring survival after septic infection.** Fathers were fed one of 5 diets varying in the protein-to-carbohydrate ratio (PC ratio) and offspring injected with either PBS or the bacterium *Serratia marcescens*. Survival was followed 6 days after infection for male and female offspring.

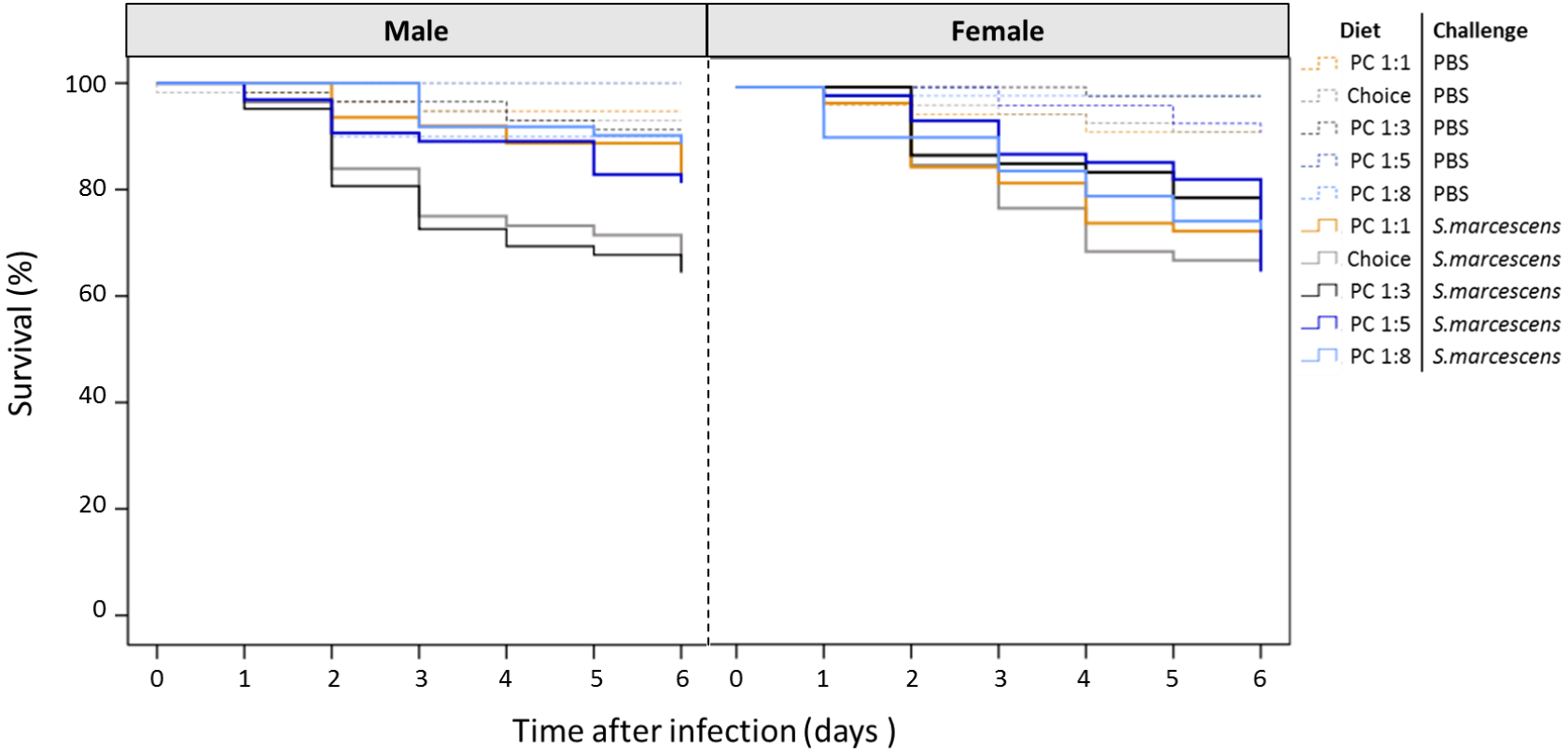


Figure S4. **Effect of parental diet manipulation on offspring survival after septic infection.** Both fathers and mothers were fed the same of one of 5 diets varying in the protein-to-carbohydrate ratio (PC ratio) and offspring injected with either PBS or the bacterium *Serratia marcescens*. Survival was followed 6 days after infection for male and female offspring.

Chapter 5

No evidence of oral immune priming in the fruit fly *Bactrocera tryoni*

No evidence of oral immune priming in the fruit fly *Bactrocera tryoni*

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Competing interests

The authors have no competing interests to declare.

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Author's contributions

All the author designed the experiments, collected the data, analysed the data and contributed to the writing of the manuscript.

ABSTRACT

Immune priming is defined as an enhanced protection of the host upon a secondary exposure to the same pathogen and has been observed widely in invertebrates. In the present study, we aimed to investigate oral priming across developmental stages (i.e., ontogenic priming) and in adult stage using the fruit fly *Bactrocera tryoni* and the pathogenic bacterium *Serratia marcescens*. In a first experiment, we measured ontogenic immune priming by feeding larvae either heat-killed or live *S. marcescens* and injecting them with the same pathogen at adult stage. In a second experiment, we measured oral immune priming at adult stage by feeding adult females with either live or heat-killed *S. marcescens* and injecting them with the same bacterium two days following priming. In both experiments, survival rate of challenged flies was used as a proxy of pathogen resistance. We observed no evidence of immune priming, with survival rates are comparable between primed and non-primed flies. Further explorations of the effects of immune priming on individuals' performance might help to better understand this phenomenon in insects.

Keywords: Immune priming, Ontogenic priming, Oral infection, Septic injection, Survival rate.

INTRODUCTION

Immune responses are typically characterized as being either innate or adaptive (Dempsey et al., 2003; Iwasaki and Medzhitov, 2015). While adaptive immune responses are characterised by the ability of the host to remember the infection by a specific pathogen (Litman et al., 2010), innate immune responses are described as first and rapid defence responses against a wide range of pathogens (i.e. bacteria, viruses, fungi, and macroparasites) (Hoffmann and Reichhart, 2002). Innate immunity has been therefore considered as broad, with only weak specificity (Medzhitov and Janeway, 2000). Research in the last decades has shown however that innate immunity is more complex than traditionally considered (Netea et al., 2015) with innate responses being essential for the stimulation of the acquired immune system (Iwasaki and Medzhitov, 2015) as well as showing some memory in its ability to fight infections (Kurtz, 2005; Kurtz and Franz, 2003).

The memory characteristics of innate immunity have been widely described in insects as "immune priming" which is defined as an enhanced protection of the host upon a secondary exposure to the same pathogen [for review, see (Milutinović et al., 2016; Moret and Siva-Jothy, 2003)]. Protective effects of immune priming are often characterized by a higher survival rate of primed individuals upon a second challenge with the same pathogen [e.g., (Futo et al., 2017; Moret and Siva-Jothy, 2003; Roth et al., 2009; Sadd and Schmid-Hempel, 2006)] and/or the capability of the host to clear pathogens (Pham et al., 2007; Sadd and Schmid-Hempel, 2006). An increased number of haemocytes (López et al., 2014; Zhang et al., 2014), greater phenoloxidase (PO) activity (Shi et al., 2014) or higher amount of reactive oxygen species (ROS) (Mikonranta et al., 2014) have also been reported in primed individuals when re-infected.

Septic and oral routes of infection are commonly used to study immune priming. Septic infection involves introducing pathogens into the insects body cavity by pricking or injecting [see for instance, (Apidianakis and Rahme, 2009; Dinh et al., 2019)]. Septic infections have been widely employed to study immune priming, such as pricking or injection of inactivated pathogens in mealworm beetles, *Tenebrio molitor* (Dubuffet et al., 2015), red flour beetles (*Tribolium castaneum*) (Schulz et al., 2019; Tate and Graham, 2015), vinegar flies *Drosophila melanogaster* (Pham et al., 2007) and honeybees, *Apis mellifera* (López et al., 2014). Besides, experimental priming can also be done by injecting animals with a sublethal dose of live bacteria as used in the bumblebee *Bombus terrestris* (Sadd and Schmid-Hempel, 2006) or pricking insect with living bacterial solution as used in the beetle *Tribolium castaneum* (Ferro et al., 2019). On the other hand, oral infection is considered to be a more natural way of infection, and it often involves feeding animals with a pathogen-contaminated diet (Blaser and Schmid-Hempel, 2005; Milutinović et al., 2013; Rafaluk et al., 2015). Priming via oral route has been previously tested by using bacterial

spore-culture supernatants (Futo et al., 2017; Greenwood et al., 2017; Milutinović et al., 2014; Schulz et al., 2019) or sublethal dose of living bacteria or virus (Mikonranta et al., 2014; Tidbury et al., 2011) (Moreno-García et al., 2015). Septic and oral infections induce different physiological and immunological responses. For instance, while young larvae of the red flour beetle had shorter developmental time after septic priming (Roth and Kurtz, 2008), those with oral priming showed a delayed larval development (Milutinović et al., 2014). Additionally, some transcriptomic sequencing data have revealed clear distinct immunological and physiological processes underpinning the two different routes of infection (Behrens et al., 2014).

Despite a diversity of studies investigating within generational immune priming in insects, there are still important questions to be answered such as: (i) Oral priming has mainly been performed only in larvae and, to our knowledge, there is no current study investigating oral priming in adults. Considering that larval behaviours may not predict adult behavioural traits in holometabolous insects (Monceau et al., 2017) probably because of great differences in metabolism, physiology, and immunity between developmental stages (Tetreau et al., 2019), studies investigating oral priming in adults are needed. (ii) Ontogenic immune priming has only been tested using living bacterial cells (Moreno-García et al., 2015) (Thomas and Rudolf, 2010). While a live pathogen can interact with its host in a complex manner (Canny and McCormick, 2008; Piña-Vázquez et al., 2012; Sansonetti, 2002), infections with heat-killed pathogens only induce activation of the immune responses without metabolic interactions between the host and its pathogens. Using both types of infections (i.e., with live and inactivated pathogens) might provide insights into using immune priming as an immune stimulation without the need of infections with live pathogens.

In the present study, we aimed to address these questions through two experiments using the fruit fly *Bactrocera tryoni* and the pathogenic bacterium *Serratia marcescens*. In a first experiment, we measured ontogenic immune priming. For this purpose, *Bactrocera tryoni* larvae were orally exposed to either heat-killed or live *S. marcescens*, and injected with the same bacterium at adult stage. In a second experiment, we measured immune priming at adult stage by feeding adult female flies either heat-killed or live bacteria and injecting them with the same bacterium two days following priming (i.e., challenged flies). In both experiments, survival rate of challenged flies was used to measure priming effect. Here, we provide the first investigation of the effects of oral priming at adult stage and across developmental stages in fruit flies.

MATERIALS AND METHODS

1. Literature searching method

The online search for literature was conducted in the ISI Web of Science and Scopus (as suggested by Catherine et. al., 2018) using the following keyword combinations to search for:

(1) Immune priming in adult stage: (oral immune priming*OR priming) AND (adult*OR adult stage): No records found
(2) Priming that uses heat-killed bacteria: (oral immune priming*OR priming) AND (heat-killed*OR heat killed*OR dead pathogen*OR heat treatment): No records found

To increase the number of articles, a simpler keyword "Oral immune priming" was used. There were 3 records found in the ISI Web of Science and 28 records in the Scopus (including 3 references found in the ISI Web of Science). Based on these initial 28 records, we excluded references that were either "review paper" or "transgenerational immune priming" or "priming via septic injury" or "papers that just mentioned oral immune priming". Finally, we selected 7 references belonging to "within transgenerational immune priming via oral route" category. These 7 references were then subcategorized into "studies that used heat-killed pathogens to measure immune priming" and "studies in which immune priming was performed at adult stage".

2. Study insect

Flies were collected from our lab-adapted stock of fruit fly (*Bactrocera tryoni*). Flies were reared on a gel-based larval diet (Moadeli et al., 2017) and raised on a 1:3 ratio of hydrolysed yeast-to-sugar (Y:S) [Sugar (CSR® White Sugar), hydrolysed yeast (MP Biomedicals Cat. no 02103304)] at adult stage. The fly colony was maintained at 25^0C and 65% humidity with a 12-hours light/dark cycle for 26 generations.

Twelve diet trays containing 150 mL larval gel-based diet were prepared. A volume of 150 µl egg solution (around 2250 eggs) collected from the fly stock was seeded in each tray. Half of these trays were used to generate second instar larvae (i.e., 4 days after seeding eggs) to examine ontogenic immune priming. The other half was used to generate adult flies. For this purpose, seven days after seeding the eggs, the trays were moved into boxes containing 500 mL of fine vermiculite to allow the larvae to jump and pupate in this substrate. Vermiculate was sieved, pupae collected, transferred to 12.5mL plastic cages and allowed to develop until adult stage.

3. Bacterial preparation

Serratia marcescens (ATCC 13880, Thermo Scientific) was cultured on Nutrition broth (Oxoid, CM0001) at 26^0C shaking at 200 rpm for approximately 12 h (overnight). The liquid culture was centrifuged at 10,000 x *g* for 2 min at 4^0C to remove residues of the culture medium, and the

pellet was washed twice using 1X Phosphate Buffered Saline (PBS, Sigma). Bacterial cells were resuspended in sterile PBS and optical density (OD_{600}) was measured using a spectrometer (Eppendorf). The heat-killed bacteria treatment was prepared by heating the bacterial solution in a water bath at 85°C for 15 min and stored at -80^{0}C. The heat-killed bacterial solution was spread on agar plates at a volume of 10µl (5 replicates), incubated at 26 °C for 48 h and no growing colonies were observed. The bacterial solutions were diluted to OD_{600}=200 (around 3.43×10^{11} cells per ml) for oral priming (Vodovar et al., 2005). Septic injection was used to challenge adult flies. This method of priming and challenging was applied to study immune priming in *Aedes aegypti* mosquitoes (Moreno-García et al., 2015). For the injection, bacterial cells were diluted to OD_{600}=0.025 (around 4.29×10^7 cells/ml) (Dinh et al., 2019).

4. Experiment 1: Effect of oral priming across development stages

Groups of approximately 1000 second instar larvae were removed from the larval gel diet, washed with water to eliminate diet remnants and assigned to one of the 3 priming treatments: 1) 5% sucrose solution and live bacteria solution at a ratio of 1:1, 2) 5% sucrose solution and heat-killed bacteria solution at a ratio of 1:1, and 3) 2.5% sucrose solution. Larvae were dipped in the priming solution and fed for 2hrs. Primed larvae were then washed with sterile water and transferred to standard gel-based diet (20 ml) in 100mm petri dishes. Each treatment was replicated nine times, yielding 900 primed larvae in total per treatment. Three days after, the larvae were transferred to 12.5 litre plastic boxes containing 500mL fine vermiculite and allowed to jump out of the diet and pupate. Vermiculate was sieved, pupae collected and transferred to 12.5 L plastic cages, and allowed to emerge into adult flies. One-day-old female flies were injected with 0.2 µL of live bacteria. PBS-injected flies were used as controls for the injury of the injection. Dead flies were removed daily from the cages and survival recorded for 12 days.

5. Experiment 2: Effect of oral priming at adult stage on survival after infection

Groups of 100 one-day-old female flies were kept in 12.5 L plastic cages and deprived of water and food for 24 hrs. Three cages were prepared for each priming treatment, yielding a total of 300 flies per priming treatment. Flies were then fed one of the three priming solutions as previously described. Priming treatments were delivered to the flies via filter paper soaked with 2 mL of one of three treatment solutions. We observed that almost all flies immediately approached the bacterial solution within a minute. To ensure successful priming, we removed flies that were outside the bacterial solution after 1 minute. These flies accounted for less than 10% of the total flies and can be either flies that did not approach the bacterial solution or flies that left the bacterial solution too quickly. Flies were allowed to feed for 2 hrs and the filter

paper was then removed from the cage. After the priming treatment, flies were provided with water and *ad libitum* food. Two days after priming, flies were cold anesthetized in a −20°C refrigerator for 2 min and injected with 0.2 µL of a solution of live bacteria (OD_{600}=0.025) using a MP4 microinjection system (World Precision Instruments). PBS-injected flies were used to control for the injury. Survival was recorded daily for 12 days. We did three replicates for each injection treatment (30 flies/replicate).

6. Statistical analysis

We fitted GLMs with binominal distribution to test for the effects of priming, injection treatment, time post-infection and their interactions on the survival of adult flies. P-values were obtained from χ^2 test. Following GLM, SNK tests were used to test for differences of survival rate between priming treatment. Significance was considered at $P<0.05$. All analyses were performed in R (R Development Core Team, 2017), and all plots were done using BM SPSS Statistics 25.0.

RESULTS

1. Experiment 1: Oral priming at larval stage does not promote survival after infection at adult stage

We only detected an effect of the injection treatment on the survival of flies between one and 12 days post-infection (PI) (Table S1). Flies injected with live bacteria died at a greater rate compared to PBS-injected ones (Fig. S1). Similar results were observed when we only looked at the survival rate at 12 days PI, with the survival rate of flies not being affected by either the priming treatments or the interaction between priming and injection treatment (Table S2). However, again, we found that PBS-injected flies survived at a greater rate than those injected with live bacteria, irrespective of the priming treatment (Fig.1). When we only considered individuals injected with live bacteria, we also did not detect any significant effect of priming on their survival (Binomial GLM, Resid. Dev =177.890, Resid. Df=178, P=0.265).

2. Experiment 2: Priming at adult stage does not promote survival after infection

Survival of flies between one and 12 days PI was only affected by the injection treatment (Table S3), with flies injected with live bacteria dying at a greater rate than flies injected with PBS (Fig. S2). At 12 days PI, survival was not affected by priming as well as the interaction between the injection treatment and priming (Table S4). But we found again a significant effect of the injection treatment on survival (Table S4), with flies injected with PBS surviving at a greater rate than flies injected with live bacteria (Fig. 2). When we only considered individuals injected with live

bacteria, we also did not detect any significant effect of priming on their survival (Binomial GLM, Resid. Dev =334.887, Resid. Df=296, P=0.379).

DISCUSSION

In the present study, we found no evidence of a positive effect of pre-exposure to pathogenic bacteria on the survival of adult *B. tryoni* after re-infection with the same pathogen. It has been previously shown that oral immune priming at the same developmental stage (Futo et al., 2017; Greenwood et al., 2017; Mikonranta et al., 2014; Milutinović et al., 2014; Tidbury et al., 2011) or across developmental stages (Moreno-García et al., 2015) can protect individuals upon a secondary exposure to the same pathogen. However, immune priming seems not to always effectively enhance survival of insects after re-infection. Works on immune priming in ant (*Formica selysi)* and the red flour beetle (*Tribolium castaneum)* have shown, for instance, similar survival rates in primed and non-primed individuals upon a subsequent infection with a lethal dose of fungal/bacterial pathogens (Futo et al., 2017; Reber and Chapuisat, 2012). These results might suggest a complex process of priming on immunity (Cooper and Eleftherianos, 2017) which potentially leads to different outcomes after re-infection.

Several possibilities may explain for the lack of immune priming across life stages in our experiment. Firstly, oral priming might only induce protection against oral infection while the flies in our experiment were challenged via septic infection. This is partly supported by a study in *Drosophila melanogaster* showing that the route of infection can shape adaptive responses that will be specific to either oral or septic infection (Martins et al., 2013). Secondly, maintaining specific immune responses to a large array of different pathogens may come at a high energetic costs, the host may therefore develop defences against only a restricted set of pathogens, for instance, the ones that are the most abundant in their environment. Finally, the absence of ontogenic immune priming can also fit into three catagories described in (Tate and Rudolf, 2012): (i) exposing *B. tryoni* larvae to heat-killed or living bacteria do not increase their resistance; (ii) previous exposure to heat-killed or living bacteria might increase resistance in larvae but not in adults (i.e., resistance is not carried over developmental stages); and (iii) ingestion of bacteria induces changes in immune responses that can be transferred to the adult stage; however, changes in immune status do not necessary lead to a better survival (Adamo, 2004) (Saejeng et al., 2010). Investigating innate immune responses after an oral ingestion of bacteria as well as using the same route for priming and challenging would shed more light into theories mentioned above.

Although we could not detect any effects of oral priming on the survival of re-infected adult flies, it would be interesting to examine the reproductive performance of the primed hosts after re-infection. In the *Anopheles albimanus* mosquitoes, primed females that successfully fought the infection (i.e., low parasite level) were unable to produce eggs while females with a higher parasite load produced eggs (Contreras-Garduño et al., 2014). Also, the degree of resistance seems to depend on the level of reproductive investment. There is evidence from a previous study in flour beetles (*Tribolium castaneum*), primed with the natural pathogenic bacteria (*Bacillus thuringiensis*), that females with a lower survival benefit likely invest more resources in reproduction (i.e., produced more offspring) compared with those with a higher survival benefit (Khan et al., 2018).

In conclusion, our data showed no evidence of a positive effect of priming on the survival rate of larvae and adult *B. tryoni* infected with the pathogenic bacterium *S. marcescens*. Due to the complexity of immune priming processes which might depend on the interactions between different factors including life-stages (Tetreau et al., 2019), priming routes [e.g., (Roth and Kurtz, 2008), (Milutinović et al., 2014) (Behrens et al., 2014)], sex (Moreno-García et al., 2015), pathogen characteristics [i.e., pathogen species and strains (Dhinaut et al., 2018) (Futo et al., 2017) and pathogen status (active vs inactive)], more investigation is required to explain for the absence of immune priming in this tephritid flies, including measurement of other life history traits of primed hosts.

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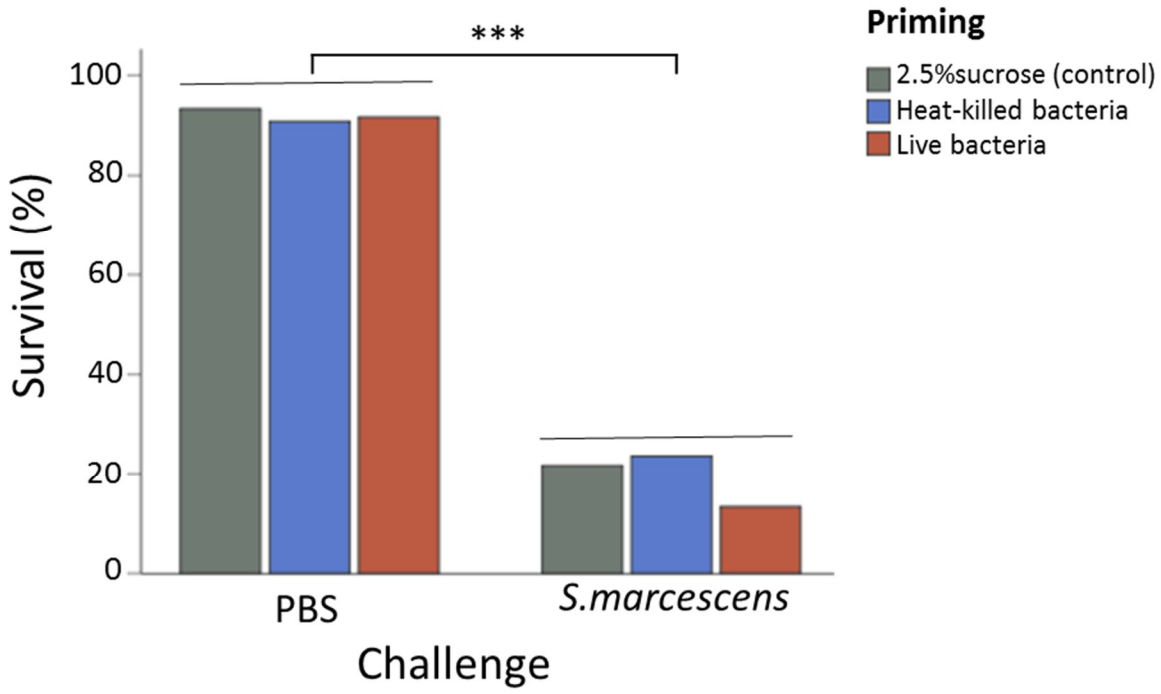
403 **FIGURES**



404

405 **Figure 1. Effect of oral priming across developmental stages**. *Bactrocera tryoni* flies were
406 immune primed at larval stage and challenged at adult stage with the bacterium *Serratia*
407 *marcescens*. Flies were primed with a 2.5% sucrose or heat-killed or live *S.marcescens* solution
408 and re-infected with live *S. marcescens*. PBS-injected flies were included to control for injury
409 damage. Differences in survival rate between priming treatments were assessed by SNK tests and
410 significance was considered at $P<0.05$**.**

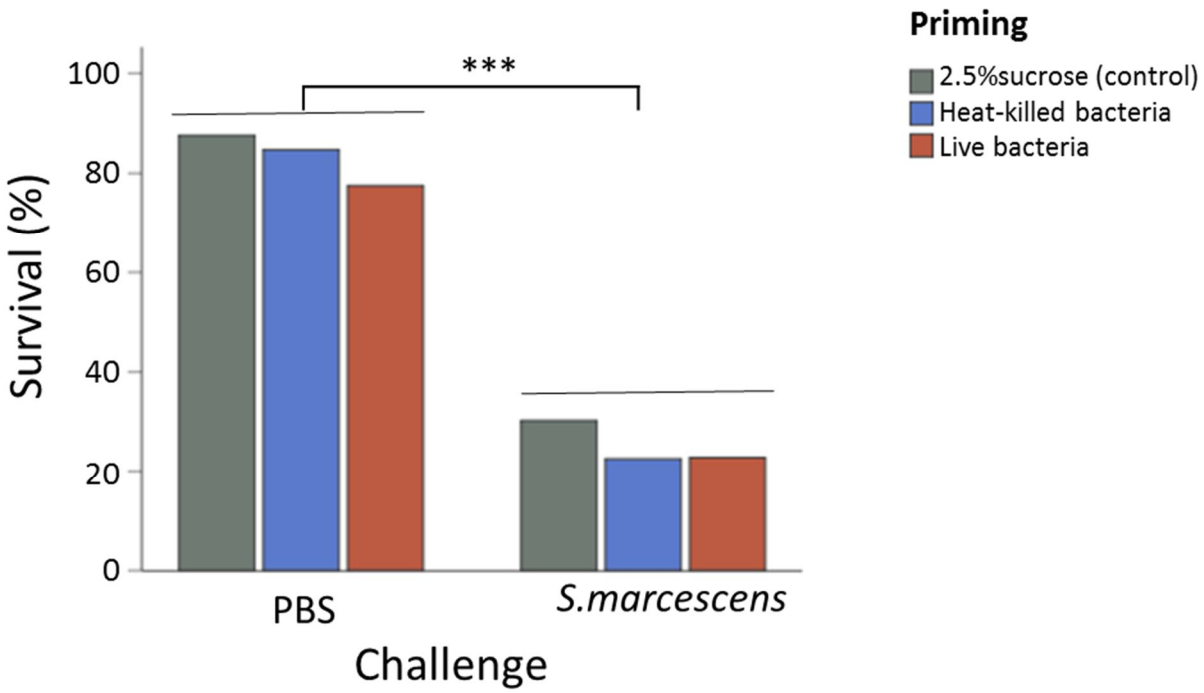


Figure 2. Effect of oral priming in adults. Adult *Bactrocera tryoni* were primed with a 2.5% sucrose or, heat-killed or live *Serratia marcescens* solution. Flies were infected with live *S. marcescens* two days after priming. PBS-injected flies were included to control for injury damage. Differences in survival between priming treatments were assessed by SNK tests and significance was considered at $P<0.05$.

SUPPLEMENTARY MATERIALS

Figures

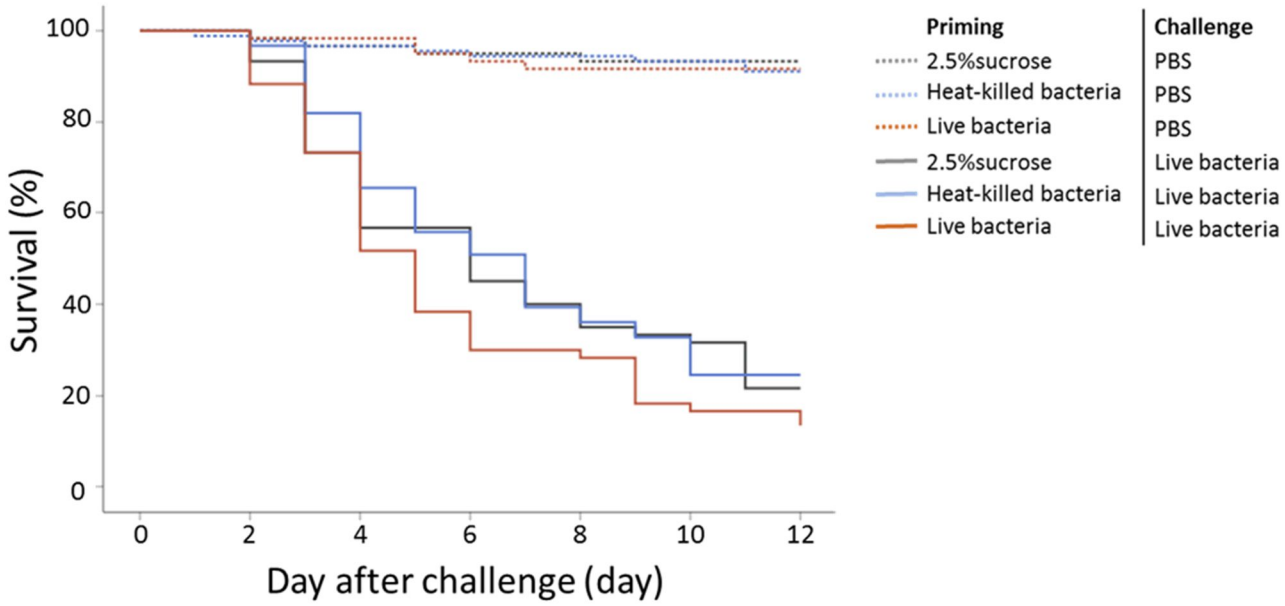


Figure S1. Kaplan-Mier survival curves of adult flies that were primed at larval stage and challenged with the bacterium *Serratia marcescens* at adult stage. *Bactrocera tryoni* larvae were primed with a 2.5% sucrose or heat-killed or live *S. marcescens* solution and challenged with live *S. marcescens* when reaching adulthood. PBS-injected flies were included to control for injury. Survival was monitored for 12 days after the immune challenge.

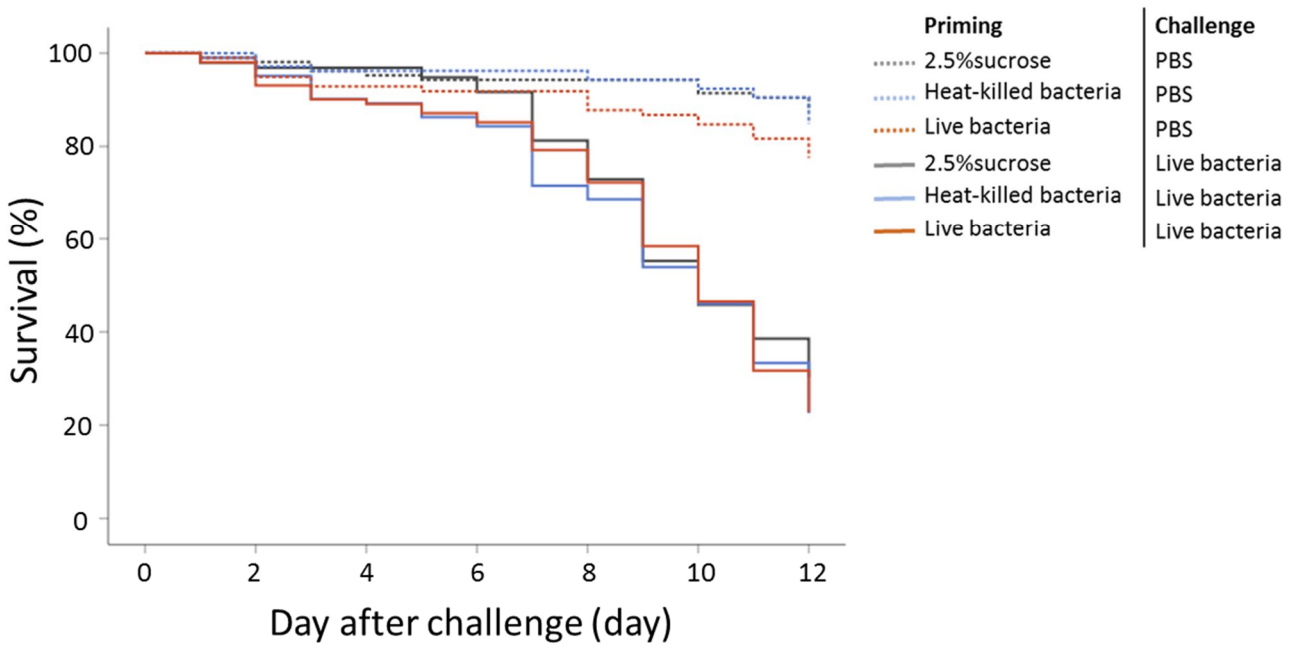


Figure S2. Kaplan-Mier survival curves of primed adult *Bactrocera tryoni* after being challenged with the bacterium *Serratia marcescens.* Adult *B. tryoni* were primed with a 2.5% sucrose or heat-killed or live *S.marcescens* solution and re-infected with the same bacteria two days after priming. PBS-injected flies were included to control for injury damage. Survival was monitored for 12 days after the immune challenge.

Tables

Experiment 1: Ontogenic immune priming

Table S1. Results of a Binomial GLM to test for the effects of priming, injection, time and their interactions on the survival of flies between one and 12 days post-infection. Significance are in bold.

Tested effect	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL	-	-	390	530.503	-
Priming	2	3.889	388	526.615	0.143
Injection	1	229.712	387	296.902	**0.000**
Time	1	286.894	386	10.008	**0.000**
Priming × Injection	2	0.000	384	10.008	1.000
Priming × Time	2	0.000	382	10.008	1.000
Injection × Time	1	0.000	381	10.008	1.000
Priming × Injection × Time	2	0.000	379	10.008	1.000

Table S2. Results of a Binomial GLM to test for the effects of priming, injection and their interactions on the survival of flies at 12 days post-infection. Significance are in bold.

Tested effect	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL	-	-	390	530.503	-
Priming	2	3.889	388	526.615	0.147
Injection	1	229.712	387	296.902	0.000
Priming × Injection	2	1.208	385	295.695	0.552

Experiment 2: Immune priming within the same developmental stage

Table S3. Results of a Binomial GLM to test for the effects of priming, injection, time and their interactions on the survival of flies between one and 12 days post-infection. Significance are in bold.

Tested effect	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL	-	-	606.000	836.120	-
Priming	2	4.464	604	831.656	0.107
Injection	1	223.101	603	608.555	**0.000**
Time	1	385.896	602	222.660	**0.000**
Priming × Injection	2	0.039	600	222.621	0.981
Priming × Time	2	0.000	598	222.621	1.000
Injection × Time	1	0.000	597	222.621	1.000
Priming × Injection × Time	2	0.000	595	222.621	1.000

Table S4. Results of a Binomial GLM to test for the effects of priming, injection, time and their interactions on the survival of flies at 12 days post-infection. Significance are in bold.

Tested effect	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
NULL	-	-	606	836.120	-
Priming	2	4.464	604	831.656	0.110
Injection	1	223.101	603	608.555	0.000
Priming × Injection	2	1.027	601	607.529	0.602

Chapter 6

General discussion

6.1. General discussion

6.1.1. Infected fruit flies shift their diet choice to promote their survival after infection and contain the growth of pathogens

In this thesis, I first demonstrated that, following septic infection with pathogenic bacteria, adult *Bactrocera tryoni* flies modulated their macronutrient intake and shifted their nutritional choice to a carbohydrate-biased diet (Chapter 2, Dinh et al., 2019). Interestingly, this change in feeding behaviour promoted the survival of flies after infection and contained the growth of pathogens. We called this modulation of feeding following infection "self-medication through food balancing" which meets three important criteria as described in (Clayton and Wolfe, 1993). Firstly, the shift in PC ratio toward carbohydrate-biased was only found in infected flies, but not in other control groups (i.e., deliberately ingested). Secondly, carbohydrate-biased diet resulted in a reduced bacterial load, at least at day 3 and 5 after infection (i.e., detrimental to pathogen). Finally, carbohydrate-biased diet increased flies' ability to survive the infection during the first 5 days post-infection (i.e., the detrimental effect on parasites leads to increased host fitness). Finally, the carbohydrate-biased diet selected by infected flies does not maximise lifetime egg production in non-infected individuals (Fanson et al., 2009). This has also been considered as a criterion for self-medication (Singer et al., 2009). These findings are consistent with a previous study in Drosophila showing that flies under septic infection, with the pathogenic Gram-positive bacterium *Micrococcus luteus*, shift their macronutrient intake toward a carbohydrate-biased diet (Ponton et al., In Press). Although the mechanisms underlying the effects of this dietary shift are still unclear in *B. tryoni*, the fly's nutritional status might influence the infection by inducing changes in immunity. In Drosophila, the expression of antimicrobial peptide genes is increased when flies are fed a sugar-biased diet (Ponton et al., In Press). The same molecular mechanisms might be found in *B. tryoni*, it is however difficult to measure dietary effects on immune gene expression in this model, at this stage, since the transcriptome of *B. tryoni* is not yet publicly available. The shift in nutritional choice might also modulate the resources available to the pathogens (Pike et al., 2019) and do not provide the pathogens with the adequate nutrition, restricting their growth. This can be tested by feeding infected flies and their pathogens single diets ranging from high to low protein-to carbohydrate ratios and measure the variance in pathogen growth between these diets.

Further investigations on the potential effects of the interaction between sex and nutrition on infection might also help to better understand the underlying mechanisms. Studies in tephritid

flies and crickets have shown that nutrition interacts differently with immunity in males and females. For instance, while the phenoloxidase activity and encapsulation ability are influenced by both carbohydrate and protein intakes, these immune components are only affected by protein intake in males (Fanson et al., 2013) (Rapkin et al., 2018). These results might be linked to a different allocation of resources between life history traits and immunity in males and females, as well as different metabolic costs that might occur between life history traits (Hanssen et al., 2004; Lochmiller and Deerenberg, 2000; Rauw, 2012) when flies shift their diet choice. Further studies might explore not only survival after infection but also other life history traits such as the reproduction and longevity of males and females under different dietary conditions.

6.1.2. Carry-over effects of the macronutrient balance on infection

I next investigated whether unbalanced diets have long-term effects on pathogen resistance across developmental stages and generations. In chapter 3, I found that both juvenile and adult traits were modulated by larval diets. Importantly, larval diets affected the ability of flies to resist an infection in a sex-dependent manner with a higher survival rate in infected females – but not in infected males – when fed sugar-biased larval diets compared to individuals fed protein-biased and standard diets. While it has been previously shown that the larval diet affects a range of life history traits during adulthood [e.g., (Bauerfeind and Fischer 2009; Colasurdo, Gelinas, and Despland 2009; Dmitriew and Rowe 2011; Kaspi et al. 2002; Runagall-Mcnaull, Bonduriansky, and Crean 2015)], there is only a limited number of examples on the effects of larval diet on adult resistance to infection (Kelly and Tawes, 2013; Linenberg et al., 2016), and the mechanisms underlying these effects are still unknown.

In holometabolous insects, pupation is a key step in reaching adulthood and it has been suggested that bacteria can be transmitted to adults through surviving metamorphosis (Duneau and Lazzaro, 2018). In my study, I focused on the adult resistance to pathogenic bacteria, however it would be interesting to also assess the effects of larval diet on the composition of adult microbiota. It is now well established that the composition of the microbiota influences the immune status and therefore the capability to fight infections (Belkaid and Hand, 2014; Pickard et al., 2017). Our understanding of the differences in microbiota and infection prevalence in adulthood, between sexes, is however limited. Investigating how larval nutrition influences the composition of the microbiota and how the microbiota influences immunity and resistance to infection in males and females might help

to decipher the mechanisms underlying the sex-specific effects of larval diets on adult resistance.

In chapter 4, I also observed sex-specific effects of parental diet on the ability of *B. tryoni* offspring to resist septic infection. Sons – but not daughters – from mothers fed unbalanced diets survived the infection at a lower rate compared to those from mothers fed balanced diets. This might indicate that mothers in low physiological state produce sons that immune system is less efficient at fighting or tolerating infections. However, why only sons are affected remains to be explored. Surprisingly, when both parents were fed unbalanced diets, it provided their sons – but not daughters – a protective effect after infection. This is the first time to my knowledge that effects of parental dietary manipulation in term of nutritional quality are reported on the disease resistance of both male and female adult offspring. Environmental conditions experienced by parents can served as a cue to predict conditions that offspring will be experiencing (Shea et al., 2011) and parents might adjust their investment to optimize offspring fitness (Mousseau and Fox, 1998). Nutritional stress experienced by parents can prime their offspring to better resist an infection as observed in the Indian meal moth (*Plodia interpunctella*) and *Daphnia magna* (Boots and Roberts, 2012) (Ben-Ami et al., 2009) (Mitchell and Read, 2005). These observations suggest that parental nutrition might serve as a non-specific cue of adverse environmental conditions that can include pathogen infection. Further investigations into the effects of parental diets under different offspring dietary conditions would provide more information regarding the transgenerational plasticity driven by early-life nutrition. Moreover, ancestral nutrition can affect offspring phenotype for several generations unequally (Deas et al., 2019). Hence, examining transgenerational effects through multiple generations might shed more light into better understanding how long the effects of past nutritional conditions persist for, and how these effects may vary between generations.

Considering the importance of carry-over effects of the macronutrient balance on infection and development, it would be interesting for future studies to construct a multi-dimensional nutritional study to investigate developmental and transgenerational effects across a large number of diets varying in both the PC ratio and macronutrient concentration. Such studies would increase our understanding of long-term effects of environmental conditions experienced in the past on subsequent life-history traits, a central question in Ecology (Sutherland at. al., 2013).

6.1.3. No evidence of oral immune priming in *B. tryoni*

In chapter 5, my results showed that oral immune priming with heat-killed or living bacteria did not provide a survival advantage to adult flies after re-infection. Although a large majority of studies have demonstrated a survival benefit of immune priming, our results are supported by studies in *Formica selysi* ants and *Tribolium castaneum* beetles showing no evidence for immune priming (Futo et al., 2017; Reber and Chapuisat, 2012). These results might reflex (i) The complexity of the priming mechanism with various factors affecting the outcome; (ii) Priming might benefit other traits than survival after infection (e.g., priming might increase host's reproductive output); or (iii) Oral infections do not stimulate immunity in tephritid flies. The absence of survival benefit of oral immune priming on pathogen resistance in our work prevented us from further examining the impact of diet quality on the effectiveness of oral priming. Future investigations of priming in *B. tryoni* might involve priming individuals through injection of killed pathogens or sub-lethal doses of bacteria at larval and adult stages. Also, investigation of priming in *B. tryoni* should now focus on measuring other life history traits in primed individuals such as reproduction.

6.2. Perspectives

Climate change is considered to be a major cause of species extinctions partly driven by decreases in food availability along with increases in disease prevalence through species interactions [(Cahill et al., 2013) and references therein]. Hence, better understanding the direct and carry-over effects of nutrition on infection outcomes would provide useful insights for species conversation in a changing world. Additionally, our result support the search for alternative ways to control harmful pest insects. The insect model used in our study, *B.tryoni*, is the most damaging pest insect in Australia causing large economic losses estimated at $28.5 million/annum in 2000 (Clarke et al., 2011) (Sutherst et al., 2000) (Hancock et al. 2000). The use of chemicals has previously been demonstrated to effectively control outbreak of this species but later banned because of their cost to human health and environment (Reynolds et al., 2017). Better understanding the biology and ecology of this species is essential to develop environmentally friendly methods to manage its populations. More importantly, results obtain during my PhD might also provide important knowledge for biomedical science with regard of finding alternative ways to treat infections. Since the Penicillin was discovered in 1928 by Scottish scientist and Nobel laureate Alexander Fleming, antibiotics have come a long way to effectively cure various kind of infectious diseases caused by microbes (Bennett and Chung, 2001). However, drug resistance has now

reached alarming levels that threatens public health and poses new challenges to researchers (Dong et al., 2007; Livermore, 2004; Williams, 2002). In an attempt to address this problem, scientists have been intensively looking for new ways to cure infections that include, for instance, the use of predatory bacteria or antimicrobial peptides from amphibians and reptiles (Reardon, 2015). Interestingly, the use of a common dietary element (i.e., iron) to cure lethal infections in mice has also been reported (Sanchez et al., 2018). Better understanding how nutrition influences the functioning of immune systems and the outcome of infections by different types of pathogens can contribute to developing new approaches to cure and/or decrease the negative effects of infections on host traits. In this thesis, I showed that the balance of macronutrients in the diet is an important modulator of survival after infection not only at the individual level but also across developmental stages and generations. More investigations about the mechanisms that link the metabolic and immune pathways resulting in an enhanced resistance would contribute important knowledge to one of the most exciting areas of translational research- Nutritional Immunology.

6.3. References

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Appendix I: Biosafety approval letter

Appendix I of this thesis has been removed as it may contain sensitive/confidential content

Appendix II: List of oral presentations at conferences during PhD candidature

- National Fly Meeting (Sep, 2019) "An integrated approach of nutritional immunology in fruit flies", Warburton, Australia.

- 2019 Higher Degree Research (HDR) conference (June 2019) "Effects of larval diet on adult fitness and pathogen resistance", Macquarie University, Sydney, Australia.

- 2018 HDR conference (June 2018) "Effect of parental diet on offspring development and pathogen resistance in Queensland fruit flies"- Macquarie University, Sydney, Australia

- 2018 ESA, ESC and ESBC Joint Annual Meeting (November 2018) "Nutritional effects on host defence induced by *Serratia marcescens* in *Batrocera tryoni*", Vancouver, Canada

- 6th Australian Biology of Tephritid Fruit Flies Meeting (March 2018): "Transgenerational effects of parental diet on offspring pathogen resistance" – Canberra, Australia.

- 5th Australian Biology of Tephritid Fruit Flies Meeting (June 2017). Studies on nutritional immunology in Queensland fruit flie" – Sydney, Australia.

- 2017 HDR conference (June 2017) "Nutrition and infection in Queensland fruit flies"- Macquarie University, Sydney, Australia

Appendix III. List of scholarships/ travel funds during PhD candidature

- International Macquarie Research Excellence Scholarship (iMQRES) that covers tuitions fee ($113,991 AUD) and allowance ($79,000 AUD) for 3 years to study PhD at Macquarie University, Australia (2016-2019).

- PhD Top-Up Scholarship ($15,000 AUD) for 3 years at Macquarie University, Australia (2016-2019).

- Travel fund ($800 AUD) to attend National Fly Meeting in Warburton and visit research laboratory at Monash University, Australia (2019).

- Travel fund ($5000 AUD) to attend ESA, ESC and ESBC Joint Annual Meeting in Vancouver, Canada (2018)