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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

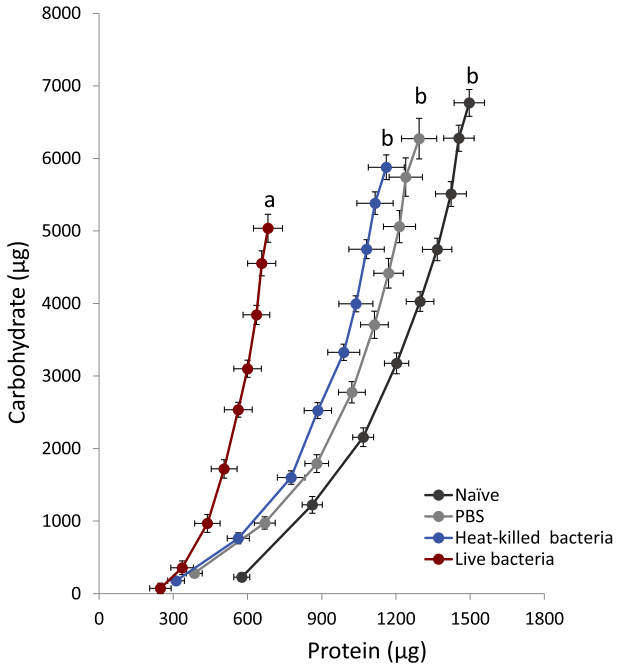

Fig. 1. Cumulative macronutrient intakes in the diet choice experiment. Cumulative carbohydrate and protein intake for naïve flies (n = 19), flies injected with PBS (n = 17), heat-killed bacteria (n = 22) or live bacteria (n = 13) was measured for 9 consecutive days (each data point representing 1-day 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.

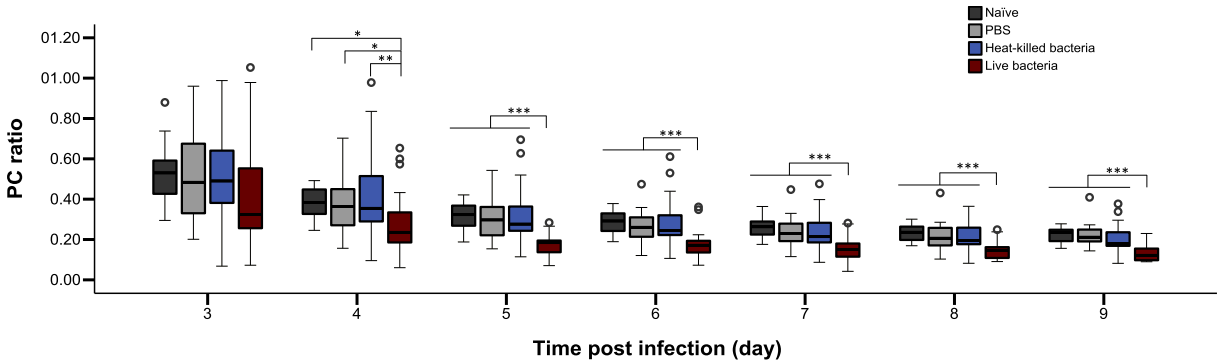

Fig. 2. Dietary protein-to-carbohydrate (PC) ratio in the diet choice experiment. Daily dietary PC ratio ingested by naïve flies (n = 19), flies injected with PBS (n = 17), heat-killed bacteria (n = 22) or live bacteria (n = 13). Box-and-whisker plots show median and interquartile range (IQR); whiskers 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).

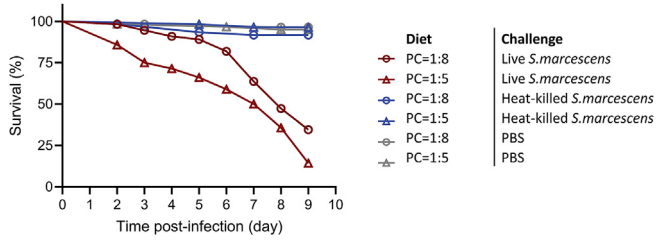

Fig. 3. Effect of diet on flies' survival after injection. Percentage of survival of flies injected with live bacteria (red), heat-killed bacteria (blue) or PBS (grey) when fed two diets varying in the protein-to-carbohydrate (PC) ratio: PC 1:8 (circle) vs PC 1:5 (triangle). (For interpretation of the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

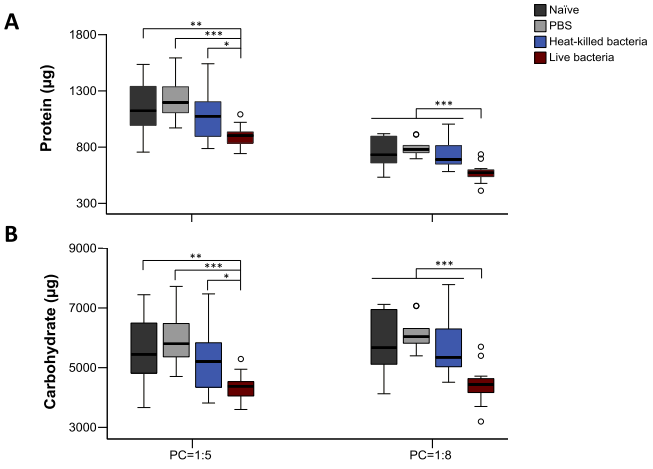

Fig. 4. Macronutrient intake in the no-choice experiment. Quantity of protein (A) and carbohydrate (B) consumed by flies for 9 days when restricted to a single diet, PC 1:8 (naïve flies n = 11, flies injected with PBS n = 11, heat-killed bacteria n = 12 or live bacteria n = 15) or PC 1:5 (naïve flies 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).

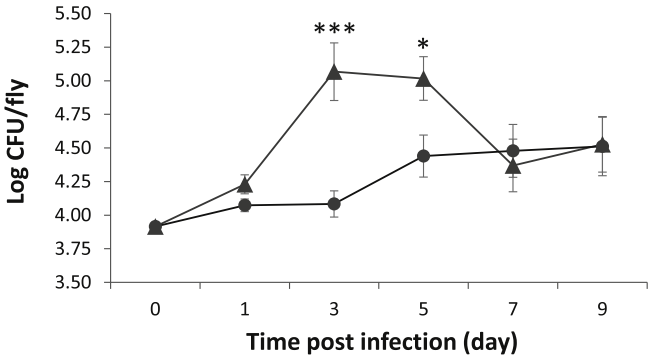

Fig. 5. Bacterial load of infected flies in the no-choice experiment. Bacterial load of flies injected with live bacteria when restricted to a single diet [PC 1:8 (circle) or PC 1:5 (triangle)]. Bacterial load was measured at 0, 1, 3, 5, 7 and 9 days post-infection. Plots show means of bacterial loads 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).

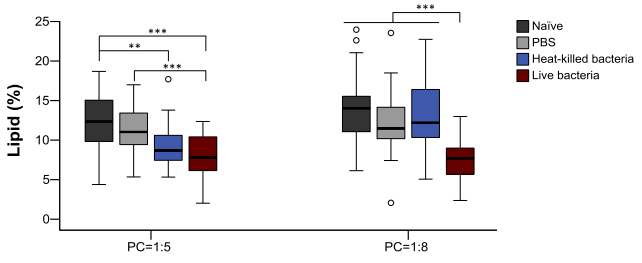

Fig. 6. Effect of diet and infection treatment on percentage of body lipid under diet restriction. Percentage of body lipid for naïve flies, flies injected with PBS, or heat-killed bacteria or live bacteria when restricted to a single diet (PC 1:8 or PC 1:5). Box-and-whisker plots show median and interquartile 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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