Received: 22 August 2016 | Revised: 8 February 2019 | Accepted: 11 February 2019

DOI: 10.1111/mec.15057

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

No evidence that gut microbiota impose a net cost on their butterfly host

Alison Ravenscraft[1,2] | Nicole Kish[2,3] | Kabir Peay[1] | Carol Boggs[2,3,4]

[1]Department of Biology, Stanford University, Stanford, California

[2]Rocky Mountain Biological Laboratory, Crested Butte, Colorado

[3]Department of Biological Sciences, University of South Carolina, Columbia, South Carolina

[4]School of the Earth, Ocean & Environment, University of South Carolina, Columbia, South Carolina

Correspondence
Alison Ravenscraft, Center for Insect Science, University of Arizona, Tucson, AZ.
Email: aravenscraft@gmail.com

Funding information
Rocky Mountain Biological Station, Grant/Award Number: Graduate Fellowship; University of South Carolina; National Science Foundation, Grant/Award Number: DGE - 1147470; American Society of Naturalists, Grant/Award Number: Student Research Award; Stanford University; National Institutes of Health PERT, Grant/Award Number: K12GM000708

Abstract

Gut microbes are believed to play a critical role in most animal life, yet fitness effects and cost–benefit trade-offs incurred by the host are poorly understood. Unlike most hosts studied to date, butterflies largely acquire their nutrients from larval feeding, leaving relatively little opportunity for nutritive contributions by the adult's microbiota. This provides an opportunity to measure whether hosting gut microbiota comes at a net nutritional price. Because host and bacteria may compete for sugars, we hypothesized that gut flora would be nutritionally neutral to adult butterflies with plentiful food, but detrimental to semistarved hosts, especially when at high density. We held field-caught adult *Speyeria mormonia* under abundant or restricted food conditions. Because antibiotic treatments did not generate consistent variation in their gut microbiota, we used interindividual variability in bacterial loads and operational taxonomic unit abundances to examine correlations between host fitness and the abdominal microbiota present upon natural death. We detected strikingly few relationships between microbial flora and host fitness. Neither total bacterial load nor the abundances of dominant bacterial taxa were related to butterfly fecundity, egg mass or egg chemical content. Increased abundance of a *Commensalibacter* species did correlate with longer host life span, while increased abundance of a *Rhodococcus* species correlated with shorter life span. Contrary to our expectations, these relationships were unchanged by food availability to the host and were unrelated to reproductive output. Our results suggest the butterfly microbiota comprises parasitic, commensal and beneficial taxa that together do not impose a net reproductive cost, even under caloric stress.

KEYWORDS
dietary restriction, fitness, gut bacteria, life history, life span, microbiome

1 | INTRODUCTION

Bacteria are ubiquitous inhabitants of the animal gut. Research on the function of these gut mirobiota has focused primarily on benefits to the host (e.g., Nicholson et al., 2012), which can include enhanced uptake and synthesis of nutrients, breakdown of toxic food byproducts and protection against pathogen invasion (Clemente, Ursell, Parfrey, & Knight, 2012; Dillon & Dillon, 2004). However, widespread interest in their benefits can obscure the complex and varied outcomes of these relationships. Species differ in the degree to

which they depend upon their gut symbionts: for some hosts, they are essential to survival (e.g., Eutick, Veivers, O'Brien, & Slaytor, 1978; Coon, Brown, & Strand, 2016), but for others they may be largely superfluous (e.g., Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017; Shelomi, Lo, Kimsey, & Kuo, 2013). Understanding what determines the direction and strength of these relationships for different hosts requires careful comparative work not only at the level of physiological impacts, such as nutrient synthesis, but also at the level of net impacts, such as the gut flora's effects on host life history and reproductive output.

Only a handful of studies have quantified the effects of the gut microbiota on host fitness. In chickens, experimentally increasing the abundance of one bacterial species, *Lactobacillus acidophilus*, results in faster growth and increased egg output (Haddadin, Abdulrahim, Hashlamoun, & Robinson, 1996). In termites, elimination or perturbation of the gut flora severely shortens the host's life span and reduces oviposition rate (Eutick et al., 1978; Rosengaus, Zecher, Schultheis, Brucker, & Bordenstein, 2011). In mosquitos and dung beetles, gut microbes are required for larval development (Coon et al., 2016; Schwab, Riggs, Newton, & Moczek, 2016). In tsetse flies, host fecundity decreases as gut microbes are eliminated earlier and earlier in a fly's life, and flies without gut flora produce no offspring (Nogge & Gerresheim, 1982). This previous work suggests that the gut microbiota can indeed impact host fitness, and stresses the need for additional investigation in a variety of hosts with diverse nutritional ecologies and life histories.

The relationship between a host and its gut microbiota is highly context-dependent. Because much of the gut flora's function documented thus far revolves around nutrient acquisition and synthesis, the quality of the diet in relation to the host's needs should determine whether the nutritive contribution of gut microbes increases or diminishes host fitness. A poor diet consists of the improper ratio or amount of the nutrients needed by the host, or contains toxins or recalcitrant compounds which the host cannot metabolize. Such diets create an opportunity for gut microbes to provide a service (e.g., nutrient biosynthesis or breakdown). On the other hand, diets that already fulfil the host's requirements may render microbial contributions irrelevant. Because gut symbionts absorb nutrients for their own metabolism and can stimulate the host's immune system, hosts that sustain gut microbes but do not gain services from them could incur a net fitness cost.

Research on this topic is limited, but generally supports these expectations. Gut microbiota increase the fecundity of female olive flies fed an unbalanced diet, but do not affect the fecundity of females fed a complete diet (Ben-Yosef, Aharon, Jurkevitch, & Yuval, 2010). On a poorer diet, thrips with gut symbionts lay more eggs than those without; on a richer diet, thrips *without* gut flora lay more eggs (de Vries, Jacobs, Sabelis, Menken, & Breeuwer, 2004). A poor diet is lethal to germ-free *Drosophila* larvae, but symbiotic larvae survive (Shin et al., 2011). These examples suggest that gut flora can benefit hosts by compensating for a poor diet, but may have negative or no effects on hosts that consume a diet that already satisfies their nutritional needs.

The prevalence of gut microbial associations and their context-dependent impacts on the host emphasize the need to investigate gut associations across a broad spectrum of comparative systems. Adult butterflies provide a previously unexplored context in which to investigate the outcomes of gut symbioses. Because lepidopterans gather most of their nutrients during the larval stage, adults' nutritional needs are simple—limited primarily to sugars, which provide energy to the adult and carbon for egg production (O'Brien, Boggs, & Fogel, 2004). This adult diet is also easy for the host to digest. Furthermore, the adult lepidopteran gut flora is highly variable in both species composition and abundance, probably because butterflies acquire most or all of their gut microbiota from the environment (Ravenscraft, Berry, Hammer, Peay, & Boggs, 2019). Gut microbiota are therefore unlikely to be nutritionally advantageous to butterflies, providing a potential opportunity to measure the costs which insect hosts—especially those with environmentally acquired gut microbiota—incur for housing bacteria in the gut.

The impact of the butterfly gut flora probably depends on host nutritional context. In the wild, adult food availability strongly affects population size in *Speyeria mormonia* (Boggs & Inouye, 2012), probably through effects on fecundity (Boggs & Ross, 1993). Variation in food availability is often extreme—flowers per butterfly shift by up to four orders of magnitude across years (C. Boggs, unpublished data). Food limitation might change the cost–benefit ratio of hosting gut flora: because the gut flora consume some portion of the host's diet to support their own metabolism, food restriction could shift a gut microbial association from a net mutualism or commensalism to a net parasitism.

To better understand the relationship between environmentally acquired gut microbiota and host reproductive fitness, we used the butterfly *S. mormonia* (Lepidoptera: Nymphalidae) to investigate whether the abdominal (mostly gut) microbiota are related to butterfly life span, fecundity and egg quality, and whether food availability to the host modulates these effects. We predicted that gut flora, as a whole, would be less costly to butterflies that were fed ad libitum than those that were semistarved.

Ultimately, the net cost or benefit of the microbiota must be the sum of the effects of the individual, often interacting, microbial taxa of which it is composed. Individual microbial species or strains may be commensal, detrimental or beneficial to their host, and the nature of each of these relationships may shift with host nutrition or environmental conditions. We therefore also performed an exploratory analysis of how food availability to the host modulated relationships between butterfly fitness and particular microbial taxa, with the expectation that a greater number of bacterial species would be negatively correlated with host fitness under semistarvation than under unrestricted feeding.

2 | METHODS

2.1 | Study system and site

The Mormon fritillary, *Speyeria mormonia*, is a univoltine, montane butterfly. Adults fly from June to September and eggs hatch in the

autumn. Eggs contain protein, triglyceride and glycogen stores, which are important nutrient sources for the offspring (Chippendale, 1978). Larvae overwinter as unfed first instars and seek out their host plant *Viola* (Violaceae) in the spring. Adults primarily feed on nectar from plants in the Compositae; males also feed at mud puddles, dung and carrion. The study population was located in a fescue grassland near the Rocky Mountain Biological Station (RMBL), Gunnison County, CO, USA (38°56′50″N, 106°58′50″W; 2,900 m asl). In midsummer, ranchers move cattle onto the site to graze, decreasing nectar availability.

Adults' nutritional ecology and nutrient allocation under different feeding regimes are well documented (Boggs, 1986,1997; Boggs & Freeman, 2005; Boggs & Ross, 1993; O'Brien et al., 2004). Because oocytes are not mature upon the female's emergence from the pupa, adult-derived nutrients are incorporated into the eggs and thus directly influence host fitness (Boggs & Ross, 1993). Up to 80% of egg carbon is derived from adult feeding (O'Brien et al., 2004), which is tightly linked to reproductive output: female fecundity decreases linearly in direct proportion to adult food availability (Boggs & Ross, 1993). Egg carbon/nitrogen (C:N) composition is unusually high for a butterfly, probably in response to the species' over-wintering strategy (O'Brien et al., 2004).

2.2 | Butterfly collection and experimental treatments

In July and August 2014, we captured 125 freshly eclosed and mated females from the field study population, brought them to the lab, and immediately weighed them and measured their wing length. Females were provided with a surface-sterilized, dried and pressed leaf of their host plant and housed in individual cages within an environmental chamber at 16:8 hr light/dark. Cages were constructed of lantern globes topped with large plastic Petri dishes with ventilation holes and were lined with wax paper. Lantern globes, Petri dishes and wax paper were sprayed with ethanol to reduce bacterial contamination. Females were fed a filter-sterilized diet similar in composition to flower nectar, following Erhardt and Rusterholz (1998). HOBO data loggers (Onset Computer Corporation) recorded the temperature of the environmental chamber at 15-min intervals.

To control for differences in butterfly size among the feeding treatments, females were paired by mass and wing length. The members of each pair were assigned to one of two feeding treatments: ad libitum or semistarved. The *ad lib* female was allowed to feed to satiation twice per day, in the morning and afternoon. The semistarved female was offered a reduced volume of diluted artificial nectar, resulting in a 25% reduction in volume and a 50% reduction in the 2-day running average of the calories and amino acids her partner consumed. Previous work has shown that egg production decreases linearly with adult female food intake (Boggs & Ross, 1993). We chose a starvation level of 50% *ad lib* directly from the results of this work to ensure that the semistarved females experienced physiological food stress. We measured how much every butterfly ate by offering a droplet of known volume, allowing the butterfly to feed, and then measuring the leftover volume with a Hamilton syringe.

Butterflies were assigned to one of three gut flora treatments: unaltered, perturbed, or perturbed and reseeded (hereafter "reseeded" for brevity). Unaltered females were fed only sterile diet; their gut communities were therefore composed of microbes they acquired in the field as larvae and freshly emerged adults, and any microbes acquired in the lab in spite of antiseptic conditions. These females served as a control for possible direct effects of antibiotic treatments on the butterflies. Perturbations in the gut flora of the other two groups were generated by antibiotic treatment. To differentiate between direct effects of antibiotics on the butterfly itself, and actual relationships between fitness and perturbation of the gut microbiota, both the perturbed and the reseeded butterflies were fed broad-spectrum antibiotics (diet containing 500 mg/L each of penicillin, gentamicin, rifampicin and streptomycin, and 667 mg/L nystatin) for their first four feedings. In an attempt to repopulate the guts of the reseeded group, we captured wild individuals of *S. mormonia*, dissected out their guts and gut contents, homogenized these in sterile artificial nectar, and fed this to the reseeded females on the day after the final antibiotic treatment. All females were then fed sterile artificial nectar (without antibiotics) for the remainder of their lives. This treatment scheme was designed to generate variation in gut community membership and total bacterial load.

We combined the feeding and gut treatments in a full factorial design, resulting in six treatment groups: (a) *ad lib* feeding, unaltered gut microbial community (N = 10); (b) semistarved, unaltered gut (N = 11); (c) *ad lib*, perturbed gut (N = 18); (d) semistarved, perturbed gut (N = 18); (e) *ad lib*, reseeded gut (N = 19); and (f) semistarved, reseeded gut (N = 16). The females included in the analyses of performance and fitness varied slightly due to missing or excluded data. (For example, six samples failed during quantitative PCR [qPCR] of bacterial 16S rRNA or host *ef1α* genes, and euthanized females were not included in the analysis of life span.) We report final sample sizes for each model in Table 4 and the number of females contributing to each model in Supporting Information Table S1.

We weighed females every other day. Eggs were removed from each cage every day and counted. Every other day, up to 10 eggs from each butterfly were dried at 50°C and stored in sealed Eppendorf tubes for later chemical analysis. Summary statistics for all life history metrics are reported in Supporting Information Table S2.

Upon death, abdomens were washed with 70% ethanol and preserved in cetrimonium bromide (CTAB) for sequencing. Because we sequenced whole abdomens, the bacteria in this study include those present in the reproductive tract and other areas of the abdomen, in addition to the gut. However, because most abdominal bacteria are located in the gut—as indicated by the fact that the microbiota derived from entire insects closely resemble samples taken specifically from their guts (Sabree, Hansen, & Moran, 2012)—we use "abdominal flora" and "gut flora" interchangeably.

Most females died naturally. However, post-reproductive butterflies that were weak and largely immobile were euthanized (N = 15).

2.3 | Egg chemical analyses

Egg mass and chemical composition were measured at the University of South Carolina following the procedures of Niitepõld and Boggs (2015). Briefly, to measure triglyceride content we crushed three or four eggs in distilled water, centrifuged this and added the supernatant to a 96-well plate with Infinity triglycerides stable reagent (Thermo Scientific). Absorbance was read at 500 nm and compared to a triglyceride standard. To measure glycogen content we crushed four eggs in 2% sodium sulphate, washed this with 1:2 chloroform–methanol followed by 80% methanol, took the supernatant, added anthrone reagent, and heated at 90°C. Absorbance was read at 625 nm and compared to a glucose standard curve. Triglyceride and glycogen data were corrected to micrograms per egg.

2.4 | Microbial community characterization

DNA was extracted at Stanford University following the protocol of Peay, Bruns, Kennedy, Bergemann, and Garbelotto (2007). Briefly, samples were homogenized via bead beating, extracted with chloroform and cleaned using the DNeasy Blood and Tissue Kit (Qiagen). Interspersed among our samples, we also performed DNA extractions on two empty tubes and two tubes of clean CTAB buffer. These four blank extractions were processed simultaneously with and identically to the samples, including all downstream lab work and sequencing.

Bacterial sequences were indexed using barcoded reverse primers (Caporaso et al., 2012). We amplified the V4 hypervariable region of the 16S rRNA gene using primer set 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′). This region amplifies bacterial taxa with few biases and is appropriate for accurate phylogenetic placement (Bergmann et al., 2011; Liu, Lozupone, Hamady, Bushman, & Knight, 2007). Because these primers also amplified butterfly 18S rRNA, we designed a PNA clamp to block amplification of host DNA (Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013). The clamp sequence was GCCCGCTTTGAGCACTCT and it was synthesized by PNA Bio. Samples were amplified in a volume of 25 μl with the following recipe: 12.5 μm PNA clamp, 0.2 μm forward primer, 0.2 μm reverse primer, 0.2 mm dNTP, 0.65 U OneTaq HotStart (New England Biolabs) and 1× Thermopol buffer (New England Biolabs). Thermocycler settings were denaturation at 95°C for 45 s followed by 35 cycles of denaturation at 95°C for 15 s, PNA clamp annealing at 76°C for 10 s, primer annealing at 50°C for 30 s and extension at 68°C for 30 s, with a final extension of 68°C for 3 min (adapted from Lundberg et al., 2013). We used Veriti (Applied Biosystems) and T100 (Bio-Rad) thermocyclers. We did not amplify our samples in triplicate; empirical research has shown that PCR replication is unnecessary (Smith & Peay, 2014).

Every 96-well sample plate included at least one blank sample of PCR water. These PCR blanks entered the workflow after DNA extraction and were subsequently processed simultaneously with and identically to the samples, including all downstream lab work and sequencing. In total, we processed five of these PCR blanks (in addition to the four DNA extraction blanks mentioned above).

To verify amplification success, PCR products were visualized using gel electrophoresis. Products were cleaned and concentrated using SequalPrep normalization plates (Life Technologies A10510-01). Samples were sequenced at the Stanford Functional Genomics Facility on an Illumina Mi-Seq platform using 2 × 300 chemistry.

Sequences were edited to remove priming sites and poor quality bases at the 5′ and 3′ ends using the program CUTADAPT (Martin, 2011), merged and clustered at a 97% similarity cutoff with UPARSE (Edgar, 2013). De novo and reference-based chimera checking were performed in UPARSE against the RDP Gold database. Taxonomy was initially assigned using the RDP classifier with Greengenes as the training set (McDonald et al., 2012; Wang, Garrity, Tiedje, & Cole, 2007). We checked and revised these assignments by searching representative sequences in the NCBI nucleotide collection.

For each of the operational taxonomic units (OTUs) observed in the negative controls (four blank extractions and five PCR blanks that underwent all sample preparation and sequencing, as described above), we performed a *t* test comparing each OTU's relative abundance in the samples to its relative abundance in the blanks. If the OTU was present at significantly greater relative abundance in the negative controls than the samples, we identified it as a contaminant. One OTU (OTU_8, an *Acidovorax* species) was classified as a contaminant by this method and was removed from the data set prior to analysis. Rare OTUs—those that did not account for at least 0.1% of the total reads of at least five samples prior to rarefaction—were also removed from the data set.

To control for differences among samples in sequencing depth, we rarefied the sequencing data to 1,000 reads per sample using the R package "phyloseq" (McMurdie & Holmes, 2013). The 1,000 read cut-off allowed us to retain an adequate number of butterflies in the data set while still profiling the dominant bacteria in their guts. At this depth the slopes of the sample rarefaction curves were shallow, relative abundances of the focal OTUs (described below) were stable, and coverage analysis indicated that we detected a median of 98.5% of true diversity in the experimental females (Supporting Information Figures S2–S4). To ensure that results were not driven by a single rarefaction, we performed each of our analyses on 1,000 rarefied data sets (described below). However, as a result of rarefying to 1,000 reads, we probably failed to reliably detect OTUs that were present in a butterfly at a relative abundance of approximately 1/1,000 raw reads or less. This may have reduced our ability to detect relationships between the focal OTUs (described below) and butterfly fitness, especially for those focal OTUs that were present at low relative abundance on average, such as *Arthrobacter* sp. (OTU_65), *Rhodococcus* sp. (OTU_58), *Methylobacterium* sp. (OTU_112), *Caulobacter* sp. (OTU_15), *Pedobacter* sp. (OTU_83) and *Stenotrophomonas* sp. (OTU_2544) (Table 3).

2.5 | Quantification of total bacterial load and absolute OTU abundances

Bacterial DNA was quantified with qPCR using SYBR green fluorescence chemistry (iCycler IQ, Bio-Rad) and primers 515f /806r. DNA was amplified in a volume of 20 μl with the following recipe:

12.5 μm PNA clamp, 0.2 μm forward primer, 0.2 μm reverse primer and 1× PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences). Thermocycler settings were denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, PNA clamp annealing at 76°C for 10 s, primer annealing at 50°C for 30 s and extension at 68°C for 30 s. To verify that amplified fragments were the expected length, we performed a melt curve ramping from 55°C to 95°C. PCR products were also visualized using gel electrophoresis.

The original number of 16S rRNA copies per sample was calculated by comparing the sample's threshold cycle to an internal standard curve. Standards were composed of full-length *Escherichia coli* 16S rRNA amplicons and ranged from an initial 10–10^7 copies/μl. (Curve generation is described below.) Each sample and standard was run in triplicate and the results were averaged.

Efficiencies across all qPCR plates ranged from 61.2% to 71.7%. Standard curve correlation coefficients ranged from 0.957 to 0.986. Low efficiencies resulted from use of the 515f/806r primer set, which is not optimized for qPCR. Use of these primers was necessary to maintain consistency with the Illumina data and allows correspondence between bacterial community composition and 16S rRNA copy counts. More specifically, it allowed us to multiply the relative abundances of the focal OTUs by the absolute bacterial loads estimated via qPCR. Had we used different primer sets for the Illumina and qPCR components of the study, the primer sets would probably have had different amplification biases for different bacterial taxa, which would have invalidated direct comparisons between the Illumina and qPCR data. Quantification with 515f/806r plus an *E. coli* standard curve has been successfully used in studies ranging from soil bacterial communities to insect gut flora (Carini et al., 2016; Hammer et al., 2017; Sanders et al., 2017). Due to the low efficiency of the 515f/806r primers, our reported bacterial counts may underestimate true bacterial titres, but any downwards bias will apply to all samples. As a result, the trends we detected, and our overall results, would not have changed if we had used more efficient bacterial primers. However, for studies that focus on the role of specific bacterial taxa, an alternative would be to use more efficient, taxon-specific primers.

To generate the standard curve, 16S rRNA was amplified from *E. coli* using primers 27f/1492r, ligated into a plasmid vector and cloned. To screen for the correct insert, colonies were PCR-amplified using primers M13f/M13r followed by gel electrophoresis and bidirectional sequencing. One colony that passed both screens was grown to saturation in LB + kanamycin media. Plasmids were extracted (Qiagen Plasmid Mini kit) and linearized with *Spe*I restriction enzyme (FastDigest, Thermo Fisher FD1253). To confirm complete linearization, DNA was purified (Qiagen PCR cleanup column) and visualized on a gel. DNA concentration was quantified with PicoGreen. We calculated copy number/μl as the molecular weight of plasmid plus insert (g/molecule = length in bp × 650 Da/bp/6.02 × 10^{23}) divided by the measured DNA concentration (g/μl). Via serial dilution of the raw extract we created standards ranging from 10^7 to 10 copies/μl.

Bacterial 16S rRNA counts per sample were normalized by the number of host *ef1α* copies per sample. The practice of standardizing by the copy number of a host gene controls for possible variation among samples in the total amount of DNA obtained from the DNA extraction and its suitability for PCR. We designed primers that amplified a 159-bp segment of the *ef1α* gene of *S. mormonia* (SpeyF: ACACCTGTGTTGGATTGCCA, SpeyR: GAGGGGCTTGGATGGTTGAA). With these primers, we quantified host *ef1α* using the same reagents and thermocycler protocol as for bacterial 16S rRNA, with the exception that we did not add a PNA clamp and omitted the clamp annealing step. To calculate the starting number of *ef1α* copies, each sample's threshold cycle was compared to an internal standard curve ranging from an initial 10 to 10^7 copies/μl of *ef1α* amplicons. Each sample and standard was run in triplicate and the results were averaged. Efficiencies of the *ef1α* qPCRs ranged from 91.8% to 98.6% and the correlation coefficients of the standard curves ranged from 0.997 to 0.999.

To generate the *ef1α* standards, we amplified one sample using primers SpeyF/SpeyR, purified the PCR product (QIAquick PCR Purification Kit, Qiagen), quantified the product (Qubit DS DNA HS assay, Qiagen), diluted this to a concentration of 2 ng/μl and re-quantified. Using the molecular weight of a 159 -bp DNA fragment (159 × 650 Da), we calculated the copy number of *ef1α*. We then serially diluted to obtain standards ranging from 10^7 to 10 copies/μl.

We calculated per-sample absolute abundances of the OTUs by multiplying each OTU's relative abundance in the rarefied data by the total number of 16S rRNA copies per *ef1α* copies in each sample.

2.6 | Statistical analyses

To assess the effects of the gut treatments, we used ordination (nonmetric multidimensional scaling [NMDS]) plots to visualize and PERMANOVA (the *adonis* test in the R package "vegan"; Oksanen et al., 2015) to test for dissimilarity in abdominal community composition, measured as the abundance-weighted Unifrac (Lozupone, Hamady, & Knight, 2006) distances between rarefied samples. The PERMANOVA test assumes that within-group distances are equivalent (Anderson & Walsh, 2013); we therefore tested for differences in dispersion among gut treatments using VEGAN's *betadisper* function.

We used linear and linear mixed effect models (Zuur, Ieno, Walker, Saveliev, & Smith, 2009) to assess whether food availability influenced the relationship between bacterial load and butterfly performance (food consumption, life span) and fitness (fecundity, egg quality). To control for additional possible covariates, we found the best-fit fixed effects structure using backwards model selection with likelihood ratio tests starting from the full models listed in Table 1. (Variables are defined in Table 2.) All models were fit in R version 3.5.1 (R Core Team, 2018). Models without random effects were fit using the *lm* command in the "stats" package; models with random effects were fit using the *lmer* command in the package "LME4" (Bates, Maechler, Bolker, & Walker, 2015). All metrics were modelled with Gaussian error and an identity link function.

TABLE 1 Variables evaluated as predictors of each performance or fitness metric

Metric	Predictors tested[a]
Whole life	
Average eaten per day	gut + winglength +mass.initial + temp.life + bacterial.load + egg.onset
Life span	gut*feed + bacterial.load*feed + winglength +mass.initial + temp.life + egg.onset
Fecundity	gut*feed + bacterial.load*feed + life span + winglength +mass.initial + temp.life + egg.onset
Daily	
Egg weight	gut*feed + bacterial.load*feed + winglength +mass.bfly + neggs +preveaten3 + temp.prevday*age + (1|femaleID)
ln(triglycerides)	gut*feed + bacterial.load*feed + winglength +mass.bfly + neggs +preveaten3 + temp.prevday*age + egg.wt + (1|femaleID)
Glycogen	gut*feed + bacterial.load*feed + winglength +mass.initial + neggs +preveaten3 + temp.prevday*age + egg.wt + (1|femaleID)

[a]Variables are defined in Table 2. A random effect for butterfly individual is indicated as (1|femaleID). Asterisks denote main terms with interactions.

TABLE 2 Predictor definitions

Age	Days since the butterfly was captured
bacterial.load	The log (base 2) of the number of bacterial 16S copies divided by the number of host *ef1a* copies per individual
egg.onset	Age at which a female laid its first egg (a proxy for how old the butterfly was when it was captured)
egg.wt	Average weight of an egg laid by a given butterfly that day
feed	Feed treatment (*ad lib* or semistarved)
foodperday	Average volume of food eaten per day
gut	Gut treatment (unaltered, perturbed or reseeded)
lifespan	The number of days a female lived in the lab
mass.bfly	Butterfly mass (measured every other day)
mass.initial	Initial mass of the butterfly at capture
neggs	Number of eggs laid on a given day
preveaten3	Average volume eaten over the previous 3 days
femaleID	Identity of the butterfly
temp.life	Average temperature the butterfly experienced over its life in the lab
temp.prevday	Average temperature experienced over the previous day
winglength	Wing length at capture (a proxy for size)

We conducted a power analysis to measure the ability of each model to detect a meaningful interaction between feeding treatment and total bacterial load, where a biologically meaningful effect size was defined to be 20% of one standard deviation of the given life history metric. For each metric, we took the final model from backwards model selection and added a term for the interaction of feeding treatment and bacterial load. We set the coefficient of this interaction term to equal our "meaningful" effect size and then applied this updated model to our original data. This produced predictions under the assumption that the meaningful effect size was the true effect size. We simulated 1,000 data sets by adding residual error to the model predictions as follows: for each data set, error was randomly drawn from a normal distribution with a mean of 0 and a standard deviation equal to the original model's estimated residual standard error. Where applicable, we also added error to account for random effects structure, assigning each female its own error drawn from a normal distribution with a mean of 0 and standard deviation equal to the estimated standard deviation of the original model's random effect term. We reran the updated model on each simulated data set. Power was calculated as the percentage of iterations in which the interaction of feeding treatment and bacterial load was detected as significant. For example, a model in which the interaction was detected as significant in 998 out of 1,000 simulations had 99.8% power to detect a meaningful effect that truly existed.

We also investigated whether food availability influenced relationships between the life history metrics and abundances of

TABLE 3 Taxonomic identities of the 20 focal OTUs

Genus	Samples[a]	Mean % reads[b]	*SD* % reads	Phylum	Class	Order	Family	OTU ID	Notes[c]
Commensalibacter	21	18.3	36.2	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	OTU_1	
Pantoea	39	6.7	19.1	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	OTU_5	
Cloacibacterium	51	4.5	5.5	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	OTU_2	
Sphingomonas	45	4.2	5.7	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	OTU_17	
Stenotrophomonas	19	2.8	12.8	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	OTU_19	
Acinetobacter	47	2.7	3.8	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	OTU_14	
[Comamonadaceae]	49	2.4	3.3	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	OTU_7	1
Sphingomonas	41	1.8	5.6	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	OTU_44	
Rhodococcus	26	1.8	4.4	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	OTU_32	
Staphylococcus	43	1.5	2.0	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	OTU_20	
Streptomyces	13	1.4	10.3	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	OTU_31	2
Orbus	15	1.4	4.7	Proteobacteria	Gammaproteobacteria	Orbales	Orbaceae	OTU_55	3
Dechloromonas	45	1.3	1.8	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	OTU_13	
Telluria	43	1.1	1.3	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	OTU_77	4
Arthrobacter	20	0.8	2.9	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	OTU_65	5
Rhodococcus	18	0.7	2.1	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	OTU_58	
Methylobacterium	40	0.7	1.1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	OTU_112	
Caulobacter	37	0.6	0.9	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	OTU_15	6
Pedobacter	22	0.6	1.7	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	OTU_83	
Stenotrophomonas	12	0.3	2.0	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	OTU_2544	

[a]Number of females (out of a possible total of 63) in which the OTU was found. [b]An OTU's mean percentage of reads per individual. [c](1) In a BLAST search, OTU_7 matched equally closely to the genera *Ottowia*, *Diaphorobacter*, *Alicycliphilus*, *Comamonas*, *Acidovorax* and *Brachymonas*. (2) RDP assigned OTU_31 to the family Streptomycetaceae with no finer resolution. Closest BLAST hit was the genus *Streptomyces*. (3) RDP assigned OTU_55 to the order Pasteurellales with no finer resolution. Closest BLAST hit was Orbales:Orbaceae:*Orbus*. (4) RDP assigned OTU_77 to the genus *Janthinobacterium*. Closest BLAST hit was the genus *Telluria*. (5) RDP assigned OTU_65 to the family Micrococcaceae with no finer resolution. Closest BLAST hit was the genus *Arthrobacter*. (6) RDP assigned OTU_15 to the family Caulobacteraceae with no finer resolution. Closest BLAST hit was the genus *Caulobacter*.

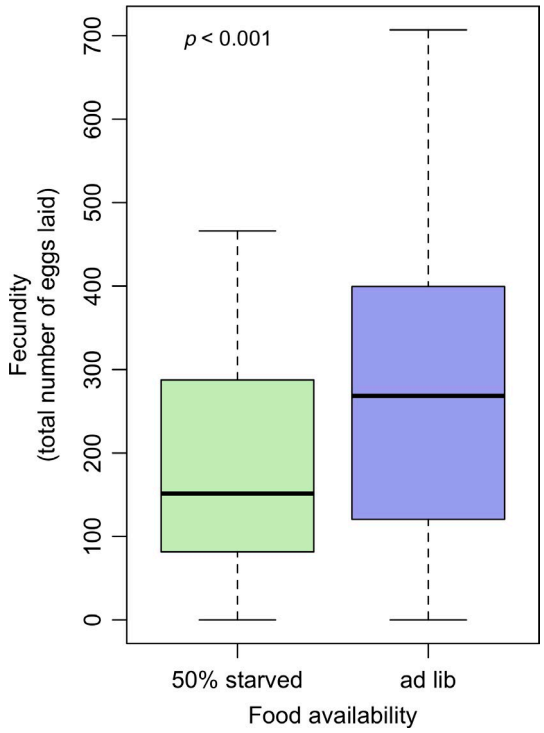

FIGURE 1 Effects of the feeding treatments on fecundity. *Ad lib*-fed females laid almost twice as many eggs over their lifetime as semistarved females. Thick black lines denote medians. The tops and bottoms of the boxes denote the lower and upper quartiles, respectively. Whiskers are placed at 1.5 times the interquartile range or, if all data fall within this range, they are placed at the most extreme value measured. The *p*-value is the result of comparing the final model for fecundity (Table 4) to a model without the feeding treatment ($df = 1$, $F = 29.8$) [Colour figure can be viewed at wileyonlinelibrary.com]

individual OTUs. We focused on OTUs that were observed in at least 13 (20%) of the females and had an average of at least three reads per butterfly after rarefaction. Table 3 lists the resulting 20 focal OTUs. For each performance or fitness metric, we used a likelihood ratio test to compare a model that included the significant covariates found during backwards model selection (the "base model") to 20 equivalent models, each with an additional term for the interaction between feeding treatment and the absolute abundance of one of the focal OTUs, calculated as (fraction of reads assigned to the OTU) × $\log_2$(16S rRNA copies/*ef1α* copies + 0.001). Because there were 20 focal OTUs, this usually resulted in 20 *p*-values per fitness metric, but in rare cases where >85% of observations were associated with a 0 count for a particular OTU, that OTU was omitted from testing. To account for multiple testing we used false discovery rate (FDR) correction as implemented by the *p.adjust* function in R. To ensure that our results were not driven by a single rarefaction, we generated 1,000 rarefied data sets, repeated the described procedure for each rarefaction (including FDR correction for the number of OTUs tested for each fitness metric), and counted the number of times the interaction between feeding treatment and an OTU was significant.

We similarly tested for direct relationships between the life history metrics and the abundances of the OTUs: we added the log absolute abundance of each focal OTU to the base model of a given fitness metric, tested for improved fit and performed FDR correction for the number of OTUs tested for that metric. This procedure was repeated over the 1,000 rarefied data sets, counting the number of times each OTU was a significant predictor of each metric.

We conducted power analyses to measure the ability of our models to detect meaningful relationships between butterfly fitness and (a) interactions between feeding treatment and absolute abundances of the focal OTUs and (b) the absolute abundances of the focal OTUs alone. A biologically meaningful effect size was again defined to be 20% of one standard deviation of the given life history metric. These analyses were performed in the same manner as the power analyses for total bacterial load except that we simulated 100 data sets instead of 1,000 for each case. More specifically, for each combination of a metric and a focal OTU, we took the metric's final model from backwards model selection and added a term either for (a) the interaction of feeding treatment and the given OTU's absolute abundance, or (b) just the given OTU's absolute abundance. We set the coefficient of this term to equal our "meaningful" effect size and applied this updated model to the original data. We simulated 100 data sets according to the parameters of the updated model, following the same method as described above for the bacterial load power analyses; the resulting simulated data sets incorporate fixed effects, error associated with random effects and residual error. We reran the updated model on each of these 100 simulated data sets. Power was defined as the percentage of these iterations in which the relevant term (either the feed × OTU term or just the OTU term) was detected as significant. Because we only performed these analyses on a single rarefied data set, power estimates for the focal OTU tests are approximate (Rarefaction was not involved in the previously described power analysis for bacterial load because it used only qPCR data, not sequencing data).

3 | RESULTS

3.1 | Effects of semistarvation

Semistarved females laid fewer eggs compared with *ad lib*-fed females (ANOVA, $df = 1$, $F = 29.8$, $p < 0.001$; Figure 1). Controlling for life span and the age at which a butterfly began laying eggs, an average *ad lib*-fed butterfly laid 333 eggs while a semistarved animal laid only 191 eggs. Food availability did not affect life span, egg mass, or egg triglyceride or glycogen content (Table 4). Gut community composition did not differ between the feeding treatments (weighted Unifrac, betadisper ANOVA: $df = 1$, $F = 0.33$, $p = 0.57$; PERMANOVA: $df = 1$, $F = 0.80$, $p = 0.50$; Supporting Information Figure S6).

3.2 | Bacterial load

Total bacterial abundances were highly variable among individuals: qPCR indicated that experimental females hosted a mean of 1.1

TABLE 4 Predictors of butterfly performance and fitness

Metric	Significant predictors[a]	OTU identity[b]	Coefficient (OTU or feed*OTU)	*N*
Whole life				
Average eaten per day	egg.onset			45
Life span	egg.onset + $\log_2$(OTU_1 + 0.001) + $\log_2$(OTU_58 + 0.001)	*Commensalibacter* sp. *Rhodococcus* sp.	0.45 −0.72	49
Fecundity	feed + life span + egg.onset			88
Daily				
Egg weight	mass.bfly + age			175
ln(triglycerides)[c]	gut*feed + temp.prevday			193
Glycogen	egg.wt + temp.prevday*age			88

[a]Variables are defined in Table 2. [b]Taxonomic identity of significant OTUs. [c]The significance of the gut term was driven by whether or not animals were exposed to antibiotics, suggesting a direct effect of antibiotic treatment.

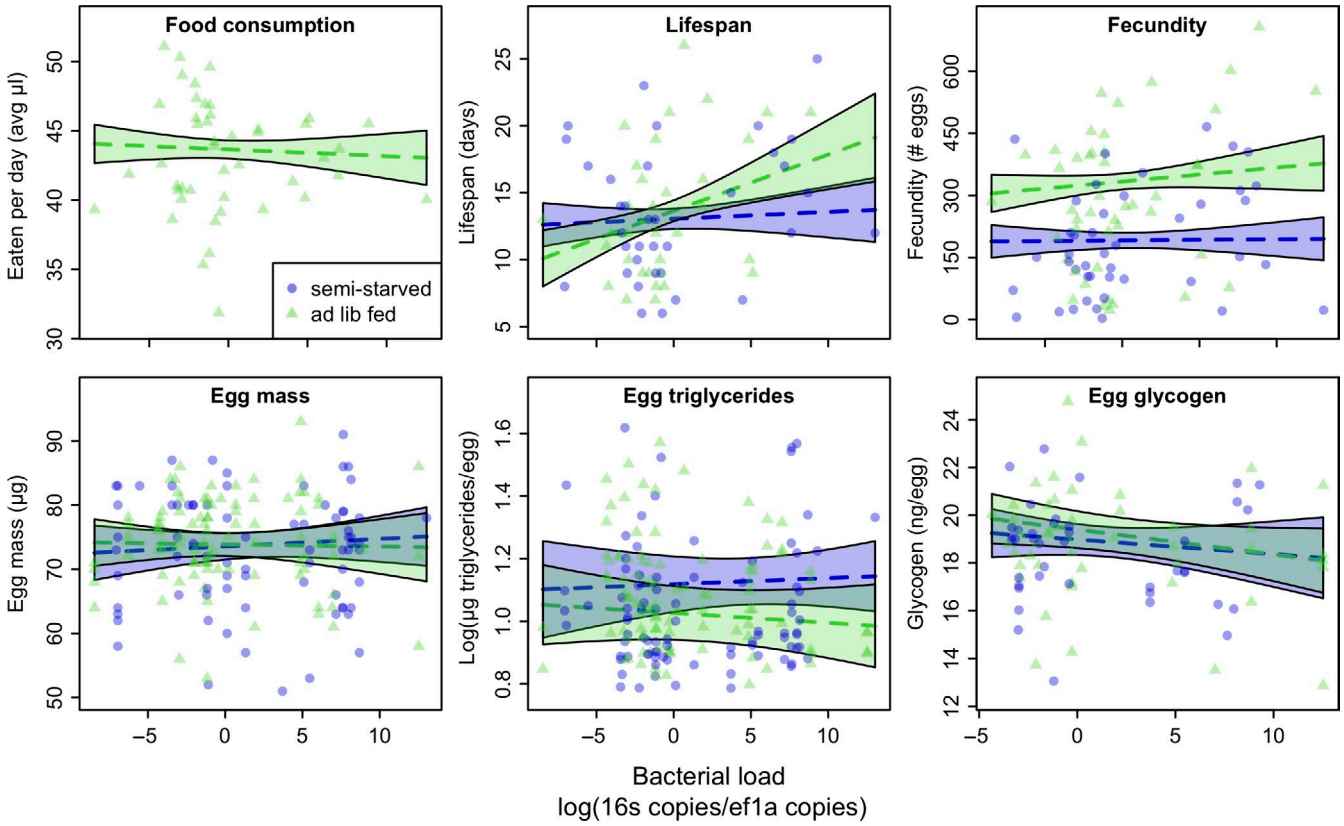

FIGURE 2 No relationships between total bacterial load and butterfly fitness or performance. Neither total bacterial load nor the interaction of bacterial load with feeding treatment was significantly correlated with butterfly fitness or performance. Dashed lines show model expectations as a function of the interaction of feeding treatment by total bacterial load while controlling for significant covariates (see Table 4). For presentation, these covariates were set to their median values. Shading indicates the model-estimated standard errors. Points are the raw data. In the case of food consumption only the *ad lib* prediction is shown because semistarved females' consumption was determined by *ad lib* females' consumption. In the lower three panels, note that egg measurements were taken at multiple time points for each female; due to differing life spans and egg laying patterns, individual females are associated with different numbers of data points. (The random effects structure of the models takes this into account.) [Colour figure can be viewed at wileyonlinelibrary.com]

and a median of 0.4 bacterial 16S rRNA copies per host *ef1α* copy, but bacterial load ranged from 0.002 to 8,396 16S rRNA copies per *ef1a* copy (interquartile range 0.13–3.9 16S rRNA/*ef1a*; Supporting Information Figure S1; calculations were performed on log base 2 abundances but are in reported here in base 10 for ease of interpretation.) Gut bacterial load did not differ between semistarved and

ad lib females (ANOVA, $F = 0.4$, $p = 0.53$; Supporting Information Figure S1), nor did it differ between gut treatments (ANOVA, $F = 1.6$, $p = 0.20$; Supporting Information Figure S1).

We had predicted that semistarved butterflies would suffer from hosting large numbers of microbes, while *ad lib*-fed animals would not. Contrary to these expectations, bacterial load was unrelated to fitness in both the *ad lib*-fed and the semistarved females (Figure 2; Table 4). Our ability to detect the influence of feeding treatment on the relationship between total bacterial load and a life history metric was high: statistical power was >95% for all models (Supporting Information Table S3).

3.3 | Composition of the gut microbiota

After sequence processing, quality filtering, and removal of rare and contaminant OTUs, we obtained 180,096 sequences from the abdomens of 92 experimental females (24 unaltered, 50 perturbed, 51 reseeded) and 142,333 sequences from the abdomens of the 46 wild donor butterflies whose gut flora was fed to reseeded females. After rarefaction to 1,000 reads per sample, a total of 519 OTUs were observed across the 63 remaining experimental females. The 20 most abundant OTUs detected across these females are presented in Supporting Information Table S4, and, for comparison, the 20 most abundant OTUs detected across donors are listed in Supporting Information Table S5. Nine OTUs were shared between experimental females' and donors' most abundant OTUs. Females' and donors' gut flora differed in community composition (weighted Unifrac, betadisper ANOVA: $df = 1$, $F = 2.2$, $p = 0.14$; PERMANOVA: $df = 1$, $F = 3.9$, $p = 0.004$; Supporting Information Figure S5). More specifically, 19 out of 519 OTUs (3.7%) differed in their frequency of detection between donors and experimental butterflies, including four of the focal OTUs (Supporting Information Figure S7). Of the focal OTUs, a *Rhodococcus* species (OTU_32), a *Pedobacter* species (OTU_83) and a *Sphingomonas* species (OTU_17) were more likely to be present in the experimental females, while a bacterium in the family Caulobactereaceae (OTU_15) was more likely to be present in the donors (Supporting Information Figure S7).

Abdominal bacterial communities were highly variable in composition. Observed OTU richness varied substantially (mean 71 OTUs per female when rarefied to 1,000 reads per sample; *SD* 48; range 2–167). No OTU was present in all 63 females. On average, each OTU was found in only nine females (*SD* = 9) (Supporting Information Figure S8). The OTU present in the greatest number of females was a *Cloacibacterium* species (OTU_2) found in 51 out of the 63 individuals. On average, a randomly selected pair of females shared 18 OTUs (*SD* = 17), or 26% of their OTUs, and the mean pairwise Bray–Curtis dissimilarity between females was 0.84 (where a score of 0 indicates that two samples' species compositions match completely, and a score of 1 indicates they share no species).

3.4 | Effects of the gut treatments

The three gut treatments did not differ in total bacterial load (ANOVA, $df = 2$, $F = 1.6$, $p = 0.20$; Figure 3a) nor in observed OTU

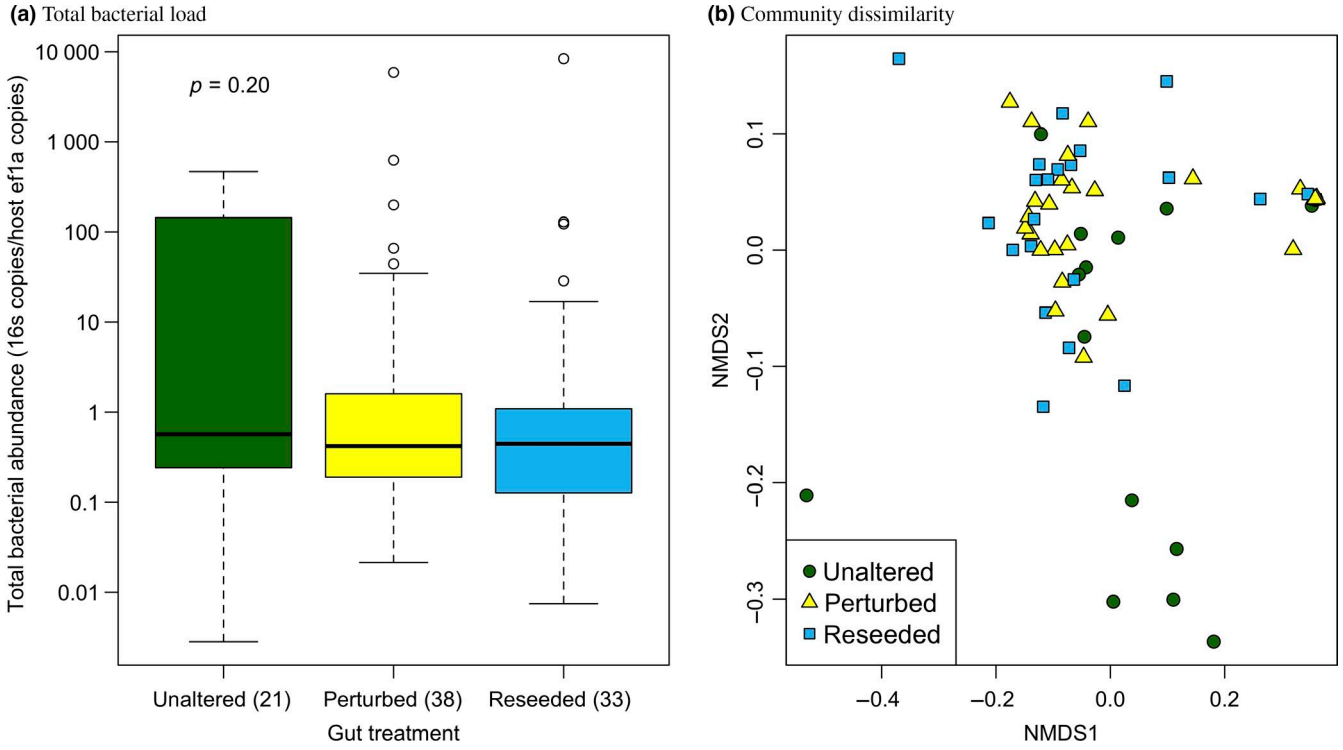

FIGURE 3 Effects of the gut treatments on abdominal flora. (a) Females did not differ in total bacterial load across the three gut treatments. Boxplot features are as in Figure 1. (b) NMDS plot of the abundance-weighted Unifrac dissimilarities between females. PERMANOVA tests (see Table 5) indicated that unaltered females differed in OTU composition from reseeded and perturbed females [Colour figure can be viewed at wileyonlinelibrary.com]

richness (ANOVA: $df = 2$, $F = 0.3$, $p = 0.60$). The treatments did not differ in dispersion (weighted Unifrac, betadisper ANOVA: $df = 2$, $F = 1.5$, $p = 0.23$) but did differ in microbial community composition (PERMANOVA: $df = 2$, $F = 2.4$, $p = 0.02$; Figure 3b; Table 5). Specifically, the gut of flora unaltered females differed from that of the perturbed and reseeded females (Table 5). Results were equivalent when differences in composition were measured with Bray–Curtis distances (Supporting Information Figure S5). Overall, these results suggest that (a) our reseeding attempt was unsuccessful and had little, if any, effect on the gut microbiota, and (b) the antibiotic treatment had only small effects on gut community composition.

The gut treatments correlated with only one life history metric: egg triglyceride content (Table 4). Females exposed to antibiotics (perturbed or reseeded treatments) laid eggs with lower triglyceride content if they were semistarved than if fed *ad lib*, while unaltered females laid eggs with *higher* triglyceride content when they were semistarved than if fed *ad lib*. These patterns were probably the result of direct effects of the antibiotics on the host. For further discussion of the gut treatments and their effects, see the Supporting Information Appendix.

3.5 | Relationships between focal OTUs and butterfly performance and fitness

We selected 20 focal OTUs (those present in the rarefied data set in at least 20% of females and with an average read count of at least three per animal) and tested for correlations between their absolute abundances and the fitness and performance of their hosts. These focal OTUs were members of the phyla Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria; most were known insect or gut associates or common environmental bacteria (Table 3; Supporting Information Table S7). Many of the focal OTUs were present at low abundance on average, but spiked to higher abundance in some females (Supporting Information Table S6).

The results of significance testing for relationships between the fitness metrics and absolute abundances of each of the 20 focal OTUs are presented in Table 6. We considered a relationship to have strong support if it was significant for at least 80% of the rarefied data sets, and moderate support if it was significant for at least 70% of the rarefied data sets. We detected only two relationships between the focal OTUs and butterfly fitness or performance: a *Commensalibacter* species (OTU_1) was positively correlated with host life span, while one of the *Rhodococcus* species (OTU_58) was negatively correlated with host life span.

The most strongly supported relationship was the positive correlation between abundance of the *Commensalibacter* species and life span (Tables 4 and 6; Figure 4a). With every doubling in the absolute abundance of *Commensalibacter*, life span increased by 0.5 days, 3.6% of the average life span of 14 days. Addition of this bacterium as a predictor increased the predictive power of the life span model by 28% (predicted R^2 for a model with only egg.onset = 0.03; predicted R^2 for a model with egg.onset plus the *Commensalibacter* species' abundance = 0.31). This relationship is confounded by the facts that (a) relative abundances of *Commensalibacter* tended to increase with total bacterial load and (b) total bacterial load tended to increase at the end of the summer (Supporting Information Figure S9). The *relative* abundance of *Commensalibacter* was not correlated with host life span (Supporting Information Table S10).

In contrast to *Commensalibacter*, absolute abundance of a *Rhodococcus* species was *negatively* correlated with butterfly life span (Tables 4 and 6; Figure 4b). With every doubling in the absolute abundance of this *Rhodococcus*, life span decreased by 0.7 days, or 5% of the average life span. This bacterium increased the predictive power of the life span model by 9% (predicted R^2 for a model with only egg.onset = 0.03; predicted R^2 for a model with egg.onset plus the *Rhodococcus* species' abundance = 0.12). Neither the absolute nor the relative abundance of this *Rhodococcus* was related to the date of butterfly capture (absolute abundance ANOVA: $F = 2.2$, $p = 0.14$; relative abundance ANOVA: $F = 1.3$, $p = 0.27$), nor was its relative abundance correlated with total bacterial load (ANOVA, $F = 2.1$, $p = 0.15$). Abundances of the *Commensalibacter* species and this *Rhodococcus* species were not strongly correlated (absolute abundance: correlation = −0.06; relative abundance: correlation = −0.14). Interestingly, a second focal OTU in the genus *Rhodococcus* (OTU_32) was not correlated with any aspect of butterfly fitness or performance (Table 6).

The statistical power of our models to detect relationships between absolute OTU abundances and butterfly life history was generally high (Supporting Information Table S9). However, our models were under-powered for 12 out of 18 OTU–food consumption relationships, four out of 20 OTU–life span relationships, and one relationship each for egg weight, egg triglycerides and egg glycogen (Supporting Information Table S9). Power was often insufficient to

TABLE 5 Differences in abdominal community composition (Unifrac) between gut treatments

		Differences in dispersion[a]			Differences in composition[b]		
Comparison	***df***	***F***	**raw *p***	**FDR *p***	***F***	**raw *p***	**FDR *p*[c]**
Overall	2	1.51	0.230		2.37	0.023	
unaltered-perturbed	1	2.62	0.114	0.197	3.34	0.019	0.029
unaltered-reseeded	1	2.38	0.131	0.197	3.43	0.009	0.028
perturbed-reseeded	1	0.03	0.859	0.859	0.38	0.880	0.880

[a]Results of *betadisper* tests (PERMANOVA assumes no difference in dispersion). [b]Results of PERMANOVA tests. [c]*p*-values of post hoc pairwise tests adjusted for multiple testing (FDR correction).

TABLE 6 Per cent significant relationships (after FDR correction) between absolute abundance of each of the focal OTUs and butterfly performance and fitness [Colour table can be viewed at wileyonlinelibrary.com]

OTU ID	Food consumption OTU	Life span feed*OTU[c]	Life span OTU	Fecundity feed*OTU	Fecundity OTU	Egg weight feed*OTU	Egg weight OTU	Egg triglycerides feed*OTU	Egg triglycerides OTU	Egg glycogen feed*OTU	Egg glycogen OTU
OTU_2	0	0	0	0	0	0	0	0	0	0	0
OTU_7	0	0	0	0	0	0	0	0	0	0	0
OTU_14	0	0	0	0	0	0	0	0	0	0	0
OTU_17	0	0	0	0	0	0	0	0	0	0	0
OTU_13	0	0	0	0	0	0	0	0	0	0	0
OTU_77	0	0	0	0	0	0	0	0	0	0	0
OTU_20	0	0	0	0	0	0	0	0	0	0	0
OTU_44	0	0	0	0	0	0	0	0	0	0	0
OTU_112	0	0	0	0	0	0	0	0	0	0	0
OTU_5	0	0	0	0	0	0	0	0	0	0	0
OTU_15	0	0	0	0	0	0	0	0	0	0	0
OTU_32	0	0	0	0	0	0	0	0	0	0	0
OTU_83	0	0	1	0	0	0	0	0	0	0	0
OTU_1	0	0	83	0	0	0	0	0	0	0	0
OTU_19	0	0	0	0	0	0	0	0	0	0	0
OTU_65	0	0	0	0	0	0	0	0	0	0	0
OTU_58	6	0	73	0	0	0	0	0	0	0	0
OTU_55	0	0	0	0	0	0	0	0	0	0	0
OTU_2544	NA	0	0	0	0	0	0	0	0	0	0
OTU_31	NA	0	0	0	0	0	0	0	0	0	0

[a]OTUs that were significant in at least 80% of runs after FDR correction are highlighted in dark red. OTUs significant in at least 70% of runs are highlighted in orange. [b]Comparison of the base model to an equivalent model with an additional term for the feed by $\log_2$(OTU absolute abundance) interaction. [c]Comparison of the base model to an equivalent model with an additional term for the log (base 2) absolute abundance of the focal OTU.

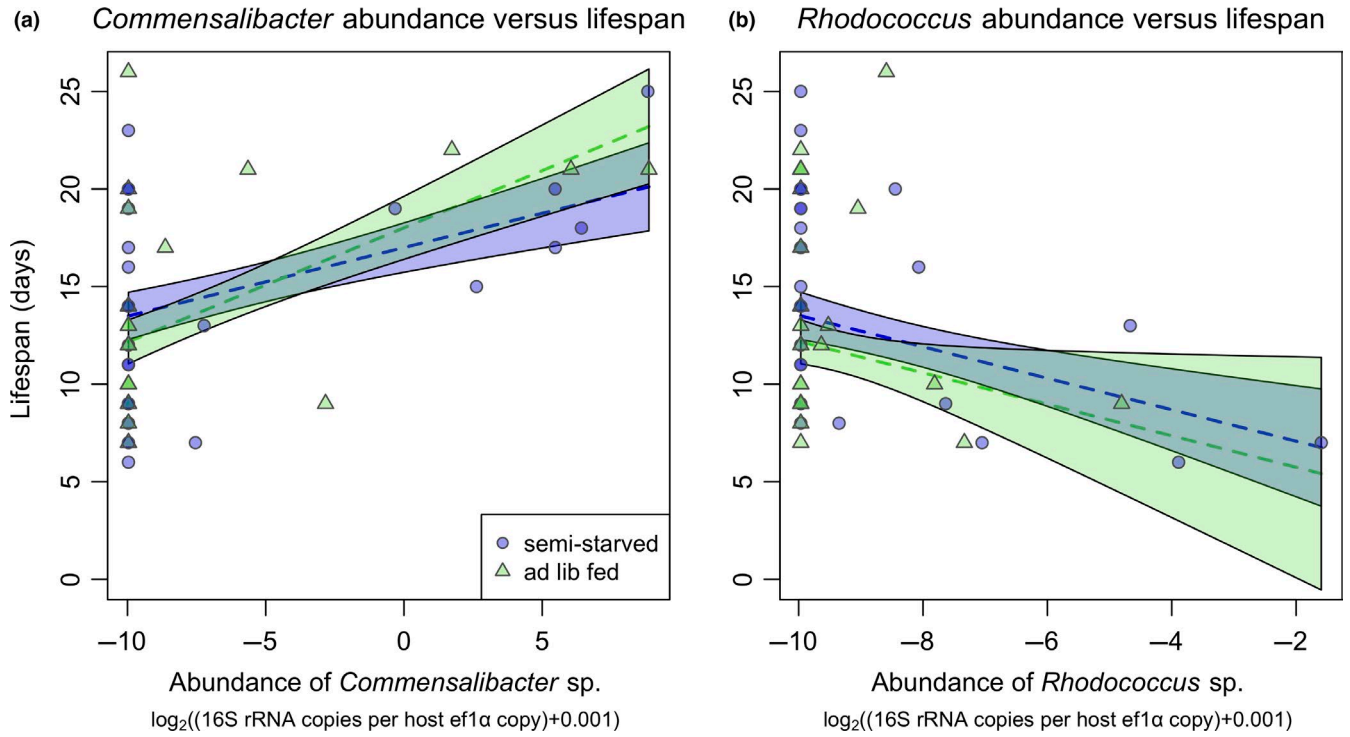

FIGURE 4 Relationship between life span and abundances of *Commensalibacter* sp. and *Rhodococcus* sp. Host life span was correlated with the abundance of (a) *Commensalibacter* sp. (OTU_1) and (b) *Rhodococcus* sp. (OTU_58). Points show the raw data (N = 49); dotted lines show model-predicted means and shading indicates model-estimated standard errors. For the model predictions, egg.onset is set to 2 days and the abundance of the unplotted OTU is set to its median observed value [Colour figure can be viewed at wileyonlinelibrary.com]

detect significant interactions between food availability and abundances of the focal OTUs (Supporting Information Table S8). This was particularly true for life span, for which we only had sufficient power to detect the interaction with OTU_1. However, we did have sufficient power for 12/20 focal OTUs for fecundity, 15/20 for egg weight, 14/20 for egg triglycerides and 10/20 for egg glycogen (Supporting Information Table S8).

We also tested for relationships between the fitness metrics and *relative* abundances of the 20 focal OTUs. We did not detect correlations between these relative abundances and butterfly fitness or performance (Supporting Information Table S10).

4 | DISCUSSION

We investigated how (a) the overall density of the gut flora and (b) the abundances of dominant bacterial OTUs were related to butterfly fitness under abundant or restricted food conditions. As predicted, in a well-fed female there was no relationship between total bacterial load and fitness or performance. Contrary to our expectations, however, semistarvation did not trigger negative consequences of hosting gut flora: under food limitation, greater bacterial loads did not correlate with decreased fitness. Abundance of a bacterial species in the genus *Commensalibacter* was positively correlated with life span and abundance of a *Rhodococcus* species was negatively correlated with life span; these relationships were also unaffected by food availability to the host. Taken as whole, these results suggest that individual gut microbial taxa may have positive, negative or neutral relationships with *Speyeria mormonia*, but overall *S. mormonia* does not incur a net cost for hosting gut microbes.

4.1 | No evidence that food availability mediates the host–gut flora relationship in butterflies

The feeding treatments induced physiological food limitation in semistarved females: a 50% reduction in calories resulted in a 51% reduction in fecundity. These results are consistent with previous work that has shown that in *S. mormonia*, female fecundity decreases linearly in proportion to food availability while life span is maintained (Boggs & Ross, 1993). However, even the densest microbial communities did not correspond with decreases in the fitness of semistarved females. Although we observed a trend in the predicted direction for life span (a tendency for *ad lib*-fed females to have increased fitness at high bacterial loads, while the fitness of semistarved females did not vary with bacterial load), this trend was not significant. Power analysis indicated that we should have been able to detect a relationship if it was present. Overall, we found no evidence to suggest that high bacterial loads impose a cost on butterfly hosts, even under food restriction.

In other hosts, gut floras have been shown to compensate for the negative effects of *qualitatively* unbalanced diets: in olive flies

and *Drosophila* larvae, germ-free hosts suffered reduced fitness on an unbalanced diet, but fitness was rescued by the acquisition of either normal gut flora, or of a diet that met the host's nutritional requirements (Ben-Yosef et al., 2010; Shin et al., 2011). We focused on adult butterflies as a comparative system to test the net effect of the gut flora on *quantitative*, rather than qualitative, dietary restriction. Because investing in gut flora can be costly when they are not needed—as has been shown for thrips supplied with a diet that met their nutritional needs (de Vries et al., 2004)—and because the nutritional needs of the adult butterfly are simple, we expected that semistarved adult butterflies would be an appropriate system in which to test for a net caloric cost to hosting environmentally acquired gut flora. However, we found no evidence that the gut flora impose a meaningful caloric burden on food-limited hosts.

Lepidopteran gut microbes could have a wide range of effects unrelated to host caloric intake, including competitive exclusion of pathogens or detoxification of plant secondary compounds (Hammer & Bowers, 2015; Mason, Couture, & Raffa, 2014). In particular, there is some evidence that gut flora confer pathogen protection to larval lepidopterans (Robinson, Schloss, Ramos, Raffa, & Handelsman, 2010). This study was not designed to test whether gut microbes serve any of these functions in butterflies, and they remain possibilities.

4.2 | Lepidopteran gut flora are highly variable

Only a few of the butterflies tested had particularly high abundances of any given microorganism. Indeed, the density and composition of the gut flora varied widely among the animals in our study. This variation may have been due in part to the gut treatments to which the animals were subjected. However, these treatments were not sufficient to generate consistent differences among gut microbial communities (discussed further in the Appendix). Wide variation in both bacterial load and taxonomic composition appears to typify the lepidopteran gut microbiota; a survey of wild butterflies of 50 Costa Rican species found that gut bacterial density varied across six orders of magnitude and, on average, a pair of adults shared less than one-quarter of their gut bacterial species (Ravenscraft et al., 2019). The lack of consistent differences between our gut treatments could have resulted, in part, from the high degree of natural heterogeneity in this system.

Three of the dominant genera in *S. mormonia* appear to be common in other adult butterflies. *Commensalibacter* is abundant in Neotropical butterflies (Hammer, McMillan, & Fierer, 2014; Ravenscraft et al., 2019), and is also found in other insects with sugar-rich diets (Crotti et al 2010). We discuss its possible relationship to host life span below. *Pantoea* is a general insect associate and was common in a survey of adult Costa Rican butterflies (Ravenscraft et al., 2019). Finally, there appears to be a widespread association between butterflies and *Orbus* bacteria: the genus has been detected in adults across a wide geographical range including Costa Rica, Panama, South Korea and, in the present study, Colorado, USA (Hammer et al., 2017; Kim et al., 2013; Ravenscraft et al., 2019). *Orbus* has also been detected in the guts of flies and bees (Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Kwong & Moran, 2013). Given that we did not detect a relationship between *Pantoea* or *Orbus* and the fitness of *S. mormonia*, it seems likely that these genera are not specific butterfly associates, but rather opportunistic tenants that are generally adapted to colonize the insect gut.

Most of the dominant bacteria in the guts of experimental and donor *S. mormonia* were common environmental bacteria (Supporting Information Table S7). This suggests that *S. mormonia* may acquire most or all of its adult gut flora directly from the environment after emergence from the pupa, and that adults of this species (and perhaps many short-lived, temperate butterfly species) do not form strong associations with particular gut microbes. This does not necessarily preclude a functional role for the butterfly gut flora: some hosts, such as mosquito larvae, depend upon environmentally acquired microbes for survival (Coon et al., 2016). However, in such generalized relationships between a host and environmentally acquired bacteria, most interactions may be commensal, rather than mutualisms or parasitisms. Evidence suggests this to be the case for a growing number of hosts (Hammer et al., 2017; Sanders et al., 2017; Shelomi et al., 2013; Šustr, Stingl, & Brune, 2014; Whitaker, Salzman, Sanders, Kaltenpoth, & Pierce, 2016). Despite the recent surge of interest in the functional roles gut flora play in animal hosts, highly variable and functionally commensal gut flora may be more common than generally assumed. In particular, they may be typical of many insects, especially those with a simple gut structure and holometabolous development (Engel & Moran, 2013).

4.3 | Correlations between host life span and abundances of *Commensalibacter* and *Rhodococcus* are intriguing but require experimental verification

Despite the unstable nature of the butterfly gut flora, two OTUs *were* related to host performance: increased abundance of a *Commensalibacter* species was related to longer life span, while decreased abundance of a *Rhodococcus* species was correlated with shorter life span. These relationships are intriguing, but only correlative in nature; the directionality of the relationships cannot be determined from our data. Furthermore, interpretation of the first relationship is difficult because relative abundances of *Commensalibacter* were positively correlated with total bacterial load, and total bacterial load itself increased at the end of summer. However, *Commensalibacter* is a beneficial mutualist in other insects. *Drosophila* with monospecific colonization of their gut by *Commensalibacter intestini* grow and develop slightly faster than conventionally reared flies (Shin et al., 2011). The bacterium may benefit the flies by suppressing a pathogenic bacterial species, *Gluconobacter morbifer* (Roh et al., 2008; Ryu et al., 2008).

Members of the genus *Rhodococcus* are common environmental bacteria and are also known insect symbionts: the kissing bug, *Rhodnius prolixus*, depends on the presence of *Rhodococcus rhodnii* in its gut for normal growth and development (Bell, Philp, Aw, &

Christofi, 1998). *Rhodococcus* has also been isolated from the gut of the larval gypsy moth (Broderick, Raffa, Goodman, & Handelsman, 2004). The negative correlation between *Rhodococcus* abundance and adult butterfly life span, even if it proves to be causal, does not necessarily imply that *Rhodococcus* is detrimental to butterfly fitness. Indeed, we found no relationship between abundance of this bacterium and host reproductive output or egg quality.

Further research on the possible effects of *Commensalibacter* and *Rhodococcus* on butterfly life history is warranted. In the meantime, the contrast between these two organisms' relationships to host life span serves to demonstrate that the net effects of the gut microbiota are ultimately the sum of the component microbes' varied commensal, mutualistic or parasitic relationships with their host.

4.4 | Limitations of this study's approach

Detangling the effects of an entire symbiotic community on a host is difficult due to the number of players involved and the difficultly of culturing many gut microbes. The approach of testing for correlation between the dominant OTUs and host fitness can yield suggestive results for further exploration. This may be especially true for hosts that have more stable gut communities (although enough variation must exist, or be created, to allow tests for correlation) and whose gut flora are more likely to serve an important functional role. Of course, follow-up studies are crucial to experimentally test the causality of any correlations found.

This approach does have limitations. First, there are limits to statistical power; some of our models were underpowered, especially to detect an influence of food availability on relationships between fitness and the focal OTUs. Furthermore, because we rarefied to 1,000 reads per butterfly, we may have failed to detect the focal OTUs in butterflies in which they were present at low relative abundance (i.e., fewer than approximately 1/1,000 raw reads). For both of these reasons, we could easily have missed relationships that were actually present. Second, we were only able to sample the microbiota at a single time point, upon death of the butterfly. The microbes found in aged individuals may not reflect the density or composition of the microbiota at the time of peak functioning and fecundity. This shortcoming could be overcome in other hosts that produce solid faeces, which would allow nondestructive sampling of the gut flora at multiple time points. Third, some of the microbes we detected in *S. mormonia* could have been metabolically dormant or dead cells passing through the gut with no effect on the host. The presence of inactive bacteria among the focal OTUs may have lessened our chance of detecting correlations with host fitness because some other, nonfocal OTUs might have been active in the gut. Fourth, although our animals were collected in the field, captivity can have a pronounced effect on the microbial community (Hammer et al., 2014). The density and taxonomic composition of our animals' microbiota did differ from that of wild (donor) butterflies; this may also have lessened our ability to detect biologically relevant relationships. Fifth, the gut microbial community is not limited to bacteria; adult butterflies are known to host other microorganisms, including yeasts (Ravenscraft et al., 2019). The potential effects of eukaryotic gut microbes on butterfly hosts are unknown. Finally, relationships between microbes and hosts can occur at taxonomic levels below or above the 97% sequence similarity cut-off which is often interpreted as defining a microbial species. At a finer scale, strains of *E. coli* can have different effects on human hosts depending on which plasmids they possess (Rasko et al., 2008; Wijetunge et al., 2014). At broader scales, whole clades of microbes can share similar functional traits, and members of a functional group may therefore be interchangeable with respect to host function. Analyses conducted at the OTU level will be relatively blind to this type of diffuse mutualism effect.

5 | CONCLUSIONS

Across a wide range of bacterial densities in *Speyeria mormonia*, we found no conclusive evidence that the gut microbiota as a whole either contribute to, or significantly detract from, host nutrition, performance or fitness. Although we expected that high bacterial loads would be detrimental to these butterflies, we did not detect any net reproductive costs incurred due to hosting gut bacteria, even at high bacterial densities and under food restriction. We also failed to detect any evidence that food availability modulates relationships between butterfly fitness and the abundances of particular gut bacteria. Mutualism theory posits that symbiotic partners often participate in a cost–benefit trade-off (Douglas, 2008). However, our work suggests that if butterflies do derive any benefits from their gut flora as a whole, or from individual members (which remains an open question; our study did not, for example, investigate the potential for gut flora to provide protection from pathogens), they may receive these benefits for "free." Future research should investigate when, and whether, the cost–benefit trade-off paradigm applies to gut microbial symbioses.

ACKNOWLEDGEMENTS

Sincere thanks to J. Hack, C. Karban and H. Slyce for field assistance and H. Slyce, L. Tran, M. Hannah, B. Reardon, J. DuRant, J. Niklas, J. Wiltes, R. Hotelling and S. Desmond for lab assistance. The staff of RMBL offered invaluable logistical support. We thank Tadashi Fukami and his 2013 Ecological Statistics class, Luke Frishkoff, Jacob Russell and several anonymous reviewers for their insightful comments and suggestions. Jon Sanders provided the 16S rRNA standard for our qPCR standard curve. This work was supported by an NSF Graduate Research Fellowship, American Society of Naturalists Student Research Award, RMBL Graduate Fellowship, and an NIH PERT postdoctoral fellowship to A.R., funds from the University of South Carolina to C.L.B., funds from Stanford University to K.P., and a Stanford Vice Provost for Undergraduate Education award to J. Hack.

AUTHOR CONTRIBUTIONS

A.R., K.P. and C.B. designed the experiments. A.R., N.K., and C.B. performed the experiments. A.R. conducted the lab work for the Illumina sequencing. N.K. performed the lab work for the egg chemical analyses. A.R. analysed the data and wrote the manuscript and N.K., K.P. and C.B. provided additional writing and editorial feedback.

DATA ACCESSIBILITY

Raw Illumina sequences are available in the NCBI Sequence Read Archive under BioProject number PRJNA516286. All other data (OTU table, bacterial abundances calculated from qPCR, butterfly fecundity, egg chemical composition, etc) are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.95p86c0

ORCID

Alison Ravenscraft https://orcid.org/0000-0003-4974-4804

REFERENCES

Anderson, M. J., & Walsh, D. C. I. (2013). PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecological Monographs*, *83*(4), 557–574. https://doi.org/10.1890/12-2010.1

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bell, K. S., Philp, J. C., Aw, D. W. J., & Christofi, N. (1998). The genus *Rhodococcus*. *Journal of Applied Microbiology*, *85*, 195–210. https://doi.org/10.1046/j.1365-2672.1998.00525.x

Ben-Yosef, M., Aharon, Y., Jurkevitch, E., & Yuval, B. (2010). Give us the tools and we will do the job: Symbiotic bacteria affect olive fly fitness in a diet-dependent fashion. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1687), 1545–1552. https://doi.org/10.1098/rspb.2009.2102

Bergmann, G. T., Bates, S. T., Eilers, K. G., Lauber, C. L., Caporaso, J. G., Walters, W. A., … Fierer, N. (2011). The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biology & Biochemistry*, *43*(7), 1450–1455. https://doi.org/10.1016/j.soilbio.2011.03.012

Boggs, C. (1997). Dynamics of reproductive allocation from juvenile and adult feeding: radiotracer studies. *Ecology*, *78*(1), 192–202. Retrieved from http://www.esajournals.org/doi/abs/10.1890/0012-9658(1997) 078%5B0192:DORAFJ%5D2.0.CO%3B2

Boggs, C. (1986). Reproductive strategies of female butterflies: Variation in and constraints on fecundity. *Ecological Entomology*, *11*(1), 7–15. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2311.1986.tb00274.x/full

Boggs, C., & Freeman, K. (2005). Larval food limitation in butterflies: Effects on adult resource allocation and fitness. *Oecologia*, *144*(3), 353–361. https://doi.org/10.1007/s00442-005-0076-6

Boggs, C. L., & Inouye, D. W. (2012). A single climate driver has direct and indirect effects on insect population dynamics. *Ecology Letters*, *15*(5), 502–508. https://doi.org/10.1111/j.1461-0248.2012.01766.x

Boggs, C. L., & Ross, C. L. (1993). The effect of adult food limitation on life history traits in *Speyeria mormonia* (Lepidoptera: Nymphalidae). *Ecology*, *74*(2), 433–441. Retrieved from http://www.jstor.org/stable/1939305

Broderick, N. A., Raffa, K. F., Goodman, R. M., & Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied Environmental Microbiology*, *70*, 293–300. https://doi.org/10.1128/AEM.70.1.293

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., … Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621–1624. https://doi.org/10.1038/ismej.2012.8

Carini, P., Marsden, P. J., Leff, J. W., Morgan, E. E., Strickland, M. S., & Fierer, N. (2016). Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. *Nature Microbiology*, *2*, 16242. https://doi.org/10.1038/nmicrobiol.2016.242

Chandler, J. A., Lang, J., Bhatnagar, S., Eisen, J. A., & Kopp, A. (2011). Bacterial communities of diverse *Drosophila* species: Ecological context of a host-microbe model system. *PLoS Genetics*, *7*(9), e1002272. https://doi.org/10.1371/journal.pgen.1002272

Chippendale, G. (1978). The functions of carbohydrates in insect life processes. In M. Rockstein (Ed.), *Biochemistry of insects* (pp. 1–55). New York, NY: Academic Press. https://doi.org/10.1016/B978-0-12-591640-0.50006-6

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: An integrative view. *Cell*, *148*(6), 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035

Coon, K. L., Brown, M. R., & Strand, M. R. (2016). Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. *Molecular Ecology*, *25*(22), 5806–5826. https://doi.org/10.1111/mec.13877

R Core Team (2018). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org/

de Vries, E. J., Jacobs, G., Sabelis, M. W., Menken, S. B. J., & Breeuwer, J. A. J. (2004). Diet-dependent effects of gut bacteria on their insect host: The symbiosis of *Erwinia* sp. and western flower thrips. *Proceedings of the Royal Society B: Biological Sciences*, *271*(1553), 2171–2178. https://doi.org/10.1098/rspb.2004.2817

Dillon, R. J., & Dillon, V. M. (2004). The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology*, *49*(98), 71–92. https://doi.org/10.1146/annurev.ento.49.061802.123416

Douglas, A. E. (2008). Conflict, cheats and the persistence of symbioses. *New Phytologist*, *177*(4), 849–858. https://doi.org/10.1111/j.1469-8137.2007.02326.x

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), 996–998. https://doi.org/10.1038/nmeth.2604

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Reviews*, *37*, 699–735. https://doi.org/10.1111/1574-6976.12025

Erhardt, A., & Rusterholz, H.-P. (1998). Do Peacock butterflies (*Inachis io* L.) detect and prefer nectar amino acids and other nitrogenous compounds? *Oecologia*, *117*, 536–542. Retrieved from http://link.springer.com/article/10.1007/s004420050690

Eutick, M. L., Veivers, P., O'Brien, R. W., & Slaytor, M. (1978). Dependence of the higher termite, *Nasutitermes exitiosus* and the lower termite, *Coptotermes lacteus* on their gut flora. *Journal of Insect Physiology*, *24*(5), 363–368. https://doi.org/10.1016/0022-1910(78)90076-8

Haddadin, M. S., Abdulrahim, S. M., Hashlamoun, E. A., & Robinson, R. K. (1996). The effect of *Lactobacillus acidophilus* on the production and chemical composition of hen's eggs. *Poultry Science*, *75*(4), 491–494. https://doi.org/10.3382/ps.0750491

Hammer, T. J., & Bowers, M. D. (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*, *179*(1), 1–14. https://doi.org/10.1007/s00442-015-3327-1

Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P., & Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(36), 9641–9646. https://doi.org/10.1073/pnas.1707186114

Hammer, T. J., McMillan, W. O., & Fierer, N. (2014). Metamorphosis of a butterfly-associated bacterial community. *PLoS ONE*, *9*(1), e86995. https://doi.org/10.1371/journal.pone.0086995

Kim, J. Y., Lee, J., Shin, N.-R., Yun, J.-H., Whon, T. W., Kim, M.-S., … Bae, J.-W. (2013). *Orbus sasakiae* sp. nov., a bacterium isolated from the gut of the butterfly *Sasakia charonda*, and emended description of the genus *Orbus*. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 1766–1770. https://doi.org/10.1099/ijs.0.041871-0

Kwong, W. K., & Moran, N. A. (2013). Cultivation and characterization of the gut symbionts of honey bees and bumble bees: Description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family Neisseriaceae of the betaproteobacteria, and *Gilliamella apicola* gen. nov., sp. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 2008–2018. https://doi.org/10.1099/ijs.0.044875-0

Liu, Z., Lozupone, C., Hamady, M., Bushman, F., & Knight, R. (2007). Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Research*, *35*(18), e120. https://doi.org/10.1093/nar/gkm541

Lozupone, C., Hamady, M., & Knight, R. (2006). UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics*, *7*, 371. https://doi.org/10.1186/1471-2105-7-371

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nature Methods*, *10*(10), 999–1002. https://doi.org/10.1038/nmeth.2634

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet. Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Mason, C. J., Couture, J. J., & Raffa, K. F. (2014). Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator. *Oecologia*, *175*(3), 901–910. https://doi.org/10.1007/s00442-014-2950-6

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., … Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*, *6*(3), 610–618. https://doi.org/10.1038/ismej.2011.139

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host–gut microbiota metabolic interactions. *Science*, *336*(6086), 1262–1267. https://doi.org/10.1126/science.1223813

Niitepõld, K., & Boggs, C. L. (2015). Effects of increased flight on the energetics and life history of the butterfly *Speyeria mormonia*. *PLoS ONE*, *10*(10), e0140104. https://doi.org/10.1371/journal.pone.0140104

Nogge, G., & Gerresheim, A. (1982). Experiments on the elimination of symbionts from the tsetse fly, *Glossina morsitans morsitans* (Diptera: Glossiniciae), by antibiotics and lysozyme. *Journal of Invertebrate Pathology*, *40*, 166–179.

O'Brien, D., Boggs, C., & Fogel, M. (2004). Making eggs from nectar: The role of life history and dietary carbon turnover in butterfly reproductive resource allocation. *Oikos*, *105*, 279–291. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.0030-1299.2004.13012.x/full

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., …Wagner, H. (2015). *vegan: Community Ecology Package. R package version 2.3-0*. Retrieved from http://cran.r-project.org/package=vegan

Peay, K. G., Bruns, T. D., Kennedy, P. G., Bergemann, S. E., & Garbelotto, M. (2007). A strong species-area relationship for eukaryotic soil microbes: Island size matters for ectomycorrhizal fungi. *Ecology Letters*, *10*(6), 470–480. https://doi.org/10.1111/j.1461-0248.2007.01035.x

Rasko, D. A., Rosovitz, M. J., Myers, G. S. A., Mongodin, E. F., Fricke, W. F., Gajer, P., … Ravel, J. (2008). The pangenome structure of *Escherichia coli*: Comparative genomic analysis of *E. coli* commensal and pathogenic isolates. *Journal of Bacteriology*, *190*(20), 6881–6893. https://doi.org/10.1128/JB.00619-08

Ravenscraft, A., Berry, M., Hammer, T., Peay, K., & Boggs, C. (2019). Structure and function of the bacterial and fungal gut microbiota of Neotropical butterflies. *Ecological Monographs*, *00*(00), 1–25.

Robinson, C. J., Schloss, P., Ramos, Y., Raffa, K., & Handelsman, J. (2010). Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microbial Ecology*, *59*(2), 199–211. https://doi.org/10.1007/s00248-009-9595-8

Roh, S. W., Nam, Y.-D., Chang, H.-W., Kim, K.-H., Kim, M.-S., Ryu, J.-H., … Bae, J.-W. (2008). Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. *Applied and Environmental Microbiology*, *74*(20), 6171–6177. https://doi.org/10.1128/AEM.00301-08

Rosengaus, R. B., Zecher, C. N., Schultheis, K. F., Brucker, R. M., & Bordenstein, S. R. (2011). Disruption of the termite gut microbiota and its prolonged consequences for fitness. *Applied and Environmental Microbiology*, *77*(13), 4303–4312. https://doi.org/10.1128/AEM.01886-10

Ryu, J.-H., Kim, S.-H., Lee, H.-Y., Bai, J. Y., Nam, Y.-D., Bae, J.-W., … Lee, W.-J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. *Science*, *319*(5864), 777–782. https://doi.org/10.1126/science.1149357

Sabree, Z. L., Hansen, A. K., & Moran, N. A. (2012). Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees. *PLoS ONE*, *7*(7), e41250. https://doi.org/10.1371/journal.pone.0041250

Sanders, J. G., Łukasik, P., Frederickson, M. E., Russell, J. A., Koga, R., Knight, R., & Pierce, N. E. (2017). Dramatic differences in gut bacterial densities correlate with diet and habitat in rainforest ants. *Integrative and Comparative Biology*, *57*(4), 705–722. https://doi.org/10.1093/icb/icx088

Schwab, D. B., Riggs, H. E., Newton, I. L. G., & Moczek, A. P. (2016). Developmental and ecological benefits of the maternally transmitted microbiota in a dung beetle. *The American Naturalist*, *188*(6), 679–692. https://doi.org/10.1086/688926

Shelomi, M., Lo, W.-S., Kimsey, L. S., & Kuo, C.-H. (2013). Analysis of the gut microbiota of walking sticks (Phasmatodea). *BMC Research Notes*, *6*, 368. https://doi.org/10.1186/1756-0500-6-368

Shin, S. C., Kim, S.-H., You, H., Kim, B., Kim, A. C., Lee, K.-A., … Lee, W.-J. (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, *334*(6056), 670–674. https://doi.org/10.1126/science.1212782

Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PLoS ONE*, *9*(2), e90234. https://doi.org/10.1371/journal.pone.0090234

Šustr, V., Stingl, U., & Brune, A. (2014). Microprofiles of oxygen, redox potential, and pH, and microbial fermentation products in the highly alkaline gut of the saprophagous larva of *Penthetria holosericea* (Diptera: Bibionidae). *Journal of Insect Physiology*, *67*, 64–69. https://doi.org/10.1016/j.jinsphys.2014.06.007

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Whitaker, M. R. L., Salzman, S., Sanders, J., Kaltenpoth, M., & Pierce, N. E. (2016). Microbial communities of lycaenid butterflies do not correlate with larval diet. *Frontiers in Microbiology*, *7*, 1920. https://doi.org/10.3389/fmicb.2016.01920

Wijetunge, D. S. S., Karunathilake, K. H. E. M., Chaudhari, A., Katani, R., Dudley, E. G., Kapur, V., … Kariyawasam, S. (2014). Complete nucleotide sequence of pRS218, a large virulence plasmid, that augments pathogenic potential of meningitis-associated *Escherichia coli* strain RS218. *BMC Microbiology*, *14*, 203. https://doi.org/10.1186/s12866-014-0203-9

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. (2009). *Mixed effects models and extensions in ecology with R*. New York, NY: Springer.

SUPPORTING INFORMATION

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

How to cite this article: Ravenscraft A, Kish N, Peay K, Boggs C. No evidence that gut microbiota impose a net cost on their butterfly host. *Mol Ecol*. 2019;28:2100–2117. https://doi.org/10.1111/mec.15057