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RESEARCH ARTICLE

Role of the gut microbiome in mediating standard metabolic rate after dietary shifts in the viviparous cockroach, *Diploptera punctata*

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ABSTRACT

Diet may be a significant determinant of insect gut microbiome composition. However, the extent to which dietary shifts shape both the composition and relevant functions of insect gut microbiomes, and ultimately impact host energy balance (i.e. metabolic phenotype), is not well understood. We investigated the impacts of diet switching on *Diploptera punctata* females maintained on a dog food (DF) diet relative to those fed a comparatively sub-optimal cellulose-amended dog food (CADF) diet for 4 weeks. After this period, dietary shift resulted in a significantly higher average mass-specific standard metabolic rate (SMR) in CADF-fed females compared with DF-fed females. We also uncovered significant ^{13}C-enrichment in DF-fed insect samples relative to CADF-fed insect samples and lowered bacterial essential amino acid (EAA) provisioning in CADF-fed samples. Differences in SMR and EAA provisioning were not accompanied by significant differences in overall microbiome composition between the two groups. However, cellulolytic and nitrogen-fixing bacterial families dominant in wild omnivorous cockroaches and wood-feeding termites were significantly enriched in CADF-fed females than in DF-fed females, at the end of the study. We propose that these changes in microbiome composition after dietary shifts are associated with changes in EAA provisioning and possibly SMR. Further studies are needed to comprehensively understand the relative importance of gut microbial functions among the complexity of factors known to underscore SMR responses in insects under varying dietary conditions.

KEY WORDS: *Diploptera punctata*, Gut microbiome, Metabolic phenotype, Nutrient provisioning, Standard metabolic rate

INTRODUCTION

Animals strive to maintain a balance between energy inputs (sources) and outputs (sinks). Foraging, ingestion, digestion and nutrient absorption represent expenditures to acquire energy, whereas growth, maintenance of homeostasis, performance and reproduction represent expenditures dependent on acquired energy (Karasov, 1986). Several factors impact energy acquisition, but dietary quality, defined as the ratios of nutritional (protein: carbohydrate:lipid ratios) and non-nutritional (allelochemicals) components of ingested food is one of the most important (Behmer, 2008; Roeder and Behmer, 2014).

Dietary quality directly influences the minimum energy expenditure of ectotherms at a given ambient temperature under resting conditions, i.e. the standard metabolic rate (SMR) (Chown and Gaston, 1999). However, the impacts of dietary quality on SMR of ectotherms are not always straightforward (Cruz-Neto and Bozinovic, 2004; Burton et al., 2011). For example, in the harvestman, *Pachylus paessleri* (Opiliones), Naya et al. (2007) found significantly lower SMR and fecundity in response to feeding on a low-quality carbohydrate-rich diet relative to a high-quality protein-rich diet. In contrast, feeding on low-quality diets increased SMR and reduced growth in fifth instar *Spodoptera eridania* larvae (Karowe and Martin, 1989), and increased SMR in the locust *Locusta migratoria* (Zanotto et al., 1997) and the American cockroach *Periplaneta americana* (Ayayee et al., 2018). However, feeding on preys varying in lipid: protein ratios did not seem to impact SMR in the predatory wolf spider *Pardosa prativa* (Jensen et al., 2010), and in some cases SMR is constrained by morphological traits (e.g. size), developmental trajectories and physiological variation (Clark et al., 2016).

Indirectly, dietary quality may impact ectotherm energy balance through the disruption of host gut-associated microbiomes. For example, diet-driven differences in gut microbiomes has been demonstrated across various insect orders (Colman et al., 2012; Yun et al., 2014), as well as various wood-feeding beetle species, such as the generalist Asian long-horned beetle *Anoplophora glabripennis* (Geib et al., 2009; Scully et al., 2014), and specialists *Monochamus alternatus* (pine tree-feeding) and *Psacothea hilaris* (mulberry tree-feeding) beetles (Kim et al., 2017), various lignocellulolytic-fed omnivorous cockroach species (Bertino-Grimaldi et al., 2013; Pérez-Cobas et al., 2015), and various wood-feeding, soil-feeding and humus-feeding termite species (Raychoudhury et al., 2013; Rossmassler et al., 2015; Mikaelyan et al., 2016; Su et al., 2016). Diet-induced differences in gut microbiome compositions can also subsequently influence host gut microbiome functions. For example, differences in essential amino acid provisioning functions by bacterial and fungal members of host gut microbiomes (a function relevant for optimal insect host peptide and protein biosynthesis) under different dietary conditions have been verified in insects, such as *A. glabripennis* fed artificial diet versus host tree diets (Ayayee et al., 2016a), *P. americana* fed an optimal dog food diet or a sub-optimal cellulose-amended dog food diet (Ayayee et al., 2016b), and various soil mesofauna (Larsen et al., 2011; Pollierer et al., 2019), Enchytraeids (Larsen et al., 2016a), as well as earthworms (Larsen et al., 2016b; Potapov et al., 2019) feeding on various environmental dietary sources. These diet-induced disruptions of gut microbiome function are attributable to changes in gut microbiome functions, which may result in altered host metabolic phenotypes, and consequently changes in host SMR.

Although the impacts of diet type and quality on SMR are well studied, our understanding of the role of gut microbiota in mediating

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Received 31 January 2020; Accepted 5 May 2020

Journal of Experimental Biology (2020) 223, jeb218271. doi:10.1242/jeb.218271

SMR responses in insects is comparatively nascent. An exception is a recent study that investigated the relationship between diet shifts and gut microbiome composition and metabolite production by host and microbiome across different genetic lines of *Drosophila melanogaster* (Jehrke et al., 2018). Diet had no significant effect on gut microbiome compositions or on investigated metabolite–microbiome associations among genetic lines or developmental stages. This observation was attributed to the relatively high quality of the diets used and the comparatively simple composition of the gut microbiome of *D. melanogaster* (Wong et al., 2011; Jehrke et al., 2018), which may have masked any substantial dietary impacts. To date, we still do not quite understand how shifts from optimal laboratory-based diets to near-sub-optimal diets impact gut microbiome composition and functions, and how these interactions may potentially mediate the manifestation of the host metabolic phenotype.

We hypothesize that the energy expenditure of hosts is mediated by differences in gut microbiome composition and function. To evaluate our hypothesis, we used the only known viviparous cockroach species *Diploptera punctata*. This cockroach was chosen because of its viviparous reproductive strategy and the scarcity of data detailing impacts of dietary quality on this strategy in sexually mature females prior to starting reproductive events. Post-mated *D. punctata* females carry developing embryos in a specialized structure called a brood sac while providing nutritive 'milk' secretions (Williford et al., 2004; Bell et al., 2007). Like most cockroach species, *D. punctata* harbors the bacterial endosymbiont *Blattabacterium* spp. in specialized cells in the fat body (Gier, 1936), and also has a diverse adult gut microbiome (Ayayee et al., 2017; Dietrich et al., 2014). We exposed cockroaches to two diets differing in quality over 4 weeks, and measured changes in SMR (a proxy for energy expenditure), body mass (a proxy for growth), the composition of the gut microbiome, and provisioning of essential amino acids, a crucial function of the microbiome. We predicted changes in the composition and function of the gut microbiomes of *D. punctata* insects fed a comparatively sub-optimal diet relative to those fed an optimal diet, underscored by changes in SMR.

MATERIALS AND METHODS

Insect rearing and study layout

Diploptera punctata (Eschscholtz 1822) females were obtained from the insectary in the Department of Entomology at The Ohio State University. Adult laboratory-reared females used in this study were obtained from an initial colony fed an optimal dog food (DF) diet (Red Flannel Hi-Protein Formula dog food, PMI Nutrition, St Louis, MO, USA). Cockroaches were maintained in closed containers and provided with ventilation and water *ad libitum* under ambient laboratory conditions (ambient temperature 22±2°C, relative humidity 45%). The sub-optimal cellulose-amended dog food diet (CADF) was made by diluting DF with cellulose (Sigma-Aldrich, St Louis, MO, USA) at a 30:70 ratio (w/w), as used in a previous study (Ayayee et al., 2018). Cellulose was mixed with the DF diet because it is not a phago-deterrent for cockroaches (Bignell, 1978), but promotes increased food consumption for nutrient acquisition from the DF fraction in the CADF diet (A. Muñoz-Garcia, personal observations). It is also relatively recalcitrant to digestion by insects without gut microbial assistance (Kane and Breznak, 1991) and is shown to impact gut microbial compositions (Bignell, 1977, 1978; Gijzen et al., 1994; Bell et al., 2007) and microbial functions (Bignell, 1978; Ayayee et al., 2016b).

To investigate the impacts of diet on SMR and gut microbiome composition, we used 22 adult unmated females of varying ages from the DF-fed laboratory colony, to avoid any confounding effects due to sex. Selected females were first kept individually in containers without food for 24 h, to ensure that they would become post-absorptive before the start of the study on day 1. On day 1, we measured SMR and body mass, after which females were randomly assigned to a DF (N=11) or CADF (N=11) group for the duration of the study. On day 28 of the experiment, we measured body mass and SMR again in the same individuals.

A separate group of females was used to study essential amino acid (EAA) provisioning by the gut microbiota in *D. punctata*. For this purpose, we used previously collected samples from mated females (head capsules and six legs) and their associated embryos, unmated females and orphaned nymphs obtained from dead females from colonies that were part of another long-term study and had been feeding on the same DF and CADF diets used in this study for 6–8 weeks. Samples from both DF and CADF dietary groups were combined into DF-insect and CADF-insect sample groups, respectively, without any distinction between adult females and embryo samples for simplicity. Samples were lyophilized for 48 h, pulverized after lyophilization, and stored at −80°C, before compound-specific stable isotope analysis of EAA.

Measurement of standard metabolic rate

On day 1 and day 28, we measured oxygen consumption using the manual bolus integration method (Lighton, 2008). Briefly, females were incubated in a 60 ml airtight syringe containing dry air for 90 min at an ambient temperature of 30±2°C. Reference air samples (from an empty airtight syringe with dry air) were also collected using the same collection method. Subsequently, 40 ml of air was drawn from the airtight syringe and injected into an Oxzilla oxygen analyser (Sable Systems International, Las Vegas, NV, USA) at a flow rate of 260 ml min^{-1}, after passing through a column of silica gel and ascarite to remove water vapor and CO_2. The amount of oxygen consumed by each female was then calculated by determining the difference in the amount of oxygen (V_{O_2}) left in the airtight syringe used for sampling insects relative to the empty reference syringe (Withers, 1977). Oxygen consumed was subsequently converted to energy expenditure using the factor 20.08 J ml^{-1} O_2 (Schmidt-Nielsen, 1995). For each female, body mass was measured before and after incubation. We calculated mass-specific SMR as the quotient of energy expenditure and body mass.

The temperature in the incubation chamber (30°C) was constant during the trials, but different from that of the laboratory (~22°C). We used this set-up to be able to compare values for SMR across individuals and treatments, avoiding the fluctuations in ambient room temperature in the laboratory, which is a common practice (Dingha, 2009; DeVries et al., 2013; Harrison and Fewell, 2016). Moreover, the temperature difference between the insect colony and the incubation temperature (5–7°C) is within the ~10°C range within which mass-specific SMR is not significantly affected by temperature changes in cockroaches (Dingha, 2009; Harrison and Fewell, 2016). *Diploptera punctata* females were incubated for 90 min to compensate for their smaller sizes relative to other cockroaches (Ayayee et al., 2018), and to ensure significant oxygen consumption given the discontinuous gas exchange cycle of cockroaches (Schimpf et al., 2012a,b).

DNA extraction and gut bacterial load determination

After SMR measurements on day 28, all females (N=22) were immobilized at 4°C for 5 min and surface-sterilized as described by Ayayee et al. (2017); the entire gut was removed and gut mass was determined. We extracted DNA from each insect sample using the

Qiagen DNeasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, CA, USA). We carried out quantification of bacterial loads out using the bacterial primers 357f and 519r (Turner et al., 1999) via qPCR using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), cockroach gut DNA (~16–18 ng μl^{-1}) and water to a final 20 µl volume. Bacterial 16S rRNA plasmid standards were run in triplicate and insect DNA samples in duplicate. Reaction conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55.3°C for 15 s and 68°C for 20 s, and a final elongation step of 62°C for 20 s, using the Realplex Mastercycler (Eppendorf, Westbury, NY, USA). Bacterial load of template samples was calculated based on the generated standard curve of plasmid standards, after examination of melting curves and divided by gut mass as copy number/gram.

Amplicon generation, processing and analyses

Amplicon library preparation of the V4 region of the bacterial 16S rRNA gene was carried out using barcoded modified forward (515f) (Parada et al., 2016) and reverse (806r) (Apprill et al., 2015) primers for multiplexing and enhanced amplification of the bacterial and archaeal groups in environmental samples (Walters et al., 2016). DNA samples from individual cockroaches from each dietary treatment (N=10 from DF-fed individuals, one dropped due to low quality; N=12 from CADF-fed individuals, another individual was added from a similar CADF colony) were used for amplicon preparation. Briefly, we carried out triplicate PCR reactions out for all samples (with two technical DNA replicates each) using the Phusion polymerase and the Phusion High-Fidelity PCR Master Mix with HF buffer, water and barcoded-primers in a final reaction volume of 20 µl. Reaction conditions consisted of an initial denaturation for 30 s at 98°C, 30 cycles of 10 s at 98°C (denaturation), 10 s at 65°C (annealing) and 8 s at 72°C (extension), with a final extension phase of 5 min at 72°C. Gel-verified amplified products were pooled, purified using the Axygen AxyPrep Mag PCR Clean-Up kit (Axygen Biosciences, Union City, CA, USA) following the manufacturer's protocol, and concentration was determined using a Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of purified PCR products (12 ng) were then pooled together (yielding a total of 44 samples), and the multiplexed sample was sequenced on the Illumina Miseq platform using V2 chemistry (2×250 PE) at the University of Minnesota Genomics Center (UMGC, St Paul, MN, USA). Raw sequence data for all 44 samples are available in the NCBI Sequence Read Archive under the following BioProject accession number: PRJNA598044.

All 44 de-multiplexed reads were first merged using the fastqmerge_pairs command in usearch (Edgar, 2010), with default quality filtering parameters, and minimum and maximum merged read lengths of 250 and 253, respectively. Primary quality checking (filtering and error learning and de-replication) of merged fastq reads was performed using DADA2 (Callahan et al., 2016) and removal of primer sequences using cutadapt (Martin, 2011). Subsequent chimera removal (removeChimeraDenovo) and taxonomy assignments of amplicon sequence variants (ASV) using the SILVA database (version Silva_v132) (Quast et al., 2012) were carried out using DADA2. The final ASV table was then formatted to include taxonomic information and exported to a biom format for downstream analysis in QIIME (version 1.9) (Caporaso et al., 2010). Technical replicates of each sample were subsequently combined (avoiding over-inflation in subsequent downstream statistical analyses), resulting in the original 22 insect samples.

Alpha (count, richness and evenness measures) (Simpson, 1949; Shannon, 1957; Chao, 1984) and beta (Bray–Curtis distance matrix) (Bray and Curtis, 1957) diversity measures were analysed on the ASV table at the ASV and family level to assess within- and between-sample diversity among dietary categories using non-parametric analyses. Dietary category comparisons were examined using MRPP (Mielke, 1984), ANOSIM (D'Argenio et al., 2014) and PERMANOVA (Anderson, 2017). An indicator species analysis at the family level, examining microbial community members most likely driving differences among cockroach gut microbiomes from both dietary groups, was done using the group_significance command in QIIME (version 1.9) followed by a Kruskal–Wallis test. Figures were generated using JMP Pro 14 (SAS, Cary, NC, USA).

Assessment of microbial EEA provisioning

Pulverized samples were analysed at the Stable Isotope Facility at the University of California, Davis (Davis, CA, USA). Briefly, samples were acid-hydrolysed in 6 mol l^{-1} HCl at 150°C for 70 min under an N_2 headspace and subsequently derivatized via methoxycarbonylation-esterification (MOC) (Yarnes and Herszage, 2017) as modified from Walsh et al. (2014). Two technical replicates per *D. punctata* and dietary samples were analysed. Exogenous carbon addition, kinetic isotope effects from derivatization reagents, and normalization to the international reference for $\delta^{13}C$, V-PDB, was conducted following the procedures outlined in Walsh et al. (2014). EAA $\delta^{13}C$ data were obtained for isoleucine, leucine, lysine, phenylalanine, methionine, threonine and valine from all samples. The mean precision of the technical replicates across EAAs was ±0.55‰, which is well below the established quality control value of ±1.25. Final accuracy, as determined by the mean absolute difference in the measured and known $\delta^{13}C$ values of EAAs from a quality assessment mixture of amino acids, was within ±0.49‰.

EAA provisioning by gut microbiota in *D. punctata* was assessed using the established stable isotope fingerprinting technique (Larsen et al., 2009) and subsequently used to assess EAA provisioning in a variety of macroinvertebrates (Ayayee et al., 2016a; Larsen et al., 2013, 2016b). Briefly, the $\delta^{13}C$ values of EAAs in consumers (in this case *D. punctata*) biomass should be similar to those of EAAs from dietary materials (typically within ±2‰ range of mean dietary $\delta^{13}C$ EAAs), as consumers rely exclusively on dietary sources for EAAs (McMahon et al., 2010; Newsome et al., 2011). Origin of consumer EAAs and the relative contribution of bacterial, fungal and plant EAAs to consumer EAAs were subsequently determined through a linear discriminant function analysis (LDA), using $\delta^{13}C$ EAAs of the original representative bacterial, fungal and plant dataset from Larsen et al. (2013) as training data, calibrated to $\delta^{13}C$ EAAs data of the same representative samples from the Stable Isotope Facility.

Statistical analyses

We first tested all measured variables for normality and examined this using the Shapiro–Wilk's W goodness-of-fit test (Shapiro and Wilk, 1965) before analyses. Non-normal data were subsequently log-transformed, and normality of transformed data was tested again using the Shapiro–Wilk test. Insect body mass, gut mass, mass-specific SMR and bacterial copy numbers failed this initial normality screening and were all log-transformed. A mixed-model analysis (for repeated measures) with diet, time and their interaction as fixed effects, and samples as random factor, was carried out with SMR and body mass as our dependent variables, followed by Tukey's honestly significant difference pairwise mean comparisons. Gut bacterial load (16S rRNA copy numbers) and gut masses by day 28 were analysed using one-way ANOVA, with diet as the main factor. All these statistical analyses were performed using JMP (SAS).

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Analyses of $\delta^{13}C$ EAA values and ^{13}C-isotopic enrichment among insect samples and dietary groups (DF and CADF) were, respectively, carried out using ANOVA with treatment groups (DF-insects, CADF-insects, DF-diet, CADF-diet) and amino acids (all seven EAAs) as factors, first without and then with normalization to respective dietary $\delta^{13}C$ EAAs in JMP (SAS). We carried out an interlaboratory calibration for $\delta^{13}C$ EAA data from this study and the representative fungal, bacterial and plant $\delta^{13}C$ EAAs from Larsen et al. (2013), before analyses. LDA in JMP was used to create a classification model based on $\delta^{13}C$ EAA values from a training data set consisting of three classifiers, fungi, bacteria and plants to predict biosynthetic origins of insect EAAs. The LDAs were run using R package MASS (R; http://www.R-project.org).

RESULTS

Effects of diet on body mass, gut mass, bacterial 16S rRNA copy number and mass-specific SMR

Diet (F=0.72, P=0.41), time (day 1 and day 28) (F=0.005, P=0.94) and their interaction (F=2.12, P=0.16) did not significantly impact body mass over the course of the study (Fig. 1A). There was a ∼3.5% body mass loss in CADF-fed females, and a ∼4.2% body mass gain in DF-fed females by day 28. Similarly, gut mass (F=0.13, P=0.72) and 16S rRNA gene copy number (F=0.10, P=0.76) were not significantly different between DF and CADF-fed females by day 28.

We found an overall significant diet by time interaction on mass-specific SMR (F=5.50, P=0.029). There was no significant difference in mass-specific SMR between dietary groups on day 1 (P=0.99; Fig. 1B). By day 28, the mass-specific SMR of the CADF-fed females, 2.06±0.35 (mW g^{-1}, mean±s.e.m.), was approximately twice that of DF-fed females: 1.03±0.09 (P=0.023; Fig. 1B). Body mass, gut mass, bacterial 16S rRNA copy number and mass-specific SMR data are provided in Table S1.

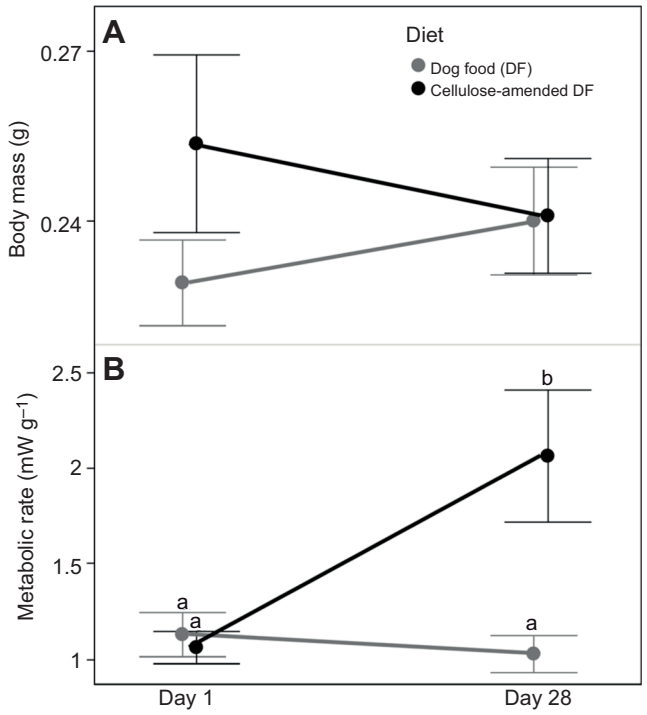

Fig. 1. Effects of diet on body mass and standard metabolic rate. Effects of dietary shift on (A) body mass and (B) standard metabolic rate of dog food-fed (DF-fed, N=11) and cellulose-amended dog food-fed (CADF-fed, N=11) *Diploptera punctata* females from the mixed model analysis. Different letters indicate significant differences. There were no significant differences in mean body mass between DF-fed and CADF-fed females on day 1 (CADF-fed females, 0.25±0.015 g, mass±s.e.m.; DF-fed females, 0.23±0.007 g) or day 28 (CADF-fed females, 0.241±0.01 g; DF-fed females, 0.24±0.01 g). Connecting lines between time points do not imply any linear changes.

Table 1. Microbial alpha diversity estimates at the taxon level (amplicon sequence variant, ASV) for DF-fed and CADF-fed *Diploptera punctata* females, following rarefaction to 17,000 reads per sample

Indices	DF-fed females	CADF-fed females	*P*-value
Chao1	778.78±229.04	916.73±271.35	0.25
Simpson's index	0.93±0.05	0.92±0.08	0.86
Shannon's evenness	5.77±1.08	6.03±1.41	0.63
Observed ASVs	566.4±193.4	665.67±212.72	0.27

Values are the means±standard deviation of the mean for each dietary group. DF, dog food diet; CADF, cellulose-amended dog food diet.

Effect of diet on microbiome composition

Quality checking (merge, multistep filtering, error learning and dereplication, removal of chimeras) via DADA2 retained 83.95% (1,160,292) of initial merged 1,382,481 reads resulting in a mean of 52,559±22,106 across samples. Filtering yielded a total of 2348 ASVs, which resulted in 165 bacterial families across dietary groups. Both family-level and ASV tables were rarefied to 17,000 reads per sample prior to all diversity analyses. None of the four alpha diversity indices measured yielded significant differences between the two dietary feeding groups at the taxon (ASV) level or family level (Table 1). We uncovered no significant differences in microbiome composition between DF-fed and CADF-fed dietary groups at the taxon (ASV) or family level (Table 2).

We found differences in the relative in abundances of 10 bacterial families between the two dietary groups (Table 3). Of these, *Xanthomonadaceae*, *Sphingobacteriaceae*, *Lactobacillaceae*, *Caulobacteraceae*, *Rhizobiaceae* and *Methylophilaceae* (in order of decreasing abundance) were significantly more abundant in DF-fed females, whereas the remaining four families [*Clostridiaceae_1*, *Paracaedibacteraceae*, Unassigned JGI_0000069-P22 and Absconditabacteriales_(SR1)] were more abundant in CADF-fed females (Table 3).

Gut microbial EAA provisioning

Despite the lack of significant differences in gut microbiome composition, we uncovered significant differences in gut microbial EAA provisioning between DF-fed females and CADF-fed females. Across all samples, threonine and lysine were more enriched than the remaining five EAAs, with leucine having the lowest raw $\delta^{13}C$ values (F=106.1, P<0.0001) (Table 4). Mean $\delta^{13}C$ of the seven EAAs in both CADF-fed and DF-fed insects were significantly more ^{13}C-enriched relative to respective diets (F=48.43, P<0.0001) (Table 4). However, the mean $\delta^{13}C$ EAA of DF-fed females was significantly

Table 2. Between-categories tests of beta diversity at the taxon (amplicon sequence variant, ASV) and family level for DF-fed and CADF-fed *D. punctata* females, following rarefaction to 17,000 reads per sample

Test	Taxon (ASV) level	Family level
MRPP	A=0.0004, P=0.42	A=−0.004, P=0.52
ANOSIM	t-statistic=0.015, P=0.33	t-statistic=−0.002, P=0.42
PERMANOVA	t-statistic =1.03, P=0.40	t-statistic=0.87, P=0.48

Values are the means±s.d. for each dietary group. DF, dog food diet; CADF, cellulose-amended dog food diet.

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Table 3. Mean abundances (read count) and calculated relative abundances of the bacterial families that were significantly different between DF-fed and CADF-fed *D. punctata* females, and their associated Kruskal–Wallis test statistics

Phylum	Family	Test statistic	*P*	DF-fed females	CADF-fed females
Proteobacteria	*Xanthomonadaceae*	4.73	0.03	**697.70 (4.1)**	195.58 (1.15)
	Caulobacteraceae	7.36	0.01	**22.40 (0.13)**	0.08 (0.0005)
	Rhizobiaceae	6.58	0.01	**12.30 (0.07)**	0.42 (0.002)
	Methylophilaceae	5.10	0.02	**3.10 (0.02)**	0.33 (0.002)
Bacteroidetes	*Sphingobacteriaceae*	5.19	0.02	**527.10 (3.1)**	33.42 (0.20)
Firmicutes	*Lactobacillaceae*	4.32	0.04	**143.70 (0.84)**	51.50 (0.30)
Firmicutes	*Clostridiaceae_1*	3.68	0.05	10.10 (0.06)	**33.17 (0.20)**
Proteobacteria	*Paracaedibacteraceae*	4.05	0.04	0.20 (0.001)	**2.33 (0.01)**
Patescibacteria	Unassigned JGI_0000069-P22	4.12	0.04	2.90 (0.02)	**12.83 (0.07)**
	Absconditabacteriales_(SR1)	3.82	0.05	2.30 (0.01)	**3.58 (0.02)**

Values are total reads (% in parentheses); 17,000 per sample. DF, dog food diet; CADF, cellulose-amended dog food diet.

higher than that of CADF-fed females (P<0.0001) (Table 4). Isotopic offset following normalization of insect $\delta^{13}C$ EAAs to dietary $\delta^{13}C$ EAAs ($\Delta\delta^{13}C_{EAA}=\delta^{13}C_{\text{insect samples EAA}}-\delta^{13}C_{\text{respective diet EAA}}$), revealed significant differences between insect samples and corresponding diets across all seven EAAs (F=32.80, P<0.0001) (Table 4). However, mean ^{13}C-isotopic offsets across all seven EAAs between DF-fed and CADF-fed females were not significantly different (Table 4). Mean ^{13}C-isotopic offsets of individual EEAs were comparable between DF-fed and CADF-fed females, except for lysine, which was more ^{13}C-enriched in DF insects (11.30±0.66‰) relative to CADF-fed insects (8.53±0.56‰; F=1.76, P=0.04; Fig. 2). The amino acid threonine had the lowest $\delta^{13}C$ value in both diets and insect samples and experienced the least isotopic offset. The $\delta^{13}C$ EAA data for samples used in this analysis are provided in Table S2.

Given the significant ^{13}C-isotopic offsets in EAAs between insect and dietary samples, we investigated the sources of isoleucine, leucine, lysine, phenylalanine and valine in the insect hosts using LDA. The model was validated by the successful separation of bacteria (N=12), fungi (N=9) and plant (N=12) into distinct groups (F=55, P<0.0001; Wilk's lambda=0.007, a test of appropriateness of separation of classifiers and for group membership prediction of non-classifier samples; Table S3). The validated predictive model was then used to predict possible sources of EAAs in insect and dietary samples, which was based on the location of insect and dietary samples in the LDA plot, and the proximity to classifier centroids (fungi, bacteria and plants). The resulting predictions of samples were subsequently validated based on the correct assignment of the test fungus, *Fusarium solanum*, to the fungal classifier group (Fig. 3). Plants were not a major source of EAAs in DF-fed and CADF-fed cockroaches (Fig. 4). The DF and CADF diets were closer to the fungal classifier than to the bacterial classifier group. This may be attributed to the high fungal protein component of the DF diet (brewer's yeast accounts for ∼26% of crude protein fraction) and the CADF diet (∼7.8% brewer's yeast in the CADF diet).

We calculated the relative contributions of bacterial and fungal EAAs to overall insect and dietary EAAs using the posterior probability scores from the validated predictive model. For DF and CADF diets, EAAs were primarily fungal in origin, with no bacterial input (Fig. 4). In contrast, both DF-fed and CADF-fed insects had bacterial and fungal EAA inputs, with a higher bacterial input in DF-insects relative to CADF-insects (Fig. 4). Bacterial EAAs may have originated from gut microbiota, whereas fungal EAAs may have originated from the diet or from gut-associated fungi.

Table 4. $\delta^{13}C$ signatures of seven essential amino acids across samples and their calculated ^{13}C-discrimination values, for DF-fed and CADF-fed *D. punctata* females and their respective diets

	Mean $\delta^{13}C_{EAA}$ (‰)	^{13}C-enrichment relative to diets
Essential amino acids		
Threonine	−6.08±0.65^{a}	
Lysine	−12.92±1.14^{a}	
Isoleucine	−18.89±0.61^{c}	
Valine	−19.80±0.88^{d}	
Methionine	−20.18±0.52^{c,d}	
Phenylalanine	−21.31±0.52^{d}	
Leucine	−23.13±0.62^{e}	
Treatment group		
DF diet	−20.94±1.76^{c}	0^{b}
CADF diet	−21.89±2.43^{c}	0^{b}
DF insects	−15.52±0.96^{a}	5.42±0.57^{a}
CADF insects	−17.21±0.83^{b}	4.66±0.41^{a}

Values are given as means±s.e.m. DF, dog food diet; CADF, cellulose-amended dog food diet. Different letters indicate significant differences (P<0.05) within groups.

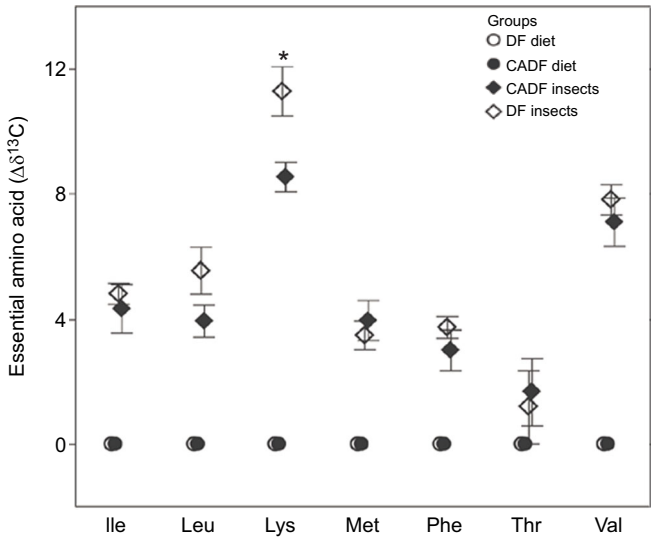

Fig. 2. Gut microbial essential amino acid provisioning. The ^{13}C-offsets ($\Delta^{13}C_{EAA}$: enrichment or depletion) of essential amino acids in DF-fed (N=5) and CADF-fed females (N=7) were normalized to respective dog food (DF) and cellulose-amended (CADF) diets. Only lysine (Lys) was significantly enriched in DF-fed insects (11.30±0.66) than in CADF-fed insects (8.53±0.56) (*P=0.04). Values represent mean values (±s.e.m.) for duplicate insect samples and dietary samples. Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Val, valine.

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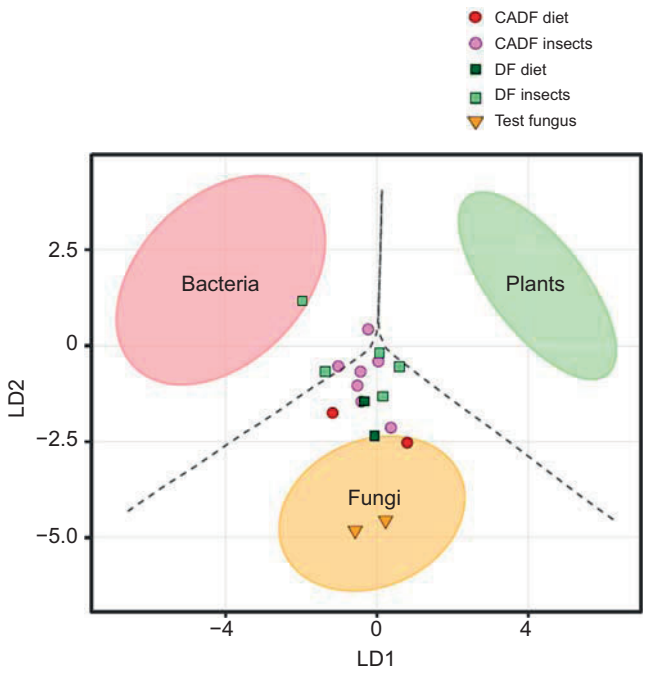

Fig. 3. Predicted possible sources of essential amino acids in insect and dietary samples. A linear discriminant function analysis (LDA) plot based on $\delta^{13}C_{EAA}$ from DF-fed (*N*=5) and CADF-fed insects (*N*=7), their respective DF (*N*=1) and CADF (*N*=2) diets, three classifier groups [fungi (*N*=9), bacteria (*N*=12) and plants (*N*=12)], and the test fungus *Fusarium solani* (*N*=2). Variation of classification accounted for 64% along LD1 and 36% along LD2. The shaded ellipses signify the 95% confidence limits for each classifier group, and the dashed lines between classifiers represent the decision boundaries for each classifier group. The essential amino acids used were isoleucine, leucine, lysine, phenylalanine, and valine.

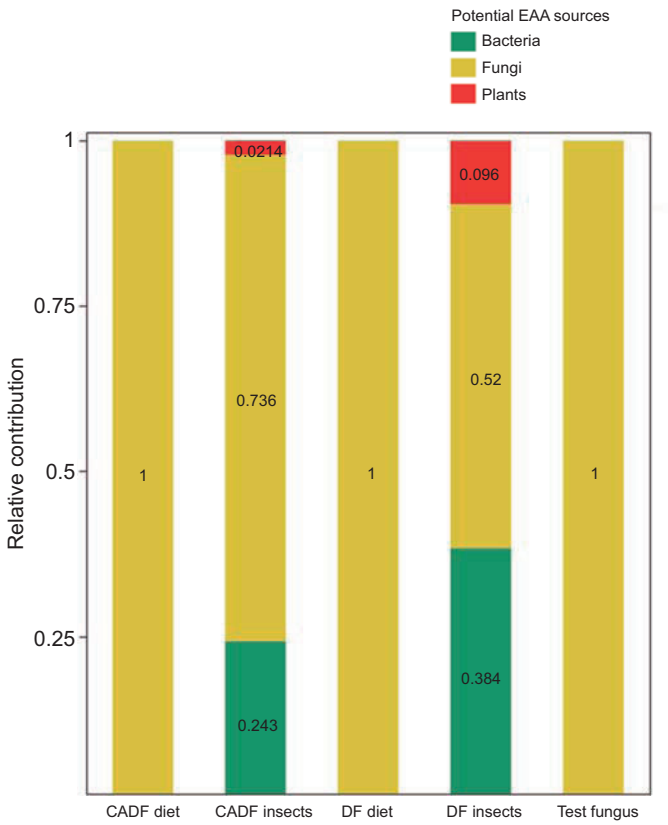

Fig. 4. Relative contributions of bacterial and fungal essential amino acids to overall insect and dietary essential amino acids. Relative input of bacterial, fungal and plant essential amino acids (EAAs) in dog food-fed (DF, *N*=5) and cellulose-amended dog food-fed (CADF, *N*=7) *D. punctata* females, relative to respective diets, based on LDA predicted probabilities.

DISCUSSION

We found that shifting *D. punctata* from an optimal (DF) to a sub-optimal (CADF) diet resulted in a significant increase in mass-specific SMR of the host. We also found that dietary shifts altered microbial EAA provisioning, a function perhaps associated with changes in relative abundances of specific bacterial families. We propose that these changes in the microbiome are concomitant with the altered metabolic phenotype of the hosts. Given that the underlying factors impacting mass-specific SMR in response to feeding on sub-optimal diets are not straightforward, potential explanations underscoring the results from this study may be a combination of host-mediated and microbe-mediated factors.

From a host-mediated point of view, the higher SMR may be attributed to higher physiological activity in the gastrointestinal tract; however, we did not observe significant differences in gut mass between the two dietary groups. Some insects may reduce food consumption on sub-optimal diets (Lee, 2007), resorting to post-absorptive processes, such as oxidation of stored reserves (lipids and proteins) (McCue et al., 2015; Zanotto et al., 1993), leading to increased SMR and relatively reduced body masses. In this study, CADF-fed females might have a higher mobilization of lipid reserves in the fat body, potentially explaining the relative reduction of body mass in the CADF-fed female group by day 28. The decrease in SMR and the lack of significant difference in body mass in response to the same sub-optimal diet (i.e. CADF diet) has been previously reported in *P. americana* cockroaches (Ayayee et al., 2018); in that experiment, animals were fed with CADF for only 7 days.

From a microbiome-mediated point of view, the higher SMR in CADF-fed females may be attributed to diet-induced changes in microbiome function, which might lead to increases in host energy expenditure. Some changes observed in response to feeding on high cellulose diets include reductions in microbe-derived metabolites for energy generation (acetate and lactate) and reduced provisioning of essential amino acids. For example, *P. americana* cockroaches fed a dog food diet had significantly higher amounts of microbe-derived acetate and lactate and higher lactic acid bacterial counts, relative to individuals fed a diet with high cellulose content (Kane and Breznak, 1991). These microbe-derived products are estimated to provide ∼14% of the respiratory requirements of cockroaches and termites (Breznak and Kane, 1990; Kane and Breznak, 1991). Although we did not quantify acetate or lactate in this study, we observed changes in ^{13}C-enrichment and EAA provisioning that support the proposed microbe-mediated SMR response in CADF-fed females. This is corroborated by the lower bacterial EAA input in CADF-fed females relative to DF-fed females (Fig. 4), despite the relatively higher fungal protein component of the DF diet compared with the CADF diet. The higher fungal EAA input in CADF-fed females may be the result of a combination of CADF diet consumption and diet-induced changes in gut fungal community membership. For example, Kakumanu et al. (2018) determined that laboratory-reared German cockroaches, *Blattella germanica*, maintained on rodent chow (with low fiber content) had significantly higher fungal abundances, and lower ciliate and protist abundances in their guts, compared with field-collected (urban apartment) omnivorous individuals. Although we did not study

the gut fungal composition in this study, and insect gut fungal–bacteria interactions are unclear, the higher fungal EAA input in CADF-fed females may have derived from the incorporation of fungal EAAs from the gut following increased gut fungal abundances and cellulolytic activity and subsequent EAA biosynthesis. Ultimately, the microbial function investigated in this study cannot be directly attributed to any bacterial or fungal taxon, regardless of the dietary group. This is because EAA biosynthesis is a feature shared by all free-living and endosymbiotic bacteria, as well as fungi. Coupling broad microbial functions, such as EAA provisioning with taxon-specific or physiology-specific microbial functions (e.g. nitrogen fixation or cellulolytic activity) using stable isotope techniques, may provide a possible approach with which to both uncover and assign microbial functions to specific gut microbiome members in similar studies going forward. Overall, results from this study indicate the potential functions of the gut microbiome in mediating host SMR responses to changing environmental conditions.

The observed differences in gut microbial EAA provisioning function were not associated with a significant difference in gut bacterial community composition in *D. punctata* females, despite the difference in diets and the duration of the feeding trial. This lack of significant difference between dietary groups adds to the growing body of work detailing the variable impacts of dietary quality on cockroach gut microbiomes. These have ranged from significant differences in composition after 9 days (Pérez-Cobas et al., 2015) and 14 days (Bertino-Grimaldi et al., 2013) of feeding, to a relatively stable gut microbiome following ∼90 days of incubation on diets varying in cellulosic content (Schauer et al., 2014; Tinker and Ottesen, 2016). However, we observed the emergence of differentially abundant and metabolically distinct taxa between DF-fed and CADF-fed females that corroborate the proposed microbiome-mediated SMR response framework in this study.

In CADF-fed females, we detected higher mean relative abundances of taxa more commonly abundant in wood-feeding termites (Hongoh et al., 2005; Köhler et al., 2012; Mikaelyan et al., 2016) and wood-feeding cockroaches (Berlanga et al., 2016). For example, in response to the presence of cellulose in the CADF diet, cellulolytic and anaerobic nitrogen-fixing Firmicutes (family Clostridiaceae_1), anaerobic Patescibacteria (families Unassigned JGI_0000069-P22 and Absconditabacteriales_SR1) that utilize citrate, malate and amino acids as both generators and recycling agents of reductive power (Pepe-Ranney et al., 2016; Sieber et al., 2019), and bacterial endosymbionts of protists Proteobacteria (family *Paracaedibacteraceae*) (Tashyreva et al., 2018; Boscaro et al., 2019) were more abundant in CADF-fed females relative to DF-fed females. These higher abundances may be indicative of different diet-induced gut conditions in CADF females, leading to the emergence of taxa that are essential to thrive on high-fiber diets. In particular, the increase in relative abundance of protist-associated endosymbiotic bacteria *Paracaedibacteraceae* may be reflective of increased abundances of gut protists in CADF-fed females. High-cellulose diets have been shown to increase the abundances of specific cellulolytic termite gut protists (*Pyrsonympha* sp. and *Microjoenia* sp.), which tend to be less abundant on more complex untreated wood diets (Duarte et al., 2017), as well as changes in protist and protist-associated bacterial symbiont abundances (Gijzen et al., 1994; Berlanga et al., 2018). Interestingly, this bacterial family has so far only been reported in free-living freshwater and marine protists (Tashyreva et al., 2018; Boscaro et al., 2019). The relationship between this bacterial species and other protist-associated bacteria from wood-feeding termites (Stingl et al., 2005; Sato et al., 2009) and cockroaches (Noda et al., 2006) remains to be investigated. In contrast, the abundant bacterial taxa in the DF-fed females were those typically detected in high abundances in other laboratory-reared cockroaches (Schauer et al., 2012, 2014; Bertino-Grimaldi et al., 2013; Tinker and Ottesen, 2016; Ayayee et al., 2017; Mohammed et al., 2018; Table 3). These bacterial members are similarly present in wild cockroaches, although with comparatively different abundances. For example, the phyla Bacteroidetes and Fusobacteria usually have lower representation in wild cockroaches, whereas the phyla Proteobacteria and Firmicutes have relatively higher representation. The higher relative abundances of these taxa in DF-fed females may be contributing to the higher bacterial EAA input.

Conclusions

In conclusion, we found that a dietary shift from an optimal diet (DF) to a relatively sub-optimal diet (CADF) for 28 days resulted in a higher SMR in CADF-fed *D. punctata* females relative to DF-fed females. We propose that the higher SMR in CADF-fed females may be attributed to diet-induced changes in microbial functions, such as lowered provisioning of microbe-derived metabolites (acetate, lactate, propionate and EAAs) for energy generation and biosynthesis. Our results support our hypothesis that such host gut microbiota functions might mediate the metabolic phenotype of insect hosts. Additionally, we found that changes in relatively low abundant taxa in the microbiome can significantly impact metabolic function, which may not necessarily be associated with significant changes in the whole microbiome composition. The results herein provide a framework within which to investigate the mediating roles of an organism's gut microbiome on several aspects of host metabolic phenotype in response to external stressors, such as diet, disease and environment.

Acknowledgements

The authors thank Jose Diaz at the Evolution, Ecology, and Organismal Biology Department, The Ohio State University, and also the University of Wyoming's Microbial Ecology Collaborative.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.A.A., G.K., C.Y., T.L., A.M.; Methodology: P.A.A., T.L., L.T.A.v.D., A.M.-G.; Validation: P.A.A., C.Y.; Formal analysis: P.A.A., C.Y., G.F.C., T.L., L.T.A.v.D., A.M.-G.; Resources: G.K., C.Y., L.T.A.v.D., A.M.-G.; Writing - original draft: P.A.A., T.L., A.M.-G.; Writing - review & editing: P.A.A., G.K., C.Y., G.F.C., T.L., L.T.A.v.D., A.M.-G.; Supervision: P.A.A., L.T.A.v.D., A.M.-G.; Funding acquisition: L.T.A.v.D., A.M.-G.

Funding

This research was supported by the University of Wyoming's Microbial Ecology Collaborative (National Science Foundation award EPS-1655726).

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.218271.supplemental

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Table S1. Body mass, standard metabolic rate (SMR), gut mass, and bacterial 16S rRNA copy number of all individual *Diploptera punctata* females fed with dog food (DF) and cellulose amended dog food (CADF) on day one and day 28 of the experiment.

Sample ID	Diet	Body mass (g)	SMR (mW/g)	Gut mass (g)	Average copy number	Time
LQ1	CADF	0.21	1.06	n/a	n/a	Day 1
LQ2	CADF	0.34	0.73	n/a	n/a	Day 1
LQ3	CADF	0.18	1.78	n/a	n/a	Day 1
LQ4	CADF	0.33	1.12	n/a	n/a	Day 1
LQ5	CADF	0.26	0.73	n/a	n/a	Day 1
LQ6	CADF	0.23	1.15	n/a	n/a	Day 1
LQ7	CADF	0.27	1.04	n/a	n/a	Day 1
LQ8	CADF	0.29	1.08	n/a	n/a	Day 1
LQ9	CADF	0.19	0.90	n/a	n/a	Day 1
LQ10	CADF	0.24	1.12	n/a	n/a	Day 1
LQ11	CADF	0.25	0.96	n/a	n/a	Day 1
DF1	DF	0.25	1.45	n/a	n/a	Day 1
DF2	DF	0.20	0.59	n/a	n/a	Day 1
DF3	DF	0.20	1.25	n/a	n/a	Day 1
DF4	DF	0.22	0.88	n/a	n/a	Day 1
DF5	DF	0.21	0.61	n/a	n/a	Day 1
DF6	DF	0.26	0.98	n/a	n/a	Day 1
DF7	DF	0.26	1.23	n/a	n/a	Day 1
DF8	DF	0.20	1.62	n/a	n/a	Day 1
DF9	DF	0.22	1.29	n/a	n/a	Day 1
DF10	DF	0.25	1.71	n/a	n/a	Day 1
DF12	DF	0.25	0.81	n/a	n/a	Day 1
LQ2	CADF	0.32	2.20	0.02	193500	Day 28
LQ3	CADF	0.22	0.61	0.05	1380000	Day 28
LQ4	CADF	0.21	1.63	0.05	748500	Day 28
LQ5	CADF	0.24	0.47	0.06	749000	Day 28
LQ6	CADF	0.23	2.53	0.02	1095000	Day 28
LQ7	CADF	0.28	2.79	0.04	261500	Day 28
LQ8	CADF	0.23	1.36	0.04	716500	Day 28
LQ9	CADF	0.20	3.21	0.06	5830000	Day 28
LQ10	CADF	0.25	4.32	0.05	385500	Day 28
LQ11	CADF	0.24	2.32	0.03	84742.5	Day 28
DF1	DF	0.29	1.58	0.04	497500	Day 28
DF2	DF	0.21	0.65	0.03	1950000	Day 28
DF3	DF	0.22	1.37	0.04	606000	Day 28
DF4	DF	0.23	0.79	0.05	1265000	Day 28
DF5	DF	0.22	0.70	0.05	2935000	Day 28
DF6	DF	0.25	1.09	0.03	548500	Day 28
DF7	DF	0.29	0.75	0.05	909500	Day 28
DF8	DF	0.19	0.80	0.04	2095000	Day 28
DF9	DF	0.25	1.19	0.05	665500	Day 28
DF10	DF	0.26	0.97	0.04	478000	Day 28
DF12	DF	0.23	1.41	0.02	433500	Day 28

Table S2. $\delta^{13}C$ of seven essential amino acids from *Diploptera punctata* individuals fed with dog food (DF) (n = 5) or with cellulose amended dog food (CADF) (n = 7), and their respective diets (DF, n = 2; CADF, n = 1) used for in the LDA analysis.

Sample ID	Sample type	Category	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine
CADF-4F	CADF-Female	CADF-Insects	-16.468	-22.07	-11.4875	-19.9455	-21.0205	-7.4745	-18.606
CADF-4N	CADF-Embryos	CADF-Insects	-15.867	-21.138	-10.0195	-17.3035	-18.807	-2.7585	-15.617
CADF-2F	CADF-Female	CADF-Insects	-18.937	-23.1615	-11.5795	-18.927	-20.688	-10.0205	-19.3735
CADF-1F	CADF-Female	CADF-Insects	-18.521	-22.602	-12.0575	-20.444	-21.078	-8.0635	-18.67
CADF-1N	CADF-Embryos	CADF-Insects	-18.0335	-22.636	-11.2295	-20.1085	-21.5725	-2.191	-18.8215
DF-4F	DF-Female	DF-Insects	-18.217	-22.6455	-10.3335	-19.549	-20.618	-6.692	-18.583
DF-4N	DF-Embryos	DF-Insects	-16.8165	-21.7805	-8.633	-18.472	-19.4285	-4.6455	-16.6205
DF-3F	DF-Female	DF-Insects	-17.6075	-20.8845	-11.0995	-19.792	-20.2355	-8.1585	-17.7675
DF-3N	DF-Embryos	DF-Insects	-16.3045	-18.3885	-7.0465	-17.217	-18.758	-1.1025	-15.849
DF-2N	DF-Embryos	DF-Insects	-17.038	-21.871	-11.0605	-18.379	-19.1005	-4.8355	-17.8205
CADF-1F	CADF-Female	CADF-Insects	-20.9645	-24.455	-13.6565	-22.561	-24.2035	-7.6895	-20.588
CADF-2F	CADF-Female	CADF-Insects	-21.2635	-25.052	-13.3065	-21.506	-22.883	-6.9335	-22.207
DF-diet	DF-diet	DF-diet	-22.1525	-26.3885	-19.258	-21.4835	-21.905	-6.9685	-24.1335
DF-diet	DF-diet	DF-diet	-21.8985	-26.951	-22.595	-22.885	-24.8705	-5.6	-26.135
CADF	CADF-diet	CADF-diet	-22.9285	-26.9745	-20.4345	-24.0895	-24.497	-8.1215	-26.221

Table S3. Predicted classification of bacteria, fungal, plant classifiers, dog food (DF) or cellulose amended dog food (CADF) fed *D. punctata* females, DF samples, CADF samples, and test fungus (*Fusarium solani*), and associated probabilities of belonging to each category estimated from the LDA analysis.

Groups	Predicted	Bacteria	Fungi	Plants
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Fungi	Fungi	0.00	1.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Bacteria	Bacteria	1.00	0.00	0.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
Plants	Plants	0.00	0.00	1.00
CADF-insects	Fungi	0.00	1.00	0.00
CADF-insects	Fungi	0.00	1.00	0.00
CADF-insects	Fungi	0.06	0.88	0.06
CADF-insects	Fungi	0.01	0.99	0.00
CADF-insects	Fungi	0.08	0.92	0.00
DF-insects	Fungi	0.14	0.66	0.20
DF-insects	Fungi	0.00	0.72	0.28
DF-insects	Bacteria	0.78	0.22	0.00
DF-insects	Bacteria	1.00	0.00	0.00
DF-insects	Fungi	0.00	1.00	0.00
CADF-insects	Bacteria	0.67	0.33	0.00
CADF-insects	Bacteria	0.88	0.03	0.09
DF-diet	Fungi	0.00	1.00	0.00
DF-diet	Fungi	0.00	1.00	0.00
CADF-diet	Fungi	0.00	1.00	0.00
Test fungus	Fungi	0.00	1.00	0.00
Test fungus	Fungi	0.00	1.00	0.00